Diagnostic Procedures in Veterinary Bacteriology and Mycology

Fifth Edition

Editors

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Preface

It is now more than 20 years since the first edition of this book appeared. In the second, third, and fourth editions, efforts were made to adhere as much as possible to the original purpose of the book, which was to provide a compilation of practical, effective methods for diagnostic veterinary microbiology laboratories both small and large. We have attempted to maintain this purpose in this fifth edition.

Given the rapid increase in knowledge in both diagnostic bacteriology and mycology, it was considered necessary to increase the number of contributors. Although most of these individuals are specialists, they were urged to emphasize procedures that are within the capability of most diagnostic laboratories. Workers who wish to amplify or vary the procedures described will find sources for additional information in the references.

There have been many changes in methods and techniques in diagnostic bacteriology and mycology in recent years as a result of the application of new technologies. The identification of some bacteria and fungi is being simplified and accelerated by the use of genetic probes and new procedures made possible by monoclonal antibodies and enzyme immunodiagnostics. Rapid commercial systems and kits for identification and serotyping have supplanted in many laboratories the slower traditional approaches. Automation will be increasingly applied to identification and antimicrobial susceptibility testing. A chapter titled "Rapid Methods of Identification" has been added to assist workers in keeping abreast of recent developments. In spite of these advances in the more affluent laboratories, however, many laboratories in the less developed countries will continue to depend, for the most part, upon traditional procedures.

The book has become increasingly dependent upon contributing authors and we are much indebted to them for their efforts. Thanks are expressed to Dr. M. M. Chengappa and Mr. Lloyd Thacheray for their many suggestions, to Ms. Linda Compton and staff for word processing services, to Mr. Harold McAllister for many of the figures, to Mr. Don Massie and staff for various illustrations, and to Ms. Sandy Brown for her diligence in detecting typographical and

spelling errors and preparation of the index. Finally, we express our appreciation to the staff of Academic Press for their efficient and always cooperative efforts.

G. R. Carter
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Classification, Normal Flora, and Laboratory Safety

G. R. Carter and John R. Cole, Jr.

Classification

Bergey's Manual of Systematic Bacteriology, Volumes 1 and 2 (1,2), provides a comprehensive listing of many established species and their characteristics. Generally speaking, the names used in the aforementioned volumes will be employed in this manual. For the most part these bacterial names are the ones listed in the *Approved Lists of Bacterial Names* (3) published by the American Society for Microbiology. As new information becomes available, changes in names and occasionally reclassification are recommended. The new names and reclassifications are generally accepted by the scientific community after publication or listing in the *International Journal of Systematic Bacteriology*.

An outline of the current Bergey classification with a listing of genera associated with animals and humans is provided below. The various bacteria are discussed in the text, usually in the same order in which they appear in the outline.

Kingdom Procaryotae[1]
Division 1. Gracilicutes
Section 1. Spirochetes
Order I. Spirochaetales
Family I. Spirochaetaceae (4 genera)
Genus I. *Spirochaeta*

[1]Reproduced by permission from *Bergey's Manual of Systematic Bacteriology*, Vols. 1 and 2. Baltimore, Williams & Wilkins, 1984, 1986.

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Genus III. *Treponema*
Genus IV. *Borrelia*
Family II. Leptospiraceae (1 genus)
Genus I. *Leptospira*

Section 2. Aerobic/Microaerophilic, Motile, Helical/Vibrioid Gram-negative Bacteria (7 genera)
Genus *Spirillum*
Genus *Campylobacter*

Section 4. Gram-Negative Aerobic Rods and Cocci
Family I. Pseudomonadaceae (4 genera)
Genus I. *Pseudomonas*
Family VI. Acetobacteraceae
Genus I. *Acetobacter*
Genus II. *Gluconobacter*
Family VII. Legionellaceae
Genus I. *Legionella*
Family VIII. Neisseriaceae
Genus I. *Neisseria*
Genus II. *Moraxella*
Genus III. *Acinetobacter*
Genus IV. *Kingella*
Other Genera (16 genera)
Genus *Flavobacterium*
Genus *Alcaligenes*
Genus *Brucella*
Genus *Bordetella*
Genus *Francisella*

Section 5. Facultatively Anaerobic Gram-Negative Rods
Family I. Enterobacteriaceae
Genus I. *Escherichia*
Genus II. *Shigella*
Genus III. *Salmonella*
Genus IV. *Citrobacter*
Genus V. *Klebsiella*
Genus VI. *Enterobacter*
Genus VII. *Erwinia*
Genus VIII. *Serratia*
Genus IX. *Hafnia*
Genus X. *Edwardsiella*
Genus XI. *Proteus*
Genus XII. *Providencia*
Genus XIII. *Morganella*
Genus XIV. *Yersinia*

Other Genera of the Family Enterobacteriaceae (6 genera)
Genus *Obesumbacterium*
Genus *Xenorhabdus*
Genus *Kluyvera*
Genus *Rahnella*
Genus *Cedecea*
Genus *Tatumella*
Family II. Vibrionaceae
Genus I. *Vibrio*
Genus II. *Photobacterium*
Genus III. *Aeromonas*
Genus IV. *Plesiomonas*
Family III. Pasteurellaceae
Genus I. *Pasteurella*
Genus II. *Haemophilus*
Genus III. *Actinobacillus*
Other Genera (7 genera)
Genus *Chromobacterium*
Genus *Gardnerella*
Genus *Eikenella*
Genus *Streptobacillus*

Section 6. Anaerobic Gram-Negative Straight, Curved, and Helical Rods
Family I. Bacteroidaceae (13 genera)
Genus I. *Bacteroides*
Genus II. *Fusobacterium*
Genus III. *Leptotrichia*
Genus IV. *Butyrivibrio*
Genus V. *Succinimonas*
Genus VI. *Succinivibrio*
Genus VII. *Anaerobiospirillum*
Genus VIII. *Wolinella*
Genus IX. *Selenomonas*
Genus X. *Anaerovibrio*
Genus XII. *Acetivibrio*
Genus XIII. *Lachnospira*

Section 7. Dissimilatory Sulfate- or Sulfur-Reducing Bacteria (7 genera)

Section 8. Anaerobic Gram-Negative Cocci
Family I. Veillonellaceae
Genus I. *Veillonella*
Genus II. *Acidaminococcus*
Genus III. *Megasphaera*

Section 9. Rickettsias and Chlamydias
- Order I. Rickettsiales
  - Family I. Rickettsiaceae
    - Tribe I. Rickettsieae
      - Genus I. *Rickettsia*
      - Genus II. *Rochalimaea*
      - Genus III. *Coxiella*
    - Tribe II. Ehrlichieae
      - Genus IV. *Ehrlichia*
      - Genus V. *Cowdria*
      - Genus VI. *Neorickettsia*
    - Tribe III. Wolbachieae
      - Genus VII. *Wolbachia*
      - Genus VIII. *Rickettsiella*
  - Family II. Bartonellaceae
    - Genus I. *Bartonella*
    - Genus II. *Grahamella*
  - Family III. Anaplasmataceae
    - Genus I. *Anaplasma*
    - Genus II. *Aegyptianella*
    - Genus III. *Haemobartonella*
    - Genus IV. *Eperythrozoon*
- Order II. Chlamydiales
  - Family I. Chlamydiaceae
    - Genus I. *Chlamydia*

Section 10. Mycoplasmas
- Division Tenericutes
- Class I. Mollicutes
  - Order I. Mycoplasmatales
    - Family I. Mycoplasmataceae
      - Genus I. *Mycoplasma*
      - Genus II. *Ureaplasma*
    - Family II. Acholeplasmataceae
      - Genus I. *Acholeplasma*
    - Family III. Spiroplasmataceae
      - Genus I. *Spiroplasma*
    - Other Genera
      - Genus *Anaeroplasma*
      - Genus *Thermoplasma*
      - Mycoplasmalike Organisms of Plants and Invertebrates

Section 11. Endosymbionts

Section 12. Gram-positive Cocci
- Family I. Micrococcaceae

Genus I. *Micrococcus*
Genus II. *Stomatococcus*
Genus III. *Planococcus*
Genus IV. *Staphylococcus*
Other genera
Genus *Streptococcus*
Pyogenic Hemolytic Streptococci
Oral Streptococci
Enterococci
Lactic Acid Streptococci
Anaerobic Streptococci
Other Streptococci
Genus *Leuconostoc*
Genus *Pediococcus*
Genus *Aerococcus*
Genus *Gemella*
Genus *Peptococcus*
Genus *Peptostreptococcus*
Genus *Ruminococcus*
Genus *Coprococcus*
Genus *Sarcina*

Section 13. Endospore-Forming Gram-positive Rods and Cocci (6 genera)
Genus *Bacillus*
Genus Sporolactobacillus
Genus *Clostridium*

Section 14. Regular, Nonsporing, Gram-positive Rods
Genus *Lactobacillus*
Genus *Listeria*
Genus *Erysipelothrix*
Genus *Brochothrix*
Genus *Renibacterium*
Genus *Kurthia*
Genus *Caryophanon*

Section 15. Irregular, Nonsporing, Gram-positive Rods (21 genera)
Genus *Corynebacterium*
Plant-Pathogenic Species of *Corynebacterium*
Genus *Gardnerella*
Genus *Arcanobacterium*
Genus *Brevibacterium*
Genus *Microbacterium*
Genus *Aureobacterium*
Genus *Arachnia*

Genus *Rothia*
Genus *Propionibacterium*
Genus *Eubacterium*
Genus *Lachnospira*
Genus *Butyrivibrio*
Genus *Actinomyces*
Genus *Bifidobacterium*

Section 16. Mycobacteria
Family Mycobacteriaceae
Genus *Mycobacterium*

Section 17. Nocardioforms (9 genera)
Genus *Nocardia*
Genus *Rhodococcus*
Genus *Oerskovia*

The Normal Flora

It is important that the clinical microbiologist have some familiarity with the kinds of organisms encountered normally in and upon animals. Such knowledge is necessary in the interpretation of the results of microbiologic examinations.

The so-called normal flora consist of the wide variety of bacteria and fungi that live in or upon normal animals without producing disease. Included in this flora are many potential pathogens and opportunistic organisms. The term *normal flora* is a convenient concept, but it should be kept in mind that the kinds and numbers of bacteria present vary greatly with different circumstances. The intestinal flora of the young animal differs markedly from that of the older animal. The flora are also influenced by geographic location, nutrition, and climate. The technical procedures employed to recover pathogenic organisms frequently give a distorted idea of the kinds and numbers of bacteria present. The older studies of the normal flora of domestic animals have often neglected the obligate anaerobes, which in the intestine make up by far the largest number of bacteria (see Chapter 15).

The normal flora of domestic animals have not been studied in as detailed a fashion as that of human beings. What little information that is available, as well as firsthand experience in the diagnostic laboratory, indicates a considerable similarity between the normal flora of humans (4) and domestic animals.

Some of the kinds of bacteria that can be expected to occur normally in and upon domestic animals are tabulated below.

Mouth, Nasopharynx

Micrococci (aerobic and anaerobic, pigmented and nonpigmented); staphylococci; hemolytic and nonhemolytic streptococci; *Bacillus* spp.; lactobacilli; fusiform bacilli; *Actinomyces; Veillonella* and other gram-negative cocci; coliforms and *Proteus* spp.; spirochetes; mycoplasmas; *Pasteurella* spp.; diphtheroids; pneumococci; yeasts, including *Candida albicans; Haemophilus* spp.; *Simonsiella.*

Jejunum, Ileum

Only small numbers of bacteria are present in this portion of the intestinal tract of animals.

Large Intestine

Fecal streptococci; *Escherichia coli; Klebsiella; Enterobacter; Pseudomonas* spp.; *Proteus* spp.; staphylococci; clostridia: *Clostridium perfringens, Cl. septicum,* and other species; gram-negative anaerobes; spirochetes; lactobacilli.

Trachea, Bronchi, Lungs

Few, if any, bacteria and fungi reside in these structures.

Vulva

Diphtheroids; micrococci; coliforms and *Proteus* spp.; enterococci; yeasts; gram-negative anaerobes. The same kinds of organisms and others can be recovered from the prepuce of the male.

Vagina

The numbers and kinds of bacteria vary with the reproductive cycle and age. The cervix and anterior vagina of the healthy mare possess few bacteria. Some of the organisms recovered from the vagina are hemolytic and nonhemolytic streptococci; coliforms and *Proteus* spp.; diphtheroids and lactobacilli; mycoplasmas; yeasts and fungi.

Skin

Animals, by virtue of their habits and environment, frequently possess a large and varied bacterial and fungal flora on their hair and skin. *Staphylococcus epidermidis* and *S. aureus* occur commonly, as do other micrococci. Of the many other organisms isolated, it is not known which make up the resident flora and which are "transients."

Milk

Micrococci, staphylococci, nonhemolytic streptococci, mycoplasmas, and diphtheroids including *Corynebacterium bovis* are frequently shed from the apparently normal mammary gland.

Laboratory Safety

Many of the bacteria, fungi, and viruses encountered in the diagnostic veterinary microbiology laboratory have the potential for causing disease in humans. Over the years there have been a number of reports of human infections acquired in veterinary diagnostic laboratories.

In the United States since 1970, employers have had a legal obligation for safety in their places of work. In addition to Federal Law, several states have their own regulations. These regulations in essence require "standard conditions, or the adoption or use of practices, means, methods, operations, or processes, reasonably necessary or appropriate to provide safe or healthful employment and places of employment." The regulations require employees to familiarize themselves with the law and observe its provisions for their own protection and that of their co-workers.

In view of the above requirements many veterinary diagnostic laboratories have prepared general guidelines that cover the laboratory as a whole, as well as guidelines that apply to particular disciplines such as microbiology and toxicology.

Space does not permit inclusion of a complete set of guidelines for a veterinary diagnostic laboratory. However, it is thought that a list of some of the important safety measures as they apply to a veterinary clinical microbiology laboratory may be useful. It should be kept in mind that these are a part of comprehensive guidelines that apply to the veterinary diagnostic laboratory as a whole.

Some Safety Recommendations for Veterinary Microbiology Laboratories

All employees working with agents potentially pathogenic for humans should be instructed in proper safety procedures. They should be encouraged to read safety literature provided, including the kind cited in the supplementary references.

1. Eating, drinking, and smoking: There will be no eating, smoking, application of cosmetics, or storage of food in laboratory areas where infectious materials are used or stored. Food should not be stored in laboratory refrigerators or freezers.
2. Laboratory gowns, coats, or uniforms must be worn while working in those laboratory areas where infectious materials are present.
3. Pipetting: Mechanical pipetting aids must be used for all pipetting procedures. *Mouth pipetting is prohibited.*
4. Each laboratory should contain a handwashing sink, preferably foot or elbow operated. Persons should wash their hands after handling infectious materials and when they leave the laboratory.
5. Work surfaces should be disinfected at least once a day and after each spill of potentially infectious material.
6. Baseline serum samples and blood smears will be collected from and stored for all laboratory and other at-risk personnel. Additional serum specimens will be collected periodically, depending on the agents handled or the function of the facility.
7. Certain immunizations should be required.
   a. It is recommended that employees working in the microbiology laboratory be vaccinated against rabies and tetanus and given the recommended booster vaccinations. Other immunizations may be indicated.
   b. A skin test for tuberculosis should be conducted annually.
8. Employees carrying out procedures and tests with agents known to be dangerous to humans must carry out such operations in a biological safety hood (Class II).

   Among the agents that are particularly infectious or noxious for human beings are the following: (a) bacteria: *Brucella, Leptospira,* Mycobacteria, *Francisella tularensis, Salmonella, Bacillus anthracis, Clostridium botulinum, Chlamydia;* (b) pathogenic fungi: particularly *Coccidioides immitis;* and (c) viruses: rabies virus, Eastern equine encephalomyelitis virus.

   There are many others that have been known to cause infrequent and usually not as severe infections.

   The Centers for Disease Control publishes a booklet,

"Classification of Etiologic Agents on the Basis of Hazard," that lists many of the agents that may infect human beings. They are classed on the basis of the severity of the disease produced and appropriate precautionary measures are recommended. This publication should be available in the Safety Reference Materials.

9. Eye washes and a first aid station should be available and regularly inspected and maintained.
10. Every attempt should be made to prevent animal bites and to wash thoroughly after handling animals or animal tissues and biproducts.
11. A report of any sickness, injury, eye contamination, etc. acquired in the laboratory should be given to the supervisor and recorded in an "accident/illness book."
12. A record of compliance or lack of compliance with the above recommendations, along with details of vaccinations, tests, serum samples, reports of illness or exposure, etc., will be kept in the laboratory office.
13. The laboratory will arrange for tests for such diseases as brucellosis, leptospirosis, mycobacteriosis and possibly others if employees working with the agents of these diseases request such tests.

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2

Selection and Submission of Clinical Specimens

G. R. Carter

A recurring problem in clinical veterinary microbiology results from the submission of unsatisfactory specimens with little or no history or clinicians' comments. Improvements in the selection and submission of specimens can usually only be obtained by a constant educational effort. Veterinarians should be supplied with an adequate submission and history form with instructions on the selection and shipment of specimens. Instructions of the kind given below have been found to be of value.

Just prior to death, and shortly thereafter, a number of intestinal bacteria may invade the host's tissues. The significance of these organisms, some of which are potential pathogens, is difficult to assess when tissues have been taken even a short time after death. Live, sick animals presented for necropsy are usually the best source of specimens. In all instances, the importance of fresh tissues taken as soon as possible after death cannot be overemphasized.

Specimens for Bacterial and Mycological Examination

Preservation and Shipment

Tissues and Organs

Asepsis should be practiced as much as possible in collecting and handling materials for culture. Place tissues in individual plastic bags or leakproof jars. Portions of intestines should be packed separately. Specimens can be conveniently shipped in a Styrofoam® box or ice chest containing a generous amount of ice. Dry ice with plenty of insulation is preferred for longer preservation.

Brains sent for examination should be halved longitudinally. One

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half is refrigerated or frozen over dry ice, and the other is placed in 10% formalin for histopathological examination. Tissues in formalin should not be frozen.

Postmortem invasion of tissues by intestinal and other bacteria can be rapid, particularly in warm weather. The bone marrow is less accessible than other tissues to this invasion. An opened rib from a small animal or a 4–5-in aseptically cut piece of rib from a large animal will often yield the causative bacterium in pure or nearly pure culture. The muscle or periosteal tissue should be removed from the rib before submission. If the rib is unopened the marrow can be exposed with a small bone saw.

Swabs

Swabs are of value in many instances for the transportation of infectious material to the laboratory. However, because many bacteria are susceptible to desiccation during shipment, it is advisable to place the swab in nonnutritional transport medium (see Appendix B). Swabs that utilize a transport medium are available commercially. The survival rate of bacteria on the conventional cotton swabs is improved if the swabs are boiled for 5 min in Sörensen's buffer, pH 7.5, before autoclaving. Treatment with buffer is not required for calcium alginate swabs.

Equine Cervical Swabs

Special swabs are required for swabbing the cervices of mares and other large animals. These can be prepared by attaching absorbent cotton to the end of an 18–24-in length of wire with a rubber band. Then approximately a foot of the portion containing the cotton is enclosed with paper or a pipette paper cover and autoclaved. Sterile swabs with long handles, especially designed for swabbing cervices and obtaining material for cytologic examination, are available commercially. A multiuse, self-contained, self-sealing culture collection and transport system for anaerobic, aerobic, and cytological specimens is available for horses and cattle.[1]

Specimens for Anaerobic Culture

See Chapter 15.

Diseases Requiring Special Consideration

Not all of the diseases requiring special consideration are listed below. For those not listed consult the appropriate chapter.

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[1] Accu-Med Corporation, 270 Marble Avenue, Pleasantville, New York 10570

Clostridial Infections (Blackleg, malignant edema, etc.)
Fresh affected tissue is especially important in that clostridia rapidly invade tissues after death. The muscle tissue involved may be difficult to locate.

Enterotoxemia (Clostridia)
Several ounces of fresh intestinal contents are required. This can be submitted in a jar or plastic bag, or a section of affected intestine may be tied off and submitted. This material should be refrigerated and dispatched to the laboratory as soon as possible.

Campylobacteriosis (Cattle and Sheep)
To make possible the isolation of the causal agents, semen, preputial washings, fetal stomach content, or cervical mucus should reach the laboratory under refrigeration within 5 hr of collection. Procedures for the collection of specimens are described in Chapter 6. Failing recovery of live organisms, dead *Campylobacter* can be detected by a fluorescent antibody procedure.

Anthrax
Cotton swabs are soaked in exuded blood, or blood taken from a superficial ear vein, in acute or peracute anthrax. In swine, because the organisms may not be present in the blood, material should be aspirated from swollen lymph nodes.

Johne's Disease
The most suitable specimens are 1-2 ft of the terminal sections of the ileum with the ileocecal junction (ileocecal valve) and a similar length of the adjacent cecum, flushed free of intestinal content. Several mesenteric lymph nodes of the ileocecal region should also be included. For fecal culture, 0.5 oz of feces should be submitted in a nonrefrigerated, sealed container such as a 1-oz ointment tin or a 50-ml plastic centrifuge tube (see also Chapter 23).

Tuberculosis
See Chapter 23.

Swine Dysentery
Six to eight inches of spiral colon from an acutely affected pig should be submitted. The specimen should be fresh, and although it should reach the laboratory as soon as possible, it may be held at 4°C for 2–3 days.

Fungi (Ringworm)
Scrapings or epilations should be made at the edge of active lesions. Submit in a cotton-plugged test tube or paper envelope. Saprophytic fungi will frequently proliferate rapidly in a sealed tube because of the moisture.

Serum Samples
No anticoagulant should be used. Samples are allowed to clot and are shipped preferably in wet ice but not frozen.

Wet needles, syringes, and tubes will cause hemolysis and spoiling of blood for serological examination. Blood samples for serological examination that become overheated will also hemolyze. Care should be taken to prevent overheating from the time the samples are drawn. This is especially important with samples for complement fixation tests.

Swine blood is especially susceptible to hemolysis. It is advisable to pour the serum from clotted samples into clean, dry tubes for shipment.

Urine, Blood
See Chapter 4.

Milk
See Chapter 34.

3

Cultural Procedures Employed for Clinical Specimens

G. R. Carter and John R. Cole, Jr.

The kinds of specimens submitted to veterinary microbiology laboratories are various, and the procedure to be followed in processing each depends upon the disease or organism suspected. One of the problems is knowing, often in the absence of any clues provided by the pathologist or clinician, just what pathogen or disease to suspect. Because so little information is usually available on a given specimen and because the frequency of submissions does not always allow for special efforts, routine procedures are established by the laboratory for the bulk of specimens. Procedures for the processing of the more common kinds of specimens are summarized in Tables 3-1 and 3-2. It is recommended that a CO_2 incubator be used to provide 5–10% CO_2 for the routine incubation of plates at 37°C.

Special cultural procedures may be required for some agents. Workers should consult the appropriate chapter for specific recommendations.

Direct Examination of Materials

The materials most frequently submitted for examination are tissues, feces, swabs, milk, urine, pus, discharge, fetal stomach contents, cervical mucus, and skin scrapings. The microbiologist should carry out a direct examination for the agent that the veterinarian or veterinary pathologist may suspect. These examinations are dealt with in the manual under the appropriate pathogen or disease. The examination of stained smears and wet mounts should be routine with most materials. The findings may aid in the selection of appropriate media. When indicated, fluorescent antibody procedures are carried out on smears of tissues, fluids, or exudates.

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Table 3-1
Summary of Some Routine Cultural Procedures

Specimens	Organism	Media	Atmosphere	Incubation temperature
Organs, tissues, pus, urine, swabs, etc.	Aerobes[a] (not enterobacteria)	Blood agar, Schaedler or thioglycolate broth, MacConkey agar	Aerobic[b]	37°C
Feces, fecal swabs, intestine	Enterobacteria	Selenite, (18 hr) to MacConkey agar, brilliant green. Direct: MacConkey, brilliant green or Hektoen agar, and blood agar	Aerobic	37°C
Milk	Aerobes	Blood agar	Aerobic	37°C
Organs, tissues, pus, swabs, etc.	Microaerophiles (*Brucella, Campylobacter*)	Blood agar and selective media	10% CO_2; see special requirements for *Brucella, Campylobacter*	37°C
Organs, tissues, intestinal contents, fecal swabs	Anaerobes (Clostridia)	Blood agar, cooked meat, Schaedler broth; FA procedures	Anaerobic, aerobic	37°C
Intestinal content: suspect enterotoxemia		Mouse or rabbit inoculations: blood agar		
Organs, tissues, pus, swabs, etc.	Anaerobes (Gram-negative and *Actinomyces bovis*)	Blood agar, cooked meat medium semisolid; Schaedler broth	Anaerobic and aerobic (blood) agar	37°C
Organs, tissues, pus, swabs, etc.	Fungi in general	Sabouraud agar, Sabouraud with inhibitors, blood agar, brain–heart infusion semisolid	Aerobic	25°C or room temp. and 37°C
Skin scrapings, epilations, hair	Dermatophytes	Sabouraud agar (with inhibitors)	Aerobic	25°C or room temp.
		Blood agar, brain–heart infusion semisolid	Aerobic	37°C

[a]Including facultative anaerobes.
[b]Air with 5–10% CO_2 preferred.

Table 3-2
Additional Routine Cultural Procedures

Ear and cervical swabs		
Plate on blood agar and MacConkey agar		37°C
semisolid or Schaedler broth		37°C
Sabouraud agar		25°C
For *Listeria*[a]		
Grind tissue in broth → store at 4°C and culture on blood agar as follows:		
↓ culture on blood agar	initially	all at 37°C
	end of first week	
	end of third week	
	end of sixth week	
	end of twelfth week	
For Mycoplasmas [b]		37°C
For *Haemophilus* spp.[c]		
Streak β-hemolytic *Staphylococcus aureus* over streak lines of clinical material on blood agar		37°C

[a] See also Chapter 20.
[b] See Chapters 26 and 27.
[c] See also Chapter 13.

4

Isolation and Identification of Bacteria from Clinical Specimens

G. R. Carter

Steps followed in the isolation and identification of bacteria from clinical specimens are listed in Table 4-1. The selection of routinely used media for primary inoculation was referred to in Tables 3-1 and 3-2.

Primary Inoculation of Media

Two procedures are frequently used to obtain material for inoculation from tissues and organs. One is to sear the surface of the specimen with a hot spatula, then incise with a sterile scalpel. From this incision, material is transferred to media with an inoculation loop or a Pasteur pipette. If the tissues are fresh, fluid media such as thioglycollate or semisolid brain–heart infusion are inoculated directly. This procedure is especially indicated if antimicrobial agents have been employed.

Another procedure, which is more convenient with small specimens, is to sterilize the external surfaces of the specimen by holding it with sterile forceps and passing it through a Bunsen flame several times. It is then sectioned with sterile scissors, and the exposed surface is impressed on the agar surface. The inoculum is then spread with an inoculating loop. Occasionally, media are inoculated from a pool of tissues previously ground in broth, especially if the tissues or organs are too small to carry out the other manipulations.

The two goals of primary inoculation are (1) to cultivate the organisms and (2) to obtain discrete colonies. From the latter, pure cultures are obtained. These aims are usually best accomplished by the inoculation of solid media in Petri dishes; however, there are instances in which pour plates and agar shake cultures are useful. A useful procedure after the inoculation of plate media is to place all swabs,

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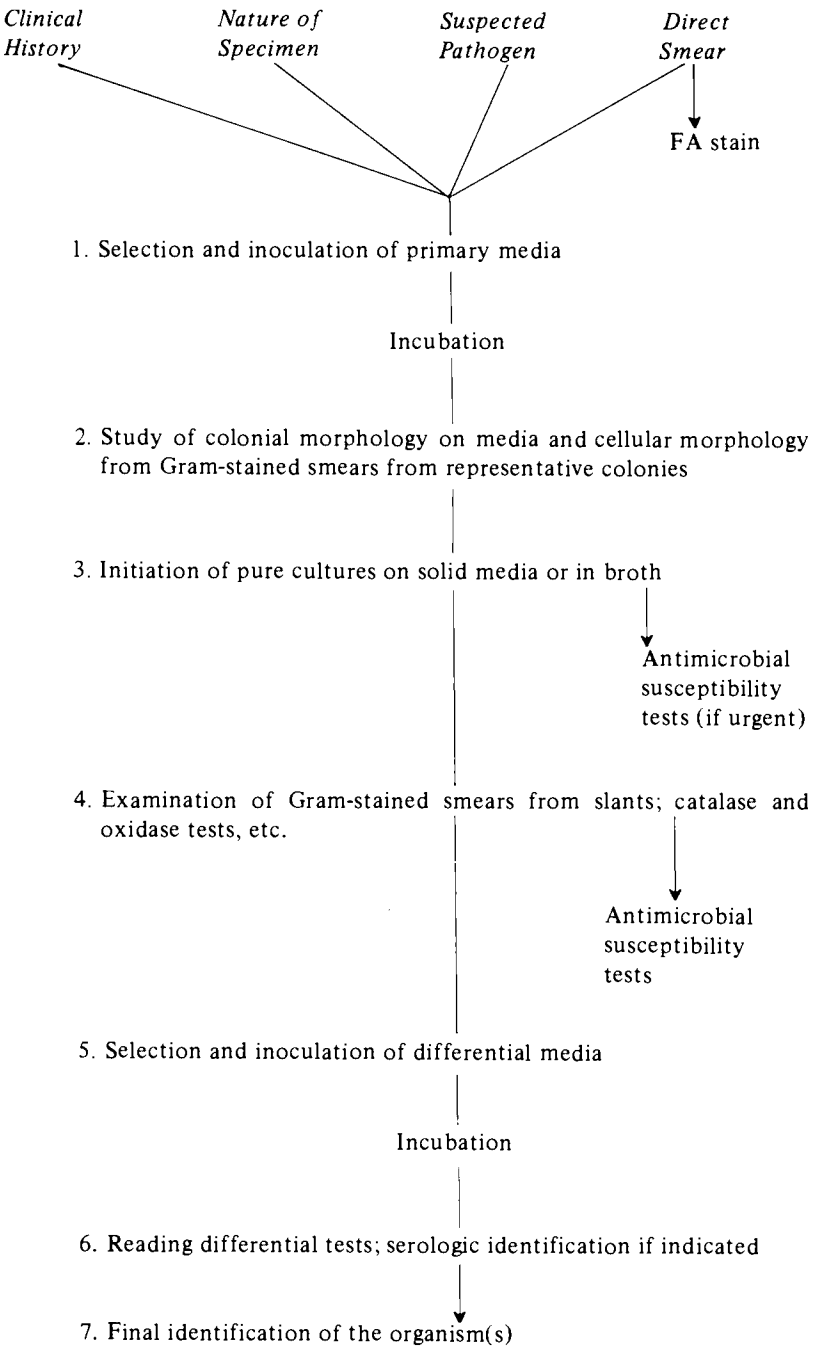
Table 4-1
Steps Usually Followed in the Isolation and Identification of Bacteria from Clinical Specimens



except fecal swabs, in a tube of semisolid brain–heart infusion (BHI) broth or Schaedler broth. This medium supports the growth of many fastidious organisms, including aerobes, facultative anaerobes, microaerophiles, and anaerobic bacteria. It has the disadvantage that the pathogen, if present, may be overgrown by other bacteria. A smear is made from the semisolid broth culture and stained by the Gram method. If indicated, the culture is inoculated onto plate media, usually blood agar.

Special directions for the cultivation of the certain pathogens are provided in the chapters dealing with these particular organisms. Blood agar is the most useful and widely employed medium. Material is streaked out on the solid medium with an inoculating loop, a swab, or a glass spreader. Media for primary culture (not anaerobic) should be placed in an incubator with 5% carbon dioxide and adequate humidity.

Urine Culture

Most of the urine samples submitted for culture are from catheterized dogs. If not catheterized, midstream urine should be caught when feasible in a sterilized container. Centrifugation is carried out if a direct examination is required. The sediment is stained by the Gram method.

Generally speaking, bacteriological examinations of animal urine have not been quantitated. In order to carry out a semiquantitative procedure, known amounts of urine can be plated on blood agar and other media. A loop that delivers 0.01 ml may be used. The inoculum is spread thoroughly, and after incubation, the number of colonies is estimated.

A quantitative procedure that provides more precision involves the use of pour plates of trypticase soy agar or other media. Generally, the inoculum used for each of two pour plates is 1.0 ml of 10^{-3} dilution.

Results may be given as the approximate number of bacteria per milliliter of urine. In humans, clinical bacteriuria is indicated by the presence of 100,000 organisms per milliliter. A count less than 1000/ml is not considered significant, while counts between 10,000 and 100,000 are suggestive of infection. It is now generally conceded that broth cultures are of little or no value.

For additional information on the laboratory diagnosis of bacteriuria, readers are referred to the *Manual of Clinical Microbiology* listed in the Supplementary Readings.

Blood Culture

Blood cultures are made whenever there is reason to suspect a clinically significant bacteremia. Because bacteremia may be intermittent,

it is advisable to culture more than one blood sample. In human beings, as many as three to four blood cultures are recommended in the initial 24-hr period (1). The author recommends the same regimen for animals.

Because only a small number of bacteria may be present in the blood of an animal with a bacteremia, 3–10 ml of blood, depending upon the size of the animal, is taken aseptically. If there is no anticoagulant in the medium, an anticoagulant should be added when the blood is taken. Suitable media may be prepared or purchased (see Appendix B).

The author has found the Vacutainer® culture tube (Becton-Dickinson, Rutherford, New Jersey) particularly convenient for small animals. It contains an anticoagulant and supports the growth of aerobes, facultative anaerobes, and anaerobes, and because the blood is inoculated directly from the animal, the chances for contamination are reduced. It is recommended that at least four culture tubes be used for a dog during a 24-hr period.

Examination of Plate Media

The kind and number of colonies or amount of growth is studied and recorded. Tissues taken from animals some time after death may yield a variety of colonies, usually indicating postmortem invasion from the alimentary tract. It is not always feasible to identify all of the different bacteria. Generally, only those colonies thought to represent the more significant organisms are identified. Smears are made from representative colonies and stained by the Gram method.

Before discarding plates, it is advisable to examine them for minute colonies with a stereoscopic microscope. By this procedure, colonies that cannot be discerned with the unaided eye (e.g., dwarf colonies, those of *Haemophilus* spp., and mycoplasmas) can be seen. All plates should be incubated, when feasible, for 3–4 days or longer if no growth is detected earlier. To prevent dehydration, plates should be placed in an airtight container, or individual plates should be sealed with tape or a large rubber band. It is advisable to hold plates at room temperature for 1 week before discarding. Some organisms such as *Yersinia enterocolitica* grow better at this temperature than at 37°C.

Pure Cultures for Identification

A small tube of broth is inoculated from one colony of the culture to be identified. Because it may be difficult to initiate a broth culture from one colony, several colonies are sometimes used if it is evident that the plate culture is pure. The inoculated tube is placed in a container of water in the incubator at 37°C in order to accelerate growth. Tubes thus inoculated, if pure, can sometimes be inoculated into dif-

ferential media the same day with a Pasteur or serologic pipette. An alternative procedure is to inoculate a slant of a suitable medium such as tryptose agar, a sector of a blood plate, or a TSI (triple sugar iron) slant. Differential media are then inoculated from these with an inoculating needle. The usual sequence of procedures is summarized in Table 4-1.

Procedures Followed in Identification

If bacteria have invaded tissues after death or if specimens have been insufficiently refrigerated, a considerable variety of bacteria will be recovered unless selective media are used. Contaminants of diverse origins are frequently encountered. Attempts to identify these organisms as to species are pointless, and the experienced veterinary bacteriologist will take into consideration the condition of the specimen, the number and variety of bacteria, the gross pathology, and the history in assessing their significance.

Experienced bacteriologists and technologists can generally tentatively recognize the colonies of the more commonly occurring bacteria, such as *Pasteurella multocida*, *Corynebacterium pseudotuberculosis*, *Actinobacillus equuli*, *Streptococcus* spp., *Bacillus* spp.. This ability, along with knowledge of the Gram reaction and morphology of a particular organism, will guide them in the selection of differential media and frequently make for a saving in the time required for final identification.

A simplified guide for the presumptive recognition of common groups of bacteria is presented in Table 4-4, which, because of its length is placed at the end of this chapter. Drs. Isenberg, Washington II, Balows and Sonnewirth, who prepared this very useful table for the *Manual of Clinical Microbiology*, kindly consented to our reproducing it.

In order to determine the genus involved, some basic characteristics must be determined. These are dealt with below.

Gram-Positive Aerobic Bacteria

The genera of some important gram-positive aerobes is determined by characteristics listed in Table 4-2. The mycobacteria, although gram-positive, are not included in Table 4-2. They are presumptively identified as to genus by the acid-fast stain. *Nocardia* spp. are partially acid-fast and display characteristic branching and beading. *Erysipelothrix rhusiopathiae* and *Listeria* can be distinguished from *Actinomyces* on the basis of microscopic morphology, colony characteristics, and oxygen requirements, and from *Lactobacillus* by the fact that the latter

Table 4-2
Determination of Some Genera of Gram-Positive Aerobes and Facultative Anaerobes[a]

Type	Motility	Catalase	Oxidase	Urease	Glucose (acid)	Esculin	O-F Test (Glucose)	Aerobic	Anaerobic	Remarks
Cocci										
Streptococcus	−	−	−	NR	+	V	NR	+	+	
Micrococcus	−	+	+	NR	+	NR	O	+	−	
Staphylococcus	−	+	−	NR	+	NR	F	+	+	
Rods										
Corynebacterium	−	+	−	V	+	−	NR	+	+	
Listeria	+	+	−	−	+	+	NR	+	+	
Erysipelothrix	−	−	−	−	+	−	NR	+	+	
Bacillus	+[b]	+	+	V	+	+	NR	+	V	Spores common
Lactobacillus	−	−	−	−	+	V	NR	+	+	
Kurthia	+	+	−	+	−	−	NR	+	−	Strict aerobe
Nocardia	−	+	−	+	+	NR	O	+	−	Partially acid-fast
Actinomyces	−	−[c]	+	−	+	NR	F	+	+	Branching
Rhodococcus equi	−	+	+	V	+	−	NR	+	+	Pink, coral pigment

[a] +, >90% Positive; −, <10% positive; V, variable; O, glucose oxidized; F, glucose fermented; NR, not required.
[b] Asporogenous strains occur.
[c] *Actinomyces viscosus* is positive.

have a characteristic morphology and different growth requirements. The *Clostridium* spp. are anaerobic and catalase negative.

Some gram-positive organisms of lesser importance are referred to in Chapter 24.

When the genus is determined, reference should be more to the appropriate generic tables, in which the essential reactions are indicated.

Gram-Negative Aerobic Bacteria

The most frequently recovered aerobic gram-negative bacteria are members of the family Enterobacteriaceae. They are rod-shaped organisms producing colonies of moderate size. They are fermenters, and it is customary to inoculate TSI agar slants from colonies of suspected "enterics." As well as fermenting one or more sugars of the TSI slants, they grow on MacConkey agar and are oxidase negative. The reactions of various gram-negative organisms on TSI are given in Table 10-4. Identification of enteric bacteria as to species or group is accomplished by the criteria listed in Table 10-5. The remaining genera of gram-negative aerobes can be recognized by the characteristics listed in Table 4-3. Not included in Table 4-3 are the genera *Campylobacter, Vibrio,* and *Francisella.* Members of the genera *Vibrio* and *Campylobacter* are recognized by their characteristic microscopic morphology and cultural characteristics. The animal source, history, and lesions usually indicate the likelihood of a culture being *Francisella tularensis.* The requirement of the latter for cystine is highly supportive.

As one gains experience, the different genera become more easily recognizable. A number of features aid in the tentative recognition of gram-negative genera:

1. *Pseudomonas aeruginosa* produces a characteristic colony with hemolysis and a fruity odor on blood agar.
2. *Acinetobacter* and *Neisseria* are predominately coccal in morphology.
3. *Aeromonas* spp. are usually hemolytic and resemble species of the *Enterobacteriaceae.*
4. *Chromobacterium* may produce a characteristic pigment.
5. *Flavobacterium* spp. usually produce colonies containing yellow pigment.
6. *Pasteurella multocida* has a characteristic odor, and strains from the lungs of cattle and swine frequently produce mucoid colonies.
7. *Pasteurella haemolytica* usually produces β-hemolysis on bovine blood agar.
8. *Actinobacillus* spp. are most frequently recovered from the

Table 4-3
Determination of Some Genera of Gram-Negative Bacteria[a]

	O-F Test	MacConkey's	Oxidase	Catalase	Motility	Species and remarks
Predominantly Rods:						
Enterobacteriaceae	F	+	−	+	(+)	See Table 10-5
	F	+	−	+	−	
	F	+	−	−	−	
Yersinia	F	+	−	+	−	*Y. pestis*
	F	+	−	+	+	*Y. pseudotuberculosis* and *Y. enterocolitica*
Pseudomonas	O	+	+	+	+	*Ps. aeruginosa*
	O	+	(+)	(V)	+	*Ps. pseudomallei*
	O	+	−	+	−	*Ps. mallei*
Aeromonas	F	+	+	(+)	+	*A. hydrophila*
	F	+	+	(+)	−	*A. salmonicida*
Plesiomonas	F	+	+	+	+	*P. shigelloides*
Chromobacterium	F		−	+	+	Purple or violet pigment
Flavobacterium	O	−	+	+	−	Yellowish pigment
Pasteurella	F	−	+	+	−	*P. multocida, P. pneumotropica*
	F	+	+	+	−	*P. haemolytica*
	−	−	+	+	−	*P. anatipestifer*
Actinobacillus	F	+	+	+	−	
Bordetella	I	+	+	+	+	*B. bronchiseptica*
Alcaligenes	I	+	+	+	+	*A. faecalis*
Brucella	NR	−	+	+	−	Oxidase may be weak
Moraxella	I	V	+	(+)	−	See Table 4-4 and Table 8-1
Haemophilus	NT	−	(+)	(+)	−	Fastidious; require X and/or V factors
Predominantly Coccobacilli:						
Acinetobacter	O	+	−	+	−	*A. calcoaceticus* ssp. *anitratus*
	I	(+)	−	+	−	*A. calcoaceticus* ssp. *lwoffii*
Neisseria	O	−	+	+	−	Usually not pathogenic to animals

[a]For explanation of symbols see Table 4-2; NT, not testable; I, inactive.

horse and from lesions of actinobacillosis; colonies of *A. equuli* frequently have a "sticky" or tenacious character.

9. The common pathogenic *Campylobacter* are slow-growing, fastidious organisms requiring reduced oxygen tension for growth.
10. *Bordetella bronchiseptica* is most commonly recovered from respiratory infections of swine, rabbits, and guinea pigs.
11. *Moraxella bovis* is recovered almost exclusively from the bovine eye.
12. *Haemophilus* spp. require V factor and produce minute colonies.
13. *Brucella* spp. grow slowly and are usually recovered from blood, fetal, and genital tissues.
14. Gram-negative anaerobes are often encountered in pus and necrotic tissue, which may have an offensive odor. Cultures of *Fusobacterium necrophorum* have a foul odor, and colonies of *Bacteroides melaninogenicus* have a characteristic dark pigment.

General Comments on Identification

The different characteristics of the organisms referred to in the manual are listed by the particular organism's name and in the corresponding differential tables.

A perusal of various textbooks, scientific papers, and *Bergey's Manual* discloses some inconsistencies. Many of these may be attributed to strain variation and the use of different methods. Variation in fermentation or oxidation of carbohydrates may depend upon the time of incubation. Some species may require incubation for a week or two before sufficient fermentation or oxidation takes place. Organisms requiring supplemented media may give negative results if the test medium is not supplemented. The considerable variation among strains of bacteria should be kept in mind when using tables of differential characteristics.

In practical diagnostic bacteriology, the great majority of bacteria encountered from fresh specimens are those listed in the manual. The methodical keying-out of an organism is only occasionally required.

The differential tables in *Bergey's Manual of Systematic Bacteriology*, Vols. 1 and 2 [2,3] may be helpful in the identification of an unknown organism. This work is also a useful reference work for the diagnostic laboratory. The book by Cowan [4] contains much valuable information on the identification of bacteria of medical and veterinary significance. The most complete practical reference, whose various chapters are of great value in the identification of both typical and unusual bacteria, is the *Manual of Clinical Microbiology* [5].

Commercial Identification Systems

There are many convenient and relatively rapid miniaturized systems available commercially for the identification of bacteria of veterinary and medical significance. A number have been specifically designed for the identification of enterobacteria, anaerobic bacteria, yeasts, and other categories of organisms. Cost precludes their use in many laboratories, but they are particularly useful for the small laboratory that does not find it economical or practicable to provide and maintain the necessary conventional differential media.

In addition to these rapid systems, instruments are now available for rapid identification and susceptibility testing. Many of the rapid methods and instruments are listed and discussed in Chapter 36. Some of the commercial kits and systems are discussed in appropriate chapters.

L-Forms of Bacteria

L-type colonies indistinguishable from colonies of mycoplasmas are occasionally seen on culture media, especially from clinical materials. Although these colonies appear spontaneously with considerable frequency from certain species (e.g., *Fusobacterium necrophorum* and *Streptobacillus moniliformis*), they are also produced as a result of phage activity, penicillin, antibody, and various antimicrobial substances. Most L-forms revert to the parent bacterium on subculture, but occasionally they do not.

In diagnostic work, one occasionally notices bizarre and highly pleomorphic forms in smears from solid media or broth cultures. Some of these are the forms that under certain circumstances give rise to L-type colonies. They may consist of long filaments that show beading. Some filaments break up and produce large bodies and coccal forms. Sometimes single bacilli will give rise to large round or pyriform structures. Further subcultures will usually produce a preponderance of the bacillary form of the organism. Workers are referred to the Supplementary Readings for further information on L-forms.

Generally speaking, L-forms do not pose problems for the practical microbiologist, but the possibility of their occurrence, particularly with certain bacterial species, should be kept in mind.

Occurrence of Pathogens and Potential Pathogens in Animal Species

In many clinical microbiology laboratories, the greater part of the routine work is carried out by technicians with little knowledge of animal diseases. The experienced workers will have gained considerable

knowledge as to the occurrence of different organisms in the tissues of various animal species, but novices may be at a loss as to the probable organisms involved. In order to help cope with this deficiency, some information is provided below on the kinds of organisms most frequently associated with infections in various organs and systems of the more important animal species.

Those bacteria such as coliforms, *Pseudomonas aeruginosa, Salmonella,* streptococci (except those with a species predilection), and staphylococci are not always referred to because of their wide distribution. Not all fungi, mycoplasmas, and chlamydial agents are included. Gram-negative anaerobes that are frequently present in purulent materials have not been included.

Organisms Recovered from the Respiratory System

Bovine
Pasteurella multocida, P. haemolytica, Actinomyces pyogenes, Bordetella bronchiseptica, Haemophilus somnus, mycoplasmas.

Ovine
Pasteurella haemolytica, P. multocida, A. pyogenes, mycoplasmas, *Chlamydia psittaci.*

Porcine
Pasteurella multocida, P. haemolytica, A. pyogenes, Haemophilus parasuis, Mycoplasma hyorhinis, M. hyopneumoniae, Actinobacillus suis, Bordetella bronchiseptica, Actinobacillus pleuropneumoniae.

Equine
Streptococcus equi, Str. equisimilis, Rhodococcus equi (foals), *Actinobacillus equuli* (foals), *P. multocida, Pseudomonas mallei, Klebsiella, Bord. bronchiseptica, Cryptococcus neoformans, Aspergillus.*

Canine
Bordetella bronchiseptica, P. multocida, Klebsiella, Str. canis, Nocardia asteroides, Mycoplasma cynos and other mycoplasmas, *C. neoformans, Blastomyces dermatitidis, Actinomyces viscosus.*

Feline
Pasteurella multocida, N. asteroides, Bord. bronchiseptica, Chlamydia, C. neoformans.

Chickens and Turkeys
Haemophilus paragallinarum, P. multocida, P. gallinarum, and other *Pasteurella* spp., various mycoplasmas, *Aspergillus fumigatus, Bordetella avium.*

Organisms Associated with Canine Skin Infections

Staphylococcus intermedius, pyogenic streptococci, dermatophytes, *Pseudomonas aeruginosa*, *Candida albicans*. Various bacteria are probably opportunists: fecal streptococci, coliforms, *Proteus* spp., diphtheroids.

Bacteria Associated with Infections of the Gastrointestinal Tract

Bovine
Salmonella, *Clostridium perfringens* types B and C, *Mycobacterium paratuberculosis*.

Porcine
Salmonella (especially *S. choleraesuis*), *Cl. perfringens* type C, *Campylobacter* spp., *Treponema hyodysenteriae*.

Equine
Salmonella, *Rhodococcus equi* (foals), *A. equuli* (foals).

Canine
Salmonella, possibly other enteric bacteria, *Staph. intermedius*, *Borrelia canis*, *Spirillum* spp., *Campylobacter jejuni*, *C. uppsaliensis*.

Feline
Salmonella, *C. jejuni*, *Candida albicans* (kittens), *Chlamydia psittaci*.

Organisms Associated with Abscesses and Ulcers of the Skin and Subcutis

Streptococci and staphylococci are the most common causes of abscesses involving the skin and subcutis of most animal species. *Actinomyces bovis* and *Actinobacillus lignièresii* occur rarely as causes of abscesses in species other than the bovine. Likewise *Nocardia asteroides* is an infrequent cause of abscesses in domestic animals other than the dog and cat. *Pseudomonas aeruginosa* may be associated with abscesses in all of the domestic animals.

Bovine
Actinomyces pyogenes, *Actinomyces bovis*, *Actinobacillus lignièresii*, *Sporothrix schenckii*.

Ovine
Corynebacterium pseudotuberculosis.

Porcine
Group E,P,U,V streptococci (jowl abscesses), *A. pyogenes, Streptococcus porcinus.*

Equine
Corynebacterium pseudotuberculosis (chest abscesses), *Histoplasma capsulatum* var. *farciminosum, S. schenckii.*

Canine
Nocardia asteroides, Blastomyces dermatitidis, S. schenckii, A. viscosus.

Feline
Pasteurella multocida, N. asteroides.

Organisms Associated with Genital Infections

Streptococci, staphylococci, enteric bacteria, and *P. aeruginosa* are commonly associated with genital infections in all species.

Bovine
Campylobacter fetus subsp. *fetus* and *venerealis, Brucella abortus, Chlamydia, Mycoplasma bovigenitalium, Listeria monocytogenes, Actinomyces pyogenes*

Ovine
Brucella ovis, Br. melitensis, C. fetus subsp. *fetus, Listeria monocytogenes, Actinobacillus seminis, C. pseudotuberculosis, C. psittaci*

Porcine
Brucella suis, mycobacteria, *Pseudomonas aeruginosa, Actinomyces pyogenes, P. multocida,* pyogenic streptococci

Equine
Actinobacillus equuli, Klebsiella, Corynebacterium equi, Salmonella abortus-equi, C. albicans, pyogenic streptococci, *Taylorella equigenitalis*

Canine
Brucella canis, other brucella species (rare), *Klebsiella, Enterobacter, Proteus* spp., *C. albicans, Ps. aeruginosa,* mycoplasmas

Organisms Associated with Mastitis

Bovine
See Chapter 32.

Ovine
Staphylococcus aureus, P. haemolytica, P. multocida, Streptococcus agalactiae, Str. uberis, Str. dysgalactiae, A. pyogenes, C. pseudotuberculosis, mycobacteria, mycoplasmas

Porcine
Streptococci, *Staph. aureus, Fusobacterium necrophorum, A. bovis, Actinobacillus lignièresii, A. pyogenes,* mycobacteria, coliforms

Equine
Streptococci, *Staph. aureus,* mycobacteria

Canine and Feline
Streptococci, *Staph. intermedius*

Organisms Recovered from the Central Nervous System

Bovine
Listeria monocytogenes, Haemophilus somnus, streptococci, *Pasteurella multocida, Chlamydia psittaci, Staph. aureus*

Ovine
Listeria monocytogenes, Staph. aureus

Porcine
Listeria monocytogenes, P. multocida, streptococci

Equine
Listeria monocytogenes, Str. equi, Staph. aureus

Canine and Feline
Bacteria rarely involved.

Organisms Recovered from Urinary Tract Infections

Urine from dogs is most commonly submitted. Some of the organisms implicated in dogs are also recovered from the urine of other species on the infrequent occasions that such specimens are submitted.

Canine
Proteus (usually *mirabilis*), *Ps. aeruginosa, Staphylococcus intermedius,* enterococci, *E. coli, Enterobacter,* pyogenic and fecal streptococci

Feline
Urinary infections uncommon

Bovine
Corynebacterium pilosum, C. cystitidis

Ovine
Urinary tract infections infrequent; various bacteria.

Porcine
Eubacterium suis

Equine
Urinary tract infections infrequent; various bacteria.

Organisms Recovered from Joints

Bovine
Escherichia coli, pyogenic streptococci, *Salmonella, Actinomyces* pyogenes, *Staphylococcus aureus, Chlamydia psittaci,* mycoplasmas, *Haemophilus somnus*

Ovine
Escherichia coli, pyogenic and fecal streptocci, *Erysipelothrix rhusiopathiae, Haemophilus agni, C. psittaci, Str. dysgalactiae, Mycoplasma agalactiae, A. pyogenes*

Porcine
Erysipelothrix rhusiopathiae, A. pyogenes, various pyogenic streptococci, *M. hyorhinis, M. hyosynoviae, Staph. aureus, H. parasuis, B. suis, E. coli, A. suis*

Equine
Actinobacillus equuli, Staph. aureus, pyogenic and fecal streptococci, *E. coli, R. equi, Klebsiella, Salmonella*

Organisms Recovered from Canine Otitis Externa

Staphylococcus intermedius, Ps. aeruginosa, various streptococci, *C. albicans, Malassezia pachydermatis, Proteus* spp., *Cl. perfringens*

Organisms Recovered from Equine Cervix

Staphylococcus aureus, various streptococci, *Klebsiella*, *Ps. aeruginosa*, *S. abortus-equi*, various fungi, *R. equi*, *C. albicans*, *Enterobacter*, *E. coli*, *A. equuli*, *T. equigenitalis*

Organisms Recovered from Eyes

Not all bacteria have been included.

Bovine
Moraxella bovis, *Neisseria ovis* (or closely related species), *C. psittaci*

Ovine
Neisseria ovis, *Moraxella* spp., *C. psittaci*, *Mycoplasma conjunctivae*

Equine
Streptococcus equi, *Str. equisimilis*, *Staph. aureus*

Canine and Feline
Staphylococcus intermedius, *Staph. epidermidis*, *Ps. aeruginosa*, *Cl. perfringens*, *C. albicans*, mycoplasma (*M. felis* from the cat), *Moraxella* spp., *C. psittaci*

Bacteria Associated with Infections in Laboratory Animals

Rats and Mice
Salmonella, pyogenic streptococci, *Bacillus piliformis*, *Pasteurella pneumotropica*, *P. multocida*, *Corynebacterium kutscheri*, *Bord. bronchiseptica*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, *Mycoplasma pulmonis*, *M. arthritidis*, *M. neurolyticum*, *Yersinia pseudotuberculosis*

Guinea Pigs
Salmonella, *Bord. bronchiseptica*, *Str. pneumoniae*, pyogenic streptococci, *Klebsiella pneumoniae*, *Y. pseudotuberculosis*, *S. moniliformis*

Rabbits
Salmonella, *Bord. bronchiseptica*, *Str. pneumoniae*, pyogenic streptococci, *K. pneumoniae*, *Y. pseudotuberculosis*, *S. moniliformis*. *P. multocida*, *Haemophilus* spp. *Bacillus piliformis*, *Treponema cuniculi*, and *Fusobacterium necrophorum*

Table 4-4
Simplified Guide to the Presumptive Recognition of Common Groups of Bacteria[a]

Aerobic Cultures

I. Gram-positive cocci
A. Catalase positive
1. Arranged in clusters, large colonies: *Staphylococcus* spp. (Chapter 16); perform coagulase test; glucose fermented anaerobically
2. In pairs or small clusters; use glucose oxidatively or not at all: *Micrococcus* spp. (Chapter 16)
B. Catalase negative
1. Short and long chains, pairs; fermentative (anaerobic) utilization of sugars: *Streptococcus* spp. (Chapter 17)

NOTE: β-Hemolytic streptococci may belong to the pyogenic or enterococcus groups; α-hemolytic streptococci may belong to the pyogenic, species incertae sedis (SIS), "other," or enterococcus groups; nonhemolytic streptococci (γ) may belong to the pyogenic, "other," or enterococcus groups

a. Pyogenic group: usually, but not always, β-hemolytic; do not grow at 45°C; do not survive 30 min at 60°C; serological groups A, B, C, F, G, J, K, L, M, O, P, U, and V (Lancefield grouping); group A susceptible to bacitracin

b. Viridans (oral or "other") group: α-hemolytic or nonhemolytic; not soluble in bile; not inhibited by optochin; usually grow at 45°C

c. Enterococcus group: some β-hemolytic, others α- or γ-hemolytic; grow in 0.1% methylene blue milk, in 6.5% NaCl, and at pH 9.6; grow at 45°C and survive 30 min at 60°C; usually grow on MacConkey agar; bile-esculin positive

d. Usually lancet-shaped, pairs, single, or short chains; bile soluble; inhibited by optochin; α-hemolytic; no growth at 45°C; virulent for mice: *Streptococcus pneumoniae*

2. Primarily clumps and tetrads; no acid from glucose anaerobically: *Aerococcus* spp.

II. Gram-negative cocci, mostly in pairs
A. *Neisseria* spp. (all oxidase positive) (Chapter 14)
1. No growth at 22°C or on nutrient agar; growth on modified Thayer–Martin agar (MTM)

a. Requires enriched medium; acid from glucose and maltose; agglutination by antimeningococcal serum: *N. meningitidis*

b. Requires enriched medium; acid from glucose only: *N. gonorrhoeae*

2. Growth on MTM; ferments glucose, maltose, and lactose: *N. lactamica*
3. Growth at 22°C and on nutrient agar; light inoculum yields no growth on MTM

a. Growth on ordinary media; no acid from glucose, maltose, sucrose, or lactose: *Branhamella catarrhalis*

b. Yellow pigment; no acid from glucose, maltose, sucrose, or lactose: *N. flavescens*

c. Good growth on ordinary media; acid from glucose and maltose: pharyngeal group

B. Rule out *Acinetobacter* (former *Mima–Herellea* group, *Achromobacter* spp., *Bacterium anitratum*) (see III,A,5 below)

III. Gram-negative rods
A. Good growth on ordinary media, including MacConkey agar[b]
1. Fermentative,[c] oxidase negative,[d] nitrate reduced to nitrite
a. Lactose usually fermented,[e] phenylalanine deaminase not produced
(1) Voges–Proskauer (V-P), citrate, urease, and usually H_2S negative; indole and methyl red positive: *E. coli* (Chapter 10)

(*continues*)

Table 4-4 (*continued*)
Simplified Guide to the Presumptive Recognition of Common Groups of Bacteria[a]

(2) V-P and citrate positive; growth in KCN; usually indole negative, urease positive or delayed; motile: *Enterobacter* spp.; nonmotile: *Klebsiella* spp. (Chapter 10)

(3) H_2S and citrate positive; lysine decarboxylase not produced; V-P, urease, and indole negative; growth in KCN: *Citrobacter* spp. (Chapter 10)

b. Lactose usually not fermented; phenylalanine deaminase not produced

(1) H_2S, citrate, and lysine decarboxylase positive; indole, V-P, urease, and KCN negative: *Salmonella* spp. (Chapter 10) (exceptions: no gas, little H_2S: *S. typhi* and others) (use specific antisera)

(2) H_2S, citrate, V-P, and urease negative; nonmotile; usually no gas: *Shigella* spp. (Chapter 10) (or nonmotile *Escherichia* spp.); some *Shigella* spp. ferment lactose slowly

(3) V-P and citrate positive; urease delayed positive; motile; pigment often formed, especially at room temperature: *Serratia* spp. (Chapter 10)

(4) H_2S, indole, lysine, and methyl red positive; urease, V-P, citrate, and KCN negative; most sugars (except glucose and maltose) not fermented: *Edwardsiella* spp. (Chapter 10)

c. Phenylalanine deaminase produced

(1) Urease positive: *Proteus* spp. (Chapter 24)

(a) H_2S positive; indole positive: *P. vulgaris*

(b) H_2S positive; indole negative: *P. mirabilis*

(c) H_2S negative; citrate negative: *Morganella morganii*

(d) H_2S negative; citrate positive: *Providencia rettgeri*

(2) Fermentative; oxidase negative; growth on MacConkey agar, usually on salmonella–shigella (SS) agar (exceptions); motile at 20–25°C but not at 35°C; urease positive, phenylalanine negative: *Yersinia pseudotuberculosis, Y. enterocolitica* (Chapter 24)

(3) Fermentative; oxidase positive

(a) Catalase positive; nitrate reduced; usually motile; arginine dihydrolase produced: *Aeromonas* spp. (Chapter 7)

(b) Cells spiral or comma-shaped; motile; fermentative; lysine and ornithine decarboxylase produced: *Vibrio* spp. (Chapter 7) (identify *V. cholerae* with specific antisera, biochemical tests)

(c) No growth on MacConkey agar: see *Pasteurella* spp. (*P. multocida, P. ureae,* etc.). (Chapter 11); *Cardiobacterium* (no veterinary significance)

(d) Growth on MacConkey agar: *Pasteurella haemolytica* (Chapter 11)

(4) Oxidative utilization of sugars [oxidation–fermentation (O-F) medium, no fermentation][f]

(a) Oxidase usually positive; no gas from sugars; motile; grow on MacConkey, SS, and cetrimide agars; many have soluble pigments (green or yellow): *Pseudomonas* spp. (Chapter 7)

(b) Oxidase positive (or variable); growth variable on MacConkey agar; oxidative or no utilization of sugars; yellow pigment; no reduction of nitrate: *Flavobacterium* spp. (Chapter 14)

(c) Oxidase negative; growth on MacConkey agar; malonate negative; nonmotile; no reduction of nitrate; no decarboxylases; 10% lactose, citrate positive; majority malonate positive: *Acinetobacter* spp. (other former names and groups: *Achromobacter anitratus, Bacterium anitratum, Mima–Herellea* group) (Chapter 14)

(5) Carbohydrates not attacked (no oxidation or fermentation, O-F medium)

(a) Oxidase positive; growth on MacConkey and cetrimide agars; nitrate reduced to nitrite; urease negative; no decarboxylases; motile: *Alcaligenes* spp. (Chapter 8)

Table 4-4 (*continued*)
Simplified Guide to the Presumptive Recognition of Common Groups of Bacteria[a]

(b) Oxidase positive; nonmotile; usually no growth on MacConkey agar (exceptions); penicillin susceptible; citrate and urease negative: *Moraxella* spp. (Chapter 14)

(c) Oxidase negative; growth on MacConkey agar

(i) *Acinetobacter calcoaceticus* subsp. *lwoffii* (Chapter 14)

(ii) *Pseudomonas maltophilia* (Chapter 7)

(iii) *Bordetella parapertussis* (Chapter 8)

(d) Oxidase positive; growth on MacConkey agar; microaerophilic; slow growth: *Campylobacter fetus* (Chapter 6)

B. Some grow on ordinary media, others need enriched media; fermentative; no gas produced; oxidase usually positive, nitrate reduced, catalase positive: *Pasteurella* spp. (Chapter 11)

C. Slow growth; pleomorphic cells; nonmotile; fermentative; no gas; often polar staining; oxidase and catalase variable; slow coagulation of milk; no decarboxylase: *Actinobacillus* spp. (Chapter 12); growth on MacConkey agar; oxidase positive: *A. lignieresii, A. equuli*; no growth on MacConkey agar; oxidase negative: *A. actinomycetemcomitans; A. pleuropneumoniae* requires V factor and is CAMP positive; oxidative, no growth on MacConkey agar; oxidase negative: *P. mallei* (Chapter 7)

D. Some requirement of special media (no growth on MacConkey agar) and conditions; no capsule; nonmotile: *Brucella* spp. (members of this group have to be differentiated by CO_2 requirement, H_2S production, dye inhibition test, and agglutination tests) (Chapter 9)

E. No growth or poor growth without special factors in media; capsule variable, nonmotile

1. Require factors X and/or V: *Haemophilus* spp. (Chapter 13); characteristic satellitism along *Staphylococcus* streak or other colonies; no growth on plain agar.

2. Do not require factors X and/or V; growth improved by addition of serum or ascitic fluid; sugars not attacked; oxidase positive: *Moraxella* spp. (Chapter 14); oxidase negative: *Gardnerella vaginalis* (no veterinary significance)

F. Primary isolation best on complex media with blood; oxidase positive; shows characteristic colonies on Bordet–Gengou agar; agglutination by specific antiserum: *B. pertussis* (rough variant of *B. pertussis* grows on ordinary media); *B. parapertussis* and *B. bronchiseptica* grow on MacConkey agar; closely related antigenically (Chapter 8)

G. No growth on ordinary media; requires special media (cystine–glucose–blood agar): *Francisella tularensis* (Chapter 11)

H. Grow best on enriched media, crescent-shaped or spiral cells (long screws or portions of a turn): *Spirillum* spp. (Chapter 24); no growth on artifical media: *S. minus* and *S. volutans*

I. Primary isolation on complex media with cysteine and iron, 5% CO_2; difficult to stain with Gram stain in tissues, but reacts with Dieterle technique or direct fluorescent antibodies: *Legionella* spp. (doubtful veterinary significance)

IV. Gram-positive rods

A. Catalase positive; no spores formed; no growth on MacConkey agar

1. Nonmotile; arranged in Chinese figures; stain unevenly with bands and granules: *Corynebacterium* spp. (Chapter 21); toxin production; fermentative: *C. diphtheriae* and *C. ulcerans*; other corynebacteria and diphtheroids differentiated by biochemical and toxigenicity tests; some fermentative, others do not attack sugars

2. Motile (at 20°C but usually not at 37°C); short, diphtheroid-like rods; often narrow zone of β-hemolysis: *Listeria monocytogenes* (use biochemical and pathogenicity tests; Chapter 20)

(*continues*)

Table 4-4 (*continued*)
Simplified Guide to the Presumptive Recognition of Common Groups of Bacteria[a]

3. Motile; do not attack sugars; indole production and nitrate reduction negative; long rods; some form filaments and coccoid bodies in broth: Kurthia spp. (Chapter 24)

B. Catalase negative; no spores formed

1. Nonmotile; frequently form long filaments; usually no growth in litmus milk; fermentative (glucose, lactose); H_2S in butt of triple sugar iron agar; nonbranching: *Erysipelothrix* spp. (Chapter 20)

2. Form chains; growth is better on tomato juice agar; H_2S negative in butt of triple sugar iron agar: *Lactobacillus* spp. (Chapter 15, 24)

C. Acid fast; no branching; no spores

1. Nonmotile; no branching; no hyphae: *Mycobacterium* spp. (Chapter 23)

a. Special media required; slow growth: *M. tuberculosis, M. bovis, M. avium,* etc. (distinguish by biochemical and culture characteristics); nontuberculous mycobacteria (formerly atypical) (use culture characteristics, pigment formation, physiological and biochemical tests)

b. Rapid growth: "saprophytes"; various species

D. Nonmotile; some branching; some acid fast; hyphae, but no true conidia produced; oxidative: *Nocardia* spp. (Chapter 22)

E. Catalase positive; spores formed, many motile: *Bacillus* spp. (Chapter 18); *B. anthracis:* characteristic colonies, nonmotile, usually nonhemolytic (for identification see Chapter 18)

Anaerobic Cultures

I. Gram-positive cocci

A. Occurring mainly in clusters but also in pairs: *Peptococcus* spp.; identification by gas chromatography, biochemical reactions (Chapter 15)

B. Occurring mainly in pairs and chains: *Peptostreptococcus* spp.; identification by gas chromatography, biochemical reactions (Chapter 15)

II. Gram-negative cocci occurring in irregular masses, small cocci: *Veillonella* spp. (Chapter 15)

III. Gram-negative rods

A. Usually motile of varying sizes and shapes; nonsporing; often foul-smelling; cells larger than 0.6 μm; butyric acid not produced: *Bacteroides* spp. (Chapter 15)

B. Some with pointed ends, effuse colonies, many very pleomorphic; butyric acid produced: *Fusobacterium* spp. (Chapter 15)

C. Spiral organisms: *Borrelia* spp., *Treponema* spp. (Chapter 5)

IV. Gram-positive rods; nonsporing

A. Mycelium produced which fragments; true branching; non-acid fast: *Actinomyces* spp.; usually catalase negative; slow-growing, dry, crumbly colonies on solid media; granules adhere to walls of tube in broth; when crushed, granules show typical clubs: differentiate from among anaerobic diphtheroids and lactobacilli (Chapter 22)

B. Catalase and indole negative; nonsporing: *Bifidobacterium* spp. (Chapter 15)

C. Catalase and indole positive; nitrate reduced; gas from glucose; propionic acid produced: *Propionibacterium* spp. (Chapter 15)

D. No branching; nonmotile; lactose not fermented; butyric acid produced: *Eubacterium* spp. (including *Eubacterium suis*) (Chapter 15)

V. Gram-positive rods; spores formed; some species may appear gram-negative; motile and nonmotile; endospores that distort cell shape; some microaerophilic (e.g., *Clostridium perfringens*); catalase negative: *Clostridium* spp. (many are saprophytes,

Table 4-4 (*continued*)
Simplified Guide to the Presumptive Recognition of Common Groups of Bacteria[a]

but some are pathogenic); identification by fluorescent antibody, biochemical reactions, recognition of exotoxins, pathogenicity tests, and gas liquid chromatography (Chapter 19)

[a]With minor modifications from: Isenberg, H. D., Washington, J. A., Balows, A. and Sonnewirth, A. C.: Collection, handling and processing of specimens. In Lennette, E. H. (Ed.-in-chief): *Manual of Clinical Microbiology*, 4th ed. Washington, D. C., American Society for Microbiology, 1985.
[b]Some do not grow on MacConkey agar; exceptions listed.
[c]O-F medium; fermentation observable in triple sugar iron medium.
[d]Cytochrome (indophenol) oxidase test.
[e]Lactose is valuable in the case of prompt fermenters (on differential plates overnight, or in 24–48 hr in fermentation media); some strains of the groups listed show delayed or no fermentation of lactose. See Enterobacteriaceae (Chapter 10).
[f]Usually no change in TSI medium or on carbohydrate-containing differential plate media.

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5

Spirochetes

John R. Cole, Jr.

The spirochetes are classified as bacteria in the order Spirochaetales and contain two families, the Spirochaetaceae and the Leptospiraceae. Four genera (*Spirochaeta, Cristispira, Treponema,* and *Borrelia*) are included in the Spirochaetaceae family. The genus *Leptospira* is currently placed in the family Leptospiraceae. Two of these genera, *Spirochaeta* and *Cristispira,* are considered free-living and commensal, respectively. The other three genera (*Treponema, Borrelia,* and *Leptospira*) contain pathogenic species.

Spirochetes are slender, spiral in shape, round on cross section, and multiply by transverse fission. Movement is active and accomplished by spinning and flexing about the long axis.

They are found in water, soil, decaying organic matter, plants, animals, and humans. These microorganisms are relatively inactive biochemically, and identification is based on staining reactions, growth requirements, morphology, pathogenicity, and serology.

Distinguishing characteristics of the three pathogenic genera, *Leptospira, Treponema,* and *Borrelia,* are shown in Table 5-1. They are gram-negative but are observed best by darkfield or phase microscopy or by staining with silver impregnation or Giemsa stain. Only *Borrelia* stains with aniline dyes.

Leptospira

The genus *Leptospira* is divided into saprophytic and pathogenic groups. The pathogens are included in the species *L. interrogans* and separated into approximately 180 serovars (serotypes) on the basis of cross-agglutination and agglutinin adsorption reactions. Some

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important serovars and their hosts are summarized in Table 5-2. Those most commonly associated with diseases of domesticated animals are listed in Table 5-3.

Pathogenicity

Leptospirosis is generally characterized by having two distinct phases: (1) leptospiremia and fever for about 7 days, followed by (2) leptospiruria, which may persist 2–3 months. Organisms may be recovered from the blood during the first phase and the kidney or urine during the second phase.

Canine

The four clinical syndromes recognized are the acute hemorrhagic, icteric, subacute or uremic, and inapparent forms. The first two forms are caused primarily by *icterohaemorrhagiae*, while the latter two are caused by *canicola*. In the initial stages of the disease, the first three forms of leptospirosis are usually clinically indistinguishable; all are characterized by depression, anorexia, vomiting, and diarrhea or constipation. Signs of the specific clinical syndrome appear in later stages of the disease.

Bovine

Clinical signs are fever, diarrhea, depression, anorexia, infertility, and sometimes abortion. Hemoglobinuria, icterus, and decreased milk production may also develop.

Table 5-1
Distinguishing Characteristics of *Leptospira*, *Treponema*, and *Borrelia*[a]

Characteristic	*Leptospira*	*Treponema*	*Borrelia*
Morphology			
Length	6–20 μm	5–20 μm	3–20 μm
Width	0.1–0.2 μm	0.09–0.5 μm	0.2–0.5 μm
Ends	A semicircular hook on one or both ends	Pointed, may have terminal filaments	Taper terminally to fine filaments
Spirals			
Number	Many, fine, tight	6–14, regular, angular	4–8, loose
Amplitude	0.4–0.5 μm	1 μm	3 μm
Motility	Spinning, undulating	Rotating, undulating, stiffly flexible	Lashing, corkscrewlike
Growth conditions	Aerobic	Anaerobic	Anaerobic

[a]Modified from Carter, G. R.: *Essentials of Veterinary Bacteriology and Mycology*. Courtesy of Michigan State University Press, East Lansing, Michigan, 1976.

Table 5-2
Important Leptospiral Serovars and Their Hosts[a,b]

Serovar	Known host	Occurrence in[c]			
		Humans	Dogs	Cattle	Swine
icterohaemorrhagiae	Rat, mouse, raccoon, opossum	Common	Occasional	Reported	Reported
canicola	Dog, cattle, swine, skunk	Common	Common	Rare	Occasional
pomona	Cattle, swine, skunk, raccoon, wildcat, deer, opossum, horse	Occasional	Rare	Common	Common
autumnalis	Opossum, raccoon, mouse	Rare	?	?	?
ballum	Mouse, gray fox, rat, opossum, raccoon, wildcat, skunk, gray squirrel, rabbit	?	?	?	?
grippotyphosa	Raccoon, mouse, fox, squirrel, rabbit, bobcat	Rare	Reported	Sporadic	Sporadic
bataviae	Rat, field mouse	Rare	?	?	?
hardjo	Cattle	Rare	?	Common	?
sejroe	Opossum, raccoon, mouse	?	?	Sporadic	?
hebdomadis	Opossum, raccoon	?	?	?	?
australis	Opossum, raccoon, fox	?	?	?	?
szwajizak	Cattle	?	?	Reported	?
balcanica	Cattle	?	?	Reported	?
bratislava	Swine	?	?	?	Reported

[a]Modified from Carter, G. R.: *Essentials of Veterinary Bacteriology and Mycology*. Courtesy of Michigan State University Press, East Lansing, Michigan, 1976.
[b]Data principally applicable to the United States.
[c]Based in some instances on serological evidence.

Table 5-3
Principal Leptospiral Serovars Associated with Diseases of Domesticated Animals

Animal species affected	Serovars
Cattle	*pomona*
	hardjo
	grippotyphosa
	icterohaemorrhagiae
	canicola
Sheep	*pomona*
Swine	*pomona*
	grippotyphosa
	canicola
	icterohaemorrhagiae
Horse	*pomona*
Dog	*canicola*
	icterohaemorrhagiae

Porcine

Infections are usually subclinical or asymptomatic. Abortions late in pregnancy are sometimes the only sign of infection. Occasionally, metritis, icterus, anemia, fever, and meningoencephalitis are observed.

Equine

The disease is characterized by fever, depression, anorexia, and icterus. Periodic ophthalmia or abortion may occur after the fever subsides.

Laboratory Examination

Direct Examination

Leptospires can be demonstrated in tissue and body fluids by darkfield, phase, or fluorescence microscopy (Fig. 5-1). A fluorescent conjugate to the five basic serovars is available from the National Leptospirosis Reference Center, National Veterinary Services Laboratory, Ames, Iowa. Leptospires observed by direct examination should be confirmed by isolation procedures.

Blood

Five milliliters of blood drawn during the febrile phase is mixed with 0.5 ml of a 1.0% solution of sodium oxalate or 0.1 ml of a 1.0% solution of heparin (sodium citrate may be inhibitory).

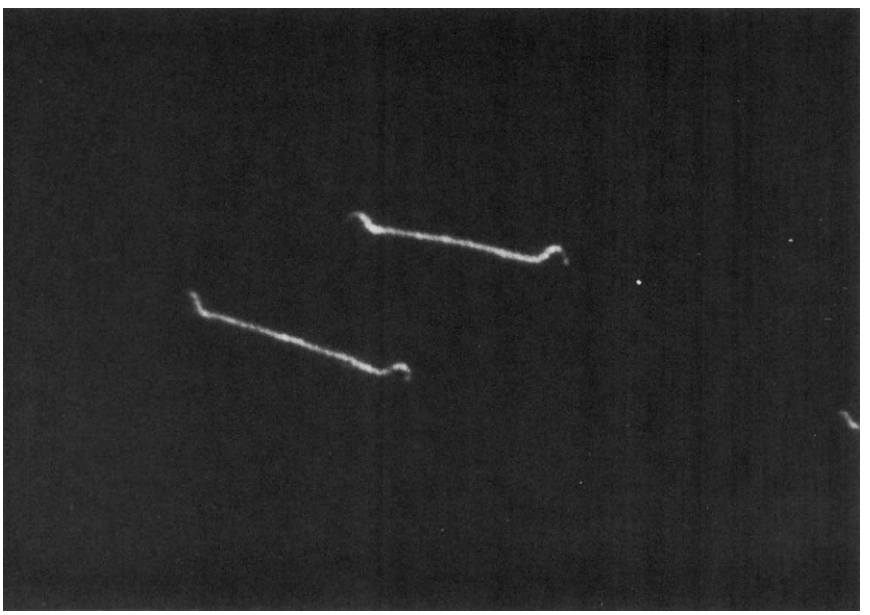

Figure 5-1. *Leptospira pomona.* Darkfield illumination, × ~845.

1. Centrifuge at 1500 rpm for 15 min.
2. Transfer supernatant fluid to another tube and centrifuge at 3000 rpm for 30 min.
3. Discard the supernatant fluid and prepare a wet mount from the sediment.
4. Examine by darkfield or phase microscopy. *Caution:* Protoplasmic extrusions from blood cells and other artifacts may be confused with leptospires.
5. Smears can be prepared from the sediment for fluorescent antibody staining. The procedures included with the fluorescent antibody conjugate should be followed.

Urine

Fresh urine is neutralized with *N*/10 HCl or *N*/10 NaOH.

1. Centrifuge at 3000 rpm for 10 min.
2. Discard supernatant fluid and prepare a wet mount from the sediment.
3. Examine as described for blood.

Tissues

A portion of kidney or liver must be taken aseptically shortly after death.

1. Grind a weighed portion of tissue in a stomacher, mortar and pestle, or Ten Broeck tissue grinder.
2. Prepare a 10% tissue suspension in Stuart's (Difco Laboratories, Detroit, Michigan) or EMJH (Ellinghausen, McCullough, Johnson and Harris) (Difco) liquid medium or 1% bovine serum albumin (BSA, Fraction V® powder) (Miles Laboratories, Inc., Elkhart, Indiana) and mix well.
3. Follow steps 1 through 5 for blood.

Isolation Procedures

Leptospires can be isolated from blood, urine, and tissue suspensions by inoculation into any of the following media: (a) EMJH medium; (b) Fletcher semisolid medium (Difco); (c) Tween 80®–albumin medium (OAC) (1). The semisolid forms of EMJH and OAC (oleic albumin complex) media are recommended for the isolation of leptospires. They are prepared by adding 1.5 g agar per liter of medium.

Samples that are contaminated with other microorganisms should be filtered through a 0.45-μm bacteriological filter and inoculated into medium containing 5-fluorouracil, in a concentration of 200 μg/ml, or neomycin sulfate, 300 μg/ml. A microsyringe filter holder (Millipore Corporation, Bedford, Massachusetts) attached to a syringe can be used.

Animal inoculation can also be used when samples are contaminated or when leptospires are present in small numbers.

Blood

One to two milliliters of blood are drawn aseptically during the febrile phase.

1. Inoculate 1–2 drops of oxalated or heparinized blood (see Direct Examination: Blood) into each of three tubes of EMJH, Fletcher, or OAC semisolid medium.
2. Incubate at 28–30°C or at room temperature in the dark.
3. At 7-day intervals, examine a drop of medium from each tube by darkfield or phase microscopy. Tubes are discarded if growth does not appear within 6 weeks.

Urine

Aseptically collect a minimum of 1.0 ml of urine. The sample is inoculated both undiluted and diluted. However, if urine cannot be inoculated into the medium immediately, dilute the urine 1:10 with 1% BSA to maintain viable leptospires (2). The diluted material may be held at room temperature.

1. With a 2-ml syringe and 20-gauge needle, inoculate 1–2 drops of fresh undiluted urine into each of three tubes of EMJH, Fletcher, or OAC semisolid medium.
2. Discard all but 0.1 ml of urine from the syringe.
3. Draw 0.9 ml of EMJH liquid medium or 1% BSA into the syringe (1:10 dilution).
4. Discard a few drops (2–3) from the syringe.
5. Inoculate 1–2 drops of the 1:10 dilution into each of three tubes of semisolid medium.
6. Prepare and inoculate four additional 10-fold dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) as in steps 2–5.
7. Incubate and examine as described for blood.

Tissues

The kidney, liver, and brain are the organs preferred for isolation attempts.

1. Grind a weighed portion of tissue in a stomacher, mortar and pestle, or Ten Broeck tissue grinder.
2. Prepare a 10% tissue suspension in EMJH liquid medium or 1% BSA and mix well.
3. Follow steps 1–6 as described for urine.
4. Incubate and examine as described for blood.

Isolation attempts should be made as soon as possible after death because leptospires survive only a short time in autolytic tissues. After 4 hr, recovery attempts become impractical.

Laboratory Animals

Weanling hamsters, gerbils, or guinea pigs are preferred for the isolation of leptospires associated with infection in domesticated animals, especially serovar *hardjo*.

Two or three animals are used for each isolation attempt.

1. Inoculate laboratory animals intraperitoneally with 0.5–1.0 ml of unclotted blood, neutralized or buffered urine, or a 10% tissue suspension in EMJH medium or 1% BSA.
2. Take cardiac blood aseptically on postinoculation days 5, 8, 10, and 14, or when an increase in temperature is detected.
3. Immediately inoculate at least two tubes of Fletcher, EMJH, or OAC semisolid medium with 2–3 drops of blood.
4. Prepare and examine wet mounts from unclotted blood as described in steps 1–5 of Direct Examination: Blood.
5. Collect blood samples for serology and kill animals surviving 21 days postinoculation.
6. Attempt isolation from tissues and urine as previously described.

Identification of isolated leptospires is based on serologic reactions with specific antiserum and is usually performed by the WHO/FAO Collaborating Laboratory for the Epidemiology of Leptospirosis, Centers for Disease Control, Atlanta, Georgia, and the National Leptospirosis Reference Center, NVSL, APHIS, USDA, Ames, Iowa.

Serologic Procedures

Macroscopic and microscopic agglutination tests are the most commonly used procedures for detection of serum leptospiral antibodies. The enzyme-linked immunosorbent assay (ELISA) technique has been reported to be of value in the detection of specific immunoglobulins (3–8). It is not widely used as a routine procedure for diagnosis of leptospirosis at this time.

The macroscopic agglutination test is used for screening either single or pooled serum samples and employs commercially available killed antigens. The macroscopic test is used because of availability of antigens, ease of performance, and safety. A major disadvantage of the test is its lack of specificity. Sera may react with multiple serovars, especially if the samples are obtained during the acute phase of the disease.

The microscopic agglutination test, which utilizes live leptospires as antigen, is highly sensitive and serovar-specific. The time and

attention required to maintain viable, pure cultures of several serovars is the major disadvantage of this test. However, every effort should be made to use this procedure.

Sera that are positive on screening with the macroscopic test should be confirmed by the microscopic test. These positive sera may be negative by the microscopic test because of low serum antibody levels and nonspecific reactions.

Macroscopic Agglutination Test

1. Antigens:

The Galton antigens (Lee Laboratories, Grayson, Georgia) are available as single serovars or as pools designated I, II, III, IV, V, and VI. The pools most commonly used are I through IV, which contain the following serovars:

Pool I	Pool II	Pool III	Pool IV
ballum	*bataviae*	*autumnalis*	*australis*
canicola	*grippotyphosa*	*pomona*	*hyos* (currently *tarassovi*)
icterohaemorrhagiae	*pyrogenes*	*wolffi*	*mini georgia* (currently *georgia*)

2. Plate screening test
   A. On a glass plate, place 0.01 ml serum to be tested on a separate square for each antigen or pool.
   B. Using supplied dropper, add one drop of each single or pooled antigen to each drop of serum.
   C. Using a clean portion of applicator stick, mix antigen and serum.
   D. Rotate the plate by hand 5–10 times.
   E. Place on an electric rotator for 4 min at 125 rpm.
   F. Observe reaction over indirect light.
   G. Record reaction as follows:
      Positive: Agglutination (peripheral[1])
      Negative: Even suspension
   H. Use known positive and negative sera as controls.
   I. Confirm positive samples with the microscopic agglutination test.
3. Determination of titer by macroscopic methods:

The plate dilution test may be used for the determination of titers. Initial serum–saline dilutions of 1:5 and subsequent twofold dilutions

[1]Nonspecific clumping due to bacteria and cell debris usually occurs in the center of the drop. Specific agglutination occurs at the edge of the drop. Confidence in reading this test is obtained with experience and use of adequate control antiserum.

are prepared. Specific instructions for conducting this test are included with the antigens.

4. Evaluation of macroscopic methods for serum titrations:

The macroscopic tests may be helpful in establishing a presumptive diagnosis if a significant titer increase is detected with acute and convalescent serum samples or if a high titer is obtained with a single sample. Agglutination at a dilution of 1:60 suggests current infection.

The microscopic agglutination test is preferred for serum titrations because of its greater specificity.

Microscopic Agglutination Test

1. Antigens:

The antigens are 5-day-old cultures grown in EMJH, Stuart, or OAC medium. The serovars used will depend upon those suspected of being prevalent in the animal population in the particular location. Suggested serovars of *pomona, hardjo, grippotyphosa, icterohaemorrhagiae, bratislava, canicola,* and *autumnalis.*

2. Antigen preparations
   A. Examine cultures microscopically for purity, homogeneity, and density.
   B. Transfer sufficient antigen for the test into tubes (13 × 100 mm).
   C. Centrifuge at 1500 rpm for 15 min to remove debris.
   D. Transfer supernatant to another tube and adjust to an antigen concentration of 100–200 organisms per high-power field (×450). This concentration is equivalent to a McFarland number 0.5, a light transmission of 60–70% on a Spectronic 20 or equivalent spectrophotometer set at 400 nm, or a Nephelometer set to 25 with either dry well or wet well.

3. Microtiter plate:

This technique (9) is a modification of a procedure initially reported by workers at the Centers for Disease Control (10). A disposable microtiter pipette equipped with a 0.025- ml dropper (Linbro Scientific Company, New Haven, Connecticut) is used for dispensing the serum and antigen into microtiter plates with flat-bottom wells (Linbro or Microtest II®, Falcon Plastics, Oxnard, California). A multimicrodiluter handle equipped with 0.025-ml microdiluters (Cooke Engineering Company, Alexandria, Virginia) is used to dilute the serum.

   A. Serum dilution (twofold)
      (1) In a test tube (13 × 100 mm), prepare a 1:25 dilution using 2.4 ml PBS and 0.1 ml serum.
      (2) Add 1 drop (0.025 ml) of PBS to each well in the plate except for the wells in the first row (row H).[2]

[2]Standard microtiter plates are labeled top to bottom, row A through H.

(3) Add 2 drops (0.05 ml) of the 1:25 serum dilution to wells in row H. One well is used for each antigen tested.
(4) Using the 0.025-ml microdiluter, mix the dilutions in row H by twirling the diluters 10–15 times.
(5) Transfer diluter to row G and mix.
(6) Repeat step 5 for the desired number of dilutions.
(7) After mixing the last dilution, rinse diluters by twirling in distilled water and blot dry.
(8) Include known positive and negative sera for controls.

B. Addition of antigen (see section 2 for preparation of antigen)
(1) Using a 0.025-ml dropper, add 1 drop of antigen to each dilution in the first column of each serum sample.
(2) Repeat for the remaining antigens in their corresponding columns.
(3) Gently shake the plates to mix contents, cover with plastic lid to exclude debris, and incubate at room temperature for 2 hr.

C. Reading of test: The plate is placed on the stage of a darkfield microscope equipped with a long-working-distance 10× objective (No. 519-438 or 559-003, E. Leitz, Inc., Rockleigh, New Jersey; or No. A0 1019 or A0 1076, American Optical Corp., Buffalo, New York) and 10× eyepieces, and the wells are examined for agglutination (Fig. 5-2). A 3.5× objective with 15× eyepieces may be used; however, at this magnification, only agglutination will be observed.

D. Interpretation of test results: The end point is the highest dilution in which at least 50% of the organisms are agglutinated. End points of 1:100 are suspicious, and those greater are positive. Lysis may occur at low dilutions with some serovars.

The vaccination history of the herd must be considered in evaluating results. End points of 1:1000 or greater can be detected in animals that have been recently vaccinated (11,12).

E. Screening test: If a large number of sera are to be tested, it may be desirable to screen them first at the 1:50 dilution to eliminate the negatives. When screening, 0.025 ml of the 1:25 dilution is placed in each well of a column (e.g., serum 1 in column 1, etc.). The antigens are added to the rows (e.g., *pomona* in row A, *hardjo* in row B, and so on). This procedure allows 12 sera to be screened against 8 antigens on one plate. Sera that are positive against one or more serovars are then titered only against the serovars to which they are positive.

F. A revision of this microtiter procedure, which provides for options of volume used (0.025 ml or 0.05 ml), establishes the baseline dilution of 1:100, and gives detailed media preparation,

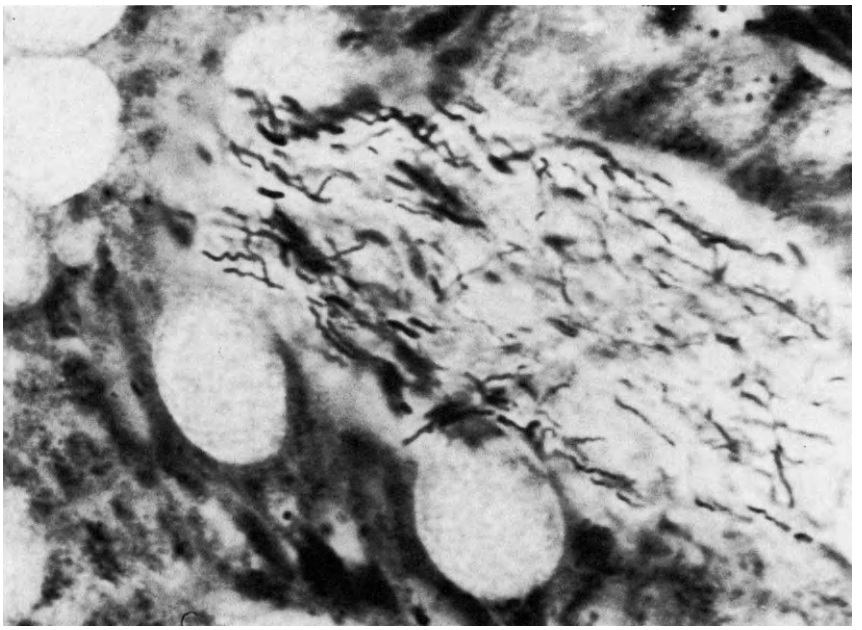

Figure 5-2. Leptospiral agglutination: negative reaction and degrees of positive reaction.

has been prepared by personnel at the National Leptospirosis Reference Center. A copy of this technique can be obtained by writing the National Leptospirosis Reference Center, National Veterinary Services Laboratory, Box 844, Ames, Iowa 50010.

G. Transfer plate system: A modification of the microtechnique using the transfer plate system (Cooke Laboratory Products, Alexandria, Virginia) for direct reading on microscope slides using a 10× objective, 10× eyepieces, and dry, darkfield condenser has been described (13).

4. Maintenance of cultures: Leptospires are sensitive to hydrogen peroxide; therefore, rabbit serum containing catalase from hemolyzed erythrocytes must be used in the culture medium.

Stock cultures in EMJH, Fletcher, or OAC semisolid medium should be maintained, since leptospiral transfers occasionally do not grow.

Treponema

Treponema hyodysenteriae is the name that has been proposed for the large spirochete responsible for swine dysentery. This disease has been

recognized since 1921 and was initially thought to be caused by *Vibrio coli*. However, in the early 1970s, workers in the United States and Great Britain demonstrated that *T. hyodysenteriae* was the primary etiologic agent.

Pathogenicity

Swine dysentery is usually observed in 15–70-kg pigs but may affect suckling as well as adult swine. There is marked catarrhal hemorrhagic enteritis, which is confined to the large intestine. Death may occur, but high morbidity leading to poor weight gain is the usual finding.

Laboratory Examinations

Direct Examination

Treponema hyodysenteriae can be observed in the mucosal lesions of the large intestine by darkfield or phase-contrast microscopy using the following procedure (14).

1. Rinse or lightly scrape a portion of the affected mucosa to remove debris.
2. Suspend a portion of a deep scraping from the mucosa in a drop of saline or water on a microscope slide.
3. Examine by darkfield or phase-contrast microscopy at a magnification of 400–1000.
4. Observe three to five spirochetes per high-power field.

It is important to differentiate *T. hyodysenteriae*, which is 7–8 μm long, loosely coiled, motile by flexing movements, and tapered at the ends, from the smaller, tightly coiled spirochetes normally found in swine. Mucosal or fecal smears may be stained with crystal violet, carbolfuchsin, or Victoria blue 4-R stains, although wet mount preparations to observe motility are preferred. These spirochetes may be observed in histologic sections of the colonic mucosa stained with the Warthin–Starry, Goodpasture's, or Victoria blue 4-R stains (Fig. 5-3).

Rectal swabs or feces may be utilized for examination from a live animal, but numbers of organisms in the feces may be small and the spirochetes not readily detectable (15).

Isolation Procedures

Treponema hyodysenteriae can be isolated from the intestinal mucosa using the following procedure (16,17).

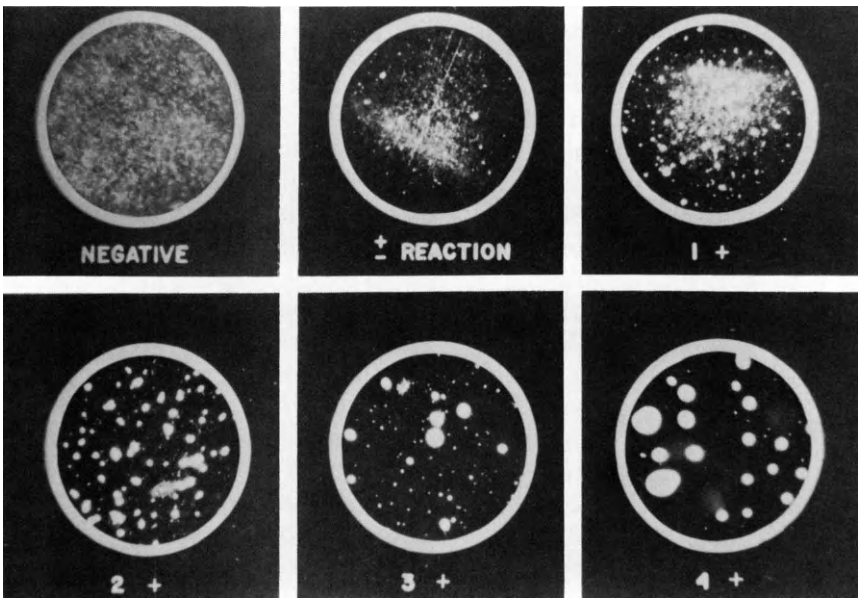

Figure 5-3. *Treponema hyodysenteriae* in a section of swine intestine. Warthin–Starry stain, × ~1000.

1. Take 6–8 in of spiral colon from an acutely affected animal. The sample may be held for 2–3 days at 4°C before isolation attempts. Do not freeze.
2. Open the colon longitudinally and remove the mucosa with a sterile swab.
3. Alternatively, prepare a 1:10 suspension of the colonic mucosa in saline.
4. Centrifuge slowly for 10 min to remove the large particulate matter.
5. Pass the supernatant through a series of cellulose acetate filters: prefilter, 8.0 μm, 3.0 μm, 1.2 μm, 0.8 μm, 0.65 μm, and 0.45 μm.
6. Streak the mucosal swab or the material from the filtrate that passed the 0.8 μm, 0.65 μm, and 0.45 μm filters onto freshly prepared or prereduced trypticase soy agar (TSA) containing 5% defibrinated bovine or equine blood. The addition of 400 μg/ml of spectinomycin (The Upjohn Company, Kalamazoo, Michigan) and 1% sodium ribonucleate (United States Biochemical Corp., Cleveland, Ohio) to the medium suppresses most of the contaminating flora, does not adversely affect the isolation of *T. hyodysenteriae*, and enhances the degree of hemolysis

   A medium (BJ) which further suppresses fecal contamination and enhances growth of *T. hyodysenteriae* has been described

(18). In addition to the basic medium (TSA) and 5% blood, it contains five antibiotics (spiramycin, rifampin, vancomycin, colistin, and spectinomycin), and a 5% pig feces extract.

7. Incubate the plates at 42°C in an anaerobic container. A vented Gas-Pak® jar with cold palladium catalyst (BBL, Cockeysville, MD) may be used to obtain a hydrogen and carbon dioxide atmosphere of 80:20 H_2–CO_2 by evacuation and refilling. A H_2–CO_2 generator envelope (GasPak-BBL) is also acceptable.

Cultural Characteristics and Identification

Growth of *T. hyodysenteriae* on blood agar is evidenced by a zone of clear (β) hemolysis, which may contain small, white translucent colonies. This is in contrast to *Treponema innocens*, which is weakly β-hemolytic and considered nonpathogenic (14). *Treponema hyodysenteriae* is gram-negative but is more readily observed using the stains listed under Direct Examination. *Treponema hyodysenteriae* is 6–8.5 μm long, 0.32–0.38 μm in diameter, loosely coiled, motile, cytochrome oxidase negative, catalase negative, stimulated by hydrogen, and anaerobic.

Treponema paraluis–cuniculi is pathogenic for animals and the cause of rabbit syphilis. Diagnosis is based on lesions around the genitalia and demonstration of the organisms in these lesions by staining or darkfield microscopy.

Treponema suis has been observed in washings of ulcerated preputial diverticula in pigs (19).

Treponema succinifaciens, a small anaerobic spirochete, has been isolated from the colon of a pig and is considered nonpathogenic (17).

Serologic Procedures

Tests that have been adapted for use in the diagnosis of swine dysentery or for detection of carrier animals are the tray agglutination test (20), microtitration agglutination test (21), and ELISA (22). The ELISA procedure appears to be the most sensitive of these tests, and it may be useful for determining the status of a herd by diagnostic laboratories that have personnel and equipment necessary to perform this test.

Borrelia

Borrelia anserina causes fowl spirochetosis, a highly fatal disease in chickens, turkeys, geese, and other fowl. The disease is characterized by acute septicemia with concomitant fever, diarrhea, listlessness, and emaciation. Anemia is commonly present. The spleen is usually enlarged and mottled.

The spirochete is transmitted primarily by fowl ticks. Other arthropods and feces of infected birds may also transmit *B. anserina*.

The disease is diagnosed by demonstration of the spirochetes, which are 3–20 μm in length, in blood, spleen, and liver smears stained by Giemsa's slow method (23). *Borrelia anserina* can be isolated from the blood and grown in 6–12-day-old embryonated chicken or turkey eggs (24,25) (Fig. 5-4).

Borrelia hyos has been observed in the blood of swine but has not been identified with a specific disease. A *Borrelia* species, possibly *B. hyos*, has been demonstrated in the large intestine of pigs with swine dysentery (26).

A spirochete that has been tentatively classified as *B. suilla* causes ulcerative granuloma in wounds of pigs and can be observed in sections by silver impregnation techniques (27).

The association of spirochetes with intestinal disorders of dogs has been observed by several workers. The organisms, which probably represent different species although they have not been well characterized,

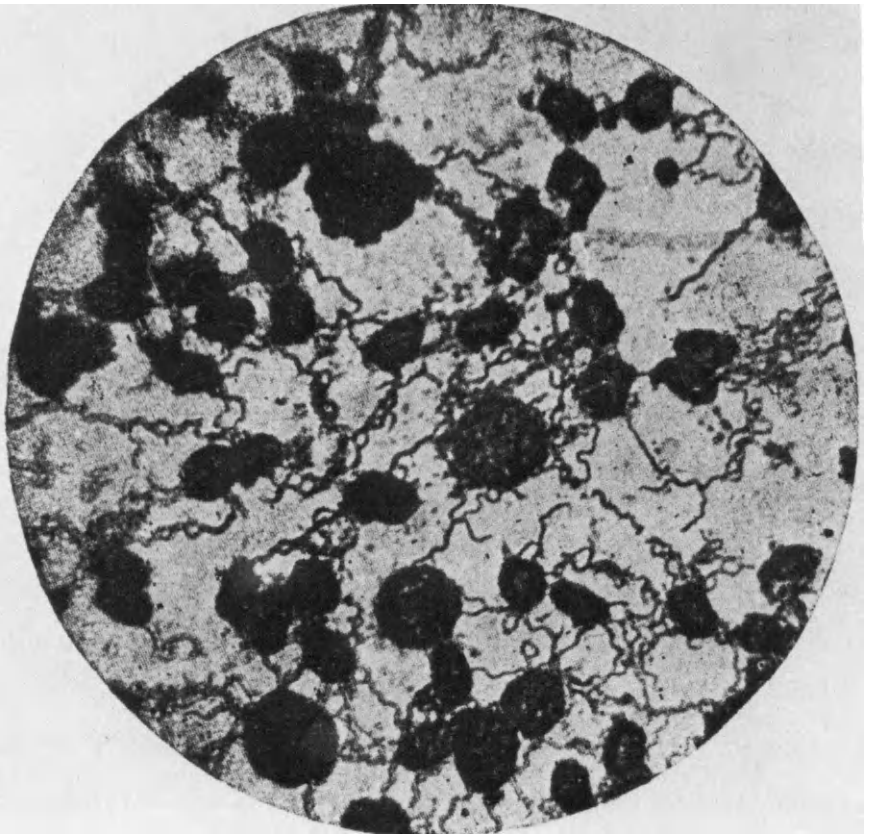

Figure 5-4. *Borrelia anserina:* Smear of chicken embryo blood, ×1300 (38).

have been given the tentative names *Borrelia canis, Spirillum eurygyrata,* and *Spirillum minutum.* Their occurrence and significance have been discussed by Craige (28).

Borrelia vincentii can be found in the mouth of normal dogs. In combination with *Fusobacterium fusiforme, B. vincentii* is thought to cause ulceromembranous stomatitis in debilitated dogs (29).

Borrelia burgdorferi, the causative agent of Lyme disease, was first recognized and described in 1975 following an illness occurring in children from Lyme, Connecticut (30). The organism causing the disease was first cultured in 1981 from an ixodid tick, *Ixodex dammini,* in New York. Although initially found in the northeastern United States, it is now widespread throughout various sections of the country.

Pathogenicity

The disease has been reported from both wild and domesticated animals, especially dogs (31,32). Usually the only clinical sign observed in dogs is transient or recurrent arthritis. Clinical signs including uveitis, encephalitis, and arthritis have been reported in horses and cows (33,34).

Laboratory Examinations

Serologic techniques are currently the most reliable methods for diagnosis of this disease. Culture of the spirochete is extremely difficult. The indirect fluorescent antibody (IFA) procedure can be used to detect the organism in renal cortical tissue and antibodies in serum (32). It has been reported, however, that the enzyme-linked immunosorbent assay yielded less variable results, had greater sensitivity, and was more easily standardized than the IFA (32). Procedures for performing the IFA and ELISA have been described (35–37).

An example of each of these tests (37) follows:

Indirect Fluorescent Antibody

1. Grow spirochetes in suitable medium, such as Barbour–Stoenner–Kelly (BSK), for 5–7 days (see Johnson, *et al.* 1984).
2. Harvest cells by centrifugation at 21,000 *g,* 15°C, for 30 min.
3. Wash cells three times in 0.01 *M* phosphate-buffered saline (PBS), pH 7.2.
4. Dilute cells in PBS to yield approximately 100 organisms per 40× microscopic field.
5. Apply antigen suspension to glass slides and allow to air dry. It is convenient to use acetone-resistant multiwell fluorescent

antibody slides. Place 20 μl of antigen in each well and remove excess liquid with a Pasteur pipette. After the slide has dried, fix in acetone for 10 min and allow to air dry.

6. For each serum to be tested, prepare twofold dilutions in PBS from an initial 1:16 dilution in 3% normal hen's yolk sac suspension (NYS). The NYS decreases nonspecific fluorescence that interferes with reading endpoints.
7. Place a drop (20 μl) of each serum dilution ≥1:64 on an antigen-coated well. Incubate in a moist chamber for 30 min at 37°C.
8. Rinse slides, then soak them in PBS for 10 min. Blot dry.
9. Place a drop (20 μl) of FITC-labeled antihuman immunoglobulin (Ig) (polyvalent conjugate) on each well. Incubate, rinse, and dry as above. Alternatively, use Ig class-specific conjugates, but only if class specificity has been evaluated.
10. Cover slide with carbonate-buffered mounting fluid, pH 9, and coverslip.
11. Read fluorescence intensity on a fluorescence microscope equipped for fluorescein. AT CDC, the titer is expressed as the reciprocal of the highest dilution of serum giving 1+ (barely visible) blue-green fluorescence of at least 50% of the spirochetes per microscopic field. A Leitz Dialux 20 fluorescence microscope equipped with a HBO-100 mercury incident light source, I cube filter system, 40× dry objective, and 6.3× binoculars (final magnification, 315×) can be used.
12. A titer ≥256 is considered a positive test result.

Enzyme-Linked Immunosorbent Assay

1. Grow spirochetes in suitable medium, such as BSK, for 5–7 days. For the ELISA, approximately 350 ml of broth medium is required.
2. Harvest cells by centrifugation at 21,000 *g*, 15°C, for 30 min.
3. Wash cells three times in 0.01 *M* phosphate-buffered saline (PBS), pH 7.2.
4. Resuspend washed cells in 15 ml of PBS and mix with an equal volume of 2 *M* NaCl in PBS.
5. Sonicate the suspension in an ice bath for 10 min at 60% percent of maximum setting on a Biosonik IV Sonifier (or comparable settings on different models).
6. Centrifuge sonicate at 21,000 *g*, 4°C, for 30 min.
7. Wash sediment once with 5 ml of 1 M NaCl. Pool both supernatant fluids and dialyze against several changes of distilled water and then against PBS.
8. Determine protein concentration of antigen, and adjust its concentration to 5 μg of protein per ml of 0.1 *M* carbonate buffer, pH 9.6.

9. Place 25 μl of antigen in each well of a U-bottom microtiter plate (Dynatech, Cambridge, Massachusetts). Incubate overnight at 4°C.
10. Wash plate three times with 0.9% NaCl containing 0.05% Tween 20 and 0.02% NaN_3.
11. To each well, and 25 μl of a 1:500 dilution of test serum in PBS containing Tween 20 and 0.02% NaN_3 (PBS-TA). Incubate at 37°C for 1 hr.
12. Wash plate three times as above.
13. To each well, add 25 μl of antihuman immunoglobulin alkaline phosphatase conjugate, diluted in PBS-TA. Incubate at 37°C for 3 hr and wash as before.
14. To each well, add 25 μl of *p*-nitrophenyl phosphate substrate (2 mg/ml in 0.05 *M* carbonate buffer, pH 9.8, containing 0.001 *M* $MgCl_2$). Incubate at 37°C for 1 hr.
15. To stop the reaction, add 25 μl of 5 *N* NaOH to each well. Read OD at 405 nm.
16. At CDC, the ratio of the OD of the test serum to the OD of a high-titered serum from a patient with Lyme disease is determined (OD ratio). An OD ratio ≥ 0.41 (≥ 2 SDs above the mean OD ratio of 100 normal control sera) is considered a positive test result. Results are expressed as the mean of duplicate tests.

Kits are commercially available for the IFA (Diagnostic Technology, Inc., Hauppauge, New York 11788; Wampole Laboratories, Cranbury, New Jersey 08512); and ELISA and IFA (Whittaker M. A. Bioproducts, Walkersville, Maryland 21793; Hillcrest Biological, Cypress, California 90630).

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6

Campylobacter

J. F. Prescott

Organisms in this genus are thin (0.2–0.8 μm × 0.5–5 μm) gram-negative, motile, curved rods. The cells are often S-shaped or seagull shaped, but are occasionally long (8 μm) spiral rods. They are vigorously motile by a single polar flagellum. Microaerophilic (3–15% O_2) conditions are generally required for growth, and optimal conditions are 6% O_2. Some species, however, (e.g., *C. mucosalis*) grow best initially anaerobically, while others (e.g., *C. cryaerophila*) are aerobic. All species are cytochrome oxidase positive.

The genus contains species causing important genital and intestinal infections of animals, as well as saprophytic species. The recent interest in *C. jejuni* as an important zoonosis in humans has resulted in improved classification of the genus, for long generally neglected in veterinary bacteriology. At one time *Campylobacter* were classified with *Vibrio*, but the former are nonoxidizers and the latter fermenters, and for this and for other reasons the two genera are recognized as distinct. Isolates can be broadly distinguished into catalase positive and negative species (Tables 6-1, 6-2).

Campylobacter fetus

Classification and Pathogenicity

The taxonomy recommended in the Approved List of Bacterial Names (1) is that *C. fetus* is divided into 2 subspecies, *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*. The former organism is found in the prepuce of carrier bulls and the genital tract of infected cows and is an important cause of infectious infertility and sporadic abortion. A

Diagnostic Procedures in Veterinary Bacteriology and Mycology, Fifth Edition

Table 6-1
Differentiation of Catalase Positive *Campylobacter* Species

Species	H_2S in		Sensitivity to[b]		Growth in			Hippurate hydrolysis	Growth at		Nitrate reduction	Growth on TTC agar[d]
	Lead acetate strip[a]	TSI	Nalidixic acid	Cephalothin	1% glycine	TMAO[c]	1% bile		25°C	42°C		
C. fetus subsp. *venerealis*	–[e]	–	R	S	–	–	+	–	+	–	–	–
C. fetus subsp. *fetus*	+	–	R	S	+	–	+	–	+	–	–	–
C. jejuni	+	–	S	R	+	–	+	+	–	+	–	+
C. coli	+	–	S	R	+	–	+	–	–	+	–	+
C. cryaerophila	–	–	S	R	–	–	–	–	+	–	+	–
C. faecalis	+	+	R	S	+	+	Var	–	–	+	+	–
C. laridis	+	–	R	R	+	+	+	–	–	+	NP[f]	–
C. hyointestinalis	+	+	R	S	+	+	+	–	+	poor	+	–

[a]Medium with 0.02% cysteine.
[b]30-μg disks.
[c]Anaerobic growth in presence of trimethylamine-*N*-oxide.
[d]Growth on triphenyl tetrazolium chloride agar (0.4 g/liter).
[e]Biotype *intermedius* positive.
[f]No data.

Table 6-2
Differentiation of Catalase Negative *Campylobacter* Species

Species	Growth in			Dirty yellow colonies	Growth on TTC agar[a]
	1% Glycine	1% Bile	3.5% NaCl		
C. sputorum subsp. *sputorum*	–	–	–	–	ND[b]
C. sputorum subsp. *bubulus*	+	–	+	–	ND
C. mucosalis	–	+	–	+	ND
C. upsaliensis[c]	ND	ND	ND	–	–
C. concisus	–	ND	–	ND	ND
Sheep colitis isolate	–	Var	– (3.0%)	ND	ND

[a]Tolerance to triphenyl tetrazolium chloride (0.4 g/liter).
[b]No data.
[c]Weakly catalase positive.

biotype of *C. fetus* subsp. *venerealis*, biotype *intermedius*, is recognized (2), but there is evidence that this may be *C. fetus* subsp. *fetus*. *Campylobacter fetus* subsp. *fetus* is found in the intestine of cattle, sheep and humans; it may cause gastroenteritis in humans. Use of cephalosporins in selective media for isolation of *C. jejuni* from diarrheic stool inhibits the recovery of *C. fetus* subsp. *fetus* and may have led to underestimation of its role in diarrhea in animals. In cattle and sheep it is isolated sporadically from cases of abortion and in people from cases of septicaemia in immunosuppressed patients.

The distinction between the subspecies on the basis of pathogenic potential is important epidemiologically, but there is evidence that it may be somewhat arbitrary since there are a few reports of the ability of some *C. fetus* subsp. *fetus* strains to cause localized genital tract infection in cows (3). Subspeciation should, however, be attempted. The differentiation between the subspecies on biochemical grounds (Table 6-1) is also not precise since there may be variants (especially in the laboratory) of *C. fetus* subsp. *venerealis* that are glycine resistant (4).

Diagnosis of *Campylobacter Fetus* Subsp. *venerealis* Infection

A definitive diagnosis of genital campylobacteriosis can be difficult. In the past, diagnosis has been made on the basis of cervical mucus agglutination, fluorescent antibody (FA) staining, and culture. The introduction of a selective transport medium has improved diagnosis in carrier bulls.

Direct Examination

Campylobacter can be demonstrated in Gram-stained smears of fetal stomach contents.

Fluorescent antibody procedures have been widely employed for the identification of *C. fetus* in preputial washings, cervical mucus, and fetal stomach contents. Smears are prepared from the sediment of centrifuged preputial washings and from other materials in the conventional manner. Filtration of cervicovaginal mucus through a cellulose acetate disc (5-μm pore size) improves the quality of the smears and contributes to greater accuracy in the FA test (5). The conjugate does not distinguish between subsp. *venerealis* and subsp. *fetus*. The FA reagent is not available commercially but is available through the courtesy of some laboratories. The FA procedure has been found to be highly effective, probably because both dead and living campylobacter are stained, and distinguishes *C. fetus* from the nonpathogenic *C. sputorum* subsp. *bubulus* sometimes present in preputial washings of bulls.

Collection of Cervical Mucus

The test is convenient and accurate if animals are tested between 2 and 7 months after infection, are not in estrus, and no blood is present. The test is done on a herd basis, so that 10 cows in the above category are tested, or 20 if no such selection is possible.

A number of methods have been described. The following simple procedure is given by Laing and associates (6). The mucus is collected by means of a glass tube about 50 cm in length and 1 cm in diameter. This pipette has a slight bend about 10 cm from one end, which is lightly plugged with cotton wool at one end; the other end is also lightly plugged with cotton wool, to act as a stop for the mucus. Before use, the pipettes are wrapped in greaseproof paper and sterilized by autoclaving.

A piece of rubber tubing about 50 cm long is attached to the straight end of the pipette, the plug at the bent end is removed, and this end is passed into the vagina as far as the cervix. Then by sucking on the free end of the rubber tubing and moving the pipette backward and forward in the vagina, one can loosen a portion of mucus and draw it into the pipette. The suction is maintained as the pipette is withdrawn from the vagina; a small, sterile rubber stopper is inserted into the pipette and a label identifying the animal is attached. In the laboratory, the mucus is forced from the pipette into a test tube by applying pressure on the cotton wool plug at the straight end of the pipette with a length of flexible wire.

Hoerlein and Kramer (7) describe the collection of cervical mucus using sterile artificial insemination pipettes (large bore 1.5 ml) introduced through a 12-in-long (8-mm diameter) Pyrex® speculum. The mucus is drawn into the pipette by means of a small syringe attached by a short length of rubber tubing. If mucus cannot be cultured within 4 hr after collection, it is recommended that it be frozen on dry ice while in transit to the laboratory.

Mucus Agglutination Test

For the vaginal mucus agglutination test, 0.2–0.3 g of mucus are extracted overnight with seven times its volume of phenolized (0.37%) physiological saline. A four-tube final dilution series (1/32–1/256) is recommended. The antigen and the test is prepared according to the method described by Moynihan and Stovell (12). A serum agglutination test for the diagnosis of genital campylobacteriosis has been described but is unreliable.

Collection of Preputial Washings

Bartlett and others (8) suggest the following method. A plastic or Perspex® glass pipette of approximately 8 mm outside diameter and 1.5-mm-thick wall is used. It should be about 21 in long with a 15° bend in the tube 3 in from the end, to which a 2-oz rubber bulb is attached. The other end is shaped so that a blunt chisel edge is formed, the bevel being on the same side as the reflex angle located near the opposite end of the tube (3).

Sterile pipettes should be used for each bull. Samples are obtained by inserting the pipette into the prepuce and surrounding preputial membrane with the chisel edge, and using the rubber bulb to suck into the pipette the 1 ml or so of smegma which can be obtained. The pipette is flushed gently with 4 ml of sterile saline on withdrawal from the prepuce.

Preparation of Transport Medium

The transport medium of Clark and Dufty (9) allows excellent recovery of *C. fetus* and is convenient because it can be transported in air at room temperature for 2–3 days without loss of organisms. The medium consists of freshly prepared bovine serum with 300 μg 5-fluorouracil, 100 units polymixin B sulfate, 50 μg brilliant green, 3 μg of nalidixic acid, and 100 μg of cycloheximide per ml. It is dispensed in 10-ml quantities in widemouth vaccine bottles (30-ml capacity) and the rubber stoppers inserted. The bottles are placed for 2 min in a boiling water bath so that the serum solidifies. After cooling, the medium is dispersed by stirring with a sterile glass rod. An 18-gauge needle is inserted through the firmly fixed rubber stoppers, the vials placed in an anaerobe jar, and the air evacuated and replaced with a mixture of 2.5% O_2, 10% CO_2, and the balance N_2. On opening the jar the needles are immediately removed and the vials then stored at 4°C for up to 3 months. The vials should be stored for 1 week at 4°C before use; during this time the medium becomes dark green in colour.

Inoculation of Preputial Fluid into Transport Medium

Preputial fluids are washed into 4 ml of physiological saline. The sample is let stand for 15–20 min to allow epithelial cells to settle and then 1 ml of supernatant liquid withdrawn by sterile syringe and injected

through the rubber stopper of the vial. Duplicate vials are recommended. The sample is mixed into the medium by shaking. The vial is then transported to the laboratory at temperatures between 18–37°C (*not* cooled). At the laboratory the container is incubated at 37°C for 4 days. Then 2–3 ml of physiological saline is mixed thoroughly with the sample and all fluid removed by pipette to a sterile tube. The fluid is then examined for *C. fetus* by culture and immunofluorescence. For culture the fluid is filtered through a 0.65-μm Millipore® filter as described below, or swabbed directly onto the plates.

Isolation Procedures

Filtration is recommended to reduce contaminating bacteria. After prefiltration to remove debris, preputial fluid, cervical mucus, or enrichment broth is filtered through a Millipore® filter, pore size 0.65 μm. A 0.1-ml volume is distributed over the surface of solid media using a swab.

Isolation media should be rich. Examples are brucella, serum dextrose (Oxoid), Columbia, or brain–heart infusion agar, with 5–10% blood. The basal media are available as dry powders from suppliers such as BBL, Difco, or Oxoid (except serum dextrose agar). A plate of both nonselective and selective medium should be inoculated with each sample. Media is made selective by the addition of polymixin B sulfate (2 units per ml), novobiocin (2 μg per ml), and cycloheximide (20 μg per ml).

Plates are incubated at 37°C for 4–6 days under a microaerophilic atmosphere of 6% 0_2, 10% CO_2, and 84% N_2. This atmosphere can be obtained ready-mixed from gas suppliers. An alternative is the use of a CampyPak II (BBL) gas generator envelope in an anaerobic jar with the palladium catalyst. An anaerobic gas-generating envelope in an anaerobic jar without the catalyst may be satisfactory. The use of 10% CO_2 in air is not satisfactory; the O_2 content must be reduced. A candle jar is also not satisfactory. Other methods of producing a microaerophilic environment are described under *C. jejuni*.

Identification

Campylobacter fetus produces fine, nonhemolytic, round, 1-mm, slightly raised, smooth, translucent colonies. A few colonies may be rough or granular. Colonies contain gram-negative, motile, curved rods (Fig. 6-1). Some cultures may show long filaments. *Campylobacter fetus* can be readily distinguished from *C. sputorum* subsp. *bubulus*, a saprophyte, by FA staining of smears from colonies or by biochemical tests (Table 6-1). Tests for growth in glycine and for growth temperature effect should be done in thioglycollate medium and for H_2S production by the lead acetate strip method after heavily inoculating semisolid (0.16% agar) brain–heart infusion or brucella broth, each

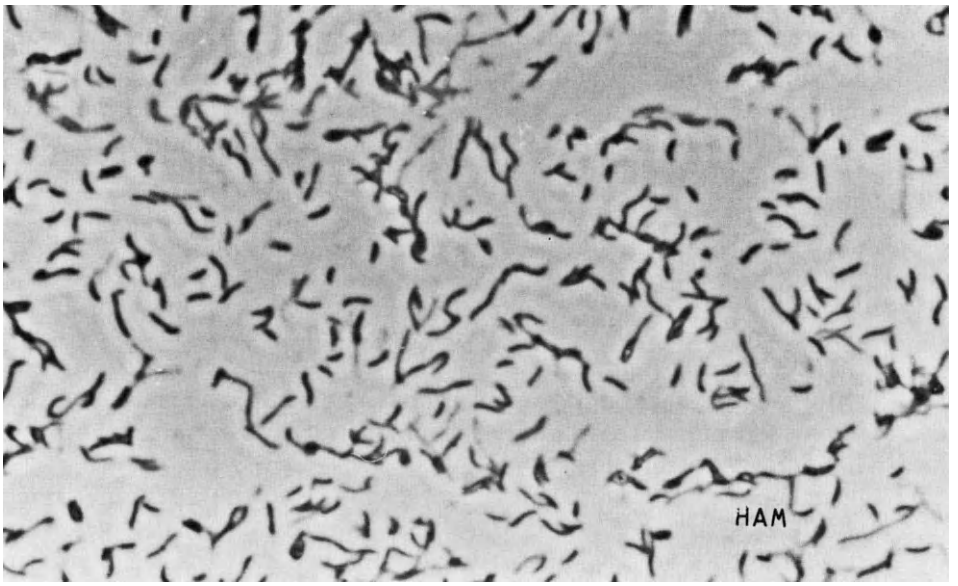

Figure 6-1. *Campylobacter fetus* subsp. *fetus* from a blood agar plate culture. Darkphase illumination, ×2440 (H. A. McAllister).

supplemented with 0.02% cysteine and incubated for 4–6 days. The *intermedius* biotype of subsp. *venerealis* gives a positive test for H_2S in this medium.

Karmali and others (10) have reported distinguishing the two subspecies of *C. fetus* (and also *C. jejuni*) by measurement of cell size and the wavelength and amplitude of the spirals. A trained observer can distinguish the three organisms by size.

Atypical *C. fetus* subsp. *fetus* isolate from humans have been described in which strains grew at 42°C, were H_2S negative, or were resistant to cephalothin (11).

Campylobacter jejuni

Classification and Pathogenicity

Campylobacter jejuni is a common and important cause of acute diarrhea in people, and an important zoonosis. Infection in humans have been reported to be acquired, directly or indirectly, from all common domestic animals. *Campylobacter jejuni* is established as a cause of diarrheic illness, which is usually mild, in a wide variety of animal species including cattle, dogs, cats, pigs, lambs, mink, and ferrets.

Apart from enteric disease the organism is a cause of abortion in many species and of vibrionic hepatitis of laying chickens. The organism can be isolated from feces or intestinal tracts of many endothermic animals, particularly birds and mammals kept under conditions of poor hygiene. Its carriage by healthy animals thus makes a diagnosis of *C. jejuni* enteritis based solely on isolation of the organism somewhat dubious.

Laboratory Procedures

Direct Examination

Diluted fecal smears may be examined by darkfield or phase-contrast microscopy for large numbers of characteristically darting, motile, corkscrewed organisms.

In aborted fetuses the organism can be shown in Gram-stained smears of stomach contents as a slender curved rod, usually present in large numbers. In chickens with vibrionic hepatitis the organism can be demonstrated in the bile by Gram staining or by phase-contrast microscopy after diluting bile 1:1 with saline.

Isolation Procedures

Rectal swabs or feces are suitable for culture and should be stored at 4°C before plating. If feces are not cultured soon, storage of swabs in Cary Blair medium at 4°C is recommended (13). This medium is made by adding 1.5 g sodium thioglycollate, 1.1 g Na_2HPO_4, 5 g Nacl, and 1.6 g agar to 991 ml of water. Heat until the agar is dissolved, cool to 50°C, and add 9 ml of a fresh solution of 1% $CaCl_2$. Adjust to pH 8.4 and steam heat for 15 min. Store at 4°C.

Fecal specimens should be inoculated onto selective plate media as soon as possible; selective media is not, however, necessary for isolation of *C. jejuni* from fetuses, placenta or chickens with vibrionic hepatitis.

Several selective media have been described (13), and Blaser's Campy-BAP medium has been preferred. It is available commercially. The medium contains brucella agar base (BBL), 5% sheep blood, and the following antibiotics: vancomycin (10 μg per ml), polymixin B sulfate (2–5 units per ml), trimethoprim lactate (5 μg per ml), cephalothin (15 μg per ml), and amphotericin B (2 μg per ml). Isolation of *C. jejuni* is improved if more than one type of selective plate medium is used (14).

Media incorporating cefoperazone as a selective agent, however, appear to be superior to Blaser's Campy-BAP (14). Examples are Bolton and Hutchinson's charcoal–cefoperazone–deoxycholate agar and Karmali's charcoal-based medium (15,16). Preenrichment with selective

broth is useful for specimens containing small numbers of *Campylobacter*, such as environmental or milk samples, but enrichment is not recommended for routine use in isolation from intestinal tracts or feces. Some selective media for *C. jejuni* contain a cephalosporin and are thus unsuitable for isolating *C. fetus* or *C. hyointestinalis*. This has led to an underestimation of the prevalence of these species in diarrheic stools.

Plates should be incubated in evacuatable anaerobe jars under an atmosphere of approximately 6% O_2, 10% CO_2, and 84% N_2. Such gas mixtures are available commercially. The jars should be evacuated and then filled with the gas mixture on three occasions. Alternative means of producing suitable microaerophilic conditions are the use of anaerobe jars with disposable gas-generating envelopes specifically designed to yield microaerophilic conditions, such as Campy-Pak (BBL). Candle jars are least preferable but if they are to be used they must be incubated at 42°C. Hydrogen stimulates the growth of *C. jejuni* and *C. coli*, explaining in part why a candle jar is not recommended. Maximum isolation of *Campylobacter* is ensured if no more than six plates are incubated in each jar.

For laboratories without access to anaerobe jars, suitable conditions can be gained by incubating the selective plate with a second blood agar plate streaked with a pure culture of a facultative anaerobe, such as *Proteus*. If the two plates are incubated in an airtight plastic bag the facultative anaerobe will lower oxygen concentrations and produce a suitable environment (17).

Campylobacter jejuni grows best at 42°C and this temperature should be used; 37°C is suboptimal. Plates should be incubated for 48 hr and then examined; reexamination at 72 hr will improve yield slightly.

Colonies are usually flat, nonhemolytic, watery, gray (sometimes with a pink tinge), spreading and often large. At times they may appear like drops of water, spreading along the streak marks. If plates are not moist, then colonies do not spread, but rather will appear as discrete (2 mm), convex, raised, round, and mucoid.

Identification

Campylobacter jejuni appear as slender, curved-to-spiral gram-negative rods, 0.2–0.5 μm × 1.5–5 μm. Colonies older than 48 hr or those exposed to air for several hours show coccoid transformation, although some typical corkscrew *Campylobacter* will still be present. *Campylobacter jejuni* is cytochrome oxidase and catalase positive and shows motility if saline hanging drops are made of plate cultures and examined by darkfield or phase-contrast microscopy.

Criteria necessary to confirm identity as *C. jejuni* (Table 6-1) are growth at 42°C but not 25°C, sensitivity to nalidixic acid but

resistance to cephalothin, and a positive hippurate hydrolysis test. Cultures grow better at 42°C than at 37°C.

The rapid hippurate hydrolysis test generally distinguishes *C. jejuni* from *C. coli* (18). For this test a 1% aqueous solution of sodium hippurate is prepared and distributed into glass tubes in 0.4-ml amounts, capped and frozen at −15°C or lower until use. After thawing, a large loopful of organisms from a 24–48-hr culture is emulsified in the substrate and incubated for 2 hr at 37°C. Then 0.2 ml of a ninhydrin solution is added and tubes observed after 10 min at 37°C for development of a deep purple colour. Ninhydrin solution is prepared by adding 3.5 g of ninhydrin in 100 ml of a 1:1 mixture of acetone and butanol. Hippurate hydrolysis is not, however, fully reliable in differentiating *C. jejuni* from *C. coli*, even using more sensitive techniques than rapid tube methods (19).

Biotypes of *C. jejuni* have been described (20). For epidemiological purposes, *C. jejuni* and *C. coli* can also be typed antigenically. The methods of Penner with heat-stable antigens (21) and of Lior with heat-labile antigens (22) are widely accepted.

In view of the presence of *C. jejuni* in the intestines of many healthy animals, their recovery from diarrheic animals is not convincing evidence of their role in disease. If required, demonstration of a rising agglutinating antibody titre (fourfold increase) would confirm their involvement (23).

Campylobacter coli

Campylobacter coli is occasionally a cause of *Campylobacter* enteritis in people. It is found commonly in the intestines of pigs, where it may be an unimportant cause of mild diarrheic illness. At one time it was thought to be the cause of swine dysentery, now known to be caused by *Treponema hyodysenteriae.*

Isolation and identification is as described for *C. jejuni,* with the exception that *C. coli* is hippurate negative (Table 6-1). The isolation of cephalothin-susceptible *C. coli* has been described (24). Roop and others suggested that H_2S production in TSI medium and growth in a minimal medium also usefully distinguished *C. coli* from *C. jejuni* (25).

Campylobacter fecalis

"Campylobacter fecalis" has been isolated from ovine feces and is regarded as a nonpathogen but may be found as a contaminant in bovine semen and vaginas. It has been reported to cause enteritis experimen-

tally in cattle (23), although the organism used in the experiment may have been misidentified. It differs from *C. jejuni* or *C. coli* in production of large quantities of H_2S in TSI medium and variable resistance to 3.5% NaCl, as well as ability to reduce nitrate to nitrite (26). Roop and others suggested that *"C. faecalis"* is a biovar of *C. sputorum* (27).

Campylobacter cryaerophila

Occurrence and Pathogenicity

Ellis, Neill, and O'Brien and others have isolated *Campylobacter* other than *C. fetus* subspecies from aborted bovine and porcine fetuses (28), and more recently from bovine mastitis (29). It has also been isolated from the prepuce of a bull (30). The role of these organisms in abortion in animals needs to be defined. They are isolated microaerophilically, usually in media used to isolate *Leptospira*, but grow readily in air on subculture.

Laboratory Procedures

Isolation Procedures

The method described is that of Neill (31). Leptospira EMJH (Ellinghausen, McCullough, Johnson, and Harris) medium with 100 μg/ml of 5-fluorouracil with added 1% rabbit serum and 0.15% agar is used as an initial enrichment medium; 0.25 ml of fluids (e.g., fetal fluids, preputial or vaginal washings) are inoculated directly into 2 ml of this medium. Fetal tissues or placentas can be homogenized (10% w/v) in quarter-strength Ringer's solution and 0.25 ml of suspension inoculated. The inoculum is placed in the bottom part of the enrichment broth. This is done gently to avoid undue aeration of the medium.

The medium is incubated in air at 30°C and growth of microaerophilic organisms is detected as a distinct zone beneath the surface. A wet mount is prepared after 48 hr and examined by darkfield or phase-contrast for the characteristically shaped and motile *Campylobacter*. Growth generally occurs within 2 weeks, but cultures should not be discarded for 5 weeks. Growth may sometimes be detected microscopically rather than macroscopically.

The second stage in isolation requires that the enrichment broth be subcultured to an enriched agar medium (e.g., Oxoid No. 2, brucella broth, brain–heart infusion) with 7% lysed horse blood and carbenicillin (125 μg per ml). Plates are inoculated with material drawn from the microaerophilic zone beneath the broth surface by Pasteur pipette and streaked in the usual manner. Plates are incubated at 30°C in a microaerophilic environment (see *C. jejuni*) for 48–72 hr.

Identification

Colonies are 1 mm, nonpigmented, and convex, but become more irregular on subculture. Once the organism has been recovered on solid medium at 30°C microaerophically, it can then be subcultured aerobically at 37°C, although 30°C gives optimal growth. Only a small proportion of isolations will be made directly on solid media without going through the enrichment procedure. Organisms have typical *Campylobacter* morphology and are catalase and cytochrome oxidase positive (Table 6-1). They do not react with antisera prepared against *C. fetus*. They differ from *C. fetus* (Table 6-1) in their ability to grow in air without added CO_2 and in their sensitivity to nalidixic acid, among other characteristics (32).

Campylobacter hyointestinalis

Gebhart and others (33) isolated *Campylobacter hyointestinalis* from swine with lesions of proliferative ileitis, but it has not been shown that this organism is the cause of the disease (34). The organism has been isolated from the intestine of other species, including cattle, hamsters, and humans. The organism is a microaerophilic curved rod, which grows well at 25°C but poorly at 43°C; 2-mm circular, convex, slightly mucoid, yellow colonies are produced at 48 hr. Differentiating characteristic are shown in Table 6-1.

Campylobacter mucosalis

Occurrence and Pathogenicity

This organism has been isolated from the lesions of porcine intestinal adenomatosis and its manifestations. The other manifestations of the infection are proliferative hemorrhagic enteropathy, necrotic ileitis, and regional ileitis. In these infections, large numbers of slender *Campylobacter* are seen on silver stains within the apical cytoplasm of the proliferating epithelial cells. Failure to reproduce the disease with pure cultures of the organism, its widespread distribution in pigs, and the recovery of *Campylobacter hyointestinalis* from the lesions has led to doubt about its etiological importance. There is also doubt about the etiologic role of *C. hyointestinalis* in this disease.

With the current uncertainty as to the etiological role of *C. mucosalis* and because of difficulty in its isolation, diagnosis of intestinal adenomatosis is best made by histopathological examination and use of Warthin–Starry silver staining, or by direct examination.

Laboratory Procedures

Direct Examination: Modified Acidfast Stain

Heat-fixed smears are made from mucosal scrapings of affected areas of intestine. Smears are stained with 5% freshly diluted carbolfuchsin for 5 min, decolorized with 0.5% acetic acid for 30 sec, and counterstained with 1% methylene blue for 10 sec. Examination shows large numbers of bright pink-staining, very slender, curved rods, predominantly intracellularly (33). Some *Campylobacter* will be seen unassociated with cells but the diagnosis should be made on the presence of intracellular tangled clumps of organisms.

Isolation Procedures

Mucosal surfaces of affected intestine are washed repeatedly in sterile saline to remove extracellular organisms. The mucosa is then scraped with a sterile scalpel and cells in the scrapings washed five times in 20 volumes of nutrient broth, centrifuging at 1000 *g* for 10 min between washings. After the final washing the cells are suspended in 10 volumes of nutrient broth and homogenized in a glass Ten Broeck homogenizer. Homogenates are plated onto Columbia blood agar, with and without 1:60,000 brilliant green as a selective agent. Plates are incubated at 37°C for 2–5 days anaerobically, which gives better isolation results than microaerophilic incubation, possibly because of the organism's requirement for hydrogen. Once the organism has been recovered it will grow readily microaerophilically.

Identification

Colonies are about 1.5 mm at 48 hr and are circular and raised, with a flat surface and shiny grey appearance. Scrapings on the bacteriological loop appear dirty yellow; *C. coli* shows a pink-tan pigment. They are cytochrome oxidase positive, although the test may take 60 sec to be positive. In young cultures strains are gram-negative, short, irregularly curved rods 0.25–0.30 μm × 0.95–2.8 μm. Older cultures showed more coccoid and filamentous forms. Differentiation of the catalase-negative *Campylobacter* is shown in Table 6–2. Slide agglutination using antisera prepared in rabbits against one of the three serological types will allow rapid diagnosis; type A is the most frequently encountered serological type (36). A detailed description is available (37).

Campylobacter uppsaliensis

Thermotolerant *Campylobacter* with no or weak catalase activity have been isolated from healthy and diarrheic dogs (38) and humans. Isolation is generally on media and under conditions used for *C. jejuni*.

Identifying properties are shown in Table 6-2 Isolates are microaerophilic, thermotolerant, and hippurate negative, produce H_2S in the lead acetate test but not in triple sugar iron, and reduce nitrate. This organism is strongly alkaline phosphatase positive (27).

Campylobacter sputorum subsp. *bubulus*

A nonpathogenic saprophyte of the bovine genital tract which must be distinguished from *C. fetus*.

Sheep Weaner Colitis *Campylobacter*

Stephens and colleagues have described the involvement of a catalase-negative *Campylobacter* in a syndrome of weaner colitis of sheep (39,40). Isolations were improved by increasing the pH of Mycoplasma agar base with 5.0% horse blood to 8.0 (38). Strains grew at 42°C but not at 25°C, failed to hydrolyze hippurate and to produce hydrogen sulfide and were resistant to nalidixic acid; most were sensitive to cephalothin. Isolations were made only under hydrogen-enriched atmospheric conditions (65% H_2, 10% CO_2, 10% 0_2).

Campylobacter pylori

Campylobacter pylori has a strong association with gastritis and pyloric ulcers in humans and has only been isolated from the stomach of people (41,42). Nevertheless, *Campylobacter*-like organisms have been identified in the stomachs of ferrets (41), and it may be worth looking for them in lesions of gastritis or ulceration in other species.

Campylobacter pylori is difficult to isolate. Brain–heart infusion agar with 7% horse blood is recommended, both as a nonselective medium and made selective with nalidixic acid (10 μg/ml), trimethoprim (5 μg/ml), vancomycin (3 μg/ml), and amphotericin (2 μg/ml). Plates must be kept very moist by storage in an airtight container after pouring. Culture should be at 37°C under 10% CO_2 or microaerophilic conditions; *C. pylori* grow as sparse, transparent 1-mm colonies after 3–6 days, and plates can be discarded after 6 days.

Atypical *Campylobacter* Isolates

In addition to newly described species of *Campylobacter* such as *C. fennellae* and *C. cinaedi*, isolated from humans, *Campylobacter* that cannot be identified using the criteria listed in Tables 6-1 and 6-2 may

be isolated in veterinary laboratories. This may be either because biochemical properties are atypical of existing species or because these are new species (42). Such isolates should be referred to reference laboratories.

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7

Pseudomonas, Aeromonas, Plesiomonas, and *Vibrio*

G. R. Carter

The genera *Aeromonas, Plesiomonas,* and *Vibrio* belong in the family Vibrionaceae. Species of these genera are gram-negative, aerobic and facultatively anaerobic, motile by polar flagella, oxidase positive (most species), and some have a curved cellular morphology *(Vibrio).* All occur widely in nature including freshwater and seawater and are also frequently associated with animals. Characteristics that differentiate *Aeromonas, Plesiomonas,* and *Vibrio* are given in Table 7-1.

Pseudomonads

Organisms of the genus *Pseudomonas* are aerobic, nonsporing, glucose nonfermenting, gram-negative rods. They are catalase positive and split sugars by oxidation; all have polar flagella except *Pseudomonas mallei,* which is nonmotile. Only the three species listed immediately below are recognized as important pathogens or potential pathogens of animals. Several other species, listed in Table 7-1, occur occasionally in clinical specimens but rarely cause disease.

Pseudomonas aeruginosa. This important potential pathogen occurs widely in nature, especially on mucous membranes.

P. pseudomallei. This potential pathogen is found in soil and water in Southeast Asia, Australia, and central Africa.

P. mallei. This species differs from the others in that it is an obligate pathogen that does not occur in nature. In previous years, it has been included in different genera, including *Actinobacillus.*

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Table 7-1
Differential Characteristics of Some Oxidase-Positive Fermentative Gram-Negative Genera[a]

Differential feature	*Aeromonas*	*Plesiomonas*	*Vibrio*
Susceptibility to 0/129 (Oxoid)			
10 μg	−	+/−	+/−
150 μg	−	+	+
Acid from			
Sucrose	+	−	+/−
Mannitol	+	−	+
Inositol	−	+	−
Gelatin liquefaction	+	−	+
Growth in nutrient broth without NaCl	+	+	−[b]

[a]Adapted from Finegold, S. M., and Baron, E. L. (1986) (7). Vibrionaceae family. In *Diagnostic Microbiology*, 7th ed. St. Louis, Missouri, Mosby, 1986, Chapter 30.
[b]Exceptions are *V. cholerae* and *V. minicus*.

Pseudomonas aeruginosa

Pathogenicity

P. aeruginosa is a frequent opportunistic pathogen. Because of its relative resistance to drugs, it may persist in infectious processes from which other more susceptible organisms have been eliminated by treatment. This organism has been reported responsible for wound infections in various animals species, listed as an occasional cause of bovine mastitis and abscesses, and incriminated in abortions in cows and mares, endocarditis in dogs, otorrhea in dogs and cats, septicemia in chickens, and in urinary tract infections and a variety of other infections in animals and humans. It is an important nosocomial agent in humans and animals. Both human and animal patients with metabolic, immunologic, and malignant disease are particularly susceptible. Because of its widespread occurrence in the environment, it is a frequent contaminant, and consequently its recovery from clinical specimens is not always significant.

Pseudomonas pseudomallei

Pathogenicity

The disease in humans is referred to as melioidosis or pseudoglanders. It may assume a benign, chronic, or septicemic form in humans and animals. Infections have been reported in humans, primates, cattle, sheep, goats, pigs, horses, dogs, cats, and rodents. In the more common

chronic form, nodules and abscesses occur in the lungs, liver, spleen, lymph nodes, and subcutis.

Pseudomonas mallei

Pathogenicity

P. mallei is the cause of glanders, a disease principally of the Equidae. It has been eradicated from North America and from central and western Europe, but it still occurs in eastern Europe and Asia. The disease in the horse, mule, and donkey has three forms: pulmonary glanders, nasal glanders, and cutaneous glanders or farcy. Infections are characterized by the formation of encapsulated nodules that contain yellow caseous pus. Carnivores and humans become infected as a result of contact with infectious materials. Swine, cattle, rats, and birds are considered to be resistant.

Other Pseudomonads

There are numerous free-living pseudomonads. They occur occasionally as contaminants in clinical materials. Several species other than *P. aeruginosa* have been incriminated in infrequent infections, mostly in humans. The most common of these, *P. maltophilia, P. stutzeri, P. fluorescens, P. acidovorans,* and *P. putida,* are usually contaminants in clinical specimens. Readers should consult the Supplementary Readings for information on other species.

Isolation Procedures

Pseudomonas spp. grow well on blood, tryptose, or trypticase soy agar, and on less complex media. They may be recovered on various enteric media. Glycerine stimulates the growth of *P. mallei,* and for this reason, a glycerol agar is sometimes used. *P. mallei* grows slowly on blood agar. Several of the less frequently occurring pseudomonads grow poorly at 37°C.

If glanders is suspected, pus or caseous material is removed from a nodule aseptically and inoculated onto blood agar. The plates are placed in a sealed container to prevent dehydration, incubated up to 9 days, and examined daily.

Selective Media

A medium for the recovery of *P. mallei* from contaminated materials has been described by Miller *et al.* (1).

Animal Inoculation

Hamsters and guinea pigs are highly susceptible to *P. mallei*. The guinea pig is favored because of the well-known *Straus's phenomenon*. Cultures are inoculated into male guinea pigs intraperitoneally, and pathological material is inoculated subcutaneously. The glanders organisms produce a septic orchitis, Straus's phenomenon, in 3–4 days. The organism is readily recovered from the testicle. Lesions are also found in the spleen, liver, and other visceral organs.

Cultural Characteristics

Pseudomonas aeruginosa

Colonies are large and grayish with irregular spreading margins. Some cultures are markedly mucoid. On blood agar, colonies are frequently β-hemolytic. Cultures have a characteristic fruitlike odor. Greenish and/or yellowish-green pigments may diffuse throughout clear media. On MacConkey and SS agar, colonies are colorless, while on brilliant green agar, they are red.

P. aeruginosa produces two pigments: pyocyanin, which is green or blue-green and water and chloroform soluble, and fluorescein (pyoverdin), which is water soluble, chloroform insoluble, yellow to green in color, and fluoresces under ultraviolet light. A similar pigment is produced by *P. fluorescens*, a nonpathogenic organism that does not usually grow at 37°C. Some strains of *P. aeruginosa* produce the pigments pyorubin (red) and pyomelanin (brown-black).

P. maltophilia

Colonies are round, smooth, nonpigmented, and glistening, with regular margins. They are not hemolytic but may display a greenish discoloration of blood agar.

P. pseudomallei

Colonies are evident after 24-hr incubation on blood, tryptose, or trypticase soy agar. After 48-hr incubation, colonies obtain a size of 1–2 mm in diameter and are smooth, umbonate, and cream colored. Colonies enlarge markedly when left at room temperature for about a week. Cultures have an earthy or ammoniacal odor.

P. mallei

Colonies are shallow, round convex, opaque becoming yellowish green or brown on aging. Growth is slow on blood agar. The colonies have a tendency to be slimy and tenacious in consistency.

Identification

The species referred to above, with the exception of *P. mallei,* are motile, and stained smears disclose gram-negative, straight or slightly curved rods.

P. aeruginosa

Characteristic colonies, sometimes with a metallic sheen, are usually β-hemolytic. A blue-green or yellowish-green pigment is often produced in clear media. Other characteristics include alkaline reactions on TSI with no gas or H_2S produced, positive oxidase reaction, and glucose used oxidatively in the OF test.

Other reactions include the following:

- Nitrates are reduced to nitrites.
- Indole is not produced.
- Gelatin is liquefied.
- Litmus milk first becomes alkaline, followed by coagulation and peptonization.
- Alkaline reaction is seen in TSI agar.

A selective medium, Cetrimide, is available for the isolation of *P. aeruginosa;* other pseudomonads are generally inhibited.

To demonstrate pyocyanin production, infusion broth or a tryptose agar slant is inoculated and incubated overnight. If the organism is *P. aeruginosa,* the bluish-green pigment pyocyanin may diffuse into the media. Identity of the pigment as pyocyanin is confirmed by the chloroform solubility test. Add 1–2 ml of chloroform to the broth culture or the slant culture and shake or agitate. If pyocyanin is present, it will be evident in the chloroform layer. Not all strains of *P. aeruginosa* produce pyocyanin. This test is not usually carried out as routine procedure. The production of fluorescein is enhanced on *Pseudomonas* agar F and Flo agar. Only a small number of strains produce pyorubin or pyomelanin. These pigments may obscure pyocyanin production.

Serotyping and pyocin typing have been used mainly for epidemiological studies and investigations involving humans. The bacteriocins of *P. aeruginosa* are called pyocins, and pyocin typing has also been useful in epidemiological studies (3).

Characteristics that separate *P. aeruginosa* from other *Pseudomonas* spp. are summarized in Table 7-2.

***P. maltophilia, P. pseudomallei,* and some other pseudomonads**

See Table 7-2 for identification.

Table 7-2
Differentiation of Some *Pseudomonas* Species[a]

	Oxidase	Growth at 42°C	Gelatin hydrolysis	Acid O-F glucose	Acid O-F lactose	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	Other
P. aeruginosa	+	+	+ or −	+	−	−	+	−	Most strains: pyocyanin & fluorescein
P. maltophilia	−	+ or −	+	+	(+)	+	−	−	Brown pigment
P. cepacia	+	+ or −	(+)	+	+	(+)	−	(+)	
P. stutzeri	+	(+)	−	+	−	−	−	−	Gas from nitrate
P. putida	+	−	−	+	(−)	−	+	−	Some strains: fluorescein
P. acidovorans	+	(−)	−	−	−	−	−	−	
P. putrefaciens	+	(+)	+	+	−	−	−	+	
P. paucimobolis	(+)	−	−	+	+	−	−	−	
P. alcaligenes	+	+ or −	−	−	−	−	(−)	−	
P. fluorescens	+	−	+	+	(−)	−	+	−	Fluorescein
P. pseudomallei	d	−	d	+	NA	−	+	−	Pigment: white to yellow

[a]Symbols: +, >90% of strains; −, <90%; (+), >50%; (−), <50%; d, different reactions; NA, not available.

P. mallei

Identification is based on the features listed below. The production of the characteristic infection in the guinea pig is confirmatory.

- Nonmotile
- Growth is enhanced by glycerin.
- Growth on glycerol potato agar is characteristic: A yellow viscid growth appears, which progresses to a yellowish-brown and ultimately a dark brown.
- Growth on blood agar is relatively slow.
- Carbohydrates for the most part are not broken down, although several may be split by oxidation.
- Slight acidity in litmus milk
- Indole is not produced; oxidase test is negative.
- Small amounts of H_2S, catalase, and ammonia are produced.
- Nitrate is reduced.
- Gelatin is not liquefied.

Aeromonads and Plesiomonads

These facultatively anaerobic, gram-negative rods with polar flagella (except *A. salmonicida*) are recovered occasionally from clinical specimens and feces of animals and humans. They occur widely in water, sewage, and soil. The name *Plesiomonas shigelloides* has replaced *Aeromonas shigelloides.* The former is the only species in the genus *Plesiomonas.* These genera and *Vibrio* are distinguished by the characteristics listed in Table 7-1.

The three species, *A. hydrophila, A. salmonicida,* and *P. shigelloides* are only occasionally clinically significant. There are no reports of the other *Aeromonas* species causing significant infections in animals.

Pathogenicity

Aeromonas hydrophila and *P. shigelloides* have been isolated from several of the following human specimens: normal stools, bile, blood, throat swabs, osteomyelitic pus, feces from cases of dysentery and gastroenteritis, and cerebrospinal fluid (4,5). *Aeromonas hydrophila* is a pathogen of reptiles, fish, and amphibians, while *A. salmonicida* causes disease in fish (see Chapter 37).

Reports of the isolation of these organisms from animals are scant. It would seem that, as in humans, *Aeromonas* can, on occasions, account for infections in animals. The following are among the sources

of *Aeromonas* that have been identified in the laboratories of the editors:

A. hydrophila	*A. salmonicida*	*P. shigelloides*
Various avian species, cattle, swine, dog, horse; wild, zoo, and laboratory animals	None	Penguin, cattle, swine, dog

They were isolated from a variety of specimens, and fewer than 5% of the isolations were obtained in pure culture.

Isolation Procedures

The organisms grow well on the common laboratory media, including media used for the enteric bacteria. Although they grow best at room temperature or lower, most can be cultivated satisfactorily at 37°C.

Cultural Characteristics

Aeromonas strains initially produce grayish-white, translucent, moist, and stippled colonies. The appearance of colonies on enteric media varies with the capacity and rapidity with which strains ferment lactose. Some strains of *A. hydrophila* produce a soluble brown melaninlike pigment in 2–3 days on infusion agar.

Identification

As mentioned above, *Aeromonas* and *Plesiomonas* colonies resemble those of enterobacteria. They can be distinguished from the latter by their capacity to produce oxidase. Reactions on TSI are given in Table 10-4. Many strains of *A. hydrophila* are β-hemolytic. The various species are identified by the characteristics listed in Table 7-3.

Vibrio

This genus belongs in the family *Vibrionaceae*. It includes *Vibrio cholerae*, the cause of human cholera, and a number of species that are recovered occasionally from human clinical specimens. They are found in seawater and fresh water, where they can cause disease in aquatic animals. One species, *V. parahaemolyticus*, can cause human intestinal disease after the consumption of sea food.

Only one species, *V. metschnikovii*, has been reported to cause an infrequent disease of young birds and poultry. The disease, which does

Table 7-3
Some Differential Characteristics of *Aeromonas* and *Plesiomonas* Species

	KCN broth	Esculin hydrolysis	Decarboxylases		Arginine dihydrolase	Flagella	Inositol	Mannitol
			Lysine	Ornithine				
A. hydrophila	+	+	+	−	d[a]	Monotrichous	−	+
A. salmonicida	−	+	d[a]	−	+	−	−	+
A. caviae	+	+	−	−	+	Monotrichous	−	+
A. sobria	−	−	+	−	+	Monotrichous	−	+
P. shigelloides	−	−	+	+	+	Lophotrichous	+	−

[a]d, Different reactions.

not appear to have been reported in North America, is characterized by the sudden onset of severe enteritis with diarrhea and high mortality. The disease and its laboratory diagnosis is discussed in detail by Peckham (6). Readers are referred to the Supplementary Readings for the description and identification of the various *Vibrio* spp. The genus *Vibrio* is differentiated from *Aeromonas* and *Plesiomonas* by the characteristics listed in Table 7-1.

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8

Bordetella and *Alcaligenes*

R. Martin Roop II

Members of the genera *Bordetella* and *Alcaligenes* are small, nonfermentative, gram-negative rods. They are catalase and oxidase positive. These two genera are discussed together because they have very similar biochemical characteristics and have a significant degree of genetic relatedness as measured by ribosomal RNA–DNA hybridization studies (1).

Pathogenicity

The two *Bordetella* species which are important as veterinary pathogens are *B. bronchiseptica* and *B. avium*. *Bordetella bronchiseptica* plays a major role in atrophic rhinitis in swine (2) and infectious tracheobronchitis (kennel cough) in dogs (3). This species can also cause severe bronchopneumonia in young pigs (2). In guinea pigs, *B. bronchiseptica* infection can result in epizootic pneumonia (4), which is characterized by high morbidity and mortality. *Bordetella bronchiseptica* has also been isolated from respiratory infections in cats, rabbits, and horses and, rarely, in humans. Occasionally *B. bronchiseptica* is isolated from wound infections in humans (5) and animals. *Bordetella avium* is believed to be the primary agent responsible for turkey coryza (6). It has also been isolated from the respiratory tracts of other avian species and may play a role in respiratory disease in chickens (7). *Bordetella pertussis* and *B. parapertussis* are associated with whooping cough and a respiratory disease characterized as a "mild form of whooping cough" in humans, respectively. Isolation of *B. parapertussis* from both healthy lambs and lambs with pneumonia has been reported (8). *Bordetella pertussis* has been isolated from a chimpanzee

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housed in a Swedish zoo, which showed clinical signs similar to whooping cough (M. Granstrom, personal communication). However, natural infection of animals with *B. pertussis* or *B. parapertussis* appears to be rare.

Alcaligenes species are saprophytic organisms which reside in the intestinal tract of vertebrates (9). They are not ordinarily pathogenic, but may play a role as an opportunistic invader, especially in a compromised host. It was once thought that *A. faecalis* played an important role in rhinotracheitis in turkeys and chickens (10). However, more extensive examination of the bacterial strains associated with this condition revealed that they were not *A. faecalis*. These strains were subsequently given the name *Bordetella avium* (6, see above). For epidemiologic purposes, it is imperative that *B. avium* and *A. faecalis* strains be properly identified and distinguished one from the other.

Isolation Procedures

A selective and differential medium described by Smith and Baskerville (11) is very useful for isolating both *B. bronchiseptica* and *B. avium* from nasal swabs, transtracheal washings, or other clinical samples that are likely to be contaminated. *Bordetella bronchiseptica* forms blue, convex, smooth colonies, 1–2 mm in diameter after 48–72 hr of growth on this medium. Fermentative organisms such as *Escherichia coli* or *Klebsiella* spp. that may occasionally grow on this medium form yellow colonies. Some *Alcaligenes* and *Pseudomonas* species may grow on this medium; however, most of these form green colonies that are easily distinguished from those of *B. bronchiseptica*. In comparative studies with nasal swabs from swine (12), the Smith–Baskerville (SB) medium appears to be superior to MacConkey agar supplemented with 1% glucose for isolating *B. bronchiseptica*. However, when attempting to isolate *B. bronchiseptica* from dogs or rabbits, Smith–Baskerville medium should be prepared without gentamycin, as some strains of *B. bronchiseptica* isolated from these animals have been reported to be sensitive to gentamycin (J. Abell and R. Adams, personal communication). SB and SB without antibiotics have also been used successfully for isolating *B. avium* from turkeys (13).

Blood agar plates (5% sheep or horse blood) are useful for culturing *B. bronchiseptica* from tissues that are unlikely to be heavily contaminated, such as lung tissue collected at necropsy. After 24–48 hr of growth at 37°C, *B. bronchiseptica* will form convex, smooth, colonies with an entire edge. Some isolates may be hemolytic. *Bordetella avium* forms two types of colonies on blood agar (6). Type I colonies are small (approximately 1 mm), pearl-like, and glistening, with an entire edge. Type II colonies are larger, circular, and convex, with an en-

tire edge and smooth surface. *Alcaligenes faecalis* also forms two types of colonies on blood agar (9). Type I *A. faecalis* colonies are approximately 0.5 mm in diameter, low, convex, and glistening, with an entire edge. Type II colonies are 1.0–1.5 mm in diameter, flat, and umbonate, with a spreading edge. Type II colonies are characteristic of *A. faecalis* strains that were formerly known as *"A. odorans."* These latter strains also produce a distinctive odor, which has been described as resembling the smell of freshly cut apples or strawberries. *Alcaligenes xylosidans* ssp. *xylosidans* forms smooth, glistening colonies with an entire edge, approximately 1 mm in diameter, while *A. xylosidans* ssp. *denitrificans* forms colonies similar to the type I colonies of *A. faecalis* (9). *Alcaligenes piechaudii* produces circular, smooth, entire colonies, which are nonhemolytic on 5% horse blood agar (14).

Bordetella bronchiseptica forms a distinctive type of colony on Bordet–Gengou (BG) agar (15), a blood-based medium which was designed for isolating *B. pertussis* (5). *Bordetella bronchiseptica* colonies on BG medium have a smooth colony surface with a domed, "mercury droplet" appearance and are approximately 1 mm in diameter after 48 hr of growth at 37°C. Most strains are hemolytic, but this characteristic is highly dependent upon the source of the blood used in preparing the medium. Colonial characteristics of *B. bronchiseptica* on BG are best described using the nomenclature of Peppler and Schrumpf (16), which takes into account colonial elevation, colonial texture, and hemolysis. For example, most freshly isolated strains of *B. bronchiseptica* will produce domed, smooth, hemolytic colonies, which would be described as $Dom^+Scs^+Hly^+$. Upon passage in the laboratory, variants will begin to appear that have the phenotypes $Dom^+Scs^+Hly^-$, $Dom^+Scs^-Hly^-$, $Dom^-Scs^+Hly^-$, and $Dom^-Scs^-Hly^-$. Variant colonies are usually bigger than $Dom^+Scs^+Hly^+$ colonies. This phenomenon is known as *phase variation* and is associated with a reduction in virulence. For this reason, it is important to use $Dom^+Scs^+Hly^+$ colonies when testing for virulence-associated properties of *B. bronchiseptica*. Furthermore, if serial passage of *B. bronchiseptica* in the laboratory is necessary, only $Dom^+Scs^+Hly^+$ colonies should be transferred.

Identification

Gram-stained preparations of *B. bronchiseptica* and *B. avium* reveal uniform, gram-negative rods approximately 0.3–0.5 μm in diameter and 1–2 μm in length (Fig. 8-1). *Alcaligenes* species appear as gram-negative rods or cocci, 0.5–1.0 μm in diameter and 0.5–2.6 μm in length. All of these organisms are oxidase positive, catalase positive, and produce an alkaline slant with an alkaline reaction or no reaction in the butt of a TSI slant. *Bordetella bronchiseptica*, *B. avium*, and

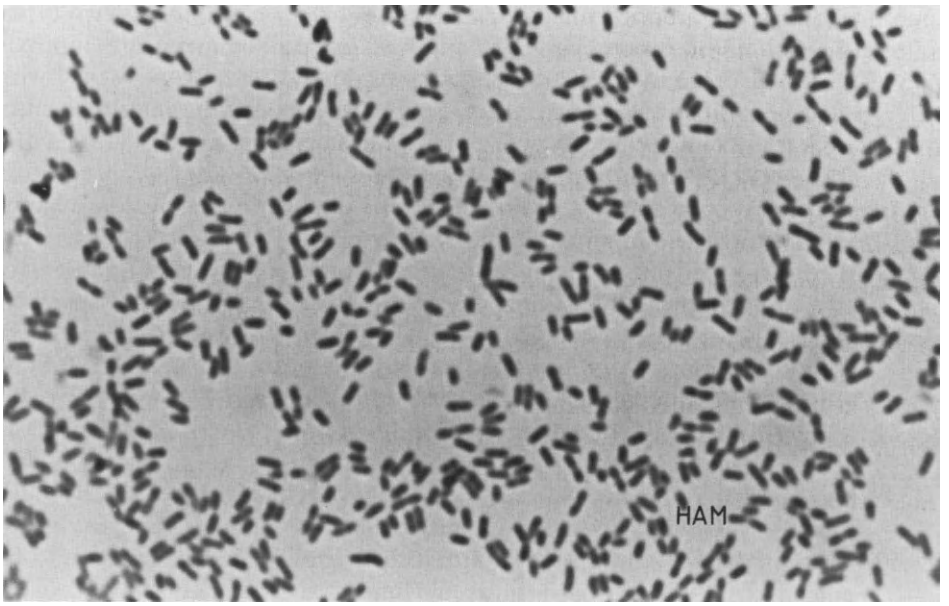

Figure 8-1. *Bordetella bronchiseptica* from a blood agar plate culture. Gram stain, ×2500 (H. A. McAllister).

all *Alcaligenes* species are motile. *Bordetella bronchiseptica* rapidly hydrolyzes urea (within 4 hr); *B. avium* and *Alcaligenes* species do not. Utilization of glycolate and malonate as sole carbon sources for growth reportedly distinguishes *A. faecalis* from *B. avium* (6). Differential characteristics for *B. bronchiseptica, B. avium,* and *Alcaligenes* species are given in Table 8-1. The API Rapid NFT system (Analytab Products, Plainview, New York) appears to be useful for identifying *B. bronchiseptica* and *Alcaligenes* isolates (17). Unfortunately, *B. avium* is not currently listed in the identification index. In the NFT system, *B. avium* strains assimilate adipate but not caprate, while *A. faecalis* strains assimilate caprate, but not adipate (17).

Two other groups of gram-negative, nonfermentative bacteria which might be confused with *Bordetella* and *Alcaligenes* species are members of CDC groups IVc-2 and IVe. These organisms have been isolated from human clinical samples (6), but their importance in veterinary medicine, if any, is presently unknown. Differential characteristics for these strains are also listed in Table 8-1. For more information on these organisms, the reader should consult Chapter 29 of the *Manual of Clinical Microbiology* published by the American Society for Microbiology (18).

Bacteria with phenotypic characteristics similar to *B. avium,* designated "*B. avium*-like," have been described (19). These organisms were

Table 8-1
Differential Characteristics of *B. bronchiseptica, B. avium, Alcaligenes* spp., and CDC Groups IVc-2 and IVe[a]

	Urease	Nitrate reduction	Gas from nitrate	Oxidation of		Sole carbon source for growth	
				Glucose	Xylose	Malonate	Glycolate
B. bronchiseptica	+	+	−	−	−	−	−
B. avium	−	−	−	−	−	−	−
A. faecalis[b]	−	−	−	−	−	+	+
A. xylosidans ssp. *xylosidans*[c]	−	+	+	D[d]	+	−	−
A. xylosidans ssp. *denitrificans*	D	+	+	−	−	−	−
A. piechaudii	−	+	−	−	−	D	?[e]
CDC group IVc-2	+	−	−	−	−	D	+
CDC group IVe	+	+	+	−	−	−	−

[a]All of the organisms listed are motile, oxidase and catalase positive, grow on citrate as a sole carbon source, and grow on MacConkey agar. Table includes information obtained from references 5, 6, 9, 14, and 18.
[b]Includes strains formerly known as *Alcaligenes odorans.*
[c]Formerly known as *Achromobacter xylosidans.*
[d]D, differs among strains.
[e]?, Information unavailable to the author.

isolated from the upper respiratory tracts of turkeys but are apparently nonpathogenic. The taxonomic status of these organisms is presently uncertain. Analyses of biochemical characteristics (19), cellular fatty acid composition (20), and electrophoretic patterns of enzymes (J. Musser, personal communication) indicate that they are not *B. avium.* However, the relationship of these organisms to *A. faecalis* is not clear. Colonial morphology, hemagglutination of guinea pig erythrocytes, growth in 6.5% NaCl, and growth on minimal essential medium (MEM) have been proposed as tests for distinguishing *B. avium* from the "*B. avium*-like" strains (13,19,21).

Some other gram-negative, nonfermentative organisms with characteristics similar to *Bordetella* and *Alcaligenes* species along with differential characteristics are listed in Table 8-2 (22).

Detection of the Dermonecrotic Toxin of *Bordetella bronchiseptica*

Bordetella bronchiseptica possesses an intracellular, heat-labile toxin, which is lethal when injected intraperitoneally into mice and produces necrosis when injected intradermally into guinea pigs. This toxin, known as the dermonecrotic toxin or DNT, is believed to be important

Table 8-2
Differential Characteristics for Gram-Negative, Nonfermentative Bacteria with Phenotypic Characteristics Similar to Those of *B. bronchiseptica*, *B. avium*, and *Alcaligenes* species[a,b]

	Glucose oxidation	Oxidase	Growth on MacConkey	Motility	Type of flagella
Bordetella[c]	–	+	+	+	Peritrichous
Alcaligenes	D	+	+	+	Peritrichous
Pseudomonas	D	D	+	+[d]	Polar
Flavobacterium	D	+	D	–	NA
Moraxella	–	D	D	–	NA
Eikenella	–	+	–	–	NA
Acinetobacter	D	–	D	–	NA

[a]Table includes information obtained from references 5, 6, 9, 14, 18, and 22.
[b]D, differs between species within a genus; NA, not applicable.
[c]Represents phenotypic characteristics for *B. bronchiseptica* and *B. avium* only.
[d]One species of *Pseudomonas*, *P. mallei*, is nonmotile (22).

in the production of atrophic rhinitis and pneumonia in swine (15). It may also play a role in respiratory disease caused by this bacterium in other animals (23). A DNT has also been proposed as a possible virulence factor for *B. avium* (21). The DNT of *B. avium* does not appear to be serologically cross-reactive with the DNT of *B. bronchiseptica* (21).

The following technique can be used for detecting DNT production in *B. bronchiseptica* (15). Inoculate a $Dom^+Scs^+Hly^+$ colony from a 48-hr-old culture grown on BG into 5 ml of freshly prepared Stainer–Scholte medium (24) and grow overnight with shaking at 37°C. Use this culture to inoculate a 1-liter flask containing 200 ml of Stainer–Scholte medium and incubate at 37°C with shaking for 6–8 hr. Harvest the cells by centrifugation at 15,000 × *g* (0–4°C) for 10 min and resuspend in 0.01 *M* Tris, pH 7.8. Adjust the optical density (OD) of the cell suspension to 1.0 at 650 nm and lyse the cells by sonication. The cell preparation should be kept as cool as possible during lysis. Cell lysis can be detected by a change from a turbid suspension to translucence. Once lysis is complete, the sonicate can be filtered through a 0.2-μm filter if the preparation is to be assayed right away. If not, the lysate should be frozen immediately at −40°C and thawed and filtered just prior to testing. Thawed preparations should be refrigerated and used within 8 hr of thawing. The lysate should not be filtered until just prior to testing, as filtering tends to cause very rapid loss of toxigenic activity. It is important that Stainer–Scholte medium be used for this assay, as the level of DNT production by *B. bronchiseptica* strains appears to be highly dependent upon the composition of the growth me-

dium. For example, *B. bronchiseptica* strains grow very well in brucella broth, but the level of DNT production in this medium is quite low (15).

To assay for DNT activity, dilute the freshly filtered cell lysate 1:100, 1:200, 1:400, and 1:800 in Tris buffer. Inject 0.1 ml of each dilution intradermally into the back of a young (≤400 g), depilated guinea pig. Include 0.1 ml of Tris buffer alone as a negative control. Examine the animal for necrotic lesions at the site of injection at 48 hr. Lesions >5 mm are considered positive. Virulent strains of *B. bronchiseptica* should yield positive reactions at dilutions of 1:200 and greater.

Methods for assaying the DNT activity of *B. avium* strains are described in detail by Gentry-Weeks *et al.* (21).

Serology

Tube agglutination, microagglutination, and enzyme-linked immunosorbence assay (ELISA) procedures have been described for *B. bronchiseptica* and *B. avium* (25–27). These tests are designed to detect primarily humoral antibodies and may be useful for monitoring the vaccination status of a herd or flock.

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9

Brucella

Paul Nicoletti

Species[1] in this genus are homogeneous in their morphology and staining characteristics. All are gram-negative coccobaccilli but are sufficiently small so that they appear to be cocci rather than short rods with rounded ends (see Fig. 9-1). They are nonmotile, nonencapsulated, and nonsporing; they grow aerobically or microaerophilically but will not grow anaerobically.

Species and their natural reservoirs:

Brucella abortus: cattle
Brucella suis: swine
Brucella melitensis: goats and sheep
Brucella ovis: especially rams
Brucella canis: dogs
Brucella neotomae: wood rats

Pathogenesis and Pathogenicity

Each species has a decided host preference for its natural reservoir, and is not readily transmitted from the preferential host to dissimilar hosts. When a species infects a nonpreferential host, it usually localizes in the mammary glands and reticuloendothelial tissue rather than

[1]This chapter is a revision of a chapter prepared by Dr. Margaret Meyer for the Fourth Edition. Much of her material has been retained and her contribution is gratefully acknowledged.

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in the uterus and fetal membranes. The organisms localize in various tissues and organs following a bacteremia in natural hosts.

Species

Brucella abortus

In cows, uterine infections often result in abortion or birth of weak calves. Retained placentas are common. The supramammary lymph nodes are frequently infected, and the organisms are shed in the milk and also may be recovered in mammary secretions of nonlactating cows. In bulls, genital infections may be inapparent. The organisms may be in semen and there may be an apparent orchitis or epididymitis. Venereal transmission appears to be rare.

Brucella suis

Brucellosis in swine is usually a more generalized and frequently a more chronic disease than bovine brucellosis. Bacteremia may persist for months with or without localization, and organisms also may persist in the uterus, causing a lingering metritis. Genital infections are more frequent in boars than in bulls and may cause necrotic orchitis. Infection in swine is characterized by abortion, stillbirths, decreased litter size, weak pigs, and focal abscessation. Spondylitis is an occasional sequela, especially in older pigs. Venereal transmission occurs readily.

Brucella melitensis

The disease in goats and sheep is similar to that in cattle. Some sheep breeds are more resistant to infection than are goats. As with cattle, venereal transmission is rare.

Brucella ovis

The primary manifestation of infection with this species is epididymitis accompanied by lesions of the tunica vaginalis and sometimes of the testicle. In ewes there is a placentitis and occasionally abortions. Infection without abortion frequently results in weak lambs of low birth weight.

Brucella canis

Dogs are the only known host for this species. Cases in free-roaming dogs and household pets are uncommon. Under kennel conditions, it spreads rapidly to males and females. It is characterized by abortions and infertility in the bitch accompanied by an intermittent but prolonged bacteremia. Genital infections in males can be silent or can result in orchitis and epididymitis.

Brucella neotomae
This species has never been found outside its natural reservoir and is of little or no veterinary significance.

Laboratory Diagnosis

Great care should be taken in working with *Brucella*: Humans are highly susceptible to this disease, and laboratory infections are common.

Direct Examination of Smears

Koster's staining procedure has been used for microscopic detection of intracellular brucellae, particularly in the chorionic epithelium. A scraping from a cotyledon is smeared on a slide, fixed, and stained. The *Brucella* organisms are stained red against a blue background. The staining procedure is outlined in Appendix A.

Isolation Procedures

Specimens most commonly submitted for isolation of *Brucella* organisms are placental membranes, aborted fetuses, vaginal swabs, semen samples, swabs of male reproductive organs, milk, lymph nodes, and blood.

The medium of choice for isolating *Brucella* from tissue and most body fluids is tryptose agar enriched with 5% seronegative equine or bovine serum. Inoculate duplicate plates for incubation in an atmosphere of air with 10% carbon dioxide. Most strains of *B. abortus* and *B. ovis* require carbon dioxide for initial isolation. Since many sources from which brucellae are likely to be isolated are apt to be contaminated, it may be necessary to use a selective medium such as W medium (see Appendix B).

A blood culture bottle containing a diphasic medium is recommended for isolation of brucellae from blood (see Appendix B).

Inoculated plates are incubated at 37°C for at least 15 days before being discarded as negative. Subcultures are made from broth onto solid medium at 3–5-day intervals.

Examination of Milk and Tissues for *Brucella*

Collect 25–50 ml of milk or secretion from each quarter into a sterile container. Centrifuge at 2000 *g* for 20 min. Decant middle milk and

inoculate aliquots of cream and sediment on separate plates. Other liquids such as fetal stomach contents or semen may be cultured directly onto plates or following centrifugation to concentrate organisms. Examine plates for *Brucella* colonies in approximately 5 days using obliquely transmitted light and a dissection (10×) microscope.

Tissues such as lymph nodes should be sectioned with sterile instruments and macerated using a tissue grinder with a small quantity of diluent. Aliquots are then placed onto solid medium. If instruments for macerating the tissue are not available, surfaces may be minced and directly inoculated.

Cultural Characteristics and Identification

Usually after from 3–5 days incubation, pinpoint, round, smooth, glistening, bluish, translucent colonies appear. As colonies age they increase in size, lose their translucence, and gradually turn from a cream to brownish color.

In addition to colonial morphology and organism morphology as determined by the Gram (Fig. 9-1) and/or Koster stains, the identity of *Brucella* at the generic level can be ascertained by mixing bacterial cells with anti-*Brucella* serum and examining for agglutination.

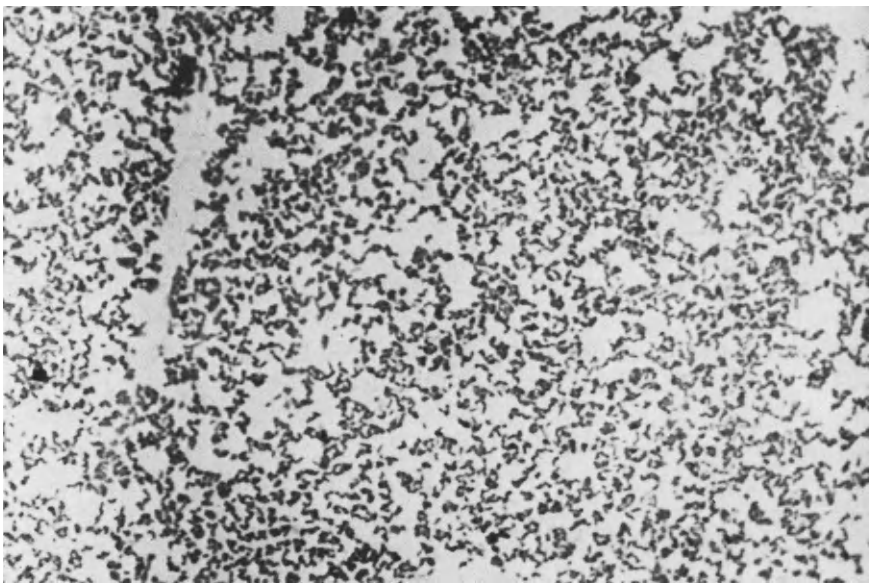

Figure 9-1. *Brucella abortus* from a culture. Gram stain, ×1000.

Smooth isolates of questionable identity can be prepared as antigens and used in a tube agglutination test. The cell density of the antigen is equilibrated to that of the United States Department of Agriculture Standard Tube Antigen.

Other characteristics that aid in the identification of the genus are the following:

- Nonmotile
- Catalase production
- Variable oxidase reaction
- Production of urease (except *B. ovis*).
- Nitrate reduction
- No change in litmus milk
- MR and VP negative
- Indole negative

The three classical species can be differentiated by their patterns of growth on appropriate concentrations of basic fuchsin and thionin and by other characteristics listed in Table 9-1.

Identification of the biovars within the species is usually carried out in reference laboratories. Workers interested in biovar identification should consult the references at the end of this chapter.

Lysis by bacteriophage is also useful for identification. Phages that can lyse members of the genus *Brucella* do not lyse members of other genera and thus can be useful in generic identification. There are several phages active or partially active upon various members of this genus. The most dependable and useful is the Tbilisi strain. At the RTD (routine test dilution), this phage lyses only smooth strains of *B. abortus*. At 10,000 × RTD it causes lysis from without on most strains of *B. suis*. Typing by other phage strains is usually done by a reference laboratory.

Antigenic Structure

The agglutination reactions observed with the classical species are considered to be due to surface antigenic determinants. These determinants are designated *A* for *B. abortus* antigen and *M* for *B. melitensis* antigen. These two antigens are distributed quantitatively in differing proportions among typical representative (biovar 1) of the smooth species and among the various biovars. By the technique of agglutinin absorption, it is possible to assay for the proportions of the two antigens in any smooth strain. This technique is of little value for routine identification purposes. In rare instances when it is essential to know the antigenic proportions, it is best to have the procedure done in a reference laboratory.

Table 9-1
Differentiation and Selected Characteristics of *Brucella* Species and their Biovars[a]

Species	Biovar	CO_2 Requirement	H_2S Production	Growth on dyes: Thionin 20 μg/ml	Growth on dyes: Basic fuchsin 20 μg/ml	Agglutination in sera: A	Agglutination in sera: M	Agglutination in sera: R	Preferred host species
B. melitensis	1	−	−	−	+	−	+	−	Goats, sheep
	2	−	−	−	+	+	−	−	
	3	−	−	−	+	+	+	−	
B. abortus	1	(+)	+	−	+	+	−	−	Bovidae
	2	(+)	+	−	−	+	−	−	
	3	(+)	+	+	+	+	−	−	
	4	(+)	+	−	(+)	−	+	−	
	5	−	−	+	+	−	+	−	
	6	−	(+)	+	+	+	−	−	
	7	−	(+)	+	+	+	+	−	
	9	−	+	+	+	−	+	−	
B. suis	1	−	+	+	−	+	−	−	Swine
	2	−	−	−	−	+	−	−	Swine
	3	−	−	+	+	+	−	−	Swine
	4	−	−	+	(−)	+	+	−	Reindeer, caribou
B. canis		−	−	+	−	−	−	+	Dogs
B. ovis		+	−	+	(−)	−	−	+	Sheep
B. neotomae		−	+	−	−	+	−	−	Wood rat

[a](+), Most isolates positive; (−) most isolates negative; +, isolates positive; −, isolates negative.

Serologic Diagnosis of Brucellosis: General

There are a variety of tests available for the serologic diagnosis of brucellosis in farm animals and dogs. Special antigens must be used for the serologic diagnosis of the nonsmooth species *B. canis* and *B. ovis*.

Herd Tests

The milk ring test is the most practical procedure for locating possible infected dairy herds. A pooled milk sample is tested from each individual herd. The sensitivity can be increased by using 2–3 ml of milk instead of 1 ml. Cattle in herds with a positive test are examined by individual serologic methods.

Blood samples may also be taken from animals at markets or at slaughter. Positive sera may indicate that the herd or flock of origin is infected.

Individual Tests

The tube agglutination test is the most widely used procedure for purposes of international trade. *Brucella abortus* antigen is used and results are expressed as titers (international units of anti-*Brucella* antibodies). The procedure has several disadvantages: It is slow; reactions to postimmunization agglutinins and sometimes to those caused by heterospecific antigens are seen; and chronically infected cattle may have titers below those considered positive. A modification of the test using 2-mercaptoethanol has been used to increase specificity.

The plate agglutination test is a modification of the tube method and is rapid. It is performed on a glass plate but has some of the same disadvantages as the tube method. To overcome some of these, the antigens may be buffered to a pH of 3.65–4.0. Modifications using buffered antigens are the *card* and *rose bengal* tests. These have been widely used as screening tests in laboratories and in the field for cattle, sheep and goats, and swine serums.

The complement fixation test (CFT) is recognized as the most specific of serologic tests of food animals. The volume of work is reduced by using the test as a definitive procedure on sera found positive by more sensitive and simple screening methods. The CFT has been the standard procedure for detection of antibodies to *B. ovis* but the enzyme-linked immunosorbent assay (ELISA) may prove to be more sensitive and specific than the CFT or gel diffusion test in the future.

A supplemental test using ethacridine (rivanol) solution is widely used in the United States for sera that are positive in sensitive screening tests. The principle of the test is to reduce the reactions caused by IgM antibodies that persist following vaccination. It is also used with swine serums to eliminate reactions caused by heterospecific antibodies.

Serologic Diagnosis of Canine Brucellosis

To aid in the diagnosis of *B. canis* infections in dogs, three serologic tests are used: rapid slide agglutination which may include the use of 2-mercaptoethanol tube agglutination (TAT), and agar gel immunodiffusion (AGID). A rapid slide agglutination test kit is produced by Pitman-Moore, Inc., Washington Crossing, New Jersey, for screening sera for antibodies; however, the tube or AGID tests, in conjunction with blood or vaginal culture should be used for definitive diagnosis.

Tube Agglutination Test

A. Media

1. Prepare 1 liter of *Brucella* agar and sterilize by autoclaving. Increase agar concentration to 2.5%. Cool to 56°C.
2. Pour 100 ml into each of 8–10 sterile cotton-stoppered Roux bottles. Allow to harden at room temperature. Invert and place bottles in the incubator. Allow to dry for 24–48 hr.

B. Seed Organism

1. Reconstitute a low passage of lyophilized *B. canis* RM666 with 1 ml of tryptose phosphate broth (TPB) and inoculate several large *Brucella* agar slants. Incubate slants for 48 hr.
2. Harvest the *B. canis*, using approximately 3–4 ml of sterile *Brucella* broth or TPB per slant. Scrape cells from slant and suspend in the broth.

C. Inoculation and Harvesting of Cells

1. Inoculate each Roux bottle with 1 ml of the *B. canis* suspension.
2. Spread the inoculum evenly over the agar with sterile glass beads by gently tilting the bottle.
3. Invert the bottles and incubate them for 48 hr at 37°C.
4. To harvest the culture, add 10 ml of PBS (pH 7.2) to each bottle. Remove the cells by agitation of glass beads across the surface of the agar, then wash the agar with 5 ml PBS. Pour the cell suspension through cheesecloth into a 50-ml sterile centrifuge tube.
5. The cell suspension is packed by centrifugation at approximately 8000 *g* in a refrigerated centrifuge. Wash packed cells three times with PBS.
6. Resuspend cells in 100 ml PBS and pour through cheesecloth into a flask.
7. Inactivate for 1 hr at 60°C, then add 1 ml of 0.01% merthiolate as

preservative. Store under refrigeration and label as "Stock Antigen."

D. Performance of the TAT Test

1. Prepare two bottles, 100 ml each, of 3.5% saline.
2. Remove 0.6 ml saline from one bottle and add 0.6 ml formalized saline [10 ml formaldehyde (37–40%) to 90 ml 0.85% saline]. Label bottle "Working Antigen." Adjust the stock antigen to an OD of .190 on the Coleman Jr. spectrophotometer at 420 nm, or .260 OD at 580 nm on the Perkin–Elmer 35, with formalized saline. Prepare as needed and store in refrigerator. Test this working antigen against the old working antigen using a known positive antiserum and antigen control.
3. Remove 0.715 ml saline from the other bottle and add 0.715 ml of 2-mercaptoethanol. Label bottle "0.1 *M* 2-ME solution" and store in refrigerator.
4. Pipette 0.04, 0.02, and 0.01 ml of each serum sample to be tested into each of three 13 × 100mm test tubes. Volumes are equivalent to dilutions of 1:50, 1:100, and 1:200, respectively.
5. Add 1 ml of 2-ME solution to each tube containing serum.
6. Add 1 ml of working antigen to each tube containing serum and 2-ME solution.
7. Shake tubes to mix serum and antigen thoroughly, and incubate (hot air) at 37°C for 48 ± 3 hr.
8. Observe for *clearing* by holding tubes in front of a fluorescent light. The background should be a dull black or dark.
9. Record the results as positive (complete clearing) incomplete clearing, or negative on the basis of the amount of *clearing* only.

Agar Gel Immunodiffusion Test

A. *Antigen Preparation*

1. *Brucella canis* cultures (48 hr) are harvested in PBS solution as described in preparation of stock antigen for the TAT.
2. The cell suspension is packed by centrifugation (8000 *g*) then washed once with PBS (pH 7.2).
3. Each milliliter of packed cells is resuspended in 3 ml PBS, and then 6 ml of a 1% Na deoxycholate solution (made up in 0.01 *M* phosphate buffer, pH 7.2) is added. The suspension is then thoroughly mixed and incubated for 4 hr at 56°C.
4. Centrifuge this suspension for 30 min at 10,000 *g*, and transfer supernatant to another centrifuge tube. In most cases it will be yellow and very viscous.
5. The supernatant is centrifuged at 20,000 *g* or 3–4 hr. This step

eliminates the viscous material, and the supernatant becomes liquid. The use of dialysis may be substituted for this step, but the antigen will be weaker.
6. Store the supernatant in 1-ml quantities at −70°C for up to 2 years. The thawed antigen can be stored under refrigeration for up to 2 weeks.

B. Performance of the Test

1. Prepare 1% Noble's agar using the following buffer: 2 gm NaOH, 9 gm H_3BO_3, and 1 liter distilled water. Adjust pH to 8.6 ± 0.2. Dissolve the Noble's agar in the buffer by boiling. Autoclave at 121°C. Dispense agar in 5 ml amounts in screw-capped tubes and store under refrigeration.
2. To prepare plates, melt agar in boiling water or microwave oven. Add 5 ml of agar to 60 × 15 mm petri dishes and allow to harden. Wells approximately 5 mm in diameter and 2 mm apart are cut in the agar and the plugs removed. Templates obtained from the National Veterinary Services Laboratory for the Equine Infectious Anemia test work well for cutting the wells.
3. Alternating outside wells are filled with positive control serum. The remaining outside wells are filled with sera to be tested. The center well is filled with *B. canis* cytoplasmic antigen.
4. Incubate in a moist environment at room temperature for 24 hr.
5. Positive sera may produce as many as four lines.

Procedures for the TAT and AGID antigen preparation and tests were compiled from information supplied by Dr. S. J. Shin, Cornell University, Ithaca, New York; Mr. Irl Long, Auburn University, Auburn, Alabama; Dr. John Cole, Jr., University of Georgia, Tifton, Georgia; and National Veterinary Services Laboratories, USDA, Ames, Iowa.

Additional sources of information on serologic procedures are listed in the references.

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10

Enterobacteria

Margery E. Carter and M. M. Chengappa

The enteric bacteria are gram-negative, facultatively anaerobic, nonsporing rods. All are fermentative, oxidase negative, and catalase positive, and many are motile with peritrichate arrangement of the flagella.

Some members of the family reside in the intestinal tract of animals and humans. Others occur in the environment associated with soil, water, plants, or insects.

The nomenclature of the Enterobacteriaceae has undergone considerable change in recent years. In the eighth edition of *Bergey's Manual* (1), the family was divided into tribes based mainly on biochemical reactions. Brenner (2) in *Bergey's Manual of Systematic Bacteriology* recognized DNA-relatedness studies and named twenty genera and more than 100 species. In the seventh edition of *Bailey and Scott's Diagnostic Microbiology*, Finegold and Baron (3) grouped the members of Enterobacteriaceae family into 24 genera. The genera contain members that are known pathogens, some that are opportunistic pathogens, others that can be isolated from clinical specimens, and species that have no known pathogenicity. Genera belonging to the first two categories are presented in Table 10-5. Organisms less commonly isolated from clinical specimens are shown in Table 10-9.

Members of the Enterobacteriaceae unlikely to be of importance in diagnostic bacteriology include *Budvicia* sp., which occurs in surface water; *Erwinia* spp. that are plant pathogens; *Obesumbacterium* sp., found only in beer; and *Yokenella* and *Xenorhabdus* spp. that are associated with insects. *Ewingella americana* has been recovered from the respiratory tract of humans but there is no evidence that it is pathogenic (4).

Diagnostic Procedures in Veterinary Bacteriology and Mycology, Fifth Edition

General Comments and Pathogenicity

Buttiauxella

The single species, *Buttiauxella agrestis,* is found in water. Although the organism is not associated with human or animal disease it has been reported from clinical specimens.

Cedecea

The three species, *Cedecea davisae, C. lapagei,* and *C. neteri,* have been isolated from human clinical specimens, mainly from the respiratory tract and from wounds. The clinical significance is unknown.

Citrobacter

The three *Citrobacter* species, *Citrobacter freundii, C. diversus* (*C. intermedius* b), and *C. amalonaticus* (*C. intermedius* a), are considered to be opportunistic pathogens in humans, and this is probably their status in animals. They have been isolated from the feces of animals and humans and from wound infections, urine, septicemias, soil, water, and sewage. *Citrobacter diversus* is known to occasionally cause meningitis in human neonates.

Edwardsiella

The three species of *Edwardsiella* have been isolated from humans, animals, and fish with various conditions. *Edwardsiella tarda* is an opportunistic human and animal pathogen associated with diarrhea, wound infections, and sepsis. *Edwardsiella tarda* and *E. ictaluri* are common pathogens of catfish.The third species of the genus, *E. hoshinae,* is not known to be pathogenic.

Enterobacter

Enterobacter is found widely in nature. *Enterobacter cloacae* and *E. aerogenes* are opportunistic pathogens. *Enterobacter cloacae* causes occasional bacteremia in humans (5), and *E. aerogenes* can be associated with bovine mastitis. *Enterobacter sakazakii* is the name given to the yellow-pigmented variant of *E. cloacae;* it is isolated from food

but only rarely from human clinical specimens. *Enterobacter gergoviae* has been recovered from human clinical specimens, cosmetics, and water. *Enterobacter agglomerans* is associated with water, soil, and sewage. It has encompassed the former *Erwinia* species, *E. herbicola.*

Escherichia

The lactose-positive member of the genus, *Escherichia coli,* is a normal intestinal inhabitant of humans and animals but is also recovered from a wide variety of diseases in all domestic animals. It may be a primary or secondary agent. Nursing and young animals, under 1 week of age, are particularly susceptible. Some important diseases are listed below.

Cattle: scours and less severe intestinal infections of calves; mastitis
Swine: scours and diarrhea in young pigs; hemorrhagic enteristis and edema disease
Chickens: airsacculitis; Hjarre's disease or coligranuloma
Dogs: urinary infections

A variety known as *E. coli* "inactive" represents the previously named A–D Group. This organism is usually lactose negative, nonmotile, anaerogenic, and does not always form indole. *Escherichia blattae* has been isolated from the hindgut of cockroaches and is not known to be pathogenic. *Escherichia fergusonii,* formerly CDC Enteric Group 10, has been recovered from veterinary clinical specimens but its pathogenicity for animals is unknown. The organism is lactose negative at 48 hr of incubation but 87% are *o*-nitrophenyl-β-D-galactopyranoside (ONPG) positive and exhibit a + + − − IMViC pattern similar to *E. coli* (4).

Hafnia

The single species, *Hafnia alvei,* is found in the feces of humans and animals, sewage, soil, water, and dairy products. It is not considered to be important as a cause of infections in animals.

Klebsiella

There are six currently recognized *Klebsiella* species (3), but only a few are known to be clinically significant in veterinary medicine.

Klebsiella ozaenae and *K. rhinoscleromatis* can cause disease in

humans and are now considered subspecies of *K. pneumoniae. Klebsiella pneumoniae* subs. pneumoniae capsular types 1, 2, and 3 cause pneumonia in humans. The organism occurs widely in nature, notably in wood products used as bedding for cattle. Severe *Klebsiella* mastitis is more prevalent when cows are kept on such bedding. *Klebsiella pneumoniae* subs. *pneumoniae* has been recovered from other infections in animals, including cervicitis and metritis in mares, wound infections, septicemia, and pneumonia in dogs.

The indole-positive biogroup of *K. pneumoniae* has been named *K. oxytoca.* This organism can be recovered from the intestinal tract of healthy animals, mastitic bovine milk samples, and from the environment.

Kluyvera

The two species, *K. ascorbata* and *K. cryocrescens,* have been isolated from human clinical specimens, feces, and food. There is no strong evidence of pathogenicity.

Koserella

Koserella trabulsii was originally known as "Enteric Group 45" or as an "atypical *Hafnia,*" as it is biochemically similar to *Hafnia.* The organism can be isolated from wound, throat, and fecal specimens of humans. It may be identical to the new genus *Yokenella* (4).

Leminorella

Leminorella grimonti and *L. richardii* have been isolated from human feces and urine. Their clinical significance is unknown.

Moellerella

The only species of the genus, *Moellerella wisconsensis,* is recovered from human feces and natural water. No pathogenic role for it has been suggested.

Morganella

Proteus morganii has been renamed *Morganella morganii* as DNA–DNA hybridization studies showed that the organism is not closely

related to the *Proteus* species. *Morganella morganii* is a well-recognized human pathogen. It has been associated with urinary tract and ear infections of animals, particularly in dogs and cats.

Proteus

The genus now contains four species. *Proteus mirabilis* occurs most frequently in clinical materials from animals. Both *P. mirabilis* and *P. vulgaris* can cause urinary materials from animals. Both *P. mirabilis* and *P. vulgaris* can cause urinary, gastrointestinal, and other sporadic infections in humans and animals. *Proteus penneri* can be a human pathogen. *Proteus myxofaciens* has been isolated only from gypsy moth larvae.

Providencia

Providencia alcalifaciens, P. stuartii, P. rustigianni, and *P. rettgeri* are not frequently recovered from animal specimens and are only rarely incriminated in animal diseases. *Providencia heimbachae* has been isolated from penguin feces and from an aborted bovine fetus (4) but the significance is unknown.

Rahnella

The single species *R. aquatilis* is a natural inhabitant of water. It has been isolated from a human burn wound. The pathogenicity for humans and animals is unknown.

Salmonella

There are more than 2000 closely related serovars. There have been various proposals for the subdivision of the genus. On the basis of different biochemical characteristics, *Salmonella* was divided into three species (6): *S. typhi, S. choleraesuis,* and *S. enteritidis.* In this scheme, all the other salmonellae became serovars of *S. enteritidis* (e.g., *S. enteritidis* serovar *typhimurium*). On the basis of numerical taxonomy and DNA-relatedness studies, another proposal (7) suggests that the genus should consist of a single species, *S. choleraesuis.* This includes all the organisms formerly known as "Arizona." The salmonellae have been placed into five subgenera on the basis of biochemical characteristics. The majority of the serovars that are isolated from clinical

specimens are in subgenus I. The Arizona serovars are included in subgenus III.

The Kauffmann–White scheme, based on the somatic, capsular, and flagellar antigens, is still acceptable because of its wide diagnostic usage. With a few exceptions, the antigenic formulae of Arizona serovars may be translated into *Salmonella* formulae and included in the scheme. The *Salmonella* serovars can be subdivided into biovars, which are strains within a serovar having different biochemical patterns (e.g., *S. choleraesuis* biovar Kunzendord). Phagovars are determined by the sensitivity of cultures to a series of bacteriophages.

Salmonellosis assumes one of the following forms: peracute septicemia, acute enteritis, chronic enteritis, or a subclinical carrier state. Some important syndromes caused by Salmonella serovars are listed below:

Group A	*S. paratyphi A*	Paratyphoid fever in humans
Group B	*S. schottmuelleri*	Paratyphoid fever in humans
	S. typhimurium	Gastroenteritis in humans; most prevalent species causing infections in various animal species
	S. abortus-equi	Abortion in mares and jennets
	S. abortus-bovis	Abortion in cattle
	S. abortus-ovis	Abortion in sheep
Group C_1	*S. choleraesius*	Enteritis in pigs; frequent secondary invader in hog cholera; infections in humans
	S. typhisuis	Infections in young pigs
Group C_2	*S. newport*	Infections in humans various animals, and especially cattle
Group D_1	*S. enteritidis*	Infections in various animals; gastroenteritis in humans
	S. gallinarum	Fowl typhoid, an acute intestinal disease of young chickens and turkeys
	S. pullorum	Severe intestinal infections of chicks and poults (pullorum); chronic infections in older fowl
	S. typhi	Typhoid fever in humans
	S. dublin	Severe infections in calves
Group E_1	S. anatum	Keel disease in ducklings

The Arizona serovars occur widely in nature, and on occasion cause severe or fatal infections in poultry (especially in turkey poults), humans, dogs, cats, and other animal species. They are frequently recovered from snakes and lizards.

Serratia

Of the seven species, only *S. marcescens* is considered a significant pathogen of humans and animals. This organism causes mastitis in cows and has been recovered from pneumonia and septicemia in humans. Roussel, Lucas, and Bouley (8) referred to infections in geckos

and tortoises, and to septicemia in the chicken. *Serratia odorifera* can be isolated from plants and food and may be a rare opportunistic pathogen in humans. *Serratia liquefaciens* is the most prevalent species in the environment and has been recovered from plants and from rodents' intestines. *Serratia fonticola* is probably not a true member of the genus.

Shigella

In this genus, the four species cause intestinal infections and dysentery in humans and primates. Their involvement as causes of disease in domestic animals is rare. *Shigella* species are closely related to *Escherichia coli*.

Tatumella

The single species, *T. ptyseos*, has been recovered from human clinical species, mainly from the respiratory tract. It may be an opportunistic pathogen. Unlike other enterobacteria, *Tatumella* has polar flagella. It grows best at 25°C.

Yersinia

The genus now contains eight species. *Yersinia enterocolitica*, *Y. pestis*, and *Y. pseudotuberculosis* are important human and animal pathogens. *Yersinia ruckeri* causes enteric redmouth of fish, and the infection usually results in a hemorrhagic septicemia. *Yersenia aldovae*, *Y. frederiksenii*, *Y. intermedia*, and *Y. kristensenii* were previously biogroups of *Y. enterocolitica*, and their role in human and animal infections has not been established.

Yersinia pestis is the casual agent of human plague. Plague is basically a disease of rats and many wild rodents, including squirrels and marmots. Humans are considered an accidental host. The organism may kill certain rodents in widespread outbreaks or survive in a latent form in others. Natural infections have been reported in dogs and cats (9).

Yersinia pseudotuberculosis is the cause of pseudotuberculosis of wild and laboratory rodents. This disease is characterized in its chronic form by the presence of small necrotic nodules in mesenteric lymph nodes, liver, spleen, and lungs. An acute septicemic form is also encountered. Other species infected include chinchilla, turkeys, rams (epididymo-orchitis), and swine. The disease in turkeys may reach

epizootic proportions. Severe infections simulating typhoid and appendicitis occur in humans.

Yersinia enterocolitica has been isolated with increasing frequency from humans and animals in recent years. It has been reported as a cause of a pseudotuberculosis-type disease in swine and in chinchilla. Sporadic infections have been observed in hares, birds, cats, minks, dogs, a bushbaby (Galago), sheep, rats, and guinea pigs. Isolations have also been made from cows, horses, feces, water, and milk. Infections in humans resemble those caused by *Y. pseudotuberculosis*. In addition, the organism has been recovered from cutaneous lesions, from cerebrospinal fluid in meningitis, from the blood in cases of septicemia and bacteremia, from liver abscesses, and from feces in cases of enterocolitis.

Laboratory Procedures

Isolation Procedures

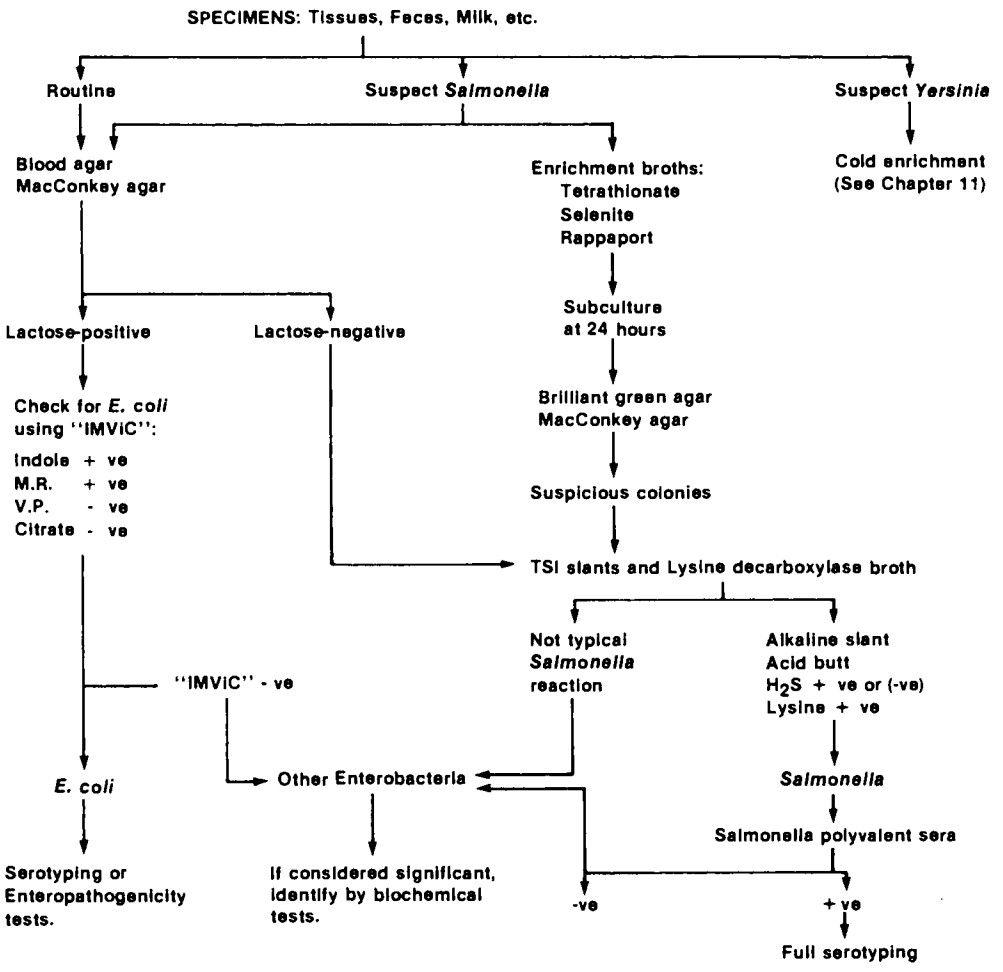
Specimens

General specimens include fecal swabs, samples of feces, tissues from a recently dead carcass, such as intestinal sections, liver, kidney, lung, spleen, or bone marrow, and milk samples from mastitis cases. Recovery of enteric bacteria from fresh tissues, in a heavy and fairly pure growth, is suggestive of a bacteremia and may be significant. The routine procedure followed in the isolation and cultivation of enteric bacteria is outlined in Table 10-1.

Samples for the recovery of salmonellae may be varied, and some need additional isolation procedures.

1. Samples from diseased or dead animals can be plated directly onto selective media and an aliquot placed in enrichment broth.
2. Feed, meat, and offal samples being checked for salmonellae are usually placed only in enrichment broth. There are often too few organisms present to warrant direct plating.
3. Samples from meat, egg, or milk products that may have undergone heat in processing are first placed in a preenrichment broth. After 12–16 hr of incubation, a portion of the nutrient broth can be placed into an enrichment broth.
4. Salmonellae may be recovered from water samples in the following ways:
   a. About 50–100 ml of the water can be added to an equal quantity of double-strength tetrathionate broth.

Table 10-1
Procedures for the Isolation and Identification of the Enteric Bacteria



b. Pass about 100 ml of water through a 0.45-μm membrane filter. The filter can be placed in 10 ml of enrichment broth.
c. A successful method of isolating salmonellae from streams or large bodies of water is with the aid of a pad of cotton wrapped in cheesecloth and tied securely with a long piece of string. This is sterilized, and when required it is thrown into the

water and attached to the bank by the string. The pad is left *in situ* for 48 hr, then recovered and placed in about 100 ml of enrichment broth.

Enrichment Broths and Selective Media for *Salmonellae*

There are many enrichment broths, and modifications of these, for the isolation of salmonellae. Some, however, can be toxic for some serovars. Strains of *S. typhisuis*, *S. choleraesuis*, *S. pullorum*, and *S. gallinarum* are inhibited by selenite and tetrathionate broths. Rappaport enrichment broth (10) successfully encourages the growth of these serovars. GN broth (BBL) supported the growth of all *S. tyhpisuis* strains tested (11).

The efficiency of selective enrichment broths is influenced by the type of specimen being examined, the proportion of inoculum to broth, and the length and temperature of incubation used. The effectiveness of selenite and tetrathionate are considerably reduced by the addition of certain products such as egg albumen. Food products containing an excess of 1% dextrose should be diluted so that the final concentration of dextrose in the broth is less than 1%.

Excessive amounts of inocula inhibit rather than enhance selectivity of the enrichment broth. Fecal samples should be added in 5–10% quantities, and food products should not exceed 15% in the broth. All enrichment broths are incubated at 37°C. Subcultures from enrichment broths onto selective media can be made after 16–24 hr of incubation. It has been found that one subculture is sufficient to isolate the great majority of the salmonellae.

Inhibition of *Proteus* species in tetrathionate broth can be obtained by the addition of 40 μg of novobiocin/ml or 0.125 mg of sodium sulfathiazole per 100 ml of the enrichment broth.

Brilliant green agar, MacConkey agar, Hektoen enteric agar, and *Salmonella*–Shigella (SS) agar are among the most generally used selective media; these are also designed to be indicator media.

Brilliant green agar can be inhibitory to *S. pullorum*, *S. gallinarum*, *S. typhi*, *S. choleraesuis*, and *S. typhisuis*. Modified brilliant green agar (Oxoid) was found to support growth of *S. typhisuis* (11). *Salmonella choleraesuis* strains will grow on MacConkey and XLD agars, while some strains of *S. pullorum* were found to be inhibited by all the common selective media except for MacConkey agar.

Sodium sulfadiazine at 8–16 mg/100 ml of brilliant green agar will inhibit *Proteus* and *Pseudomonas* species but will not affect the growth of salmonellae. The two former organisms tend to have colonies similar in appearance to *Salmonella* on brilliant green agar, that is, pinkish white with an alkaline reaction (red) in the surrounding medium.

Isolation Procedures for *Yersinia* species

Organisms of this genus are readily isolated and cultivated on nutrient, trytose, tryticase, and blood agar. They also grow on such selective and enrichment media as MacConkey, SS, and desoxycholate agar, and also in selenite and Rappaport broth. Room temperature is preferred to 37°C. *Yersinia enterocolitica* has been recovered frequently from animal feces. Large inocula and storage in the refrigerator as with listeria have been recommended for the isolation of Y. pseudotuberculosis. If one of these species is suspected, plates should be incubated at 22–25°C (room temperature) and at 37°C for not less than 48 hr. Longer incubation may be required for enrichment media for the recovery of *Y. enterocolitica*.

The best procedure for the recovery of *Y. enterocolitica* from feces is by cold enrichment as follows: Place some feces, approximately 5% by volume, in $\frac{1}{15}$ *M* phosphate buffered saline (Oxoid) and hold in the refrigerator (4°C) for 3 weeks. Plate onto MacConkey and SS agar after 7, 14, and 21 days of enrichment.

Identification

Cultural Characteristics

The colonies of the various enteric bacteria on blood agar are not sufficiently distinctive to aid appreciably in their identification, except for the propensity of *P. vulgaris* and *P. mirabilis* to swarm; *Serratia marcescens* to produce an orange-red pigment, although this is often not seen at 37°C; *Enterobacter sakazakii* to produce a yellow pigment; and *Klebsiella*, *Enterobacter*, and some strains of *E. coli* to have mucoid colonies. However, the selective and indicator media, referred to in Table 10-2, do aid in the presumptive identification of some genera, especially salmonellae.

On blood agar, strains of *Edwardsiella tarda* resemble hemolytic *Escheri chia coli*. In contrast to *E. coli*, however *E. tarda* has the appearance of a salmonella on TSI agar (see Table 10-4).

After 24 hr, colonies of *Y. pestis* are small, mucoid, and visible along the initial streak lines. On further incubation, colonies enlarge and assume a beaten copper appearance. They are raised in the center and flat at the periphery, with an umbonate edge. Colonies of *Y. pseudotuberculosis* are round, finely granular, grayish yellow, translucent, and centrally opaque, with a flat periphery showing striations. After 24 hr of incubation, colonies of *Y. enterocolitica* on blood agar are small, round, and gray. They enlarge somewhat on longer incubation. Small, round, pale colonies are produced on SS agar; round, pale colonies with slightly darkened centers are seen on MacConkey agar after prolonged

Table 10-2
Colony Appearance of the Enterobacteriaceae on Selective Agar Media[a]

Genera	Brillant green agar	SS agar	MacConkey agar
Klebsiella *Enterobacter* *Escherichia*	Yellow-green	Red or pink	Red
Providencia *Shigella*	Pinkish white with a red halo	Whitish or colorless	Whitish or colorless
Edwardsiella *Citrobacter* *Salmonella* *Arizona*	Usually pinkish white with a red halo; yellow-green if either lactose or sucrose fermented	Whitish or colorless; if H_2S is produced, a dark central spot forms within 48 hr	Whitish or colorless
Proteus	Yellow green if it is a sucrose fermenter; pinkish white with a red halo if sucrose negative	Whitish or colorless, with or without a dark central spot	Whitish or colorless

[a]Modified from McAllister, H. A.: *Procedures for the Identification of Microorganisms from the Higher Animals.* Courtesy of Lucas Brothers Publishers, Columbia, Missouri, 1970.

incubation; and growth on brilliant green agar is sparse, and colonies are green.

Biochemical Reactions

Reactions in triple sugar iron (TSI) agar slants and other biochemical reactions using either conventional or miniature systems (e.g., API or Minitek) are also used to identify the Enterobacteriaceae. Brenner (2) warns that as there are now over 100 species of enterobacteria, it is risky to identify these organisms to the species level on the basis of a few tests. Each laboratory must decide (1) what nonroutine tests to use in order to award a specific name, (2) whether it is necessary to speciate in each case, and (3) which species to ignore, as some are not known to be pathogenic for animals. A decision must also be made on the significance of the isolates, based on the handling and age of the samples. The organisms are ubiquitous, and postmortem invasion of carcasses by the enteric bacteria is rapid.

The use of TSI combined with lysine decarboxylase broth can give a presumptive identification of *Salmonella* serovars and is useful for the other enteric bacteria and for gram-negative bacteria generally. The reactions are given in Tables 10-3 and 10-4. It must be remembered that not all salmonellae produce hydrogen sulfide (for example, *S. choleraesuis* does not do so), but the biovar Kunzendorf is positive. *Salmonella typhisuis* varies in the production of H_2S and lysine–decarboxyl-

Table 10-3
Reactions Noted in Triple Sugar Iron Agar Slants

Appearance	Reactions
Acid butt yellow; alkaline slant: red	Glucose fermented
Acid throughout medium; butt and slant yellow	Glucose and sucrose and/or lactose fermented
Gas bubbles in butt and medium frequently split	Gas production
Butt shows blackening	Hydrogen sulfide produced
Unchanged or alkaline butt and slant; medium red throughout	None of the three sugars fermented

ase positive. Differentiation of important enterobacteria by biochemical tests is shown in Table 10-5.

More than 70 capsular types of *Klebsiella* have been identified. Typing is carried out by some reference laboratories.

E. Coli

In general, the serologic classifications of the enteric bacteria parallel the Kauffman–White scheme of the *Salmonella*. Identification of the serotypes of this species is not carried out routinely in most veterinary diagnostic laboratories. The antigens used to designate serotypes are as follows:

Somatic or **O** antigens: designated by Arabic numerals (e.g., **0133**)
K (surface or envelope) antigens: these thermolabile antigens are designated by the letters **L, B,** or **A** with an Arabic number (e.g., **K4 (B)**)
H or flagellar antigens: designated by **H** followed by an Arabic number (e.g., **H2**)

An example of a complete designation would be **0111: K4 (B), H2.**

Some of the serotypes and the animal species with which they have been associated are listed in Table 10-6.

It is now known that some enteropathogenic strains of *E. coli* produce one or two enterotoxins. They are referred to as the heat-stable toxin (ST) and the heat-labile toxin (LT). Procedures are now available for the demonstration of the toxins from the enterotoxic strains. These procedures include a suckling mouse test (12) and a rabbit ileal loop test (13) for ST, and a chinese hamster ovary cell culture test (14) and a mouse adrenal tissue culture test (15) for LT. These tests are somewhat involved and have limitations for animal isolates of *E. coli.*

Table 10-4
Reactions of Enterobacteriacae and Other Gram-Negative Bacteria on TSI Slants and in Lysine Decarboxylase Broth

Reactions	Organisms expected and lysine reaction		
	Lysine positive	Lysine negative	Lysine variable
Acid slant Acid butt No H_2S No gas	*Seratia* spp. *Aeromonas hydrophila* ss. *proteolytica*	*Aeromonas hydrophila* ss. *anaerogenes* *Moellerella* sp. *Actinobacillus* spp. *Shigella sonnei* *Pseudomonas pseudomallei* *Tatumella* sp. *Yersinia enterocolitica*	*Pasteurella* spp. *E. coli* inactive *Pseudomonas* spp. (some)
Acid slant Acid butt No H_2S Gas in butt	*E. coli* *Enterobacter aerogenes* *Enterobacter gergoviae* *Klebsiella* spp.	*Cedecea* spp. *Rahnella* sp. *Aeromonas hydrophila* ss. *hydrophila* *Enterobacter cloacae* *Enterobacter agglomerans* *Buttiauxella* sp. *Providencia* (some)	*Kluyvera* spp.
Acid slant Acid butt H_2S produced Gas in butt	Arizona serovars (some)	*Citrobacter* (some) *Proteus vulgaris*	
Alkaline slant Acid butt No H_2S No gas	*Obesumbacterium* spp.	*Shigella* spp. *Providencia* spp. *Yersinia pseudotuberculosis* *Xenorhabdus* spp.	
Alkaline slant Acid butt No H_2S Gas in butt	*Salmonella choleraesuis* *Salmonella sendai* *Salmonella abortus-equi* *Hafnia alvei* *Koserella* sp.	*Salmonella typhisuis* (some) *Salmonella paratyphi* A *Morganella morganii*	
Alkaline slant Acid butt H_2S produced No gas	*Salmonella pullorum* (some) *Salmonella gallinarum* *Salmonella typhi*	*Leminorella* sp.	
Alkaline slant Acid butt H_2S produced Gas in butt	*Salmonella* serovars (most) Arizona serovars (most) *Edwardsiella tarda*	*Citrobacter* spp. *Proteus vulgaris* (some) *Proteus mirabilis* *Salmonella typhisuis* (some)	

Table 10-5
Differentiation of Some Enteric Bacteria by Biochemical Tests[a]

Species	Indole production	Methyl red	Voges–Proskauer	Citrate	Hydrogen sulfide (TSI)	Urease	Phenylalanine	Lysine decarboxylase	Arginine dihydrolase	Ornithine decarboxylase	KCN (growth)	Motility	Gelatin liquefaction	Malonate	Glucose (gas)	Lactose	Sucrose	Mannitol	Dulcitol	Salicin	Adonitol	Inositol	Sorbitol	Arabinose	Raffinose	Rhamnose	Esculin hydrolysis	ONPG (no galactosidase)
Citrobacter amalonaticus	+	+	−	(+)	−	(+)	−	−	(+)	+	+	+	−	(−)	+	d	(−)	+	−	d	−	−	+	+	−	+	−	+
C. diversus	+	+	−	+	−	(+)	−	−	d	+	−	+	−	+	+	d	(−)	+	d	(−)	+	−	+	+	−	+	−	+
C. freundii	−	+	−	+	(+)	d	−	−	d	(−)	+	+	−	(−)	+	d	d	+	d	−	−	−	+	+	d	+	−	+
Edwardsiella tarda	+	+	−	−	+	−	−	+	−	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
E. ictaluri	−	−	−	−	−	−	−	+	−	d	−	−	−	−	d	−	−	−	−	−	−	−	−	−	−	−	−	−
Enterobacter aerogenes	−	−	+	+	−	−	−	+	−	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
E. agglomerans	(−)	d	d	d	−	(−)	(−)	−	−	−	d	(+)	−	d	(−)	d	(+)	+	(−)	d	−	(−)	d	+	d	(+)	d	+
E. cloacae	−	−	+	+	−	d	−	−	+	+	+	+	−	(+)	+	+	+	+	(−)	(+)	(−)	(−)	+	+	+	+	d	+
E. gergoviae	−	d	+	+	−	+	−	+	−	+	−	+	−	+	+	d	+	+	−	+	−	−	−	+	+	+	+	+
Escherichia coli	+	+	−	−	−	−	−	(+)	(−)	d	−	d	−	−	+	+	d	+	d	d	−	−	+	+	d	(+)	d	+
Klebsiella oxytoca	+	d	+	+	−	+	−	+	−	−	+	−	−	+	+	+	+	+	d	+	+	+	+	+	+	+	+	+
K. pneumoniae ss. *pneumoniae*	−	(−)	+	+	−	+	−	+	−	−	+	−	−	+	+	+	+	+	d	+	+	+	+	+	+	+	+	+
Morganella morganii	+	+	−	−	−	+	+	−	−	+	+	+	−	−	(+)	−	−	−	−	−	−	−	−	−	−	−	−	−
Proteus mirabilis	−	+	(−)	d	+	+	+	−	−	+	+	+	+	−	+	−	(−)	−	−	−	−	−	−	−	−	−	−	−
P. vulgaris	+	+	−	(−)	+	+	+	−	−	−	+	+	+	−	(+)	−	+	−	−	(+)	−	−	−	−	−	−	(+)	−
Providencia rettgeri	+	+	−	+	−	+	+	−	−	−	+	+	−	−	−	−	(−)	+	−	d	+	+	−	−	−	d	d	−
P. stuartii	+	+	−	+	−	d	+	−	−	−	+	(+)	−	−	−	−	d	(−)	−	−	−	+	−	−	−	−	−	−
Salmonella subgenus 1	−	+	−	+	+	−	−	+	d	+	−	+	−	−	+	−	−	+	+	−	−	d	+	+	−	+	−	−
subgenus III ("Arizona")	−	+	−	+	+	−	−	+	(+)	+	−	+	−	+	+	d	−	+	−	−	−	−	+	+	−	+	−	+
Serratia liquefaciens	−	(+)	(+)	+	−	−	−	+	−	+	+	+	+	−	d	−	+	+	−	+	−	d	(+)	+	+	(−)	+	+
S. marcescens	−	(−)	+	+	−	(−)	−	+	−	+	+	+	+	−	d	−	+	+	−	+	d	(+)	+	−	−	−	+	+
S. odorifera	d	(+)	(+)	+	−	−	−	+	−	d	d	+	+	−	−	+	d	+	−	d	d	+	+	+	d	+	d	+
Yersinia enterocolitica	d	+	−	−	−	(+)	−	−	−	+	−	−	−	−	−	−	+	+	−	d	−	d	+	+	−	−	(−)	+
Y. pestis	−	(+)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	d	−	−	−	+	−	−	(+)	(+)
Y. pseudotuberculosis	−	(+)	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	(−)	−	−	−	d	(−)	+	(+)	d

[a]Symbols: −, 0–10% of strains positive; (−), 11–25% of strains positive; d, 26–75% of strains positive; (+), 76–89% of strains positive; +, 90–100% of strains positive.

Table 10.6
Some Serotypes and Somatic and Pilus Antigens of *E. coli* Associated with Disease in Domestic Animals

Animal species	Serotypes	Somatic antigen	Pilus antigen
Swine	0139:K82(B); 0141:K85a,b(B) 0141:K88(L); 0141:K85a,c(B) 0149:K91; 0138:K81(B) 08:K87(B?); 08:K88(L) 018?:K?; 045:K?		K88 or F-4, K99 or F-5, 987P or F-6 F-41, Type 1
Calves		08, 09, 015, 017, 020, 021, 026, 035 055, 078, 0114, 0115, 0119, 0126, 0137 (many others reported)	K99 or F-5, F-41, Type 1[a]
Lambs	078:K80; 024:K? 078:K80(B)	08, 09, 015, 020, 026, 035, 086, 0101, 0137	K99 or F-5, F-41
Chickens		01, 02, 08, 011, 022, 078	

[a]Occasionally.

Immunological assays, such as an ELISA test for LT, are being developed, and these give some hope for a suitable diagnostic test for detecting the enterotoxigenic *E. coli* strains. Because the capacity to produce enterotoxin can be transmitted by plasmids from one strain of *E. coli* to another, it would follow that in assessing the pathogenic significance of strains isolated from cases of diarrheic disease, it is more important to determine if a given culture produces enterotoxin than to determine its serologic identity. After ingestion, enteropathogenic *E. coli* attach to and colonize the villous epithelium of the small intestine. Attachment is mediated by certain antigenic determinants present on the pili of the organism. Five important pilus antigens of *E. coli* that equate with virulence have been recognized in the diarrheal diseases of swine, calves, and lambs (see Table 10-6).

Recently, certain serotpyes of *E. coli* from calves have been shown to produce high levels of verotoxin. However, the significance of these *E. coli* serotypes in animals has yet to be determined.

Invasiveness has recently been identified as a distinct, but less common, pathogenic mechanism. Enteroinvasive *E. coli* strains can penetrate the intestinal epithelium, mainly that of the large intestine. Invasiveness can be detected by a strain's ability to produce keratoconjunctivitis in the eye of a guinea pig [Sereny test (16)], or by its capacity to penetrate cells in tissue culture (17).

Salmonella

The antigens of *Salmonella* serovars consist of:

Somatic or O antigens: Thermostable and designated by arabic numerals

Flagellar antigens: **Phase 1.** Designated by small letters of the alphabet; more or less specific for the salmonellae; **Phase 2.** Designated by Arabic numerals; less specific and duplicated in other bacterial species.

K antigens (capsular or envelope): **Vi** antigen, **M** antigen, and **5** antigen; **M** or mucoid antigen. These antigens may interfere with agglutinability of O antisera. Boiling of suspensions for 10–20 min destroys the **Vi** antigen.

The antigenic makeup of some *Salmonella* serovars commonly isolated from animals and animal sources (18) is given in Table 10-7 (19).

In most veterinary diagnostic laboratories, the salmonella isolates are examined serologically in order to determine the group to which they belong. Group identification is based on the possession of certain somatic antigens. The procedure is a simple slide agglutination test. It is usual to test an isolate first against a commercially available polyvalent **O** serum covering groups **A–I.** If this is positive, then tests are conducted with the individual group sera.

The culture of *Salmonella* to be serotyped can be taken from a TSI slant or from nutrient agar. Growth from selective media is often unsuitable for typing.

Proteus species may react with the polyvalent salmonella sera; however, *Proteus* species are urease positive and lysine decarboxylase negative, which distinguishes them from the salmonellae.

Further identification to the serovar is carried out with flagellar or **H** antisera. Many serovars are diphasic, having flagellar antigens in **phase 1** (specific) and **phase** 2 (group or nonspecific). A culture of *Salmonella* may have organisms in both phases or in just one of the phases. In the latter case, the culture, although capable of giving rise to the alternative phase, usually maintains a constant phase over several generations. Both flagellar phases must be identified to obtain the complete antigenic formula of the *Salmonella* and hence determine the serovar. This requires techniques of phase changing, which are rather involved and usually only carried out in reference laboratories.

Lysogenization by certain converting phages may produce changes in the O-antigenic formulae of the salmonellae. In antigenic groups **A, B,** and **D,** the presence of O-antigen 1 (factor 1) is associated with lysogenization, but the presence or absence of this factor in strains of these groups does not alter the name of the serovar. However, in group

Table 10-7
Some Important *Salmonella* Serovars Recovered from Animals, Birds, and Other Sources[a,b]

Serovars	Group	Somatic antigens	Flagella antigens Phase 1	Phase 2							
S. typhimurium	B	*1*, 4, [5], 12	i	1, 2	+	+	+	+	+	·	+
S. abortus-equi[c]	B	4, 12	—	e, n, x				·			
S. abortus-ovis[c]	B	4, 12	c	1, 6					·		
S. bredeney	B	*1*, 4, 12, 27	1, v	1, 7	+	·		·	·	·	·
S. derby	B	*1*, 4, [5], 12	g, f	[1, 2]	+	+	·	·	·	·	·
S. agona	B	4, 12	f, g, s	—	+	+	·	+	+	·	·
S. saint paul	B	*1*, 4, [5], 12	e,h	1, 2	+			·	·	+	
S. reading	B	*1*, 4, [5], 12	e,h	1, 5	+			·	·	+	
S. heidelberg	B	*1*, 4, [5], 12	r	1, 2	+	·	·		·	+	·
S. san diego	B	4, [5], 12	e, h	e, n, z_{15}	+	·	·			·	·
S. typhisuis	C_1	6, 7	c	1, 5		·	·				
S. choleraesuis	C_1	6, 7	c	1, 5		+					
S. choleraesuis biovar Kunzendorf	C_1	6, 7	[c]	1, 5		+					
S. infantis	C_1	6, 7, *14*	r	1, 5	+	·	·	·	·	·	·
S. oranienburg	C_1	6, 7	m, t	—	·	·	·	·	·	·	·
S. montevideo	C_1	6, 7, *14*	g, m, [p], s	—	+	·	·	·	·	·	·
S. newport	C_2	6, 8	e, h	1, 2	·	·	·	·	·	·	·
S. blockley	C_2	6, 8	k	1, 5	·			·	·	·	·
S. muenchen	C_2	6, 8	d	1, 2	·	·	·	·		·	·
S. manhattan	C_2	6, 8	d	1, 5	·	+		·	·	·	+
S. bovismorbificans[c]	C_2	6, 8	r	1, 5			·		·		
S. kentucky	C_3	8, *20*	i	z_6	·		+				
S. panama	D_1	*1*, 9, 12	1, v	1, 5	·	+	·	·			
S. gallinarum[c]	D_1	*1*, 9, 12	—	—	·				·		·
S. pullorum	D_1	9, 12	—	—	+						
S. enteritidis	D_1	*1* ,9, 12	g, m	[1, 7]	·	·	·	·	·		·
S. dublin	D_1	*1*, 9, 12, [Vi]	g, p	—		·	+		·		·
S. anatum	E_1	3, 10	e, h	1, 6	+	·	·	·	·	+	·
S. london	E_1	3, 10	1, v	1, 6	·	+	·	·	·		·
S. meleagridis	E_1	3, 10	e, h	1, w	·	·	·		·		·
S. give	E_1	3, 10	1, v	1, 7	·	·	·	·	·	·	·
S. muenster	E_1	3, 10	e, h	1, 5		·	+				
S. newingston	E_2	3, *15*	e, h	1, 6	·	·	·			·	·
S. senftenberg	E_4	1, 3, 19	g, [s], t	—	·	·	·		·		·
S. worthington	G_2	*1*, 13, 23	z	1, w	·	·		·	·		·
S. cubana	G_2	1, 13, 23	z_{29}	[z_{37}]			+				·
S. cerro	K	*6*, *14*, 18	z_4z_{23}	[1, 5]		·	+				

[a]See references 18 and 19.
[b]Number in italics, O antigen whose presence is due to phage converstion; [ ], antigen present or absent; +, commonly isolated; · = occasionally isolated.
[c]Not commonly listed as occurring in the United States.

Table 10-8
Main Characteristics Differentiating Salmonella Subgenera 1 and 111

	Salmonella subgenus 1	*Salmonella* subgenus 111 (Arizona)
Malonate	−	+
Lactose	−	v[a]
ONPG	−	+
Dulcitol	+	−

[a]v, Variable reaction.

Table 10-9
Biochemical Differentiation of *Salmonella* Biovars[a]

	S. choleraesuis	*S. choleraesuis* biovar Kunzendorf	*S. typhisuis*	*Salmonella* (most serovars)
H_2S (TSI)	−	+	v	+
Lysine	+	+	−	+
Citrate (Simmons's)	+	+	−	+
Mannitol	+	+	−	+
Inositol	−	−	+	v
Sorbitol	(+)	(+)	−	+

	S. pullorum	*S. gallinarum*	*Salmonella* (most serovars)
Glucose (gas)	(+)	−	+
Dulcitol	−	+	+
Maltose	−	+	+
Ornithine	+	−	+
Rhamnose	+	−	+

[a]v, Variable reactions; (+), most strains positive.

Table 10-10
Differentiation of the Genera *Proteus*, *Providencia*, and *Morganella*[a]

	Proteus	*Providencia*	*Morganella*
Swarming	+	−	−
H_2S (TSI)	+	−	−
Gelatin liquefaction	+	−	−
Citrate (Simmons's)	v	+	−
Mannose	−	+	+
Maltose	v	−	−
Ornithine	v	−	+
Urease (Christensen's)	+	v	+

[a]v, Variable.

Table 10-11
Some Characteristics of the Less Frequently Recovered Organisms from Veterinary Specimens[a]

	Glucose (gas)	Motility	Reaction on TSI (Slant/butt)	H_2S (TSI)	Lysine	Indole	Lactose	Sucrose	Esculin hydrolysis	Isolation from human clinical specimens
Buttiauxella	+	+	A/A	−	−	−	+	−	+	+
Cedecea	+	+	A/A	−	−	−	v	v	v	+
Hafnia	+	+	Ak/A	−	+	−	−	−	−	+
Kluyvera	+	+	A/A	−	v	+	+	+	+	+
Koserella	+	+	Ak/A	−	+	−	−	−	v	+
Leminorella	−	−	Ak/A	+	−	−	−	−	−	+
Moellerella	−	−	A/A	−	−	−	+	+	−	+
Rahnella	+	−	A/A	−	−	−	+	+	+	+
Shigella	−	−	Ak/A	−	−	v	−	v	−	+
Tatumella	−	−	A/A	−	−	−	−	+	−	+

[a]v, Variable; Ak, alkaline; A, acid reaction.

E, Phage E15 alters the O-antigen **3,10** to **3,15,** thus making *S. anatum* become *S. newington.* Other serovars in group **E** are also involved (7).

Variations can occur in salmonellae. To prevent smooth–rough (S→R) dissociation, the freshly isolated strains should be maintained on media without added carbohydrate. Rough strains may autoagglutinate in saline and are unsuitable for typing. Flagellated serovars may give rise to nonflagellated variants (OH→O variation). This change tends to be irreversible. Some serovars, such as *S. pullorum,* are permanently without flagella.

The antigenic schema for salmonellae in subgenus 111 (Arizona) is based on **O** and **H** antigens. More than 300 serovars have been identified. The antigenic formulae of most of these Arizona serovars have been translated into *Salmonella* formulae and included in the Kauffmann–White scheme (7). The key differential characteristics between *Salmonella* subgenus 1 and *Salmonella* subgenus 111 ("Arizona") are given in Table 10-8.

Salmonella biovars have the same antigenic formula, but they may differ in the disease syndrome that they cause and in certain biochemical reactions. The differential biochemical characteristics for some important veterinary biovars are given in Table 10-9.

Proteus, Providencia, and *Morganella*

Species are identified on the basis of characteristics in Table 10-10. All deaminate phyenylalanine. The main differentiating characteristics are shown in Table 10-10.

Other Enteric Organisms

Biochemical reactions for *Citrobacter, Enterobacter, Serratia,* and *Yersinia* species are given in Table 10-5. Biochemical reactions of less frequently recovered organisms belonging to the genera *Buttiauxella, Cedecea, Hafnia, Kluyvera, Koserella, Leminorella, Moellerella, Rahnella, Shigella,* and *Tatumella* are given in Table 10-11. For full identification refer to *Bergey's Manual of Systematic Bacteriology* (2) and Farmer *et al.* (20).

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11

Pasteurella and *Francisella*

G. R. Carter

The genus *Francisella* is quite distinct from *Pasteurella* taxonomically, and there is no reason other than tradition and convenience for including it in this chapter.

Pasteurella

Members of this genus are small, nonmotile, gram-negative rods or coccobacilli. They are facultatively anaerobic, nonsporing, fermentative (except *P. anatipestifer*), and oxidase positive.

Pasteurella anatipestifer is a nonfermenter and clearly does not belong in the genus *Pasteurella*. Its proper taxonomic place is still uncertain.

The taxonomy of the genera *Pasteurella* and *Actinobacillus* is in a state of flux. The names in this chapter will be those of *Bergey's Manual* (1) and a new reclassification recommended recently (2).

According to this new classification based on DNA hybridization studies, the two important biotypes of *Pasteurella haemolytica* were found to be more closely related to the genus *Actinobacillus* than *Pasteurella*. However, we will follow *Bergey's Manual* (1) and include both biotypes in the genus *Pasteurella*.

Other species which were found to have levels of homology less than 40% were *Pasteurella pneumotropica*, *P. aerogenes*, and *P. ureae*. The final taxonomic position of these species remains to be settled. We will follow *Bergey's Manual* and consider them members of the genus *Pasteurella*.

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Pasteurella multocida

This is a heterogeneous species in that it possesses strains with differences in pathogenicity, host preference, serologic and antigenic characteristics, and cultural and biochemical characteristics. A serological classification has been developed for *P. multocida* in which serotypes are identified on the basis of differences in capsular and somatic antigens (3). The capsular antigens are designated A, B, D, E, and F, while the somatic or lipopolysaccharide antigens are designated by numbers 1 through 16. The serotypes B:2 and E:2 cause hemorrhagic septicemia of cattle and water buffaloes; capsular A strains are associated with pneumonic disease in cattle, sheep and swine, usually in a secondary capacity; capsular D strains, some of which produce a dermonecrotoxin, have been considered to cause atrophic rhinitis of swine; and some serotypes, such as A:1, A:3, A:4 and A:5, cause fowl cholera. Serotyping is not usually carried out in diagnostic laboratories.

In the recommended classification of the genus *Pasteurella* (2), three subspecies of *P. multocida* have been recommended based mainly on small differences in the fermentation of carbohydrates (see Table 11-1). Given the close relationship of these subspecies, it is questionable whether such fine distinctions serve any practical purpose. However, their identification may be useful in epidemiological studies. *Pasteurella multocida* subsp. *multocida* includes those strains that cause the principal pasteurelloses referred to under *P. multocida* below. *Pasteurella multocida* subsp. *septica* includes strains from various sources including feline, canine, human, and avian. They appear to have been mainly significant in human wounds due to dog and cat bites. *Pasteurella multocida* subsp. *gallicida* probably includes the fowl cholera serotypes A:1 and some other avain strains. For our purposes we will consider them one species, *P. multocida.*

It has been known for years that many *Pasteurella* strains, usually called pasteurella-like or *P. multocida*-like, recovered from dogs, cats, and their bites and occasionally from other animals, differ in some respects from typical strains of *P. multocida* recovered from cattle, sheep, and swine. The recommended reclassification (2) has served a valuable function in assigning these organisms and some others to a number of species which are discussed below.

Pathogenicity

Pasteurella multocida occurs commonly in the upper respiratory and digestive tracts of a wide range of birds and mammals. The diseases with which it is associated are too numerous to list comprehensively. It is a frequent secondary invader or opportunist in a number of pathologic processes.

It is a primary, or more frequently a secondary, invader in pneumo-

nia of cattle, swine, sheep, goats, and other species. It is also involved frequently in bovine pneumonic pasteurellosis or "shipping fever complex" and in enzootic pneumonia of pigs. It is considered the primary cause of fowl cholera and of hemorrhagic septicemia of cattle, bison, and water buffaloes. It is one of the causes of the pleuropneumonia form of "snuffles" in rabbits and a cause of severe mastitis of cattle and sheep. Various infections have been reported from other domestic and wild animal species.

Some capsular type D strains of *P. multocida* produce a dermonecrotoxin and are thought to have an important role in the cause of atrophic rhinitis of swine. Procedures for the demonstration of the toxin are described later in this chapter.

A wide variety of infections occur in human beings (4). They have been classified as follows:

1. Superficial infections
   A. Infection of surface abrasions caused by animal bites and scratches
   B. Infections of existing lesions of the skin or mucous membranes caused by contact with an infected animal: leg and oral ulcers, corneal ulcer, conjunctivitis, etc.
2. Internal infections: pneumonia, bronchitis, pleurisy, mastoiditis, empyema, meningitis, otitis, brain abscess, etc.
3. Septicemia or bacteremia: usually secondary to an underlying chronic disease

Pasteurella multocida-like Organisms

Dogs, cats, and some other animals harbor *P. multocida* or *P. multocida*-like organisms in their oropharynx as commensals. Bites inflicted upon humans and animals by these carrier animals are frequently infected with the aforementioned pasteurellae. The recommended reclassification of the genus *Pasteurella* (2) has provided names for some of these *Pasteurella multocida*-like organisms. They are listed below and in Table 11-1.

Pasteurella canis

Many of the *P. multocida*-like strains from the oral cavities of dogs and dog-bite infections conform to this new species.

Pasteurella dagmatis

This is a commensal of the upper respiratory and digestive tracts of dogs and cats. It is a cause of local and systemic human infections

Table 11-1
Differential Characteristics of Species in the Proposed Reclassification of the Genus *Pasteurella*[a,b]

Taxon	NAD requirement[c]	Ornithine	Indole	Urease	Acid produced within 24–48 hr from Trehalose	Maltose	D-Xylose	L-Arabinose	Mannitol	Sorbitol	Dulcitol
P. multocida subsp. *multocida*	–	+	+	–	d	–	d	–	+	+	–
P. multocida subsp. *septica*	–	+	+	–	+	–	+	–	+	–	–
P. multocida subsp. *gallicida*	–	+	+	–	–	–	+	d	+	+	+
P. dagmatis	–	–	+	+	+	+	–	–	–	–	–
P. gallinarum	–	–	–	–	+	+	–	–	–	–	–
P. canis	–	+	d	–	d	–	d	–	–	–	–
P. stomatis	–	–	+	–	+	–	–	–	–	–	–
P. anatis	–	–	–	–	+	–	+	–	+	–	–
Pasteurella species B	–	+	+	–	+	+	+	–	–	–	+
Pasteurella species A	+	–	–	–	+	d	d	+	d	–	–
P. langaa	–	–	–	–	–	–	–	–	+	–	–
P. avium	d	–	–	–	+	–	d	–	–	–	–
P. volantium	+	d	–	–	+	+	d	–	+	d	–

[a]Adapted from Mutters *et al.* [2].
[b]Symbols: +, ≥90% of the strains are positive; –, ≥90% of the strains are negative; d, different results observed.
[c]NAD, Nicotinamide adenine dinucleotide.

resulting from animal bites. These strains may produce gas and they are identicial to the Henriksen biotype of *P. pneumotropica*.

Pasteurella stomatis

Strains of this species have been recovered from the respiratory tracts of dogs and cats.

Many of the *Pasteurella* strains recovered from cats and a lesser number from dogs are typical *P. multocida*. *Pasteurella* strains have a low capacity for causing disease in dogs and cats. They are most often secondary invaders and involved in mixed infections.

Pasteurella anatis

Members of this proposed new species have been recovered from the intestinal tracts of ducks.

Pasteurella langaa

Strains of this species have been isolated from the respiratory tracts of normal chickens.

Pasteurella avium* and *P. volantium

The former species, which may have a requirement for NAD, had previously been called *Haemophilus avium*. This species and *P. volantium*, which has a requirement for NAD, have been recovered from the respiratory tracts of healthy chickens.

There is little information available on the significance of the varieties *Pasteurella* A and *Pasteurella* B, listed in Table 11-1. Only a small number of strains have been studied.

Pasteurella haemolytica

Two different types of *P. haemolytica*, designated A and T, have been described based upon differences in fermentative activity, serological characteristics, and pathogenicity (5). Different serotypes have been identified based on capsular and somatic antigens. The occurrence of serotypes relative to host's disease and biotype are shown in Table 11-2 along with other differential characteristics.

Strains closely related but not identical to typical *Pasteurella haemolytica* biotypes A and T have been isolated from sheep and cattle. Bisgaard and Mutters (6) have identified six new biogroups within what they call the *P. haemolytica* complex. Kilian and Frederiksen (7) have described what they called *P. haemolytica*, third type (7). Bisgaard (8) described strains from swine that differed from typical strains of *P. haemolytica*.

Table 11-2
Differential Characteristics of Biotypes of *Pasteurella haemolytica*[a]

	Biotype A	Biotype B
Fermentation		
arabinose	+	−
trehalose	−	+
salacin	−	+
xylose	+	−
lactose	+	−
Susceptibility to penicillin	High (except serotype 2)	Low
Serotypes	1,2,5,6,7,8,9,11,12	3,4,10
Principal location in normal host	Nasopharynx	Tonsils
Principal disease association	Pneumonia in cattle and sheep; septicemia in nursing lambs	Septicemia in feeder lambs

[a]With permission from Biberstein (5).

Pathogenicity
Strains of *P. haemolytica* are frequently involved as a primary or secondary agent in pneumonias of cattle, sheep, goats, and swine. They are commonly recovered from the bronchopneumonic lungs of cattle with shipping fever or pneumonic pasteurellosis. Other important diseases in which they are involved are mastitis of ewes and septicemia of lambs.

Avian *Pasteurella haemolytica*
Synonym: *Actinobacillus salpingiditis*

Organisms isolated from chickens and turkeys that have been called *P. haemolytica* differ in several characteristics from the ruminant strains of *P. haemolytica.* They have larger zones of hemolysis and, unlike ovine and bovine strains of *P. haemolytica,* they do not usually ferment dextrin and maltose (9). Avian strains usually ferment trehalose while bovine strains do not (9). Strains similar to the avian strains have been recovered from swine and horses (5). The correct taxonomic position of these strains remains to be determined.

Pathogenicity
This organism has a low capacity for causing disease. It has been associated with salpingitis and respiratory infections. Infections are usually secondary to another disease or some predisposing condition.

Pasteurella pneumotropica

Frederiksen (10) refers to the *P. pneumotropica* complex, which included three different biotypes: Henriksen, Heyl, and Jawetz. The Henriksen biotype or *Pasteurella* "gas" is now *P. dagmatis* in the new classification. Although the Heyl and Jawetz biotypes will probably be transferred to the genus *Actinobacillus* (2), we will retain the name *P. pneumotropica*.

Pathogenicity

For the pathogenicity of the Henriksen biotype, see *P. dagmatis* above. The Jawetz and Heyl biotypes are commensals in the upper respiratory and digestive tracts mainly of mice and rats. They may be secondary invaders in respiratory infections mainly in mice and rats. *Pasteurella pneumotropica* only rarely causes human infections.

Pasteurella aerogenes

This gas-producing organism, which is probably worldwide in distribution, is most frequently isolated from the feces of swine (11). It would seem to have a low potential for causing disease. Only one of the original 25 isolates was considered to have a pathogenic role. It was recovered from swine fetuses and considered to be the cause of abortion. There have been reports of several isolations from cattle and rabbits. The correct taxonomic position of this species is still in doubt (2).

The Centers for Disease Control, Atlanta, Georgia, have received eight human isolates of *P. aerogenes*. Four were from wounds caused by swine bites, while the other two were from bite wounds of unstated origin (12).

Pasteurella gallinarum

This organism, which is now considered an official species, is found as a commensal in the upper respiratory tract of chickens and turkeys (13). Its potential for causing disease is low, and in respiratory disease processes it is usually present as a secondary invader. Mraz *et al.* (14) have described the characteristics of this species in some detail.

Pasteurella ureae

Recent DNA studies (2) with *P. ureae* indicate that it should ultimately be included in the genus *Actinobacillus*. It is recovered

infrequently from the upper respiratory tract of normal humans. It has been isolated from human patients with rhinosinusitis, bronchitis, ozena, septicemia, and postsurgical infections. Because it often occurs with other bacteria, its causal role is not always clear. There do not appear to be authentic reports of its occurrence in animals.

Pasteurella anatipestifer
Synonym: *Moraxella anatipestifer*

As mentioned previously, this organism is a nonfermenter and should not be included in the genus *Pasteurella*.

Pathogenicity

It is the cause of an acute or chronic septicemic disease (infectious serositis) of 1- to 8-week-old ducklings. Among the principal lesions are those of a fibrinous polyserositis. Infections have also been reported in pheasants, quail, waterfowl, and turkeys.

Laboratory Procedures

Isolation

The specimens from which pasteurellae are isolated are quite varied. The preferred medium for the recovery of *Pasteurella* spp. is blood agar. Selective media for the isolation of *P. multocida* (15,16), and for *P. multocida* and *Bordetella bronchiseptica* from nasal specimens have been described (15,17,18).

Pure cultures of many strains of *P. multocida* can be obtained from clinical materials containing other bacteria by the inoculation of suspensions of such materials into mice or rabbits. The isolation rate of *P. multocida* from swine nasal swabs was considerably increased when suspensions from swabs were inoculated into mice intraperitoneally (19).

Cultural Characteristics

P. multocida

Colonies appear after incubation for 24 hr at 37°C. They are usually of moderate size, round, and grayish. Some strains of capsular type A produce large mucoid colonies. Organisms from these mucoid colonies possess large capsules consisting mostly of hyaluronic acid. The various pasteurellae from dogs usually have smaller colonies than those of *P. multocida* from cattle, sheep, and swine. Colony size of the pasteur-

ellae in general depends to a great extent upon the dissociation status of the culture. Plate cultures usually have a characteristic musty odor.

The various species listed above under *Pasteurella multocida*-like organisms give rise to colonies that resemble in appearance those of nonmucoid *P. multocida.*

P. haemolytica
Satisfactory growth is obtained after 24-hr incubation. Colonies are round, grayish, and somewhat smaller than those of *P. multocida.* They are usually surrounded by a zone of β-hemolysis. This zone varies considerably and may be no larger than the colony and thus not apparent unless the colony is removed. Antibodies in media may inhibit hemolysis. Bovine blood is more suitable than that of the sheep or horse for the demonstration of hemolysis.

The various *P. haemolytica* varieties mentioned earlier, other than biotypes A and T, give rise to colonies that resemble these biotypes. However, there may be differences in hemolytic activity.

P. pneumotropica and *P. gallinarum*
The colonies of these species are indistinguishable from those of *P. multocida.*

P. anatipestifer
This organism grows best on blood or serum agar in an atmosphere of 5–10% carbon dioxide. A candle jar is satisfactory. Small dewdroplike colonies appear within 48 hr.

P. aerogenes
The colonies of this species resemble those of nonmucoid *P. multocida* except that they are somewhat smaller. On MacConkey agar, colonies resemble those of *Salmonella* in 24 hr, but in 36 hr, they develop a faint pinkish color.

P. ureae
The colonies of this organism resemble those of *P. haemolytica.*

Identification
Gram-stained smears from colonies of the species referred to above reveal small gram-negative rods or coccobacilli (Fig. 11-1). The reactions on TSI are presented in Table 10-4. Definitive identification is based upon differential characteristics listed in Tables 11-1 and 11-3. Differential characteristics of the two biotypes of *P. haemolytica* are given in Table 11-2. *Pasteurella anatipestifer* differs from the other pasteurellae in producing gelatinase, being nonfermentative, and not reducing nitrate.

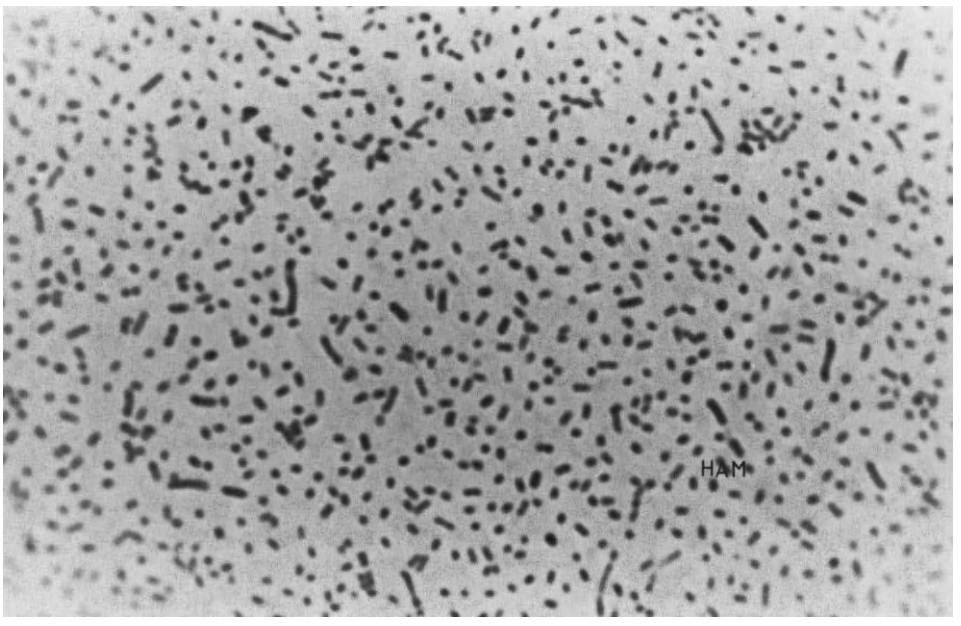

Figure 11-1. *Pasteurella multocida* from a blood agar plate culture. Gram stain, ×3000 (H. A. McAllister).

A number of cultures will be indole negative in SIM medium, peptone water, or tryptone broth. The author has found that heart infusion broth (Difco) supplemented with 2% tryptone is highly satisfactory for indole production by *P. multocida*. Clemons and Gadberry (20) described a medium supplemented with 2% peptone that gave excellent results.

The oxidase reaction may be negative with *P. multocida* strains if commercial test strips or the tetramethyl reagent containing stabilizing agents is used (12). The oxidase reaction is positive when 18–24 hr growth on a blood plate is tested with a 5% aqueous solution of tetramethyl-*p*-phenylenediamine (12).

The differential characteristics of *Pasteurella* species in the proposed reclassification are presented in Table 11-1.

Demonstration of the Dermonecrotic Toxin

As mentioned earlier, some capsular type D strains, but rarely other capsular types, produce a dermonecrotic toxin considered to be important in the etiology of atrophic rhinitis of swine.

The procedures outlined below have been effective in demonstrating

Table 11-3
Differential Characteristics of Important *Pasteurella* spp.[a]

	β-Hemolysis	MacConkey agar	Indole	Urease	Ornithine decarboxylase	Fermentation: Acid/Gas				
						Glucose	Lactose	Sucrose	Maltose	Mannitol
P. multocida	–	(–)	+	–	(+)	A	(–)	A	(–)	(A)
P. haemolytica (see Table 11-2)	(+)	(+)	–	(–)	(–)	A	(A)	A	A	A
P. pneumotropica	–	(–)	(+)	+	+	A	(A)	A	A	–
P. gallinarum	–	–	–	–	+/–	A	–	A	A	–
P. ureae	–	–	–	+	–	A	–	A	A	A
P. aerogenes	–	+	–	+	(+)	AG	–	AG	AG	–
P. anatipestifer	–	–	–	–	NA	–	–	–	–	–

[a](+), Most positive; (–), most negative; –/+, negative and positive strains; NA, not available.

the toxin. Five milliliters of brain–heart infusion broth containing 0.3% yeast extract is inoculated with the strain to be tested. Following incubation for 6–8 hr at 37°C the broth culture is cooled, sonicated in an ice bath for 5 min at an intensity of 55 (Bronwill Brosonik), then centrifuged at 2000 *g* for 20 min at 4°C. The supernatant fluid is filtered through a 0.22-μm membrane. It is used immediately or stored at −60–70°C. A strain of *P. multocida* known to be negative for the toxin should be included as a control.

Mouse Test

One-half milliliter of the filtrate is inoculated intraperitoneally into several mice weighing 20–30 gm. The mice are observed for 4 days. If appreciable levels of toxin are present, mice usually die within 24 hr.

Guinea Pig Test

One-tenth milliliter of filtrate is injected intradermally into the clipped or shaved skin of a guinea pig. Six to eight cultures may be tested for toxin in one guinea pig. Zones of necrosis are measured at 48 hr; they are usually visible within 24 hr. An enzyme-linked immunosorbent assay has been described for the recognition of toxigenic isolates of *P. multocida* (21).

Francisella

This genus contains two species, *Francisella tularensis* and *F. novicida*. The latter species was isolated from a water sample and is mainly of academic interest. Both species are small, gram-negative rods or coccobacilli that require cystine for growth. *Francisella tularensis*, a facultative intracellular parasite, is an important obligate pathogen with a wide geographic distribution.

Pathogenicity

Tularemia is principally a disease of wild animals. Humans, as well as some domestic animals and fowl, are susceptible. In nature, the disease is transmitted by insect vectors. Most human infections are acquired as a result of handling and dressing infected rabbits. Infections have been reported in many animal species, including squirrel, opossum, woodchuck, muskrat, skunk, coyote, fox, cat, sheep, deer, game birds, and domestic fowl.

The characteristic lesions observed in wild rabbits and in other animals are small necrotic foci in the liver, spleen, and lymph nodes.

Isolation Procedures

Great care should be exercised in working with potentially infectious material and cultures. A considerable number of serious and occasionally fatal laboratory infections have occurred. A medium containing cystine is essential for cultivation. Glucose cystine agar (BBL) or cystine heart agar (Difco) to which is added blood or hemoglobin is satisfactory. Material is inoculated liberally onto this medium, and onto blood agar, and incubated at 37°C for 3–5 days. Incubation in a candle jar or in 10% carbon dioxide is advantageous.

Frequently, specimens yield a variety of bacteria and in order to recover *F. tularensis*, it may be necessary to inoculate cystine media containing penicillin, polymyxin B, and cycloheximide. Animal inoculation is also useful in the recovery of *F. tularensis* from contaminated specimens. Approximately a 10% tissue suspension is prepared in broth with a tissue grinder. Graded doses are inoculated subcutaneously into several guinea pigs or mice. Infections are generally fatal within 5–10 days. The organism can then be isolated from the liver or spleen. Focal areas of necrosis in the liver and spleen strongly indicate tularemia.

In the case of guinea pigs, blood samples may be taken 1 week postinoculation and tested for agglutinins to *F. tularensis*. A positive test constitutes a diagnosis of tularemia.

Direct Examination

The best procedure for the rapid and specific identification of *F. tularensis* is direct or indirect fluorescent antibody (FA) staining of smears from exudates and affected tissue such as the necrotic foci in the liver and spleen. Preparations from formalin-fixed tissues can also be used. The FA conjugate is available commercially.

Cultural Characteristics and Identification

Francisella tularensis may grow slowly and colonies are initially small and dewdroplike. They enlarge with longer incubation and tend to become confluent. Discrete colonies may be difficult to obtain on subculture. Especially notable is the marked greening around colonies on blood agar.

The organism grows poorly in media used for biochemical tests. It is best identified by a slide agglutination test using a specific antiserum or by fluorescent antibody (direct or indirect) staining of smears.

Francisella tularensis antigen is available commercially for the detection of specific antibody in the sera of infected laboratory animals.

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12

Actinobacillus

J. E. Phillips

Survey of the Genus

The organisms of this genus are small, gram-negative, nonmotile, nonsporing bacilli and coccobacilli, often interspersed with coccal elements, some of which may lie at the poles of longer forms, giving the characteristic "morse code" form. They are aerobic, microaerophilic, or facultatively anaerobic, fermenting carbohydrates with the production of acid, but no gas.

The following species are included in the genus in *Bergey's Manual of Systematic Bacteriology: Actinobacillus lignièresii, A. equuli, A. suis, A. capsulatus,* and *A. actinomycetemcomitans. Actinobacillus seminis,* included in the genus when it was first described in 1960, appears not to belong to this group. There is, however, strong evidence that *Haemophilus pleuropneumoniae* should now be regarded as an Actinobacillus *(A. pleuropneumoniae). Actinobacillus actinoides,* another organism earlier included in the genus, is now excluded. It has similarities with *Haemophilus somnus,* which has already been described.

Pathogenicity

Most species of this genus are found characteristically as commensals on the alimentary, respiratory, and genital mucosa of several animal species and humans, but some occur only as pathogens, while the commensal species are also found in a variety of lesions.

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A. lignièresii

This organism is found mainly associated with granulomatous lesions of the soft tissues of the head and upper alimentary tract of cattle, especially the tongue, soft palate, and walls of the rumen and reticulum. The lesions in sheep are usually more suppurative than those in cattle, and are found particularly in the cutaneous areas of the head and neck, and in the lungs. The granulomatous lesions produced are similar to those caused by *Actinomyces bovis* in bone in cattle, but the "sulfur granules" are not so distinct, and the actinobacillus is spread by the lymphogenous route. *Actinobacillus lignièresii* is found infrequently in other animal species, having been reported in the tongues of dogs and horses, in lymphadenitis in laboratory rats, and in humans following horse bites.

A. equuli

Although occurring as a commensal organism in the intestinal and pharyngeal regions of normal horses, *A. equuli* is a pathogen of this host species, causing pre- and postnatal infections of foals, as well as infections in older horses. In foals, it is found associated with purulent nephritis, pneumonia, and enteritis; in mares, it can cause abortion and septicemia; it is also found as a complicating infection in verminous aneurysms. *Actinobacillus equuli* is also a pathogen of pigs, in which it causes arthritis in piglets and endocarditis in older animals. It has been recovered infrequently from lesions in other animal species, including calves, dogs, rabbits and rats.

A. suis

This porcine actinobacillus is found associated with septicemic disease with a rapid and fatal course (1) in pigs of all ages, but especially young piglets up to 3 months of age. It may also cause pneumonia and arthritis. It is recognized also as a pathogen of horses. Although it has not been recovered from normal swine, its commensal role in the upper respiratory tract has been reported (2).

A. capsulatus

Arthritis in rabbits is the only disease ascribed to this organism (3), the histology of the lesions being similar to those caused by *A. lignièresii* in cattle.

A. actinomycetemcomitans

This is an organism that was considered to be of doubtful pathogenicity when it was first reported as a secondary invader in cases of human actinomycosis. Although it is of limited importance in the veterinary field, it is now regarded as playing a considerable role in periodontal

disease in man, as well as being found as the sole bacterial agent in some cases of endocarditis. There is evidence that it may be a pathogen in animals, having been reported in epididymitis in rams (4,5).

A. (H.) pleuropneumoniae
This organism is a pathogen of swine that is responsible for a distinctive pneumonia accompanied by a fibrinous pleuritis. In some cases, it may cause meningitis and arthritis as well. It has not been recognized as a commensal organism in normal swine.

"A. seminis"
This organism has been recovered from cases of ovine epididymitis (6,7). The disease is clinically indistinguishable from the epididymitis in rams due to *Brucella ovis*.

Laboratory Procedures

Direct Examination

This procedure may be of value in the presumptive diagnosis of actinobacillus caused by *A. lignièresii*. Washing the pus or caseous material from a lesion with distilled water in a petri dish allows small, gray-white granules characteristic of actinobacillosis to be seen with a hand lens or the low power of a microscope. Such granules, transferred to a slide and crushed gently with a coverslip, will reveal club-shaped structures under low power. The coverslip can then be removed and the material spread thinly, and the smear stained by Gram's method. The presence of small, gram-negative rods suggests actinobacillosis.

Isolation Procedures

Actinobacilli of all species will grow satisfactorily from infected tissues on blood or serum agar; the addition of 5–10% carbon dioxide or the use of a candle jar often improves growth. Most strains of *A. pleuropneumoniae* require NAD (a staphylococcal streak) for isolation and cultivation. When examining granulomatous lesions, additional plates should be set up and incubated anaerobically because the causative agent of the clinically similar disease, actinomycosis, is the anaerobic organism *Actinomyces bovis*. Recovery of actinobacilli from mixed bacterial populations (e.g., the rumen) may be aided by the use of a selective medium containing oleandomycin and nystatin (8).

Cultural Characteristics

A. lignièresii

Small, bluish-white glistening colonies resembling those of *Pasteurella haemolytica* develop within 24 hr. They are usually slightly viscid or "sticky" on primary isolation, but this characteristic is lost on subculture.

A. equuli

Colonies are usually moist and extremely "sticky" and this remains even after repeated subculture. This stickiness is also a characteristic feature of broth cultures. Some strains are hemolytic.

A. suis

Colonies are similar to those of *A. lignièresii*, except that the viscid character is more apparent, but not so marked as with *A. equuli*. Broth cultures are also viscid, but less so than *A. equuli*. All strains are hemolytic.

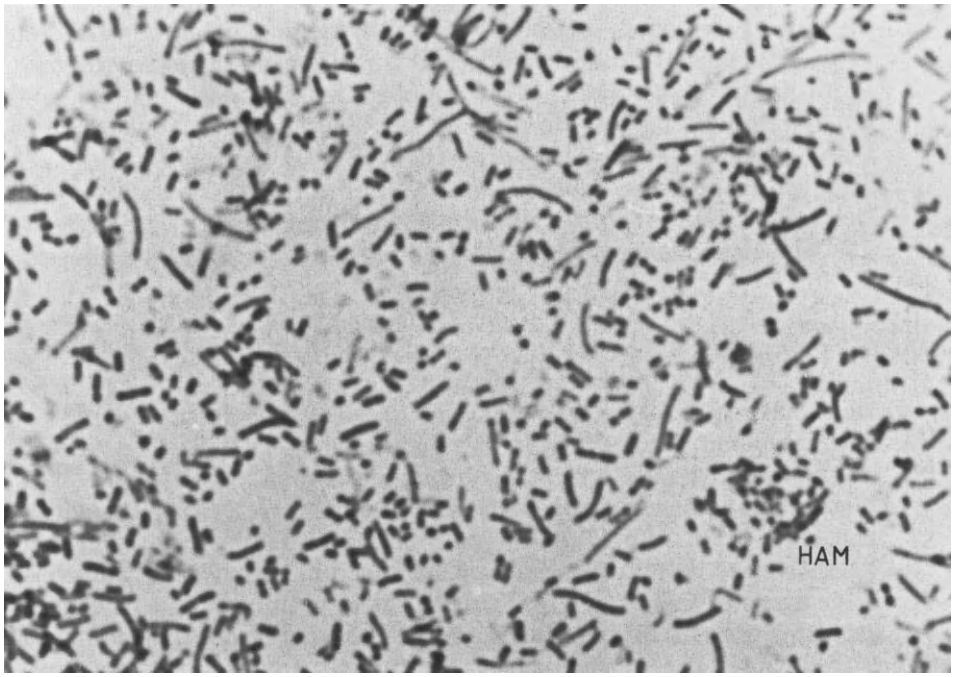

Figure 12-1. *Actinobacillus equuli* from a blood agar plate culture. Gram stain, ×2500 (H. A. McAllister).

Table 12-1
Differential Characteristics of *Actinobacillus* Species[a]

	A. lignièresii	*A. equuli*	*A. suis*	*A. capsulatus*	*A. actinomycetemcomitans*	*A. pleuropneumoniae*	*"A. seminis"*
Hemolysis (sheep)	–	v	+	–	–	+	–
Catalase	+	–	+	+	+	v	–
Hydrogen sulfide	+	v	–	–	–	+	–
Arabinose	–	–	+	+	–	–	–
Cellobiose	–	–	+	+	–	–	–
Esculin	–	–	+	(+)	–	–	–
Lactose	(+)	+	+	+	–	–	–
Mannitol	+	+	–	+	v	–	–
Melibiose	–	+	+	+	–	–	–
Salicin	–	–	+	+	–	–	–
Sorbitol	–	–	–	+	–	–	–
Trehalose	–	+	+	+	–	–	–

[a](+), Late reaction; V, variable.

A. capsulatus
Very sticky colonies are produced on blood agar and the cells are capsulated.

A. actinomycetemcomitans
Colonies are firmly adherent to the medium and difficult to break up.

A. pleuropneumoniae
Two types of colony may be found, a rounded "waxy" type, and a flatter, soft, glistening type. Both types are hemolytic, and the species give a positive CAMP reaction with a β-toxin-producing staphylococcus. Most strains require the V factor (NAD) in the medium, but some less fastidious strains may be encountered.

"A. seminis"
Small, pinpoint, round, grayish-white nonhemolytic colonies are produced on blood agar after 24 hr of incubation. They enlarge considerably after additional incubation.

Identification

All species of this genus appear as small, gram-negative rods or coccobacilli (Fig. 12-1). Occasionally, longer forms occur, especially when media contain fermentable substrates (e.g., glucose or maltose agar). All are nonmotile, reduce nitrates, are indole negative, and produce acid without gas from glucose. Most species are urease positive; most will grow on MacConkey medium (*A. pleuropneumoniae* will not). *Actinobacillus pleuropneumoniae* is CAMP positive. The main features that differentiate the members of this genus are set out in Table 12-1.

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Supplementary Readings

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13

Haemophilus and *Taylorella*

E. L. Biberstein

Haemophilus

The genus *Haemophilus* has traditionally been described as consisting of small, nonmotile, facultatively anaerobic, gram-negative pleomorphic rods or coccobacilli (Fig. 13-1), which require one or both of two defined growth factors, X and V, commonly supplied as hemin and nicotinamide adenine dinucleotide (NAD), respectively. By corollary, any organism corresponding to that description was assigned to the genus. As a result of nucleic acid hybridization studies, it has been shown that the genus *Haemophilus* as just described is extremely heterogeneous genetically and that a number of species traditionally included in the genus were actually more closely related genetically to type species of other genera than to the type species of the genus *Haemophilus*, *H. influenzae*. Consequently two species, *H. pleuropneumoniae* and *H. avium*, which are listed as *Haemophilus* species in the most recent edition of *Bergey's Manual* (1), have been reassigned to the genera *Actinobacillus* (2) and *Pasteurella* (3), respectively. The remaining *Haemophilus* species of veterinary interest are apparently no more closely related to *H. influenzae* than the two that have been ejected from the genus, but so far no specific proposals for their reclassification have been made.

Apart from their requirement for X, V, or both factors, *Haemophilus* spp. share an ability to reduce nitrates to nitrites or beyond, and, except for some strains of *H. ducreyi*, a parasite of humans only, to ferment glucose and other carbohydrates (1).

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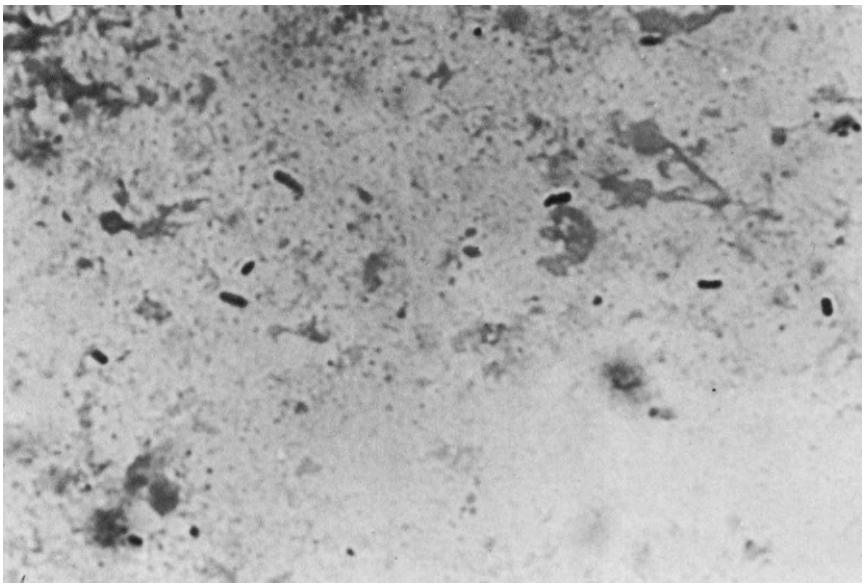

Figure 13-1. *Haemophilus paragallinarum* in sinus exudate from chicken with infectious coryza, × ~3000.

Pathogenicity

Organisms corresponding to the traditional description of *Haemophilus* occur in many host species, most commonly as apparent commensals on mucous membranes, especially of the upper respiratory and lower genital tracts. Some have a potential for pathogenicity but few are consistently pathogenic. The following have been encountered in animals.

Haemophilus parasuis (4) is a common V factor-requiring commensal of the porcine upper respiratory tract. *H. parasuis* becomes involved in swine influenza and enzootic pneumonia as a secondary invader and is an apparently primary cause of Glässer's disease (infectious polyserositis) in young or previously unexposed pigs (5,6). A much rarer bacterium, which occupies the same habitat, is all but indistinguishable from this agent, except for its additional requirement for X factor. Such an organism was first described as *H. suis* in connection with the diseases now associated with *H. parasuis* (7), but recent isolates requiring X factor lacked a clearcut pathogenic role, and their identity with the original *H. suis* is not established. The name has therefore been discontinued for the time being.

Haemophilus (para) gallinarum (4) is the agent of infectious coryza,

an upper respiratory infection of chickens. Originally characterized as requiring both X and V factors (8), the current representatives of the infectious coryza agent have been invariably V-requiring only and have therefore been named *H. paragallinarum*. The organism needs to be positively differentiated from the frequently V factor-requiring nonpathogenic *Pasteurella avium* (q.v.), with which it may coexist in the same sample. Since the exclusion of the swine pleuropneumonia agent from the genus *Haemophilus, H. paragallinarum* is the only member species that is a consistent pathogen, although the infections it causes may sometimes be subclinical, and it may persist and be shed long after cessation of clinical signs.

Haemophilus haemoglobinophilus (9), also known as *H. canis,* is a fairly common commensal parasite of the lower canine genital tract, particularly of males. On rare occasions it has been associated with vaginitis (10) and cystitis.

Haemophilus paracuniculus (11) was isolated from the gut of rabbits suffering from mucoid enteritis. Its significance in this or any other disease is not known.

Haemophilus aphrophilus (12) is a frequent member of the oral flora of humans and occasionally associated with infections, including endocarditis and brain abscesses. Its occurrence has been reported in the pharynx of dogs (13).

The following two agents were once reported as animal pathogens, but, as the original strains were lost and the organisms not encountered for many years, the names were dropped from official lists. Although the agents were claimed to have been rediscovered more recently, no formal move for reinstatement of the original names has been made.

Haemophilus ovis (14) was described in 1925 as a cause of bronchopneumonia in sheep and not recognized again for more than 50 years. Since 1978, agents resembling it have been reported as inhabitants of normal ovine nasal passages (15) and as causes of septicemic disease (16).

Haemophilus influenzaemurium (17) was isolated from the nose and pharynx of mice and implicated in epidemics of upper respiratory tract infections and conjunctivitis in mouse colonies (18).

A group of closely interrelated fastidious gram-negative bacteria includes *Histophilus ovis, Haemophilus agni,* and *Haemophilus somnus* but fits neither the past nutritional nor the emerging genetic definition of the genus. By DNA–DNA homology criteria, they are members of one species (19,20), and such bacteriologic distinctions as may exist between them are subtle and not demonstrable by routine laboratory identification procedures.

Histophilus ovis (21) and *Haemophilus agni* (22) are commensal

organisms with predilection for the genital tract, particularly of young lambs, and have been implicated in ovine septicemia, pneumonia, mastitis, and epididymo-orchitis of young rams (19).

Haemophilus somnus (23) is a commensal inhabitant of bovine mucous membranes of the upper respiratory and especially the lower genital tract. Associated diseases include septicemia with thrombotic meningoencephalitis (TEME), respiratory and genital tract infections, mastitis, abortions (24), otitis (25,26), and conjunctivitis (27) in cattle.

Isolation and Identification

Selection of specimens will be governed by signs and lesions and the organ systems implicated by these. The principles outlined in Chapter 2 generally apply. In the case of bovine abortion involving *H. somnus*, uterine caruncles have been reported the most consistent source of the agent (28).

Haemophilus spp. tend to be fragile organisms that cannot be expected to survive for long periods after removal from their hosts. While pertinent data are few and contradictory, the effects of transport media and refrigeration (3–4°C) in transit are not uniformly beneficial and, in the view of some, deleterious. Deep freezing, preferably below −60°C, preserves viability in biological materials for extended periods (29,30). In view of the uncertainties mentioned, specimens that cannot be deep-frozen should be protected from drying, heating, and chilling and forwarded to a laboratory for culturing as expeditiously as possible, preferably within 24 hr of collection.

Direct demonstration of *Haemophilus* spp. in submitted specimens is sometimes possible by Gram stain or specifically by fluorescent antibody methods (31).

For *Haemophilus* species (other than *H. somnus–agni*) X or V factors or both must be provided in any isolation medium. The X factor is minimally protoporphyrin IX or protoheme and is generally supplied as hemin or hemoglobin. It is stable to heat and storage and present in adequate amounts in the usual 5% blood agar. The V factor is the heat- and storage-labile nicotinamide adenine dinucleotide (NAD, formerly DPN), NAD phosphate (NADP, formerly TPN) or one of its mononucleotide precursors. Although V factor is present in blood, its intracellular location, lability, and susceptibility to NADase, which is present in most bloods, makes blood agar an unreliable source of V factor for *Haemophilus* spp. (32).

Both X and V factors are available in adequate amounts in chocolate agar or can be replaced by catalase-positive feeder organisms such as *Staphylococcus aureus* in deficient media. *Haemophilus* spp. will grow as satellite colonies in the immediate vicinity of such bacterial

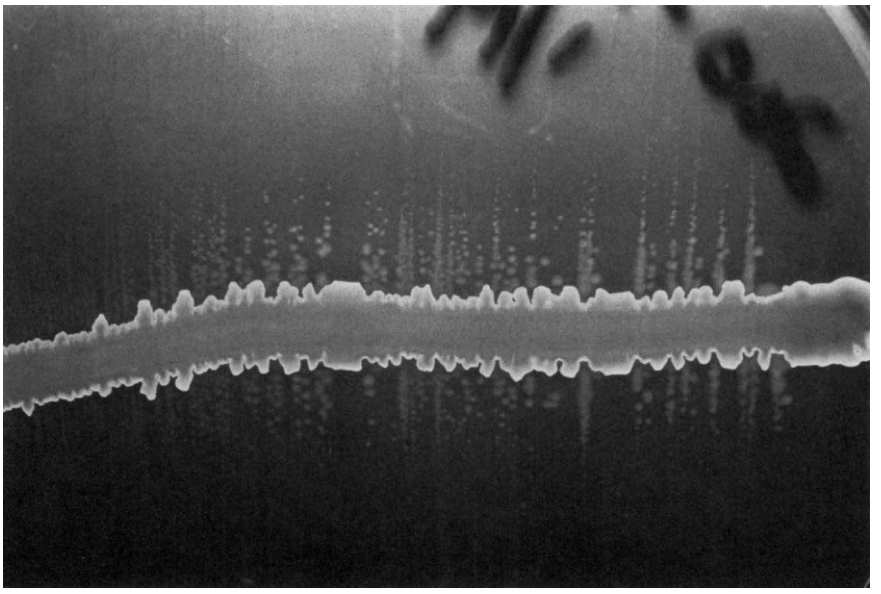

Figure 13-2. Satellite colonies of *Haemophilus paragallinarum* near growth line of *Staphylococcus* sp.

feeder cultures. The feeder organisms are inoculated across the area planted with the specimen so as to encourage satellite growth (Fig 13-2). Catalase-negative feeders (e.g., *Enterococcus faecalis*) may supply V but not X factor (33).

Mycoplasma agar (PPLO agar, Difco) containing yeast extract or hydrolysate (1–10%) and horse serum (1–10%) is a satisfactory isolation medium for most V factor-requiring *Haemophilus* spp. (34). Hemoglobin agar (Difco) supplemented with 1% IsoVitalex® (BBL) satisfies both X and V requirements. If X and V factors are supplied as hemin and NAD, respectively, 10 μg of each per milliliter of medium is ample for most of the organisms listed (35).[1] Exceptionally, X factor requirements as high as 100 μg/ml, and V factor requirements up to 25 μg/ml have been reported (1).

For isolation purposes, media incorporating the critical growth factors in adequate amounts are preferable to reliance on feeder streaks, since the success of the latter method depends on the chance landing of colony-forming units from the specimen close enough to the streak

[1]NAD is freely soluble in water. Hemin can be dissolved in triethanolamine (Eastman) at the rate of 50 mg/ml. Further dilutions can then be made in water.

to be benefited by the diffusion of growth factors. The most convenient and satisfactory isolation medium for animal haemophili is chocolate agar, that is, blood agar prepared by addition of the blood to the melted agar base when this is still at about 75–80°C and held at that temperature for several minutes before the mixture is poured into plates. *Haemophilus (para)suis* and *H. (para)gallinarum* do not grow well in the absence of serum, which is adequately supplied in chocolate and mycoplasma agars.

Inoculated media are incubated at 35–37.5°C, and there should be evidence of growth, though not maximal, within 24 hr. Raised carbon dioxide tension (5–10%) is beneficial for the growth of some *Haemophilus* species, particularly *H. (para)suis, H.(para)gallinarum, H. paracuniculus,* and *H. aphrophilus* (1), and is deleterious to none. It is a requirement for adequate growth of most strains in the *Haemophilus somnus–agni* group (22,36).

Haemophilus agni and *H. somnus* are readily isolated on either blood or chocolate agar. Little or no growth occurs on unenriched media, but there is no response to X or V factor(s). Chances of isolation are improved with uncontaminated specimens by passage of suspect material (tissue, exudate, blood) through an enrichment medium such as infusion broth (22). Embryonated eggs have also been recommended (37).

Selective media for the isolation of the *H. somnus–agni* group have been described recently (29,38,39). Their performance has varied in the hands of different investigators on different continents. A definitive evaluation must await their more extensive use under a variety of conditions.

Cultural Characteristics and Identification

All members of the genus growing on blood agar form very small colonies less than 1 mm in diameter after 24 hr of incubation. They tend to be smooth gray and not obviously pigmented, except for *H. somnus* colonies, which often appear yellowish, particularly when heaped up with a loop or growing in confluent lawns. None of the *Haemophilus* species of veterinary interest are consistently hemolytic. Variable hemolytic activity, ranging in prevalence from 27–100%, and in intensity from slight discoloration to frank clearing of blood agar (especially Columbia-base sheep blood agar) has been reported of *H. somnus* strains (40).

The diagnostic characteristics of animal haemophili are shown in Table 13-1. The requirement for X factor is best determined by the porphyrin test (41): A loopful of colonial growth from a young culture is suspended in 0.5 ml of a 2 m*M* solution of δ-amino-levulinic acid (ALA) hydrochloride (Sigma) and 0.8 m*M* magnesium sulfate in 0.1 *M*

Table 13-1
Differential Features of *Haemophilus* spp. of Animals and *Taylorella equigenitalis*[a]

	H. parasuis	*H. paragallinarum*	*H. paracuniculus*	*H. aphrophilus*	*H. haemoglobinophilus*	*H. ovis*	*H. influenzaemurium*	*Hist. ovis/ H. agni/ H. somnus*	*T. equigenitalis*
X factor required (Porphyrin test neg.)	–	–	–	+	+	+	+	–	–
V factor required	+	+	+	–	–	–	–	–	–
Indole	–	–	+	–	+	–	–	d	–
Urease	–	–	+	–	–	–	–	–	–
Ornithine decarboxylase	–	–	+	–	–	0	0	+	–
Arginine dihydrolase	–	–	+	–	–	0	0	–	–
Hemolysis	–	–	–	–	–	–	–	d	–
Gas from glucose	–	–	–	+	–	0	0	–	–
Acid from glucose	+	+	+	+	+	+	+	+	–
Fructose	+	+	+	+	–	0	+	+	–
Sucrose	+	+	+	+	+	–	+	–	–
Lactose	d	d	–	+	–	+	–	–	–
D-Xylose	–	+	–	–	+	+	–	+	–
D-Ribose	+	+	0	+	d	0	0	0	–
D-Mannitol	–	+	–	–	+	d	–	+	–
D-Sorbitol	–	+	–	–	–	0	–	+	–
Catalase	+	–	+	–	+	±	+	–	+
Oxidase	–	–	+	–	+	+	–	+	+
CO_2 enhances growth	d	+	+	+	–	–	–	+	+
Nitrates reduced	+	+	+	+	+	+	+	+	–
Nitrites reduced	–	–	0	+	–	0	0	0	–

[a]Symbols: +, ≥90% of strains positive; –, ≥90% of strains negative; ±, doubtful; d, <90% of strains positive or negative; 0, no information.

Sørensen phosphate buffer, pH 6.9. The suspension is incubated for at least 4 hr at 37°C and examined under a Wood's light (about 360 nm). If the porphyrin precursor ALA has been converted to porphyrin, a bright red fluorescence will be observed within 4–24 hr. Such a positive test indicates absence of X factor requirement. Positive and negative controls should be included. Filter paper discs impregnated with ALA are available commercially and a medium incorporating ALA has been described (42). Both offer alternative and possibly more convenient ways of performing the porphyrin test.

Still another method, dispensing with the use of the Wood's lamp, calls for the addition of 0.5 ml of Kovacs's reagent to the reaction mixture following incubation. The mixture is shaken vigorously and allowed to separate into two phases. With an X factor-independent strain, the bottom phase will be red, indicating the presence of porphobilinogen, the next intermediate after ALA on the pathway of porphyrin synthesis (41).

V factor need is adequately demonstrated by satellitic growth of suspect strains on blood agar with feeder organisms. Since the amounts of V factor in blood agar are variable and may be sufficient to obscure satellitism, media in which all ingredients have been thoroughly autoclaved and are therefore devoid of the heat-labile V factor are preferable. A common and convenient method of demonstrating the growth factor requirements is the use of three disks or strips impregnated with each and both factors, respectively, and placed on an agar plate that lacks both factors (e.g., proteose peptone) and has been inoculated for the production of confluent growth. Colonies will be clustered around the disc(s) or strip(s) containing the appropriate supplement(s) (Fig. 13-3). The method has its pitfalls: Cofactor needs, X in particular, may be obscured by "carryover" of an excess of the critical ingredient from growth on a rich medium, a problem that does not complicate the other methods. Further, the presence of any contaminant colonies on the test plates, because of their potential feeder activity, often invalidates the test, and some basal media, because of their nutritional inadequacy, may give misleading results (43).

Several of the biochemical tests are most suitably performed by micro-methods employing heavy suspensions of bacteria in a small volume (0.5 ml) of substrate solutions. Indole production, urease, ornithine decarboxylase (ODC), and arginine dihydrolase (ADH) activities can be determined in this manner. Results may be positive within 4 hr, but tubes should be held for 24 hr before being discarded as negative (1,35).

For the indole test, a 0.1% solution of L-tryptophan in *M*/15 Sørensen buffer, pH 6.8, is used. Indole is tested for by the addition of 0.5 ml of Kovacs's reagent (44).

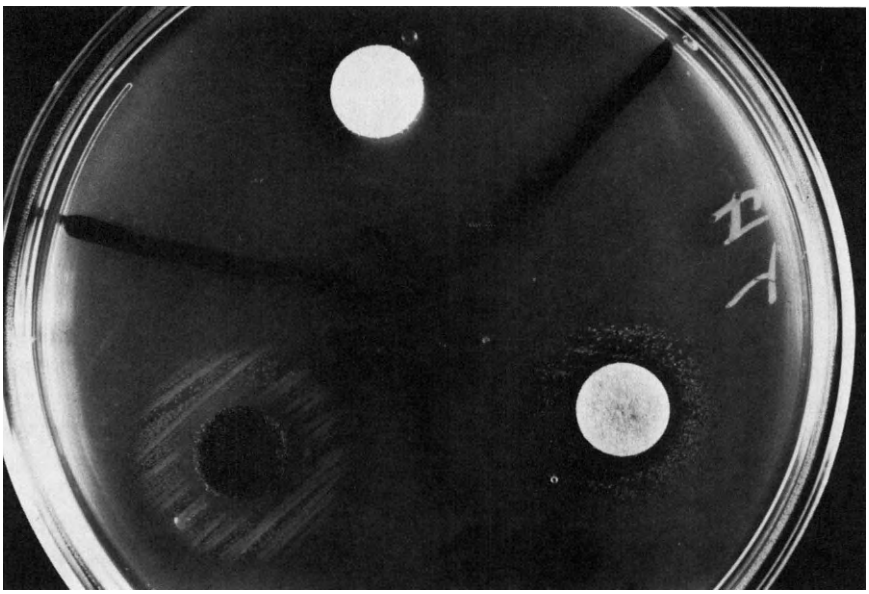

Figure 13-3. Test for cofactor requirements of *Haemophilus* spp. The plate, tryptose agar, was heavily inoculated with suspect growth prior to the application of V (*top*), X (*lower left*), and XV disks. This organism was *H. haemoglobinophilus* and grew only near the disks containing X factor.

The substrate for the urease test is

KH_2PO_4	0.1 g
K_2HPO_4	0.1 g
NaCl	0.5 g
Phenol red (0.2%)	0.5 ml[2]
Distilled water	100.0 ml

Adjust pH to 7.0 with 5 *N* NaOH, autoclave, and add 10.4 ml of a 20% filter-sterilized urea solution.

Urea hydrolysis is signaled by development of a red color (45).

ODC and ADH can be tested for in conventional Møller's medium (46).

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[2]0.2% Phenol red solution:

Phenol red crystals	0.2 g
Distilled water	92.0 ml
1 *N* NaOH	8.0 ml

Fermentation of carbohydrates is demonstrated in phenol red broth containing 1% of the substrate and 10 μg of filter-sterilized NAD and hemin per milliliter (31). Serum (1%) may have to be added for adequate growth of *H. parasuis* and *H. paragallinarum*, and a drop of defibrinated blood per 5 ml for that of *H. somnus* and *H. agni*. In these cases, it is wise to include a set of uninoculated controls and a tube lacking the fermentable substrate. *Haemophilus paracuniculus* reportedly does not grow in phenol red broth and should be tested in (bromcresol) purple broth instead (11).

The *H. somnus–agni–Histophilus* ovis group is notoriously variable in its biochemical activities, and exceptions have been noted to most of the tests shown in Table 13-1. Among the more reliable reactions are the positive oxidase test, the negative catalase test, striking growth response to increased CO_2, and the fermentation of glucose. A positive indole test is very useful. Serologic confirmation by immunodiffusion or immunofluorescence is quite definitive but rarely available (47,48). Rapid tests for preformed enzymes using commercially prepared substrates have been found useful as confirmatory procedures (49,50).

Serologic Aspects

Three major serologic subdivisions, identifiable by agglutination, hemagglutination–inhibition, or bactericidal tests (51–53), exist in the species *Haemophilus (para)gallinarum*. Since immunity is serotype-specific, determination of serotype may be important in the control of the disease.

In *Haemophilus suis* seven serotypes, A, B, C, D, (54), 1, 2, 3, (55) have been recognized and can be of interest from the epidemiologic viewpoint. They are determined by tube agglutination tests.

Serological tests for the diagnosis of *Haemophilus* infections of animals are not widely used except in poultry operations for the identification of potential carrier birds of *H. (para)gallinarum*. Antibodies are produced within 1–2 weeks of infection and may be demonstrable for over a year. Available procedures include plate and tube agglutination tests (56), agar gel precipitation (AGP) tests (57), hemagglutination–inhibition (58), and latex agglutination (59). Although there is some cross-reactivity among the serotypes, test antigens should contain all serotypes, or antibody to each serotype should be tested for separately.

Serological surveys in cattle populations utilizing agglutination and complement fixation tests and enzyme immunoassays (ELISA) have revealed widespread prevalence of antibody to *H. somnus*. So far none of the procedures employed have been discriminating enough for the indentification of ongoing problems referable to this agent in herds or individual animals.

Taylorella equigenitalis

The agent responsible for contagious equine metritis (CEM) was originally called CEM organism (CEMO) (60), and then *Haemophilus equigenitalis* (61) because of its exacting growth requirements and lack of relationship to any other genus. Subsequent studies demonstrated an equal lack of relationship to any *Haemophilus* species, genetically, physiologically, and cytochemically (62), and the genus *Taylorella* was created for its accommodation.

Pathogenicity

Taylorella equigenitalis causes an acute, self-limiting, suppurative metritis in mares that leaves no permanent effects on the breeding efficiency of recovered animals. Extremely rare instances of abortion have been reported (60). The disease is sexually transmitted, but no clinical signs develop in stallions. Both sexes may remain asymptomatic carriers for extended periods of time.

Isolation and Identification

Specimens for culture of *T. equigenitalis* include uterine and vaginal exudates, placentas, and fetal tissues. The sites routinely sampled in asymptomatic animals whose carrier status is to be determined are cervix or (if accessible) uterus, clitoral fossa, clitoral sinuses of mares, and urethra, urethral fossa and diverticulum, and prepuce in stallions. These specimens are collected on cotton swabs, which are placed into Amies's transport medium with charcoal (Difco, Remel) and must reach the laboratory under refrigeration within 48 hr of collection.

Direct examination of specimens is useful only in the case of uterine exudates from symptomatic mares, in which the agent (gram-negative rods, coccobacilli, or filaments) may be demonstrated. Swabs are inoculated on chocolate agar plates made with equine blood. Present procedures in the United States call for the use of three media with each swab: (1) Eugon (BBG) chocolate agar, prepared with 10% horse blood and containing 5 μg/ml of amphotericin B (Fungizone, Squibb) and 1 μg/ml of crystal violet; (2) a second plate of the same medium supplemented with 200 μg/ml of streptomycin; and (3) a plate of CEM Selective Medium-Timoney Formula[3]: a 5% horse blood chocolate agar supplemented with an additional 5% lysed horse blood and containing 1 μg/ml trimethophrim, 5 μg/ml clindamycin, and 5 μg/ml amphoteri-

[3]A simplified procedure utilizing only this medium is awaiting official endorsement.

cin B. The basal medium consists of BBL trypticase 1.5 g/liter, BBL phytone 5 g/liter, sodium chloride 4 g/liter, sodium sulfite 0.2 g/liter, L-cystine 0.7 g/liter, and agar 1.5 g/liter (63).

Inoculated plates are incubated at 37°C in carbon dioxide for 48 hr before being first examined and, if negative at that time, reincubated and reexamined daily for up to 7 days before being discarded as negative.

Cultural Characteristics and Identification

At 48 hr colonies of *T. equigenitalis* are under 1 mm in diameter, grayish in color and butyrous in consistency. On further incubation they may attain sizes of 1.5 mm. A Gram stain will reveal them to consist of gram-negative pleomorphic coccobacilli. They are catalase and oxidase positive. Any colony meeting these criteria is subcultured to another plate of Eugon equine chocolate agar, and the resulting growth tested for inability to grow in air, agglutinability in anti-*T. equigenitalis* serum on a slide, and alkaline phosphatase activity. The last test is performed by addition of 0.5 ml of *p*-nitrophenyl phosphate solution (1 mg/ml) to a suspension of a suspect colony in 0.5 ml of Tris buffer (pH 8.0). The mixture is incubated at 37°C for up to 2 hr. A yellow color indicates phosphatase activity.

Any suspected isolates of *T. equigenitalis* encountered in nonendemic areas should be reported to the appropriate regulatory authorities and forwarded to an official reference laboratory for confirmation.

The regulations governing the testing of horses for *T. equigenitalis* infection prior to shipment across international borders vary from country to country and are modified from time to time. Any laboratory becoming involved in this type of testing should obtain the current regulations applicable to their particular testing activity.

Serological testing is not routinely done as a diagnostic procedure. Antibody appears too late to be useful for the consistent diagnosis of the acute infection and is not sufficiently correlated with the carrier status to be a reliable indicator of convalescent or asymptomatic carriage. Its demonstration may be useful for confirming a past infection. The complement fixation test has had some limited application for this purpose (64) and proved most dependable from the third through sixth week following the infection.

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14

Miscellaneous Glucose-Nonfermenting Gram-Negative Bacteria

Thomas J. Inzana

The glucose-nonfermenting bacteria described in this chapter include organisms in the family *Neisseriaceae*, as well as the genera *Flavobacterium*, *Weeksella* (*Flavobacterium* sp groups IIf and IIj), *Agrobacterium*, and CDC groups M5, M6, and EF-4. These bacteria either utilize glucose oxidatively or not at all. The genera *Pseudomonas*, *Bordetella*, and *Alcaligenes* are also nonfermentative but are described in other chapters. Nonfermentative gram-negative bacteria that are not included in this chapter and are significant only in human medicine are *Eikenella corrodens* and CDC groups IVe, IVc-2, and EO-2.

The taxonomy of the glucose-nonfermenting bacteria seems to be under continuous revision. Therefore, newly proposed name changes for these bacteria have been included in this edition. Nonfermenting bacteria should be suspected when inoculation of triple sugar iron agar or Kligler's iron agar results in a neutral or alkaline slant and a neutral butt without gas.

Family *Neisseriaceae*

The glucose-nonfermenting members of this family consist of the genera *Acinetobacter*, *Moraxella*, *Neisseria*, and *Branhamella*. *Kingella* is also a member of this family, but is fermentative. The nonfermenting *Neisseriaceae* are aerobic and either coccal or rod-shaped. Coccal shapes may occur singly, in pairs, or in masses with adjacent sides often flattened. Cultures of *Acinetobacter* and *Moraxella* that occur in pairs may be observed as diplococci and confused with *Neisseria* or *Branhamella*. True cocci can be differentiated from coccobacilli by a Gram stain of the bacteria growing near the inhibitory zone of a

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penicillin disk; *Neisseria* and *Branhamella* will remain cocci, whereas *Acinetobacter* and *Moraxella* will be elongated. Rod shapes may occur in pairs or in short chains. Although all are gram-negative, some strains may be somewhat resistant to Gram decolorization. Species of *Moraxella* and *Acinetobacter* may give false negative reactions when the Gram reaction is confirmed by lysis with 3% potassium hydroxide. Flagella are absent, but pili may be present.

Genera that have the greatest pathogenic significance in animals are *Moraxella* and *Acinetobacter*. *Neisseria* and *Branhamella* may be isolated, however, as commensals of mucous membranes. Additional details about the family *Neisseriaceae* have been reviewed in other publications (1,2).

Acinetobacter

Acinetobacter calcoaceticus is the only species currently listed in this genus in *Bergey's Manual of Systematic Bacteriology* (1). However, Bouvet and Grimont have described 12 hybridization groups (genospecies) using DNA hybridization studies, and have proposed the genus *Acinetobacter* to contain six named species: *A. calcoaceticus*, *A. lwoffii*, *A. haemolyticus*, *A. junii*, *A. johnsonii*, and *A. baumannii*, as well as six unnamed genospecies (3). The taxonomy of acinetobacters has been unstable due to their lack of unique characteristics and because they have been isolated from a wide variety of sources by different individuals. These bacteria have been previously classified in many other genera, including *Mima*, *Herella*, *Achromobacter*, and others.

Species of *Acinetobacter* are commonly found in soil, water, sewage, food, and milk; they are also part of the normal flora of humans and animals (1). Due to their ubiquitous nature they have become important causes of nosocomial infections, particularly pneumonia and systemic infections in compromised patients. Such infections are particularly important because hospital isolates are resistant to most antibiotics except the aminoglycosides, carbenicillin, and trimethoprim-sulfamethoxazole. *Acinetobacter* species have been recovered from farm animals, pets, fowl, and laboratory and zoo animals. In most cases, *Acinetobacter* has been isolated as a commensal. However, this organism has been recovered from the blood of sick dogs (4) and associated with bronchopneumonia in mink (5). In many human and veterinary clinical laboratories, *Acinetobacter* is the second most common nonfermentative gram-negative bacterium isolated, following *Pseudomonas aeruginosa* (6,7).

Acinetobacter will grow on most laboratory media, including MacConkey agar, on which colonies are colorless to light pink. Colonies

are blue on EMB agar but do not grow on SS agar. The species proposed as *A. haemolyticus* and genospecies 6 are β-hemolytic on blood agar within 48 hr. Colonies are 2–3 mm in diameter, gray-white, and convex. Acinetobacters are gram-negative plump rods, 0.9–1.6 μm wide by 1.5–2.5 μm long, and may occur in pairs or chains. Some isolates of *Acinetobacter* may produce acid from glucose oxidatively, while other isolates are nonsaccharolytic. Nonsaccharolytic strains can be differentiated from *Moraxella* and similar bacteria by their lack of oxidase activity and resistance to penicillin; all isolates are catalase positive (1,6–8). Phenotypic characteristics of *A. calcoaceticus* and *A. lwoffii* (3) are presented in Table 14-1. For additional characteristics and differentiation of other genospecies, the reader is referred to reference 3.

Moraxella

Species in the genus *Moraxella* include *M. bovis, M. lacunata, M. phenylpyruvica, M. nonliquefaciens, M. atlantae* (formerly CDC group M3), *M. urethralis* (formerly CDC group M4), and *M. osloensis.* The latter four species have not been isolated from animals. Isolates similar to *Moraxella* have been isolated from sheep (9) and goats (10) with keratitis, and from various specimens from pigs (11), but such isolates have not yet been characterized. *Moraxella* species have also been isolated from the mouth, conjunctiva, and the respiratory, gastrointestinal, and genitourinary tracts of dogs (12–16).

Moraxella species are short, plump rods measuring 1.0–1.5 μm wide by 1.5–2.5 μm long, and therefore coccoid-appearing by the Gram stain. Most species are fastidious but will grow on blood agar. Their optimum growth temperature is 33–35°C. All species are oxidase positive, nonmotile, and nonsaccharolytic; most are catalase positive, sensitive to penicillin, and grow on MacConkey agar (1,6–8,17).

Moraxella bovis is considered the cause of infectious keratoconjunctivitis (pinkeye) of cattle and is most often isolated from infected eyes. This bacterium may also be isolated from normal eyes and from the bovine nasal cavity. Strains isolated from eyes of horses with conjunctivitis have been referred to as *M. equi* but are considered strains of *M. bovis* with different host specificity (1).

Swabs of the conjunctiva should be inoculated immediately to blood agar and incubated at 35°C for at least 2 days. Colonies are hemispherical to flat, round, and grayish-white, surrounded by a distinct narrow zone of β-hemolysis. Strains of *M. equi* are nonhemolytic. Fresh isolates are often piliated, resulting in corroding of the agar and surface spreading (1). Unlike most species of *Moraxella, M. bovis* will not grow on MacConkey agar and catalase production is variable. Litmus milk

Table 14-1
Phenotypic Characteristics of Glucose-Nonfermenting Gram-Negative Bacteria Isolated from Animals[a]

	Hemolysis	Growth on MacConkey	Motility	Acid from glucose	Oxidase	Catalase	Nitrate reduction	Urease	Gelatinase	Indole	Pigment	Penicillin sensitivity
A. calcoaceticus	−	+	−	+	−	+	−	V	−	−	−	R
A. lwoffii	−	+	−	−	−	+	−	−	−	−	−	R
M. bovis	[β]	−	−	−	+	V	[−]	−	[+]	−	−	S
M. lacunata	−	−	−	−	+	+	+	−	+	−	−	S
M. phenylpyruvica	−	[+]	−	−	+	+	V	+	−	−	−	S
B. catarrhalis	−	−	−	−	+	+	[+]	−	−	−	−	S
B. caviae	Wβ	−	−	−	+	+	+	−	−	−	−	S
B. cuniculi	−	−	−	−	+	+	−	−	−	−	−	S
B. ovis	[β]	−	−	−	+	+	[+]	−	−	−	−	S
M5	V(β)	V	−	−	+	+	−	−	−	−	−	S
M6	−	V	−	−	+	−	+	−	−	−	−	[S]
N. canis	−	−	−	−	+	+	+	−	+	−	yellow	S
N. flavescens	−	−	−	−	+	+	−	−	−	−	yellow	S
N. sicca	V	−	−	+	+	+	−	−	−	−	V(yellow)	S
N. lactamica	−	−	−	+	+	+	−	−	−	−	yellow	S
N. denitrificans	−	−	−	+	+	+	−	−	−	−	V(yellow)	S
N. mucosa	−	−	−	+	+	+	+	−	−	−	V(yellow)	S
F. meningosepticum	[α]	+	−	+	+	+	−	−	+	+	V(yellow)	R
F. indologenes	V(α)	V	−	+	+	+	V	[−]	[+]	+	yellow	R
F. odoratum	[α]	+	−	−	+	+	−	+	+	−	[yellow]	R
F. multivorum	[α]	+	−	+	+	+	−	+	−	−	yellow	R
W. zoohelcum	V(α)	−	−	−	+	+	−	+	+	+	−	S
A. tumefaciens	V(β)	+	+	+	+	+	[+]	[+]	−	−	−	R
EF-4b	V(β)	V	−	[+]	+	+	+	−	−	−	[yellow]	ID

[a]Symbols: +, ≥90% of strains are positive; −, ≤10% of strains are positive; [ ], ≥80% of strains; V, 21–79% of strains are positive; β, beta-hemolysis; α, alpha hemolysis; ID, insufficient data; R, resistant; S, susceptible; W, weak.

becomes alkaline (blue) and develops three zones: The upper third is deep blue, the middle forms a light blue soft curd, and the bottom third is white (reduced) and coagulated.

Moraxella lacunata is the type species of the genus *Moraxella* and has been isolated from guinea pigs (1), aborted equine fetuses (18), from septicemia in a goat (18), and from various diseased specimens from dogs and pigs (18). This species has also been recovered from goats with viral pneumonia and encephalitis (18). Characteristics that differentiate this species from most isolates of *M. bovis* are the lack of hemolysis and the reduction of nitrate.

Moraxella phenylpyruvica obtained its name from its capability to produce phenylpyruvic acid by deamination of phenylalanine. This organism has been isolated from the genitourinary tract and brain of sheep and cattle, the genitourinary tract of pigs, and the intestine of a goat (1). It may have pathogenic potential but is not commonly isolated. Isolates may be slightly pink and can be differentiated from other *Moraxella* species by a positive test for urease and phenylalanine deaminase.

Isolates of *Moraxella* can also be differentiated by composition of cellular fatty acids (17). Additional characteristics of *M. bovis* and other *Moraxella* species that have been isolated from animals are described in Table 14-1.

Branhamella

Species in the genus *Branhamella* were previously included in the genus *Neisseria.* They are now considered closely related to *Moraxella* and are listed in *Bergey's Manual of Systematic Bacteriology* as a coccoid subgenus of *Moraxella* (1). *Branhamella* isolates from animals include *B. caviae,* isolated from the throats of guinea pigs and the conjunctiva of dogs (1,15); *B. cuniculi,* isolated from the mouths of healthy rabbits and the nasopharynx of marine pinnipeds (1); and *B. ovis,* isolated from the conjunctiva of sheep and cattle and the upper respiratory tract of sheep (1). *Branhamella ovis* has been isolated in cultures from keratoconjunctivitis of sheep, goats, and cattle, but its pathogenicity is considered low (19–22). *Branhamella catarrhalis* is considered part of the normal nasal flora of humans and has been isolated from cases of otitis media, maxillary sinusitis, bronchitis, pneumonia, and systematic infections (1). This bacterium has also been isolated from pneumonic lungs of calves (23), and from the mouth, respiratory tract, and conjunctiva of dogs (12,15,16), but its significance in veterinary medicine is not clear.

Branhamella isolates are smaller than *Moraxella* (0.6–1.0 μm in diameter) but are otherwise similar in morphology. They can be

distinguished from *Moraxella* by remaining coccoid in the presence of subinhibitory concentrations of penicillin, whereas isolates of *Moraxella* will be elongated. Due to their inert biochemical activity, *Branhamella* isolates from animals are difficult to speciate. Reduction of nitrate and nitrite and sensitivity to various concentrations of penicillin will speciate some isolates (1).

CDC Groups M5 and M6

Bacteria in CDC groups M5 and M6 are similar to *Moraxella* species. Isolation of group M5 has been most often associated with dog bites in humans. The pathogenic significance of group M5 bacteria that occur as commensals in the canine oropharynx, and probably other animals, is unknown. Isolates of group M6 have only been reported from human clinical specimens.

Both groups are gram-negative coccobacilli, oxidase and catalase positive, nonsaccharolytic, nonmotile, and inactive in most biochemical tests. Some isolates of CDC group M5 will grow on MacConkey agar and are β-hemolytic. Analysis of cellular fatty acid composition has been used to differentiate isolates of group M5 from group M6 and other *Moraxella* isolates (17). Isolates of group M6 are easily distinguished from group M5 and many *Moraxella* isolates by lack of catalase activity (8).

Neisseria

Species in the genus *Neisseria* occur as commensals on the mucous membranes of warm-blooded animals and humans, particularly the nasopharynx and conjunctiva. They have also been isolated from the gastrointestinal and genitourinary tracts of dogs (12–16). *Neisseria canis* has been isolated from throats of dogs (24) and cats (1) and has been reported as an opportunist in a cat-bite wound of a human (25). *Neisseria denitrificans* and *N. animalis* have been isolated from the throats of guinea pigs (1). *Neisseria mucosa* has been isolated from the respiratory tract of a healthy dolphin (26). *Neisseria flavescens* and *N. sicca* have been recovered from the mouth and throat of healthy dogs (12,16). In addition, *N. lactamica* has been isolated from the conjunctival sacs of healthy dogs (15). The clearly pathogenic species (*N. gonorrhoeae* and *N. meningitidis*) have only been isolated from humans.

Three species previously listed as "false neisseriae" (*N. caviae, N. ovis,* and *N. cuniculi*) are now included in the genus *Moraxella* subgenus *Branhamella* (1). In addition, *N. animalis* is closely related to *N. denitrificans,* but its taxonomic position is at present uncertain (1).

Neisseria species are true gram-negative cocci that occur singly or in pairs with flat adjacent sides and are oxidase and catalase positive. An exception is *N. elongata*, which occurs as rods and is catalase negative. Some species may produce capsules and pili, but all are nonmotile. Primary isolation of most of the nonpathogenic species can be made on nutrient agar at 35°C. The more pathogenic species require chocolate or blood agar. Growth is greatly enhanced by an atmosphere of 3–10% CO_2 and high relative humidity. Species identification is made by colony morphology (some species may produce a yellowish pigment or polysaccharide capsule), acid from oxidative utilization of different carbohydrates, and nitrate or nitrite reduction (1,8). Some biochemical characteristics of the *Neisseria* species listed above are described in Table 14-1.

Flavobacterium and *Weeksella*

Flavobacteria are free living organisms of soil and water. Although they are not normally part of the indigenous flora of humans or animals, they have contaminated aqueous fluids in human hospitals, resulting in colonization or infection of patients. Isolation of flavobacteria from animals is probably rare, but colonization or infection of hospitalized animals is possible (6–8,27).

Flavobacteria form yellow to orange colonies, produce acid oxidatively in O-F medium, and lack motility. These bacteria are very similar to bacteria in the family *Cytophagaceae* (27), but as of yet no proposal has been made to change the classification of *Flavobacterium*. In addition to the characteristics described above, these bacteria are medium to long, narrow, gram-negative rods (0.5 μm wide by 1.0–3.0 μm long), oxidase and catalase positive, resistant to penicillin and polymyxin, and most are proteolytic. Lack of motility can be used to initially differentiate these bacteria from other pigmented and oxidative gram-negative bacteria (e.g., *Pseudomonas*).

The most pathogenic member of this group is *F. meningosepticum*, which most often causes a severe meningitis and septicemia in hospitalized human infants, as well as pneumonia and meningitis in compromised human adults. Most strains lack yellow pigment or are light yellow and are DNase positive. Other clinically significant bacteria in this group are *F. indologenes* (previously CDC group IIb; lacks DNase and produces acid from starch), *F. odoratum* (characterized by a fruity odor and strong urease production), and *F. multivorum* (positive for esculin hydrolysis and β-galactosidase). Identification of hospital isolates is particularly important because they are resistant to a wide variety of antibiotics, including amikacin and other aminoglycosides (8).

Isolates previously listed as *Flavobacterium* sp. group IIj have now

been named *Weeksella zoohelcum* and are part of the normal flora of the oral cavity and paws of dogs and cats (8,16,28). *Weeksella zoohelcum* isolates have been recovered most often from humans with infected wounds due to animal bites or scratches, but also from specimens of respiratory and systematic sites. As would be expected, these bacteria have also been recovered from animals, primarily dogs and cats (8). Their significance in veterinary medicine, however, is not clear. Bacteria previously classified as *Flavobacterium* sp. group IIf are now named *W. virosa* but have not been isolated from veterinary specimens.

Weeksella zoohelcum isolates are short to long rods and are distinguished from flavobacteria by susceptibility to penicillin, lack of yellow pigment, failure to attack carbohydrates, lack of growth on MacConkey agar, and close association with animals. Colonies are no larger than 0.5 mm in diameter and are sticky and difficult to remove from the agar. Most strains fail to grow in O-F medium but hydrolyze urea rapidly.

CDC Group EF-4

Isolates of group EF-4 are commensals in the mouth and nasopharynx of dogs and cats. They have little pathogenic significance for animals but are most commonly isolated from human wounds caused by dog or cat bites (8,16). EF-4 isolates have been further divided into groups EF-4a and EF-4b. These groups are similar except that EF-4a ferments glucose and EF-4b oxidizes glucose, but otherwise neither group can utilize any sugar other than glucose.

Group EF-4 strains are short, gram-negative rods coccobacilli. Colonies on blood agar are nonhemolytic, convex, mucoid, and may be yellow to tan. Motility, indole, and urease tests are negative, but oxidase and catalase tests are positive; nitrate is reduced. Growth on MacConkey agar is variable (8).

Agrobacterium

Isolates of *Agrobacterium* are soil inhabitants and are well known as plant pathogens. There are three proposed species of *Agrobacterium: A. rhizogenes, A. rubri,* and *A. tumefaciens.* A fourth species, previously listed as *A. radiobacter,* is identical to *A. tumefaciens* but is not a plant pathogen. Nonetheless, it is proposed that the name *A. radiobacter* be changed to *A. tumefaciens* (29). *Agrobacterium* species have not been documented as causing disease in animals. *Agrobacter-*

ium tumefaciens (radiobacter) is the only species isolated from human specimens, but it has rarely been associated with disease (8).

Agrobacterium species are gram-negative rods (0.6–1.0 μm wide by 1.5–3.0 μm long), which are motile by peritrichous flagella. Their optimum temperature is 25–28°C. Colonies are usually nonpigmented and highly mucoid due to production of an extracellular carbohydrate slime layer. Isolates are oxidase positive, and most are catalase and urease positive. They produce acid oxidatively from many commonly tested carbohydrates, including lactose. *Agrobacterium tumefaciens (radiobacter)* can be differentiated from other species of *Agrobacterium* by the oxidation of lactose to 3-ketolactose, which can be detected by addition of Benedict reagent. Data on susceptibility to antibiotics is limited, but most strains tested are resistant to penicillin (6–8,29).

Differentiation from Other Nonfermentative Bacteria

The bacteria described in this chapter can be differentiated from other oxidative and nonsaccharolytic bacteria (i.e., pseudomonads and related bacteria) by motility and a flagella stain and by glucose O-F medium. Pseudomonads are motile by polar flagella, whereas most of the nonfermenting bacteria described in this chapter are nonmotile. *Agrobacterium* is the only motile genus included in this chapter. It can be differentiated from other pseudomonads by its peritrichous flagella. *Alcaligenes*, *Bordetella*, and *"Achromobacter"* also have peritrichous flagella, but the former two genera are nonsaccharolytic and *"Achromobacter"* is negative for lactose and β-galactosidase (6–8). Furthermore, the name *"Achromobacter"* is not recognized as a valid genus name, and the name *"Achromobacter" xylosoxidans* has been changed to *Alcaligenes xylosoxidans* (30).

Identification

Commercial Identification Systems

Commercial identification systems for glucose-nonfermenting bacteria can be classified as automated or miniature. Automated systems include the Automicrobic system (Vitek Systems, Inc., Hazelwood, Missouri), Autobac IDX System (General Diagnostics, Morris Plains, New Jersey), and AutoSCAN-4 (American Microscan, Mahwah, New Jersey). Miniature systems are the API 20E and API Rapid NFT (Analytab Products, Inc. Plainview, New York), Oxi/Ferm (Roche, Inc., Nutley, New Jersey), Minitech (BBL Microbiology Systems, Cockeysville,

Maryland), and Flow NF (Flow Laboratories, Roslyn, New York). Commercial systems are fairly accurate at identification of the more commonly isolated glucose-nonfermenting gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter*. Identification of rarer nonfermenting bacteria without additional tests has been poor. This is probably due to lack of the proper substrates and inadequate data bases. For detailed description of the commercial systems listed above, the reader is referred to the 4th edition of the *Manual of Clinical Microbiology* (8) and Chapter 36, this volume.

Morphological and Biochemical Identification Systems

Identification of many of the bacteria described in this chapter can be accomplished relatively simply by their morphological properties and a few biochemical tests. Tests initially required to characterize these bacteria include odor, pigmentation, colonial and microscopic morphology from blood and MacConkey agar, KIA or TSI tubes, motility (if

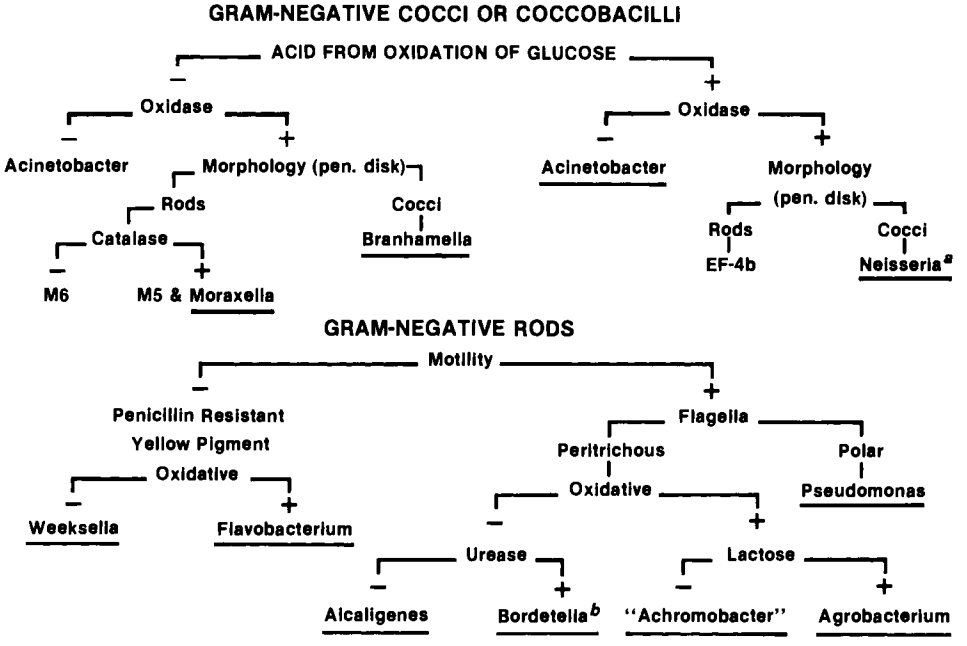

Figure 14-1. Algorithm for genus identification of glucose-nonfermenting gram-negative bacteria. This is a generalized algorithm; exceptions to this scheme will occur. [a]*Neisseria flavescens, N. cinerea, N. elongata,* and *N. canis* are nonsaccharolytic. See *Bergey's Manual of Systematic Bacteriology*, Vol. 1, for additional information. [b]*Bordetella bronchiseptica* is positive in 1–4 hr; *B. parapertussis* is positive in 24 hr, and *B. pertussis* is negative.

motile, whether flagella are polar or peritrichous), O-F media (glucose, xylose, maltose, and lactose), reduction of nitrate and nitrite, urease, indole, oxidase, catalase, and resistance to penicillin. For determination of flagellar morphology, the author's laboratory uses a silver stain procedure (31) (see Appendix A), although commercial kits are also available (Carr-Scarborough Microbiologicals, Inc., Stone Mountain, Georgia).

Two tubes of glucose O-F medium, which should be inoculated lightly, are particularly useful for differentiating fermentative, oxidative, and nonsaccharolytic bacteria. The medium of one tube is covered with stiff petrolatum or mineral oil. Acid (yellow) throughout both tubes indicates a fermentative organism, acid only at the surface of the unsealed tube indicates oxidation, and an alkaline (blue) reaction at the top of only the unsealed medium indicates the organism is nonsaccharolytic (7). Identification of nonfermentative bacteria to species may require additional biochemical tests that have been described in detail (6–8), An algorithm of key characteristics (Fig. 14-1) may be used to classify most of the nonfermenting bacteria described in this chapter to genus level.

Acknowledgments

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15

Nonsporeforming Anaerobic Bacteria

James M. Donahue

Nonsporeforming anaerobic bacteria can be isolated from various infectious processes in animals and will be overlooked unless appropriate procedures for their isolation are used. Most infections caused by nonsporeforming anaerobic bacteria are nonspecific and can involve any one or more of a number of different species. These infections may be caused by a single species but are usually polymicrobial: mixed anaerobes or anaerobes with aerobes or facultative anaerobes. *Clostridium* and *Actinomyces* are also found in nonspecific infections, but they are discussed in other chapters (*Clostridium* in Chapter 19; *Actinomyces* in Chapter 22).

This chapter deals with the laboratory diagnosis of infectious diseases involving nonsporeing anaerobic bacteria. Most of the procedures were developed by bacteriologists working in human bacteriology laboratories and have been described in detail (1–5). These procedures have been adapted by bacteriologists in veterinary laboratories to diagnose anaerobic infections in various animal species (6–16). An excellent two-part review concerning the pathogenesis, clinical significance, diagnosis, and treatment of anaerobic infection in small animals has been published (17,18).

Infections Caused by Nonsporeforming Anaerobic Bacteria

Nonsporeforming anaerobic bacterial infections can involve any region of the body where conditions in the tissue are suitable: lowered tissue oxygen and oxidation–reduction potential. Most of these infections are caused by bacteria from endogenous sources, such as the

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normal flora of the gastrointestinal tract, mouth, genitourinary tract, and skin. However, nonsporeforming anaerobic bacterial infections of exogenous origin are not uncommon. The most common are infections following bites and deep penetrating wounds. Examples of infectious processes that commonly contain nonsporeforming anaerobic bacteria are listed in Table 15-1. In a comprehensive study at the University of California at Davis, 3133 clinical specimens obtained from domestic animals contained obligate anaerobic bacteria, and about 90% of these were nonsporeforming anaerobic bacteria (10). Draining tracts, abscesses, pleural effusions, pericardial effusions, and peritoneal effusions were culture-positive for anaerobic bacteria greater than 25% of the time.

Table 15-1
Infections Likely to Involve Nonsporeforming Anaerobic Bacteria

Infectious process	Comment or example
Abscess	Any site, particularly deep
Bacteremia	All species
Head and neck	
Chronic sinusitis	Horse especially
Periodontal abscess	Tooth root abscess
Oropharyngeal abscess	All species
Pleuropulmonary	
Aspiration pneumonia	All species
Pleuritis, pleural effect	Especially chronic—cat bite empyema
Lung abscess	All species
Chronic bronchopneumonia	
Intraabdominal	
Peritonitis	Navel infection, penetrating foreign body, postsurgical
Liver abscesses	All species; cattle: *F. necrophorum*
Female genital tract	
Postparturient endometritis	All species–hard to diagnose because high numbers of aerobes and anaerobes enter uterus after parturition.
Pyometra	Dog especially
Mastitis	Chronic in cattle, summer mastitis of cattle
Soft tissue cellulitis	All species—may be crepitant due to gas
Musculoskeletal tissue	
Gas gangrene	Often involve *Clostridium*, especially *C. perfringens*
Osteomyelitis	Penetrating wounds, compound fracture, postsurgical
Foot rot	Sheep: *B. nodosus*, *F. necrophorum* Cattle: *F. necrophorum*, black-pigmented *Bacteroides*

Most infections resulting from nonsporeforming anaerobic bacteria are nonspecific. Table 15-2 summarizes the data from four studies concerning the relative frequency of anaerobic bacteria from infections in animals. In all these studies *Bacteroides* species were the most frequently isolated bacteria.

Recently, bacterial species with phenotypic reactions similar to species from humans have been described and the taxonomy of anaerobic bacteria has undergone major changes. Therefore, it is often difficult to correlate the species described in earlier literature with the present species designations.

In veterinary medicine certain infections due to nonsporeforming anaerobes can be described as specific. These include foot rot in sheep caused by *Bacteroides nodosus* and liver abscesses in cattle caused by *Fusobacterium necrophorum*. In these cases the organisms are cultured repeatedly from typical disease.

Table 15-2
Relative Frequency (Percentage of Total) of Anaerobic Bacteria from Nonspecific Infections in Domestic Animals

	Author[a]			
Genus, species	1	2	3	4
Bacteroides (total)	46	53	31	44
B. fragilis	1	2	5	7
B.-black pigmented	19	11	13	8
Other[b]	26	39	12	29
Fusobacterium (total)	6	9	11	21
F. necrophorum	1	3	5	18
F. nucleatum	1	1	—	—
Other	4	5	5	3
Gram-positive cocci (total)	17	19	12	12
Peptostr. anaerobius	12	—	8	10
Peptostr. indolicus	1	5	—	—
Other[c]	4	14	4	2
Eubacterium (total)	3	2	3	—
Propionibacterium (total)	5	1	4	—
Lactobacillus (total)	1	3	—	—
Actinomyces (total)	6	1	9	—
Clostridium (total)	8	4	30	10
C. perfringens	2	1	19	5
Other	6	3	11	5
Veillonella (total)	—	2	—	—
Other genera or not identified	10	3	—	13

[a]1, Hirsh *et al.* (9); 2, Prescott (14); 3, Berg et al. (6); 4, Hirsh et al. (10).
[b]Uncommon or species not identified.
[c]Includes *Peptostreptococcus, Peptococcus,* and anaerobic *Streptococcus.*

Selection, Collection, and Transport of Specimens

The proper selection, collection, and transport of clinical specimens cannot be overemphasized. The specimens must be taken from a site free of contamination with normal flora, and the specimens must be collected and transported in a manner that will allow the recovery of all the significant bacterial species. Contamination of the specimen will yield erroneous or noninterpretable results and will waste both money and technician time. Improper collection and transport of the specimen may result in no growth when anaerobic bacteria are the cause of the infectious process.

Selection of Specimens for Anaerobic Culture

All material from areas of the body not likely to be contaminated with normal flora can be cultured for anaerobic bacteria. The following sites should be cultured for anaerobic bacteria if the clinical findings suggest an anaerobic infection:

Abscesses and deep wounds
Body fluids (pleural, pericardial, peritoneal, joint, and cerebrospinal)
Transtracheal or direct lung aspirates
Tissue (biopsy, surgical, and necropsy)
Blood

The following specimens are generally unacceptable for the culture of nonsporeforming anaerobic bacteria:

Gastrointestinal specimens
Feces or rectal swabs
Throat and nasopharyngeal swabs
Gingival swabs
Vaginal, cervical, and urethral swabs
Voided urine
Swabs from the surface of ulcers, skin lesions, and sinus tracts
Tracheal washings

Foul odor is a significant clue that an area should be cultured for anaerobic bacteria. Other clinical features or clues suggesting infection by anaerobic bacteria are purulent exudate, sulfur granules, abscess, infection after bite, penetrating deep wound, aspiration, infection after surgery, gangrenous necrosis, tissue necrosis, and antibiotic (especially

aminoglyocides) failure. None of these clues are specific, and the specific diagnosis of anaerobic infections requires the isolation of the anaerobes involved.

Collection and Transport of Specimens

Aspirates and tissue samples are the preferred specimens for anaerobic culture. Aspirates are best taken using a needle and syringe from which air has been expelled. The surface must be decontaminated before the sample is taken. The needle must be capped for transport to the laboratory. If the specimen will not reach the laboratory within 1 hr, it should be injected into an oxygen-free vial or tube, preferably one containing a redox indicator. The specimen should be kept at room temperature, since chilling is detrimental to some anaerobes, and oxygen absorption is greater at lower temperatures (2).

Small (less than 1 cm^3) tissue samples should be processed immediately, or they can be placed in commercial anaerobic transport containers. Anaport system (Scott Laboratories, Inc., Fiskeville, Rhode Island), Bio-Bag Type A (Marion Laboratories, Inc., Kansas City, Missouri), and Vacutainer Anaerobic Specimen Collector (Becton-Dickinson and Company, Cockeysville, Maryland) are suitable for transporting small tissue samples. The maintenance of anaerobic conditions for large specimens of anaerobic bacterial infected tissue is not so critical.

Swabs are less satisfactory, but if used they should be protected from drying and exposure to oxygen or oxidizing conditions. Commercial swab transport systems for anaerobic bacteria are available (Port-A-Cul, BBL Microbiology Systems, Cockeysville, Maryland; Carr-Scarborough Microbiologicals, Inc., Stone Mountain, Georgia; Anaerobic Culturette, Marion Laboratories, Inc.; and other sources). Most of these contain a swab in an anaerobic atmosphere and a second tube containing a semisolid deep of transport medium. The swabs should be processed within 2–3 hr of collection (4). However, in one evaluation 45 of 47 anaerobic bacterial species in abscesses were recovered from swabs of the abscess that were placed in the Port-A-Cul for 24 hr (19).

Prereduced anaerobically sterilized (PRAS) modified Cary–Blair medium is a suitable transport medium for swabs and can be made as described in Appendix B. Swabs for use with PRAS Cary–Blair medium can be prepared by sterilizing them in a gassed-out, O_2-free nitrogen, airtight tube. Swabs are removed from their anaerobic environment, quickly used, and immediately submerged in the tube of PRAS Cary–Blair medium.

Laboratory Procedures

Direct Examination of Specimens

Direct gross and microscopic examination of all specimens should precede attempts at isolation. The gross appearance and odor of a specimen often suggest the possibility of anaerobes. The Gram stain will indicate the number of different morphological types of bacteria to be expected on aerobic and anaerobic culture plates. Slides should be examined carefully because certain bacteria, especially *Bacteroides* and *Fusobacterium*, often stain faintly. The presence of cellular debris or fibrin often make bacteria hard to find. Gram-positive bacteria often stain as gram-negative bacteria, especially in chronic disease processes. Many anaerobic bacteria are indistinguishable morphologically from aerobic bacteria. Exceptions include *F. necrophorum*, which is often present in clinical materials as a long, filamentous, gram-negative pleomorphic rod, and *F. nucleatum*, which is a gram-negative thin rod with tapered ends, often in pairs.

Fluorescent antisera are available to identify the *B. fragilis* group and pigmented *Bacteroides* spp. (General Diagnostics, Morris Plains, New Jersey). While there are no reports of their use in veterinary diagnostic laboratories, the experience with them in human medical laboratories is that they are specific and sensitive (20,21).

Anaerobic Culture Systems

A variety of anaerobic culture techniques are available. The method of choice depends on available space, workload, cost of equipment and media, technical capabilities of the personnel, and main purposes of the laboratory. The three major methods are the use of anaerobic jars, the use of roll–streak tubes with PRAS media, and the use of anaerobic chambers. The recovery of clinically important nonsporeforming anaerobes is equally good with anaerobic jars when compared to other techniques (22,23). Since this approach is the easiest, most economical, and most familiar to diagnostic bacteriologists, it is the recommended method. Details are available for the roll–streak tubes and anaerobic chambers (1–5).

Anaerobic jars are of two types. In both types a metal or plastic lid, usually with an O-ring gasket, is clamped to a flange at the top of the jar to create an airtight seal. In one technique the lids have vents or valves through which air can be evacuated and anaerobe gas mixture can be added (evacuation–replacement jar).

An inexpensive device for evacuating and gassing anaerobic systems has been described (24). The device can be used with an in-house vac-

uum if 20–24 in of mercury can be drawn. Evacuate the jar to 20–24 in of mercury and fill the jar with commercial grade N_2. Repeat this cycle. Evacuate the jar a third time. Instead of N_2, fill the jar with an anaerobe gas mixture (10% H_2, 5% CO_2, 85% N_2), which can be bought from commercial gas suppliers.

In the other system a vented lid is not required, and the anaerobe gas is provided by adding 10 ml of water to a sealed foil envelope (Gas Pak, BBL; Gas Gendicator, Scott; and Oxoid U.S.A., Inc., Columbia, Maryland) containing a tablet of citric acid–sodium bicarbonate and a tablet of sodium borohydride (H_2–CO_2 generator jar). Anaerobic conditions are established in jars by either technique. However, the evacuation–replacement technique is more economical and establishes anaerobic conditions more quickly than the H_2–CO_2 generator technique.

For both systems new or rejuvenated catalyst should always be used. The catalyst can be rejuvenated by heating in a drying oven at 160–170°C for at least 2 hr. To ensure that anaerobic conditions are obtained an oxidation–reduction potential indicator (Scott, BBL, Oxoid) should always be added to the jar.

Media for the Isolation of Anaerobic Bacteria

The isolation of anaerobic bacteria from clinical specimens involves the use of nonselective and selective media. The choice of media depends on the anatomical source of the specimen, findings from direct microscopic examination of the specimen, and the bacterium associated with a specific type of infection.

Nonselective media should be used for the primary isolation of most obligate anaerobes.

Enriched blood (5% v/v) agar plate media should be used. Any of the following agar bases are suitable; brucella, Columbia, Schaedler, brain–heart infusion, or trypticase soy. All these bases should be supplemented with 0.5% yeast extract, vitamin K_1 (10 μg/ml final concentration), and hemin (5μg/ml final concentration) (see Appendix B).

Selective media are sometimes useful for the isolation of obligate anaerobes or specific groups of anaerobic bacteria, especially if they are present in small numbers in a mixed bacterial infection. Phenylethyl alcohol (0.25% phenylethyl alcohol in agar bases described above) supports the growth of most anaerobic bacteria but inhibits the growth of facultatively anaerobic gram-negative bacilli. Paromomycin–vancomycin and kanamycin–vancomycin media contain 100 μg of either paromomycin or kanamycin and 7.5 μg of vancomycin per milliliter of blood agar base and are useful for the selective isolation of obligately anaerobic gram-negative bacteria, mainly *Bacteroides* and *Fusobacterium*.

Plate media should be used immediately after preparation or they can be stored then prereduced in an anaerobic jar for 6–24 hr before use. Plate media can be stored in a refrigerator (2–4°C) for up to 6 weeks if they are wrapped in cellophane bags to retard dehydration (4). Plates must be maintained in an oxygen-free atmosphere both before and after inoculation. A holding jar procedure which allows primary plating, inspection of colonies, and subculture of colonies at the laboratory bench without undue exposure of anaerobes to oxygen has been described (4). Commercial-grade N_2 or commercial-grade CO_2 are suitable for this procedure. If CO_2 is used, it must be passed through a heated copper catalyst (Sargent furnace) to remove traces of O_2. This step is not necessary if N_2 is used.

Liquid media is an important adjunct in the isolation of anaerobes, particularly where low numbers are present or antibiotics have been used. They also provide a fail-safe device in case the anaerobic jar is not set up properly or the H_2–CO_2 generator fails to activate. Thioglycollate medium without indicator (BBL-0135C, BBL) or chopped meat–glucose medium are suitable. Both should be supplemented with vitamin K_1 and hemin. All liquid media should be placed in a boiling water bath for 10 min to drive off oxygen and cooled before use or be stored with loose caps for 48 hr in an anaerobic environment before use. Liquid media should never be used as the only primary isolation media.

Incubation of Cultures

Specimens must be streaked on plates in the traditional manner using either a platinum or stainless steel loop. The jar should be incubated at 35°C as soon as possible and not be opened for 48 hr. Plates should be incubated for at least 7 days before discarding. Most anaerobes will grow in 48 hr given proper media and methods.

Liquid media should be inoculated near the bottom of the tube with 1–2 drops of material by Pasteur pipette. Tubes should be incubated in an anaerobic atmosphere with screw caps loosened and not discarded before 7 days.

Examination of Cultures

Anaerobic plates should be examined after 48 hr of incubation. Plates should be exposed to air for minimal time since certain anaerobes will die within minutes; although the majority of clinically important nonsporeforming anaerobes are more robust. All anaerobic plates, when not under examination, should be stored in holding jars with an O_2-free atmosphere.

Colonies should be examined with a dissecting microscope or hand lens, and each colony type described. Nonspecific anaerobic infections are commonly "mixed infections" and contain both anaerobic and aerobic organism. The different colony types should all be subcultured to aerobic and enriched anaerobic blood agar plates to purify the colonies and to identify which of the colony types are only facultative anaerobes. Certain facultative bacteria may at first only grow anaerobically; examples include *Listeria monocytogenes* and some strains of *Escherichia coli*. Gram-stained smears should be examined from each of the different colony types and each colony type inoculated into peptone–yeast–glucose (PYG) broth that has been previously heated for ten minutes in a boiling water bath to remove oxygen. Peptone–yeast–glucose broth is used for gas–liquid chromatographic (GLC) analysis of volatile and nonvolatile acids. It is incubated for 48 hr or until adequate growth is obtained in an anaerobic environment with the cap loosened. The method of preparation is given in Appendix B. An alternative to PYG broth is chopped meat–carbohydrate (CMC) medium prepared as described in Appendix B. The GLC profiles for CMC differ slightly from those in PYG; only PYG profiles are listed here.

Plates should be examined under UV light for the presence of the brick-red fluorescing colonies of certain *Bacteroides* species and *Veillonella* species.

The primary plates and subcultures are than reincubated for another 48 hr. At this time the anaerobic organisms will be distinguished from the aerobically growing facultative anaerobes. The black-pigmented *Bacteroides* will usually have developed pigment, and the corroding *Bacteroides* will usually show pitting of the agar.

Actinomyces species are discussed in Chapter 22. They may grow under 10% CO_2 or even be aerotolerant. *Clostridium perfringens* is discussed in Chapter 19. It is found in nonspecific anaerobic infections and is readily identified. When the Gram stain of the original specimen suggests *C. perfringens*, the organism may be isolated after approximately 12 hr of incubation. On occasion it is also aerotolerant. If the Gram reaction and morphology of the colony types recovered on plate media do not correspond to the morphological types of bacteria seen in the original specimen, the chopped meat–glucose broth or thioglycollate medium should be subcultured to an anaerobic plate.

Identification of Nonsporeforming Anaerobic Bacteria

Pure cultures of nonsporeformers must be obtained before they can be identified. They should always be subcultured onto the supplemented, enriched, and prereduced anaerobic media described.

The individual laboratory has to decide to what level it will take identification of nonsporing anaerobes. Some laboratories will be satisfied with isolation and a description based on Gram reaction and colony morphology. Other laboratories will want better identification and will base it on the use of commercially available "kit" identification systems or semiconventional media and analysis of metabolic products. A few specialist laboratories will want to use conventional media (prereduced anaerobically sterilized, PRAS) and identify isolates definitively using the classic VPI manual (1). In this description, the characteristics used in the identification of nonsporeforming anaerobes will be based on the descriptions in the *VPI Anaerobe Laboratory Manual*.

Gas–Liquid Chromatographic Analysis of Metabolic End Products

For the identification of genera of nonsporeforming anaerobic bacteria (see Table 15-3) gas–liquid chromatographic analysis of metabolic end products from PYG or CMC broth is essential. Volatile fatty acids and nonvolatile fermentation products are key characteristics of anaerobic

Table 15-3
Genera of Clinically Important Nonsporeforming Anaerobic Bacteria

I. Gram-negative rods, nonmotile or motile with peritrichous flagella	
A. Produce mixture of acids including acetic, propionic, lactic, and succinic. Butyric not usually a major product; if present, produced in combination with isobutyric and isovaleric	*Bacteroides*
B. Produce major butyric acid, little or no isobutyric and isovaleric acid, and no succinic acid; nonmotile	*Fusobacterium*
II. Gram-negative rods, polar flagella, do not ferment glucose, and are oxidase positive	*Wolinella*
III. Gram-positive rods	
A. Propionic acid major metabolic product	
1. Catalase usually positive	*Propionibacterium*
2. Catalase negative	*Arachnia*
B. Propionic acid not produced	
1. Ratio lactic acid to acetic acid greater than 1:1	
a. Lactic acid usually only major product	*Lactobacillus*
b. Succinic acid is a major product	*Actinomyces*
2. Ratio lactic acid to acetic acid less than 1:1	
a. Butyric acid not produced	*Bifidobacterium*
b. Produce butyric and other acids or no major acids	*Eubacterium*
IV. Gram-positive cocci	
A. Ferment carbohydrates with production of major amounts of lactic acid	*Streptococcus*
B. Lactic acid not a major product	*Peptococcus* or *Peptostreptococcus*
V. Gram-negative cocci, propionic and acetic acids major metabolic products	*Veillonella*

bacteria, are reproducible and reliable, and are so characteristic of some species as to act as "fingerprints" of that species.

The major problem with GLC analysis is that of initial cost of equipment. Running costs of reliable equipment will, however, be low. There are several GLC machines that have been designed specifically for use with the identification of anaerobes. Examples are the CAPCO (CAPCO, Dodeca, Fremont, California) and GOW-MAC (Gow-Mac Instrument Co., Bound Brook, New Jersey). Any laboratory attempting to identify anaerobic bacteria must have GLC facilities available to it.

Details of procedures used to extract volatile fatty acids or nonvolatile acids (e.g., pyruvic, succinic, lactic acids) from PYG or CMC are given elsewhere (1–5). The type of columns to be used, their packing material, and details on operating conditions of the GLC equipment are all described for anaerobic bacteriology (1–5). They will not be repeated here. The methods used must conform with those for the manual used and the profiles of metabolic products obtained must be compared with those shown in that book and identified and quantified by use of control solutions. Different manuals of anaerobic bacteriology (1–5) may show minor differences in metabolic products from carbohydrates; these minor differences relate to the media used and to the conditions of analysis of metabolic products. It is important to follow one set of methods and not to interchange them.

The GLC equipment designed for identification of anaerobes is easy to operate and has a long lifetime. Without such equipment anaerobe identification becomes haphazard, although some commercial "kit" systems can to some extent be used without GLC analysis to identify some of the more common anaerobic bacteria.

Commercially Available Anaerobic Identification Systems

Several identification systems are available commercially for the identification of anaerobic bacteria. All suffer from the drawback that they are designed for human medical bacteriology, and some of the common veterinary isolates are not included in their data base. For most of these systems there is little or no published information comparing the results obtained for identifying veterinary isolates to results obtained by conventional identification media. The RapID-ANA System (Innovative Diagnostic Systems, Inc., Atlanta, Georgia) was evaluated using veterinary isolates (25). Of 183 isolates, 81% were correctly identified to the genus level and 60% to the species level. Until additional studies are completed using veterinary isolates in the various identification systems, none are recommended for routine use in the veterinary diagnostic laboratory.

Identification Using Conventional Media

Two conventional systems for the characterization of isolates using biochemical test media in large tubes have been described in

laboratory manuals (1,2). These manuals should be consulted for the details of each system. The *VPI Anaerobe Laboratory Manual* (1) describes the use and preparation of PRAS medium, which is difficult to prepare and inoculate. This medium is commercially available (Carr-Scarborough, Remel, Lenexa, Kansas; and PRAS II System, Scott). A computer program is available for use with the PRAS II System.

The *Centers for Disease Control Laboratory Manual* (2) describes the use and preparation of conventional tubed media. These media are easier to prepare (see Appendix B) and inoculate. They are also commercially available (Carr-Scarborough; Remel; and Nolan Biological Laboratories, Tucker, Georgia).

In addition to the tube tests, the following tests are often helpful:

1. Catalase. With a loop remove growth from an agar plate that has been exposed to air for 30 min and smear on a slide. Add a drop of 3% H_2O_2. The appearance of gas bubbles indicates a positive test. If the growth is taken from a blood agar base ensure that no medium is taken, because erythrocytes also contain catalase.
2. Gram reaction. The Gram reaction of anaerobic bacteria is sometimes difficult to determine by traditional staining procedures, which may give false gram-negative results. The KOH test is useful in cases of doubt. Add 2 drops of 3% (w/v) KOH to a slide and add a loopful of a young culture from plate medium. The loop is stirred in a circular motion and occasionally raised from the slide surface. If the suspension becomes stringy and can be lifted from the slide within 30 sec of mixing, the organism is gram-negative.
3. Lecithinase and lipase. Inoculate an egg yolk agar plate (see Appendix B) and incubate anaerobically for 48 hr. Lecithinase production is indicated by the production of a wide zone of opalescence around colonies. Lipase production is indicated by the presence of an iridescent sheen on the surface of, and in a narrow zone around, colonies. Plates may need to be incubated an additional 48 hr to detect lipase production.

Gram-Negative Anaerobic Rods

Gram-negative anaerobic rods are the most common anaerobes isolated from clinical specimens in animals. Presently over 40 species of *Bacteroides* and 10 species of *Fusobacterium* are recognized. These numbers will increase as additional studies are completed concerning the anaerobes involved in various infections in animals and humans. Strains of gram-negative anaerobic rods similar to *Wolinella* species have been isolated from soft tissue infections in cats and dogs (26,27).

Clinically Important Species of *Bacteroides*

Bacteroides fragilis is one of the most frequently isolated anaerobes from nonspecific infections in animals. It is of particular importance because strains resistant to antimicrobials are increasing in frequency (10). Enterotoxigenic strains of *B. fragilis* have recently been isolated from young animals. There is a close association between the presence of enterotoxigenic *B. fragilis* and acute diarrheal disease in lambs, calves, pigs, and foals (28–31). Other species of the *B. fragilis* group are *B. distasonis, B. vulgatus, B. ovatus, B. thetaiotaomicron,* and *B. uniformis* (32). All are infrequently isolated from clinical specimens.

Pigmented *Bacteroides* species consist of at least nine species (33). Some strains may take as long as 21 days for pigment to develop, but pigment usually develops in 3–5 days if laked blood agar is used. Most strains will show brick-red flurorescence under long-wavelength UV light before the pigment develops. Most species are not isolated or are infrequently isolated from infections in animals. However, *B. levii* has been associated with summer mastitis in cattle and *B. salivosus* with subcutaneous abscesses and empyemas in cats (34).

Bacteroides ureolyticus forms transparent colonies that usually pit the surface of agar plates. Most strains grow poorly in broth unless supplemented with sodium formate and furmaric acid. It is isolated occasionally from nonspecific infections in animals.

Bacteroides heparinolyticus has been associated with oral-associated disease conditions of horses and cats (35). The other *Bacteroides* spp. are rarely, if ever, isolated from infections in animals.

A species requiring special attention in veterinary laboratories is *B. nodosus,* the cause of foot rot in sheep. Gradin and Schmitz (36) have described a selective medium that consists of Eugon agar (BBL) with 0.2% (w/v) yeast extract (Pfizer, Inc.), 10% defibrinated horse blood (v/v), and 1 μg/ml (w/v) lincomycin. The plates must be incubated anaerobically for 5–6 days and not discarded for 7 days. An alternative medium has been described by Thorley (37). It consists of 1% (w/v) proteose peptone (Difco), 1% (w/v) trypsin 1:250 (Difco), 1% (w/v) liver digest L27 (Oxoid), and 0.5% (w/v) NaCl. To prevent precipitation during autoclaving, the dissolved materials are brought to boiling at pH 8.5, filtered and cooled, the pH adjusted to 7.4, and then autoclaved. The agar concentration used is 5.0% (w/v); the high concentration prevents swarming. Before pouring plates, 0.05% (w/v) of a cysteine–HCl solution is added.

On the medium described by Gradin and Schmitz, certain strains produce colonies that are small and have a "snowflake" appearance. Colonies of primary isolates on the medium described by Thorley are low in profile and show central papular studding, fusing near the

colony center to give a series of shiny reticulations or a low peak. There are also clear internal bands. The outer zone consists of a fine, granular, and diffusely spreading edge made up of migrating microcolonies. Details on the colonial variation seen on subculture are available (37,38). Virulent isolates can be tested for proteolytic activity in a variety of ways (39,40), and tests to determine serotypes are done as described (37,41).

Isolations of *B. nodosus* are most likely to be made when Gram staining shows the presence of *B. nodosus* in active foot rot lesions. Typically, this is a large gram-negative rod (0.6–0.8 × 3–10 μm) with terminal enlargements at one or both ends and a general curvature to the cell, which in many cases is surrounded by radially disposed gram-negative bacilli (Fig. 15-1).

Material for culture should be taken from necrotic material at the junction of healthy with separating tissue from the feet of sheep, and transported in PRAS Cary–Blair medium to the laboratory. Those portions of necrotic material showing greatest numbers of *B. nodosus* on smears should be shaken vigorously for 30 sec in 10 ml 0.25 *M* sterile sucrose and the suspension then seeded on anaerobic media (42).

Differential characteristics of some important *Bacteroides* spp. are presented in Table 15-4.

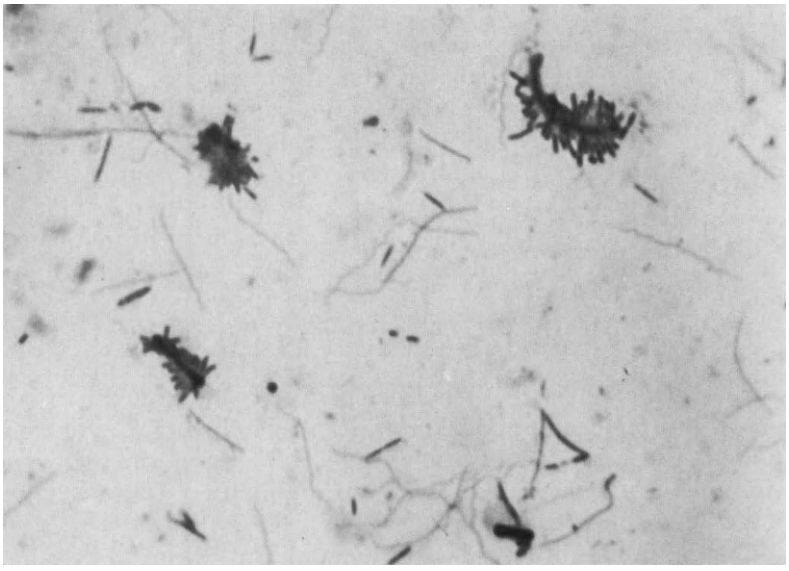

Figure 15-1. Smear from a case of foot rot showing *Bacteroides nodosus* surrounded by small bacteria. Also seen are a number of spirochetes. Courtesy of W.I.B. Beveridge.

Table 15-4
Differential Characteristics of *Bacteroides* from Veterinary Clinical Specimens[a]

Species	Growth in 20% bile	Esculin hydrolyzed	Catalase	Urease	Indole	Black pigment	Fermentation of: Arabinose	Glucose	Lactose	Maltose	Rhamnose	Salicin	Sucrose	Trehalose	Fatty acids from peptone–yeast–glucose[b]
B. fragilis	+	+	+	−	−	−	−	+	+	+	−	−	+	−	A,S,p(ib,iv,l)
Other *B. fragilis* group	+	V	V	−	V	−	+	+	+	+	V	V	+	V	A,S(p,ib,iv,l,f)
B. bivius	−	−	−	−	−	−	−	+	+	+	−	−	−	−	A,S,iV(ib,f)
B. capillosus	−	+	−	−	−	−	−	$+^{-}$	−	−	−	−	−	−	a,s(l,f,p)
B. disiens	−	−	−	−	−	−	−	+	−	+	−	−	−	−	A,S(ib,iv,f,p)
B. ruminicola[c]	−	+	−	−	−	−	+	+	+	+	−	$+^{-}$	+	−	A,S,f(p,ib,iv,l)
B. ureolyticus	−	−	−	+	−	−	−	−	−	−	−	−	−	−	A,S(l,f)
B. asaccharolyticus	−	−	−	−	+	+	−	−	−	−	−	−	−	−	A,B,iV,S,p,ib
B. intermedius	−	−	−	−	.+	+	−	+	−	+	−	−	+	−	A,S,iv(ib,p,f)
B. melaninogenicus	−	$-^{+}$	−	−	−	+	−	+	+	+	−	−	+	−	A,S(f,iv,ib,l)
B. levii	−	−	−	−	−	+	−	+	+	−	−	−	−	−	A,B,p,iv,ib,s
B. nodosus	$-^{+}$	−	−	−	−	−	−	−	−	−	−	−	−	−	a,s,p(f,iv,b,ib,l)
B. oralis	−	+	−	−	−	−	−	+	+	+	V	+	+	−	A,S,f(l)
B. heparinolyticus	−	+	−	..	+	−	..	+	+	+	..	+	+	..	S,a,p,iv
B. salivosus	−	−	+	−	+	+	−	−	−	−	−	−	−	−	A,B,ib,iv

[a]Symbols: +, positive reaction; −, negative reaction; v, variable reaction; $+^{-}$, most strains positive; $-^{+}$, most strains negative; .., not known.
[b]Metabolic products: Capital letters indicate major quantities; small letters minor amounts; parentheses indicate variable production. A, acetic; B, butyric; C, caproic; F, formic; iB, isobutyric; iC, isocaproic; iV, isovaleric; L, lactic; P, propionic; S, succinic; V, valeric.
[c]*B. ruminicola* subsp. *ruminicola* is xylose positive, subsp. *brevis* is xylose negative.

Clinically Important Species of *Fusobacterium*

The characteristics of clinically important species of *Fusobacterium* are given in Table 15-5. All the fusobacteria produce butyric acid as the major product from peptone–yeast extract–glucose broth. Major or minor amounts of acetic, propionic, and lactic acids may also be produced.

Fusobacterium necrophorum and *F. nucleatum* are the species most often encountered in veterinary specimens. *Fusobacterium necrophorum* is often recovered from organ abscesses and foot rot lesions, often in association with other anaerobic bacteria, especially species of *Bacteroides*. It is a gram-negative pleomorphic bacillus ranging in form from small coccobacilli to long filamentous rods. The colonies are usually β-hemolytic, gray, convex with an entire edge, and are 1–2 mm in diameter after 48 hr. Most strains of *F. necrophorum* are lipase positive on egg yolk agar, a biochemical property that distinguishes them from all other fusobacteria.

Fusobacterium nucleatum is occasionally found in nonspecific anaerobic infections in various animal species. It has a distinctive morphology with thin, gram-negative, pointed rods that are often in pairs arranged end-to-end.

Fusobacterium russii and *F. alocis* have been recovered from soft tissue infections of cats (43,44). Other *Fusobacterium* have been isolated infrequently from nonspecific infections in animals (9,11, 16,43,44).

Gram-Positive Nonsporeforming Rods

The identification of the more common clinically important gram-positive, nonsporeforming rods is shown in Table 15-6. *Actinomyces* speciation is discussed in Chapter 22.

The cellular morphology of these organisms is highly variable, depending on media and growth conditions. The morphology varies from small to large, long rods. Club-shaped rods or rods with bifurcated ends are often seen, and at times the organisms may occur as filaments. The organisms may resemble streptococci or may readily decolorize and appear gram-negative. Except for the *Actinomyces*, gram-positive nonsporeforming rods are infrequently recovered from clinical specimens from animals. When isolated, their significance is often not known. The following is a list of the genera of gram-positive nonsporeforming rods.

Table 15-5
Characteristics of *Fusobacterium* from Veterinary Clinical Specimens[a]

Species	Esculin hydrolyzed	Indole produced	Growth in 20% bile	Propionate from lactate	Propionate from threonine	Fermentation of			Major products in peptone–yeast–glucose[b]
						Fructose	Glucose	Mannose	
F. alocis[c]	−	−	−	−	−	−	−	−	B,a
F. gonidiaformans	−	+	−+	−	+	−	−	−	B,A,p(l,f,s)
F. naviforme	−	+	−+	−	−	−	+−	−	B,L,a(f,p,s)
F. necrophorum	−	+	−+	+	+	−+	−+	−	B,p,a(l,s)
F. nucleatum	−	+	+	−	+	−+	−+	−	B,a,p(F,L,s)
F. russii	−	−	−+	−	−	−	−	−	B,L,a(f)
F. mortiferum	+	−	+	−	+	+	+	+	B,a,p(s,f,l,v)
F. varium	−	v	+	−	+	+	+	+	B,L,a,p(s)

[a]Symbols: see Table 15-4.
[b]Metabolic products: see Table 15-4.
[c]Isolates from cats positive for growth in 20% bile and for production of propionate from lactate and threonine.

Table 15-6
Differential Characteristics of Cocci and Gram-Positive Nonsporeforming Anaerobic Rods from Veterinary Clinical Specimens[a]

Species	Gram reaction/ morphology	Indole	Nitrate	Catalase	Fermentation of: Glucose	Lactose	Maltose	Mannitol	Sucrose	Major products in peptone–yeast–glucose[b]
Eubacterium lentum	+, rod	−	+⁻	−	−	−	−	−	−	(a,l,s)
E. suis[c]	+, rod	−	−	−	−	−	+	−	−	A,F
E. tarantellus	+, rod	−	−	−	+	−	−	−	−	A,F
E. tenue	+, rod	+	−	−	+⁻	−	+⁻	−	−	A,f
Propionibacterium acnes	+, rod	+⁻	+⁻	+⁻	+	−	−	v	−	P,A,(f,l,s,iv)
Peptostreptococcus anaerobius[d]	+, coccus	−	−	−	+	−	−	−	−	A,ib,b,(iv,ic)
P. indolicus[e]	+, coccus	+	+	−	−	−	−	−	−	A,B,p,(l,s)
Streptococcus intermedius	+, coccus	−	−	−	+	+	+	−	+	L,(f,a,s)
Veillonella parvula	−, coccus	−	+	−	−	−	−	−	−	A,P,(s)

[a] Symbols: see Table 15-4.
[b] Metabolic products: see Table 15-4.
[c] *Eubacterium suis* produces acid from glycogen and starch, hydrolyzes starch, and maltose stimulates growth.
[d] *Peptostreptococcus anaerobius* is inhibited by sodium polyanethol sulfonate disk.
[e] *Peptostreptococcus indolicus* is coagulase positive.

Actinomyces

Members of this genus are the most common gram-positive non-sporeforming rods isolated from veterinary clinical specimens. This genus is discussed in Chapter 22.

Arachnia

This genus contains only one species, *A. propionica,* and it has not been isolated from clinical specimens from animals (45).

Bifidobacterium

Twenty-four species of *Bifidobacterium* are listed in *Bergey's Manual of Systematic Bacteriology* (46). *Bifidobacterium* are rarely isolated from clinical specimens. The fermentation reactions and other commonly used tests for phenotypic characterization do not reliably differentiate among all the *Bifidobacterium* spp. Therefore, the species identification of *Bifidobacterium* is not practical for clinical laboratories (47).

Eubacterium

Thirty-four species of *Eubacterium* are listed in *Bergey's Manual of Systematic Bacteriology* (48). Members of this genus are rarely isolated from clinical specimens and when isolated their significance is usually not known. Two species have been associated with disease in animals. *Eubacterium suis* is a cause of pyelonephritis in swine (49,50), and *E. tarantellus* has been associated with mortality in striped mullet (51). Other *Eubacterium* isolated from clinical specimens include *E. lentum* (9,15) and *E. tenue* (9,52). In most studies the species of *Eubacterium* isolated were not determined.

Lactobacillus

This genus is discussed in Chapter 24 under miscellaneous usually nonpathogenic bacteria.

Propionibacterium

Eight species of *Propionibacterium* are recognized (53). They are usually found in dairy products or on skin. *Propionibacterium*, mainly *P. acnes*, have been isolated from a low percentage of clinical specimens in several studies (6–9,14–16). The clinical significance of this group of bacteria is usually unknown. In humans they are usually, but not always, considered contaminants (53).

Anaerobic Cocci

The identifying characteristics of the anaerobic cocci most commonly isolated from veterinary clinical specimens are given in Table 15-6. In general they are slow-growing, and biochemical tests require 3–5 days for sufficient growth to occur before they can be read.

Recently there have been major revisions in the taxonomy of the gram-positive anaerobic cocci (see Table 15-7). Only one species of *Peptococcus* is recognized. This species, *Peptococcus niger*, is rarely if ever isolated from veterinary clinical specimens.

The genus *Peptostreptococcus* now has nine species. Two species, *P. anaerobius* and *P. indolicus*, are isolated from clinical specimens. *Peptostreptococcus anaerobius* has been isolated from clinical specimens from various animal species, and *P. indolicus* has been associated with summer mastitis in cows. The other peptostreptococci are rarely isolated from veterinary clinical specimens.

Table 15-7
Taxonomy of the Anaerobic Gram-Positive Cocci[a]

Present nomenclature	Previous nomenclature
Peptococcus niger	*Peptococcus niger*
Peptostreptococcus anaerobius	*Peptostreptococcus anaerobius*
Peptostreptococcus asaccharolyticus	*Peptococcus asaccharolyticus*
Peptostreptococcus heliotrinreducens	*Peptococcus heliotrinreducens*
Peptostreptococcus indolicus	*Peptostreptococcus indolicus*
Peptostreptococcus magnus	*Peptococcus magnus*
Peptostreptococcus micros	*Peptostreptococcus micros*
Peptostreptococcus prevotii	*Peptococcus prevotii*
Peptostreptococcus productus	*Peptostreptococcus productus*
Peptostreptococcus tetradius	"Gaffkya anaerobia"
Staphylococcus saccharolyticus	*Peptococcus saccharolyticus*
Streptococcus hansenii	*Streptococcus hansenii*
Streptococcus morbillorum	*Streptococcus morbillorum*
Streptococcus parvulus	*Peptostreptococcus parvulus*
Streptococcus pleomorphus	*Streptococcus pleomorphus*

[a]Adapted from Schaal, K. P., in Sneath, P. H. A. (Ed-in-chief): *Bergey's Manual of Systematic Bacteriology*, Vol. 2. Baltimore, Williams & Wilkins (45).

The anaerobic streptococci consist of four species: *Streptococcus hansenii, S. pleomorphus, S. morbillorum,* and *S. parvulus* (was *Peptostreptococcus parvulus)* (54). None have been reported from veterinary clinical specimens, and only *S. morbillorum* is considered a human pathogen. Two other aerotolerant species, *S. intermedius* and *S. constellatus,* have been included among the anaerobic streptococci, and *S. intermedius* has been isolated from clinical specimens (9,14).

The only gram-negative anaerobic cocci belong to the family Veillonellaceae. In one study *Veillonella* species were isolated from a low percentage of clinical specimens (14). They are characterized by the ability of colonies to exhibit a pink to red fluorescence under ultraviolet light, which is similar to that of *Bacteroides melaninogenicus.*

Antimicrobial Susceptibility Testing of Nonsporeforming Anaerobes

The antimicrobial susceptibility testing of nonsporeforming anaerobes from animals is generally unnecessary since the susceptibility is predictable. In a recent study, 97–100% of the anaerobes isolated were susceptible to chloramphenicol, clindamycin, and metronidazole; 93–100% of the anaerobes, except *Bacteroides,* were susceptible to tetracycline, ampicillin, penicillin, and cephalothin (10). A majority of the *Bacteroides,* especially *B. fragilis,* were resistant to penicillin, ampicillin, and cephalothin. Approximately one-third of these resistant isolates were also resistant to tetracycline.

Aminoglycosides are usually ineffective in the treatment of anaerobic infections. Trimethroprim-sulfamethoxazole was active *in vitro* against most anaerobes (55). However, this combination was often associated with treatment failures (8), probably due to inactivation by purulent exudates.

The susceptibility testing of pathogenic anaerobes is needed so that changing patterns of resistance can be detected. The disk diffusion technique used for rapidly growing aerobes must not be used for susceptibility testing of anaerobic bacteria. The methodology and zone size criteria have not been developed for anaerobes.

The broth–disk method described by Kurzynski and co-workers (56) is recommended. In this procedure one or more standard antibiotic sensitivity disks are added to 5 ml of thioglycolate medium without indicator (BBL 135C) in a 16 × 125-mm screwcap tube which has been preboiled 5 min and cooled. The number of disks and approximate concentration of antimicrobial obtained are listed in Table 15-8. The tubes are left for 2 hr at room temperature to allow the antimicrobial to elute into the broth. Inoculate each tube with 2 drops of an overnight chopped meat–glucose culture. Tighten the caps, and invert the tubes twice to ensure a uniform distribution of drug and inoculum. Incubate

Table 15-8
Preparation of Broth–Disk Tubes

Antimicrobial agent	Disk content	Disks per 5 ml of medium	Concent. (per ml)
Ampicillin	10 μg	2	4 μg
Carbenicillin	100 μg	6	120 μg
Cephalosporins[a]	30 μg	3	18 μg
Chloramphenicol	30 μg	3	18 μg
Clindamycin	2 μg	8	3.2 μg
Erythromycin	15 μg	1	3 μg
Metronidazole	80 μg	1	16 μg
Penicillin G	10 U	1	2 U
Tetracycline	30 μg	1	6 μg
Ticarcillin	75 μg	4	60 μg

[a]Includes cephalothin, cefoxitin, cefamandole, cefotaxime, and moxalactam.

the cultures at 35°C overnight or for 48 hr for slow-growing bacteria. Compare the turbidity of the growth in each tube containing an antimicrobial to that of a growth control tube without antimicrobial. Susceptibility to the antimicrobial is defined as either the absence of turbidity or turbidity less than 50% of that of the growth control. Resistance is 50% or more turbidity. Usually there is turbidity equal to the growth control (resistant) or no turbidity (sensitive).

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16

Micrococcus and *Staphylococcus*

John R. Cole, Jr.

The family Micrococcaceae includes gram-positive aerobic or facultatively anaerobic cocci. The following genera are listed in the family: *Staphylococcus, Micrococcus, Planococcus,* and *Stomatococcus.*

Staphylococcus

Organisms of the genus *Staphylococcus* are aerobic and facultatively anaerobic, catalase positive, nonmotile, nonsporing, fermentative, gram-positive cocci. Although they are usually seen in clusters and pairs, short chains may be seen in smears from fluid media (Fig. 16-1). They occur commonly as commensals on the skin and mucous membranes of humans and animals.

There are at least 19 recognized species of *Staphylococcus.* The principal distinguishing features of the five species considered to be important animal pathogens or frequent isolates are as follows.

Staphylococcus aureus (Staphylococcus pyogenes)

Typical strains are coagulase positive, β-hemolytic, ferment maltose and mannitol, and have pigmented colonies. In order to qualify for identification as *S. aureus,* a strain must produce coagulase. The capacity to produce this enzyme may, on occasion, be weak.

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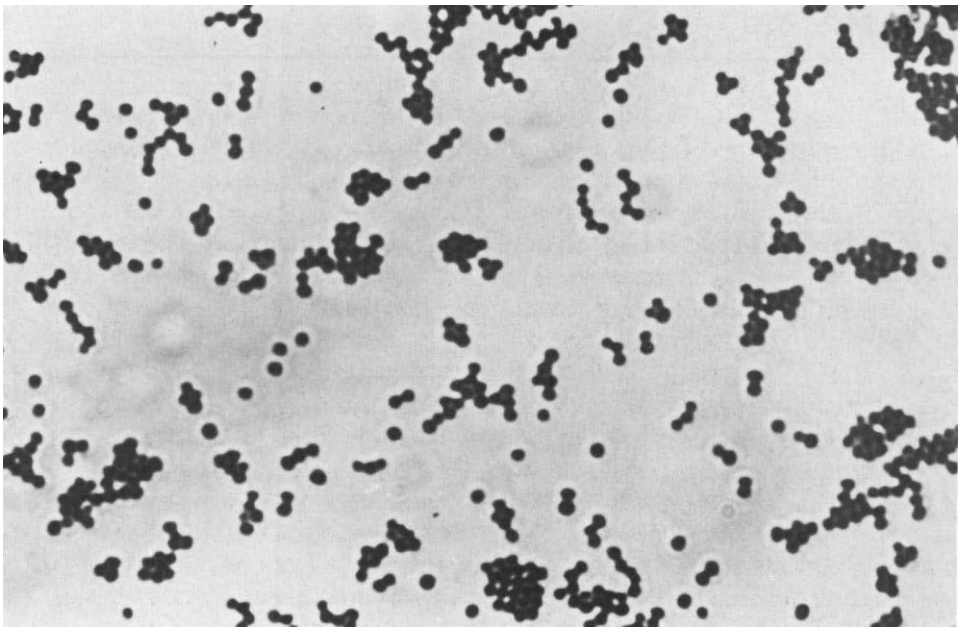

Figure 16-1. *Staphylococcus aureus* from a culture. Gram stain, ×2250.

Staphylococcus epidermidis

This species is considered an opportunistic pathogen that does not produce coagulase. Members of this species ferment maltose but not mannitol, vary in their capacity to produce hemolysis, and are usually nonpigmented.

Staphylococcus saprophyticus

This opportunistic human pathogen closely resembles *S. epidermidis* and is differentiated by its resistance to novobiocin. It is coagulase negative, is nonhemolytic, and has been isolated from air, soil, dust, and the surface of animal carcasses.

Staphylococcus intermedius

This coagulase positive, β-hemolytic, nonpigmented species is primarily cultured from dogs. It has, however, been isolated from horses, pigeons, and milk. This species may weakly ferment maltose.

Staphylococcus hyicus subsp. *hyicus*

Most strains are coagulase positive, produce no hemolysis, and are nonpigmented. It is considered an important pathogen in pigs and has been isolated from cattle and poultry.

Staphylococcus hyicus subsp. *chromogenes*

This subspecies is coagulase negative, nonhemolytic, and may produce a yellow-orange pigment. It is isolated from the skin of pigs and cattle, and from the milk of cows with mastitis.

Isolation Procedures, Cultural Characteristics, and Pathogenicity

Specimens to be cultured for staphylococci and other cocci are inoculated onto sheep or bovine blood agar, and into brain–heart infusion semisolid medium or thioglycollate broth. Washed bovine cells are recommended for blood agar for enhancing hemolysis when testing milk samples (1). The growth from semisolid or liquid media is examined for cocci by staining and is subcultured to blood agar if the plate cultures are negative. Colonies are apparent after incubation for 18–24 hr at 37°C.

Several useful selective media are of value for the isolation of staphylococci from heavily contaminated clinical materials. Two of the most frequently used are mannitol salt agar and phenylethyl alcohol agar (see Appendix B).

S. aureus

Colonies are round, glistening, convex, smooth, and opaque. β-Hemolysis is produced by most strains, and frequently a double zone is apparent in which the central clear zone is surrounded by a band of partial hemolysis. Colonies may possess white, golden, or lemon pigmentation.

Members of this species are involved in a wide variety of disease processes, such as suppurative wound infections, pyemia of lambs, mastitis, pyoderma of dogs, cats, and other animals, abscesses in many animal species, and botryomycosis of horses, cattle, and swine. Virulence has been associated with encapsulation and slime production, especially in bovine mastitis (2). These bacteria are also frequent secondary invaders and opportunists. Staphylococcal enteritis occurs in humans but has not yet been reported in animals.

S. epidermidis

The cultural characteristics of strains of this species are very similar to those of *S. aureus*. Colonies are usually white or colorless, but pigmentation, as with *S. aureus*, is sometimes present. β-Hemolysis, although not usually seen with these strains, is encountered occasionally.

These bacteria are opportunistic pathogens and have been implicated as causing mastitis in cattle, as well as abscesses and skin infections in various animal species and humans.

S. saprophyticus

Colonies of this species are often pigmented on initial isolation and are nonhemolytic.

Bacteria included in this species are considered opportunistic human pathogens. They have been cultured from a variety of human infections that involve the urinary tract or wounds and are associated with artificial prostheses.

S. intermedius

Colonies are white, circular, smooth, and glistening and do not produce pigment. They are usually β-hemolytic on blood agar.

Reports of infections due to these bacteria are confined to pyodermas in dogs and mastitis in dogs and cattle. Their significance in other animal species is not known.

S. hyicus

Pigmentation of the colonies and zones of hemolysis varies with the subspecies. Zones of discoloration may occur around the colony when "aged" blood agar is used, and this should not be confused with β-hemolysin production.

Infections by this organism occur in pigs and cattle. Exudative epidermitis, or greasy pig disease (3,4), and polyarthritis (5) due to *S. hyicus* have been reported in pigs. Both subspecies have been cultured from skin, udder lesions, and milk in cattle.

Identification

Characteristics that are recommended for use in identification of *Staphylococcus* species are shown in Table 16-1. For detection of extracellular coagulase, the tube test with rabbit plasma is recommended. Bound coagulase, or clumping factor, can be observed by mixing a culture suspension and rabbit plasma on a microscope slide. See Appendix C for descriptions of these coagulase tests. For a more

Table 16-1
Characteristics of Staphylococci and Micrococci

Genus and species	Coagulase[a]	Hemolysis (β)	Pigment	Catalase	Maltose[b]	PAB[c]	Mannitol[b]	DNase	Novobiocin[d]	Glucose[e]	Oxidase[f]
S. aureus	+	+	+	+	+	+[g]	+	+[h]	S	+	−
S. epidermidis	−	±	±	+	+	+[g]	−	−	S	+	−
S. saprophyticus	−	−	±	+	+	NA	±	−	R	−	−
S. intermedius	+	+	−	+	±	+[i]	±	±	S	+	−
S. hyicus subsp. *hyicus*	±	−	−	+	−	−[j]	−	+	S	+	−
Micrococcus sp.	−	−	±	+	−	NA	−	−	S	−	±

[a]Tube test using rabbit plasma (EDTA).
[b]Acid produced aerobically.
[c]Purple Agar Base (Difco Laboratories, Detroit, Michigan) with 1% maltose for detecting aerobic acid production.
[d]S, Sensitive, zone diameter ≥16 mm; R, Resistant; 5μg disk; P Agar medium.
[e]Anaerobic acid production (O-F medium).
[f]Reaction observed in 6% tetramethyl phenylene diamine-hydrochloride in dimethyl sulfoxide.
[g]Wide yellow zone around colony.
[h]Poultry isolates may be negative.
[i]Slight yellow to yellow-green zone under colony.
[j]Diffuse alkaline color (deep purple) around colony streak.

detailed explanation of the other procedures, see articles listed in the Supplementary Readings.

The staphylococci can be identified not only by the use of conventional procedures but also by a more rapid and readily available commercial system such as the API Staph-Ident and Staph-Trac Systems (Analytab Products, Inc., Plainview, New York) and the Minitek Gram-Positive Set (BBL Microbiology System, Cockeysville, Maryland) (6–8). These miniaturized systems will satisfactorily differentiate most of the staphylococci encountered in diagnostic laboratories.

Other Cocci

In addition to the staphylococci, other gram-positive cocci are associated with humans and animals as generally harmless commensals. Most of these are included in the genera listed below. The nomenclature is that of *Bergey's Manual* (9). They have little or no significance as causes of disease in animals, but they are encountered on occasion in clinical materials.

Micrococcus

These cocci resemble the staphylococci morphologically and occur singly and in clusters of varying size. They are gram-positive to gram-variable, and both motile and nonmotile strains are encountered. Some colonies are not pigmented. Cowan and Steel (10) distinguish staphylococci from some species of micrococci on the basis of the O-F test (see Appendix B). Most staphylococci split sugars by fermentation, while those species of micrococci that break down sugars do so oxidatively (see Table 16-1). The modified oxidase and benzidine tests, resistance to lysozyme and lysostaphin, acid production in glycerol–erythromycin medium, and growth on the selective medium of Schleifer and Kramer are additional tests for differentiating staphylococci from micrococci (11,12). These procedures should be incorporated when definitive identification of the isolate is required.

Planococcus

These are motile, nonpathogenic cocci that occur singly and in pairs, triads, or tetrads. They are found in seawater and are rarely encountered in clinical materials.

Stomatococcus

These are nonmotile, nonpathogenic cocci that occur mostly in clusters. They are considered a normal inhabitant of the mouth and upper respiratory tract of humans. The anaerobic genera *Sarcina* and *Peptococcus* will be referred to in Chapter 15. *Gaffkya* is no longer recognized as a genus (9).

Phage Typing

A large number of phage types from animals and humans have been identified. Determination of phage type is frequently of value in the study of the epidemiology of staphylococcal infections. Generally speaking, the phage types found in animals differ from those found in humans such that there appears to be host species specificity. Also, there is a tendency for the phage types important in disease outbreaks to change over an extended period of time. Phage typing is performed at the Centers for Disease Control, Atlanta, Georgia, and some other reference laboratories. For details of the procedures, consult the Supplementary Readings.

Toxins

Some strains of *S. aureus,* including those responsible for staphylococcal food poisoning, produce thermostable enterotoxins. These toxins can be detected and identified by agar gel immunodiffusion procedures. Examination of foods and cultures for enterotoxin is carried out in some public health laboratories. Additional *S. aureus* toxins that are of importance to humans are those causing the staphylococcal scalded skin syndrome and the toxic shock syndrome.

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17

Streptococcus and Related Cocci

John R. Cole, Jr.

The application of new techniques, especially DNA–DNA and DNA–rRNA hybridization, has led to the reclassification of many bacterial species. The family Streptococcaceae has undergone such a reclassification and has now been replaced in the 1986 edition of *Bergey's Manual* (1) with the heading "other genera" in the section on gram-positive cocci. Included in this group are the facultatively anaerobic, catalase negative, fermentative, gram-positive cocci. The genera that make up the aerobic cocci are *Streptococcus, Leuconostoc, Pediococcus, Aerococcus, Gemella, Enterococcus,* and *Lactococcus.* The *Enterococcus* and *Lactococcus* genera were recently included in this group. The anaerobic genera are *Peptococcus, Peptostreptococcus, Ruminococcus, Coprococcus,* and *Sarcina.*

Members of this classification occur singly, in pairs, or in chains. Many occur in nature and some are commensals in the respiratory, genital, and alimentary tracts, as well as on the skin of animals and humans. Most of the pathogens are in the genus *Streptococcus.*

Streptococcus

Organisms of this genus are characterized by their capacity to produce chains of cocci of varying lengths. The 40 species included in *Bergey's Manual* have been divided into six categories. These are pyogenic, oral, enterococci, lactic, anaerobic, and "other." Some species which could not be placed in one of the six categories were described after these categories were arranged, or have been designated Species Incertiae Sedis (SIS). Only four categories were described in the previous edition of the manual.

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A majority of the disease-producing strains are β-hemolytic and have been placed in the pyogenic category. Organisms in the oral category, which are usually α-hemolytic, are opportunists. Members of the enterococci category are found as opportunists in the intestinal tract and are currently included in the genus *Enterococcus*. The lactic category contains α- or nonhemolytic cocci, which are found in milk and are probably nonpathogenic. These are currently placed in the genus *Lactococcus*. Opportunistic, nonhemolytic cocci of the intestinal and respiratory tracts make up the anaerobic category. The "other" category includes those streptococci that cannot be conveniently placed in one of the other five.

The streptococci are further classified into groups by means of a precipitin procedure that is based on a group-specific antigen called component C. This classification is referred to as Lancefield grouping. The Lancefield groups can be subclassified to types on the basis of agglutination of specific M and T antigens. This latter test is seldom carried out in veterinary diagnostic laboratories. A list of the streptococcal species which are important in veterinary medicine is in Table 17-1.

Pathogenicity

***Streptococcus pyogenes* (A[1]; pyogenic [2])**

It has been reported as a cause of bovine mastitis. Dogs and cats have been implicated as reservoirs for this organism in cases in which recurrent infections have occurred in children (2). Primarily a human pathogen causing infections in the upper respiratory tract.

***S. agalactiae* (B; pyogenic)**

It is one of the main causes of bovine mastitis but rarely infects other animals. Group B streptococci resembling *S. agalactiae* have been recovered frequently from human infections of the urogenital and upper respiratory tracts (3). It is CAMP positive (See Appendix B).

***S. dysgalactiae* (C; SIS[3])**

It is a cause of bovine mastitis and polyarthritis of lambs. This species, or a closely related organism, has been incriminated in antepartum streptococcal infections in lambs (4). It is CAMP negative.

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[1]Lancefield Group
[2]Streptococcal category
[3]*Species Incertae Sedis*

Table 17-1
Present Classification and Grouping of Important Streptococci from Animals

Present classification	Previous classification	Lancefield group	Streptococcal category
S. pyogenes	*S. pyogenes*	A	Pyogenic
S. agalactiae	*S. agalactiae*	B	Pyogenic
S. dysgalactiae	*S. dysgalactiae*	C	SIS[a]
S. dysgalactiae subsp. *equisimilus*	*S. equisimilis*	C	SIS
S. equi subsp. *equi*	*S. equi*	C	Pyogenic
S. equi subsp. *zooepidemicus*	*S. zooepidemicus*	C	Pyogenic
S. bovis	*S. bovis*	D	Other
S. equinus	*S. equinus*	D	Other
E. faecalis	*S. faecalis*	D	Enterococci
E. faecium	*S. faecium*	D	Enterococci
E. durans	*S. durans*	D	Enterococci
S. uberis	*S. uberis*	C,D,E,P,U	Other
S. suis	*S. suis*	D,R,S	SIS
S. pneumoniae	*S. pneumoniae*	None	Pyogenic
S. porcinus	*S. sp* (E,P,U,V)	E,P,U,V	SIS
E. avium	*S. avium*	Q	Enterococci

[a]SIS - *Species Incertae Sedis*

S. dysgalactiae subsp. *equisimilis* (C; SIS)

It is associated with strangles, wound infections, genital infections, and mastitis in equine species; frequent cause of a variety of infections in swine and cattle, various infections in dogs and fowl, and human infections.

S. equi subsp. *equi* (C; pyogenic)

This is a principal cause of strangles, genital infections, mastitis, and other equine infections.

S. equi subsp. *zooepidemicus* (C; pyogenic)

This organism infects horses, cattle, and swine, and is a primary cause of cervicitis, metritis, and abortion in mares, as well as navel infections in foals. It is associated with cervicitis, metritis, and mastitis in cattle, as well as septic arthritis, abortion, and septicemia in swine. Highly pathogenic for poultry (5).

Enterococcus faecalis, E. faecium and *E. durans* (D; enterococci)

Enterococcus faecalis is highly pathogenic for poultry, but *E. faecium* is only slightly pathogenic (5). They are common inhabitants of the intestinal tract of humans and animals.

***Streptococcus bovis* and *S. equinus* (D; other)**
These occur as commensals in the alimentary tract in animals and humans. *Streptococcus bovis* and *S. equinus* have been combined under the single species, *S. equinus* (6). Isolation of *S. bovis (S. equinus)* from the blood of humans may indicate unrecognized gastrointestinal disease.

***S. suis* (D, R, S; SIS)**
This is a significant swine pathogen, which also affects humans; at least 21 serotypes have been reported. Causes meningitis, arthritis, and septicemia in swine, and meningitis and septicemia in humans.

***S. porcinus* (E, P, U, and V; SIS)**
This is a swine pathogen causing abscesses of the pharyngeal region and lymphadenitis.

***S. uberis* (C, D, E, P, V; other)**
This accounts for a small proportion of bovine mastitis and is also cultured from the vagina and tonsils of cattle.

***E. avium* (Q; enterococci)**
This has been found in the feces of chickens, animals, and humans.

***S. pneumoniae* (?; pyogenic)**
It is found occasionally in the upper respiratory tract of animals. Respiratory infections have been reported in calves, monkeys, and rabbits. The organism may be established in rat and guinea pig colonies and produce severe pneumonic disease under certain circumstances. Romer (7) has referred to isolations of pneumococci from a number of animal species, including horses, sheep, goats, swine, and a cat.

S. pneumoniae is an important cause of pneumonia, otitis, sinusitis, and meningitis in humans.

Additional Groups
Group G: Cows, mastitis; dogs, genital, skin, and wound infections (now *S. canis;* 8); cats, lymphadenitis; humans, endocarditis, pharyngitis, and arthritis (now *S. dysgalactiae;* 9)
Group L: Dogs and pigs, miscellaneous infections (now *S. dysgalactiae;* 9)
Group M: Dogs and humans, miscellaneous infections
Group N: *S. lactis* (now *Lactococcus lactis*), milk and dairy products

Laboratory Procedures

Isolation Procedures

Routine procedures for the isolation of aerobes and facultative anaerobes are recommended. Media to be used are heart infusion, trypticase soy, Todd-Hewitt, or proteose peptone, all of which are supplemented with 5% blood or serum.

After inoculation onto blood agar, swabs should be placed in brain–heart infusion (BHI), semisolid broth, or Schaedler broth, because some of the streptococci are anaerobic or microaerophilic. Some strains of *S. pneumoniae* grow best when incubated in a candle jar or in an incubator containing 10% CO_2.

Selective media especially formulated for the isolation and growth of the streptococci are referred to in Appendix B.

Cultural and Morphological Characteristics

Small, grayish colonies of gram-positive cocci are usually evident in 24 hr. Considerable colonial variation may be noted, including mucoid, smooth (glossy), and matte (rough) colonies. Cultures of *S. equi* subsp. *equi* from strangles and other infections of the horse may be strikingly mucoid. If chains are formed on solid media, they are usually short, and considerable pleomorphism may be noted. Distinct chains are produced in liquid media such as serum, BHI, or thioglycollate broth (Fig. 17–1).

Streptococcus pneumoniae produces small, round colonies with elevated edges and zones of α-hemolysis in 18–24 hr. Most cultures produce colonies with a characteristic concavity, which is especially evident on prolonged incubation. Some strains are moist and mucoid. Stained smears reveal encapsulated, gram-positive cocci occurring in characteristic pairs, singly, and as short chains.

Identification

On the basis of the results of growth on blood agar, the culture may be reported as α-, β-, or nonhemolytic streptococci. To determine the species, the differential criteria listed in Table 17-2 are employed. For the identification of those species not listed, refer to *Bergey's Manual.* The identification of streptococci usually associated with bovine mastitis is dealt with in Chapter 34.

Low concentrations of bacitracin are specifically active against

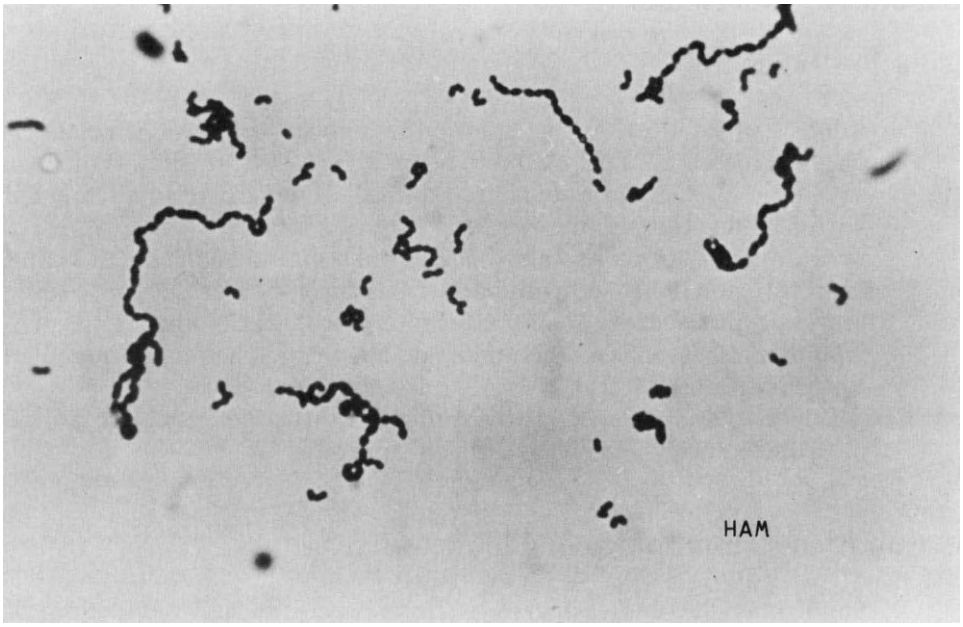

Figure 17-1. *Streptococcus* sp. from a culture. Gram stain, ×2250 (H. A. McAllister).

group A streptococci. A sensitivity disc containing 0.02 Units of bacitracin (Taxo A discs, BBL) is placed on a blood agar plate previously streaked with the organism to be examined. Zones of inhibition of 15–20 mm are obtained with group A strains.

Streptococcus pneumoniae is inhibited by optochin (Taxo P discs, BBL; see Appendix B), whereas other α-hemolytic streptococci are not. More than 80 serotypes have been identified on the basis of serologic differences in the capsular substances. Serotyping is carried out by reference laboratories; however, typing sera for the more common serotypes are available commercially (Difco).

Edward's medium and esculin agar (see Appendix B) can be used for the rapid identification of the important mastitis streptococci. The CAMP test, which is used for the rapid presumptive identification of *S. agalactiae,* is described in detail in Appendix B.

Identification systems to determine streptococcal species are commercially available. These include the API 20 S and rapid STREP (Analytab Products, Inc., Plainview, New York), and the RapID STR system (Innovative Diagnostic Systems, Inc., Atlanta, Georgia). These are discussed in greater detail in Chapter 36.

Streptococcal groups A, B, C, F, and G can be identified with considerable reliability by slide coagglutination using commercially

Table 17-2
Differential Characteristics of Important Streptococci from Animals[a]

Species	Lancefield group	Hemolysis	Fermentation							Esculin[b]	Sodium hippurate[b]	6.5% NaCl
			Trehalose	Sorbitol	Mannitol	Salicin	Lactose	Raffinose	Inulin			
S. pyogenes	A	β	+	−	V	+	+	−	−	−	−	−
S. agalactiae	B	α,β,γ	+	−	−	(+)	+	−	−	−	+	−
S. dysgalactiae	C	α,β,γ	+	−	−	−	+	−	−	−	−	−
S. dysgalactiae subsp. *equisimilis*	C	β	+	−	−	(+)	V	−	−	−	−	−
S. equi subsp. *equi*	C	β	−	−	−	+	−	−	−	−	−	−
S. equi subsp. *zooepidemicus*	C	β	−	+	−	+	+	−	−	−	−	−
S. bovis	D	α	V	−	V	+	+	+	+	+	−	−
S. equinus	D	α	V	−	−	(+)	−	+	+	+	−	−
E. faecalis	D	α,β,γ	+	+	+	+	+	−	−	+	V	+
S. uberis	C,D,E,P,V	α,γ	+	+	+	+	+	−	+	+	+	(+)
S. suis	D,R,S	α	+	−	−	+	+	(+)	(+)	+	−	−
S. pneumoniae		α	+	−	−	V	+	+	+	(+)	−	−
S. porcinus	E,P,U,V	β	+	+	+	+	(+)	−	−	+	−	+
E. avium	Q	α,γ	+	+	+	+	+	−	−	+	V	(+)

[a](+), Majority of strains positive; V, variable reactions.
[b]Hydrolysis.

available reagents (Pharmacia Diagnostics, Piscataway, New Jersey). This procedure consists of protein A containing staphylococci that are coated with antibodies specific for groups A, B, C, and G streptococci. Latex agglutination procedures are also available for identifying groups A, B, C, D, F, and G (Wellcome Diagnostics, Research Triangle Park, North Carolina; Scott Laboratories, Inc., Fiskeville, Rhode Island; Difco Laboratories, Detroit, Michigan; and Diagnostic Products Corporation, Los Angeles, Calfornia).

Hemolysis

The kind of hemolysis obtained on blood agar as a result of the growth of streptococci is given considerable emphasis, although it is frequently a variable characteristic.

For practical purposes, hemolysis is described as follows:

Alpha (α) hemolysis: Greenish (viridan) zone around colony.
Beta (β) hemolysis: Clear zone of hemolysis around colony.
Gamma (γ) or no hemolysis: No hemolysis apparent around colony.

Factors Influencing Hemolysis

Hemolysis may be influenced by (a) the basic medium employed, (b) the kind of blood used, (c) the length of the incubation period, (d) the atmosphere (anaerobiosis may result in a reduction of hemolysis), (e) location of the colonies on the plates, (f) concentration of blood, and (g) depth of the agar. Some difference may be noted between surface and subsurface colonies.

Sheep blood in trypticase soy agar is recommended for a careful study of hemolysis but, for practical purposes, media containing other kinds of blood are satisfactory.

Related Cocci

Aerobic

Leuconostoc, Pediococcus
Species of these genera have no veterinary significance.

Aerococcus
Cocci of this genus form tetrads. They have formerly been referred to as *Gaffkya*. *Aerococcus viridans* has been implicated in human uri-

nary infections and endocarditis and in a fatal disease of lobsters (gaffkemia).

Gemella

The cocci of this genus occur singly or in pairs with adjacent sides flattened. They are recovered from the human respiratory tract.

Anaerobic Cocci

Peptococcus

This genus includes anaerobic cocci that occur singly, in pairs, tetrads, or irregular masses. One species is described in *Bergey's Manual* and is probably a commensal of human beings, in which it occasionally causes infections.

Peptostreptococcus

Cocci of this genus occur singly, in pairs, and in short or long chains. All require an anaerobic atmosphere for initial isolation; however, on subsequent subculture, some strains will grow microaerophilically or aerobically. Nine species are described in *Bergey's Manual*, and all are probably commensals in humans. Some have been implicated in a variety of infections, including wound infections, puerperal fever, pleurisy, appendicitis, sinusitis, dental infections, arthritis, abscesses, and vaginitis. Two species, *P. anaerobicus* and *P. indolicum*, cause similar infections in animals.

Ruminococcus

These spherical or elongated coccoid organisms are found in the normal rumen of cattle and sheep and in the intestinal tract of rabbits. They have no pathogenic significance.

Coprococcus

These cells are round to elongated, and three species are reported: *C. eutactus*, *C. comes* and *C. catus*. All species have been isolated from human feces.

Sarcina

These spherical cells occur in packets of eight or more. One of the two species described, *Sarcina ventriculi*, has been recovered from rabbit and guinea pig stomach contents. They have not been implicated in disease.

For additional information on the significance and identification of these related cocci, refer to *Bergey's Manual* and the *Anaerobe Laboratory Manual* (10).

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18

Bacillus

G. R. Carter

Members of this genus are large, aerobic, gram-positive (old cultures decolorize easily), sporeforming rods. They are catalase positive; many are fermentative, and most are motile.

Bacillus spp. occur widely in nature, being found in the air, water, and soil. They are among the most common laboratory contaminants and are usually ignored when recovered from clinical materials. One should, however, keep in mind the possibility of encountering *B. anthracis*, particularly in areas where the disease has occurred in the past. More than 40 species of *Bacillus* have been identified. Only the more common and important species will be dealt with below.

Bacillus anthracis

Pathogenicity

Moderate to acute septicemic infections occur in cattle, sheep, caribou, elk, bison, water buffaloes, horses, mink, and other mammals. Fowl are generally resistant.

Cattle

Anthrax is most frequently seen in cattle, in which it usually causes an acute septicemic infection. The numbers affected depend upon the source of *B. anthracis*.

Swine

Pigs are less susceptible than cattle. There is frequently an acute pharyngitis with lesions and swelling in the region of the throat and neck.

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Horses
If infection is by ingestion, there is a septicemia with enteritis and colic. Infection may also be through the skin, in which case there is marked edema and lymphadenitis.

Dogs and Cats
A rare infection resembling that seen in swine.

Humans
Cutaneous anthrax, called malignant pustule or carbuncle, is the most common form. Pulmonary anthrax and intestinal anthrax resulting, respectively, from the inhalation and ingestion of spores are now rare.

Laboratory Diagnosis of Anthrax: General

Bacillus anthracis is rarely recovered except in areas where the disease is known to occur periodically. However, cases occasionally occur where there has been no history of anthrax; thus it is important to always be alert to the possibility of its occurrence.

A variety of special media and tests have been developed to aid in the isolation and identification of *B. anthracis.* These are described in detail in the *Manual of Clinical Microbiology* (1) and will be of particular interest to laboratories in areas where anthrax occurs with appreciable frequency. The procedures described below, although not dependent on special media and uncommon tests, are adequate and reliable for the isolation and identification of *B. anthracis.*

Isolation Procedures

All procedures should be carried out in a biological safety hood. The work area, after use, should be washed with an effective disinfectant. One containing hypochlorite is recommended. All infectious and contaminated materials should be autoclaved or adequately disinfected.

If anthrax is suspected, the carcass should not be opened, and a diagnosis should be attempted on blood obtained from a superficial vein. In swine and horses, fluid or exudate can be aspirated with a syringe from swollen tissues if the organism cannot be demonstrated in blood smears. If a necropsy is performed, great care must be taken not to contaminate the immediate area with spores. Spores are formed when the vegetative forms are exposed to air.

If tissues are submitted, a composite suspension is prepared with a tissue grinder or mortar and pestle using sterile physiological saline as a diluent. Blood is used as is.

1. Smears are made and stained by the Gram method. The finding of large, typical gram-positive rods indicates the likelihood of infection. Spores appear clear with the Gram stain.

   It should be kept in mind that clostridia are often found in the blood and tissues a few hours after death. The absence of square-ended capsules helps distinguish them from *B. anthracis*. Sterne (2) recommends Giemsa and Wright's stains for the demonstration of the capsule. By these procedures it is stained a reddish mauve and appears square-ended (Fig. 18-1). Another widely used procedure is to stain heat-fixed or alcohol-fixed (flame rather than let dry) blood smears with 1% polychrome methylene blue for 5–10 min (see Appendix A). The capsule and capsular material seen among the bacilli stain pink to purple (McFadyean's reaction). After fixation, smears can be immersed in 1:1000 mercuric chloride for 30 min in order to kill the spores.
2. Inoculate animals with the tissue suspension and blood if the latter is available.
   1 guinea pig: 1.0 ml subcutaneously
   1 mouse: 0.1 ml intraperitoneally
   1 mouse: 0.3 ml intraperitoneally

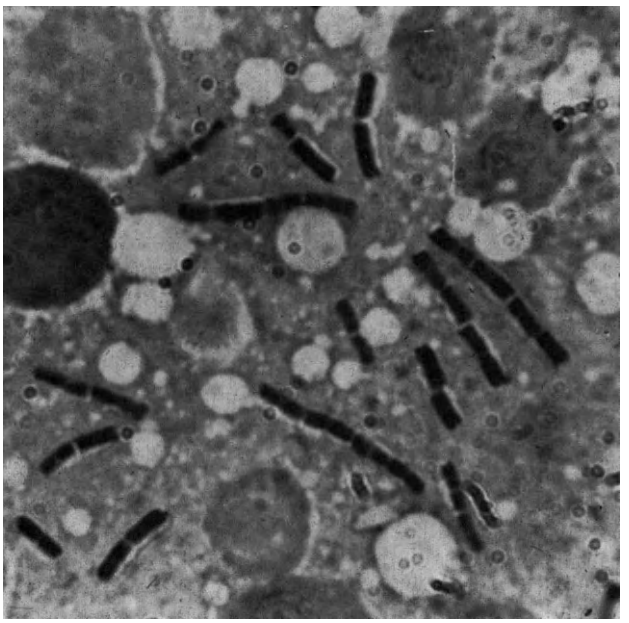

Figure 18-1. Giemsa-stained smear showing the capsule of *Bacillus anthracis*, ×2000. Courtesy of J. W. Huff and T. Vera.

1 mouse: swab blood or suspension over a scarified area at the base of the tail.

If anthrax bacilli are present, animals begin to die, usually after 24 hr, and show numerous capsulated, square-ended, large rods in stained smears from the spleen and blood.

Heart's blood and spleen of animals that died are cultured aerobically [as in (3) below] and anaerobically for clostridia. *Bacillus* spp. should be examined as indicated in (3) below. Some strains of *B. cereus* may be pathogenic for mice and guinea pigs.

3. Blood plates are inoculated from blood and tissue suspension and incubated aerobically at 37C. Weak hemolysis may be seen around areas of heavy growth. Broth cultures are checked for motility (hanging drop) and the characteristic cottonwool growth.

   As cultures of *B. anthracis* age they become rougher and have a ground glass appearance. The serrated edges and the curled peripheral projections give colonies the well-known medusa head appearance. The latter feature may also be seen in *B. cereus, B. thuringiensis,* and *B. mycoides* (1). Hemolysis is a variable characteristic with *B. cereus* and some other *Bacillus* spp. but *B. anthracis* has never been reported to be β-hemolytic as are many strains of *B. cereus.*

4. Cultures of weakly or nonhemolytic, nonmotile bacilli from the original blood and tissues are held, and if typical deaths did occur in animals inoculated with the original material, these cultures are inoculated into animals as in (2). The amounts to be inoculated are the same after the broth culture has been diluted 1:10 with normal saline.

Identification

Pure cultures of suspicious *Bacillus* organisms from (1) the original blood and tissues; (2) the dead laboratory animals that received the blood and tissues; and (3) the dead laboratory animals, if any, that had received organisms from the primary cultures that were not pathogenic for laboratory animals, are examined for the characteristics listed in Table 18-1. Experience has shown that the *Bacillus* species most likely to be confused with *B. anthracis* is *B. cereus.*

Bacillus anthracis is nonmotile and never β-hemolytic. *Bacillus cereus* is often β-hemolytic and almost always nonmotile. *Bacillus mycoides* has a characteristic rhizoid colony, is nonmotile and nonhemolytic. Both *B. cereus* and *B. mycoides* are resistant to penicillin whereas *B. anthracis* is susceptible. Virulent strains of *B. anthracis* are much more pathogenic than other *Bacillus* species. They are invasive and can kill mice when infectious materials or culture are applied to the scarified tail of a mouse.

Table 18-1
Differentiation of *Bacillus anthracis* and *Bacillus cereus*

	B. anthracis	*B. cereus*
Motility	−	Almost always +
Capsulation	+ (Pathogenic strains)	−
Penicillin (10 Units)	Susceptible	Resistant
Hemolysis	− or very weak	Often markedly hemolytic
Growth at 45°C	Slow	Rapid
Gelatin hydrolysis	Slow (3–7 days)	Rapid
Salicin (acid)	−	+
Litmus milk	Slowly coagulated and peptonized	Rapidly coagulated and peptonized
Bacteriophage	+	−
Pathogenicity (mouse, guinea pig)	+ (invasive)	Not invasive; large numbers may kill

Sterne (2) states that "a diagnosis of anthrax can be made with confidence if square-ended, robust, red-stained (Wright's or Giemsa's) and rather shaggy looking bacilli are seen in films taken at *death or shortly after.*"

If animals do not die and the characteristics do not conform to those of the anthrax bacillus, the examination for anthrax is considered negative. If the animals die and the criteria listed for *B. anthracis* (Table 18-1) are fulfilled, a positive diagnosis is warranted.

Staining with fluorescent antibodies against the cell wall or capsule of *B. anthracis* can be used for the direct examination of clinical material. Only a presumptive identification can be made by FA staining because of cross-reactions with other *Bacillus* spp. (3).

It may be difficult to identify *Bacillus* species other than *B. anthracis* with certainty. The characteristics listed in Table 18-1 allow a strongly presumptive identification of *B. cereus;* however, this should be confirmed in an appropriate reference laboratory. This also applies to the identification of other *Bacillus* species.

The variety of tests required for definitive identification of all but *B. anthracis* is beyond the scope of the average diagnostic bacteriology laboratory.

Other Methods of Identification

Bacteriophage Identification

The use of bacteriophage for the differentiation of this pathogen from the nonpathogenic *Bacillus* spp. is considered reliable (4–7); however, it has been reported that some strains of *B. mycoides* are susceptible. The latter, unlike *B. anthracis,* is resistant to penicillin. The Cherry γ

phage[1] is used as a 1:10 dilution of a frozen suspension with a titer of 10^4. The phage can be propagated on strain 14, which makes a suitable control. Known cultures of *B. anthracis* and *B. cereus* should be included as controls. The procedure is as follows:

1. On one-half of a blood agar plate, streak a culture of *B. anthracis* as a control. On the other half, streak the culture to be identified.
2. Several droplets of the phage preparation are added to each half of the inoculated blood plate.
3. Incubate the plate in the upright position for 8–12 hr. Clear zones of lysis will be seen on the control culture and on the suspected culture if it is *B. anthracis*.

String of Pearls Test

This test (8) is based upon the alteration in morphology that strains of *B. anthracis* undergo in the presence of penicillin. A tryptose agar plate containing 0.5 Units/ml of penicillin is swabbed from a 24-hr broth culture of the suspected organism. After incubation for 3–6 hr, the surface of the agar is examined microscopically under a coverslip. Anthrax bacilli swell and appear as chains of spheres, which rupture on further incubation, leaving only a spore. The morphology of the nonpathogenic and saprophytic *Bacillus* spp. remains unchanged. Known cultures of *B. anthracis* and *B. cereus* should be included as controls.

The tests mentioned above should be used along with the characteristics listed in Table 18-1 and pathogenicity for mice and guinea pigs.

Bacillus cereus

This widespread saprophyte is capable of infecting the bovine udder and producing acute and sometimes fatal gangrenous mastitis. It has also been implicated as a cause of abortion in cows and ewes (9,10). In mastitis, isolation should be attempted during the acute phase of the disease, as the organism may be absent in later samples. *Bacillus cereus* produces an enterotoxin that results in food poisoning in humans. Food poisoning due to this organism has been reported in dogs (11). It has been suggested that *B. cereus* can cause infertility in mares (12).

Bacillus cereus can be presumptively identified on the basis of the characteristics listed in Table 18-1. For confirmation, cultures should be submitted to a reference laboratory. A diagnosis of *B. cereus* food

[1]Available from the Centers for Disease Control, Atlanta, Georgia.

poisoning is confirmed if the incriminated food contains 10^5 or more organisms per gram (13).

Bacillus licheniformis

This organism has been reported as a cause of abortion in cows (14). Cultures should be submitted to a reference laboratory for definitive species identification.

Opportunistic *Bacillus* Species

There is little evidence that *Bacillus* species can cause opportunistic infections in animals. However, the possibility should be kept in mind, particularly in compromised patients. The *Bacillus* spp. that have been implicated in human infections are *B. cereus* (most frequent), *B. circulans, B. coagulans, B. brevis, B. pumulus, B. macerans, B. subtilis, B. sphaericus* and *B. thuringiensis* (1). The possibility of these and other *Bacillus* species causing infections in animals should be kept in mind.

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19

Clostridium

P. D. Walker

Organisms of this genus are anaerobic sporeforming gram-positive rods. They are motile (except *Cl. perfringens)*, fermentative, and catalase negative. The organisms concerned are widespread and are found in the intestine of animals and humans and in the soil, where they can both survive and multiply. Their ability to form spores ensures survival under adverse conditions for long periods. The ubiquity of clostridial spores results in their occasional presence as contaminants in clinical materials. There are both pathogenic and nonpathogenic species; the latter are only rarely encountered in fresh tissues.

Pathogenicity

The principal clostridia causing diseases in animals are the following:

Cl. perfringens (Cl. welchii) type A: Wound infections, gangrenous mastitis, enterotoxemia in nursing lambs in California and Oregon ("yellow lamb"). Its role in other enterotoxemias is controversial as the organism is part of the normal flora and its isolation is not conclusive evidence that it caused the disease.
Cl. perfringens (Cl. welchii) type B: Lamb dysentery; enterotoxemia in calves, sheep, goats, and foals
Cl. perfringens (Cl. welchii) type C: Enterotoxemia of sheep ("struck"), calves, lambs, and piglets

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Cl. perfringens (Cl. welchii) type D: Enterotoxemia in sheep, lambs, and goats
Cl. septicum: Malignant edema of horses, cattle, sheep and swine; gas gangrene; braxy in sheep; navel-ill in lambs; blackleg in pigs; gangrenous dermatitis of chickens
Cl. chauvoei (Cl. feseri): Blackleg in sheep, cattle, and very occasionally pigs.
Cl. novyi (Cl. oedematiens) type A: Gas gangrene in cattle, sheep and humans; septicemia in tortoises; "big head" in rams.
Cl. novyi (Cl. oedematiens/Cl. gigas) type B: Black disease (infectious necrotic hepatitis of sheep and cattle); sudden death in cattle and pigs
Cl. novyi (Cl. oedematiens/Cl. bubulorum) type C: Osteomyelitis in buffaloes (Indonesia)
Cl. novyi (Cl. oedematiens/Cl. haemolyticum) type D: Bacillary hemoglobinuria in cattle; necrotic liver disease in sheep.
Cl. sordellii: Gas gangrene from inoculation accidents. Some workers claim that it can produce a gas gangrene type of disease in cattle and also enterotoxemia in this species.
Cl. tetani: Tetanus in all species of domestic animals
Cl. botulinum types C and D: Botulism in sheep, cattle, mink, dogs, monkeys, ferrets, and several avian species, such as chicken and wild duck
Cl. botulinum of various types: Wound and infant botulism has been described in humans, but not in animals.
Cl. colinium: Ulcerative enteritis, originally called "quail disease," an acute disease of game birds, young turkeys, young chickens, quail, grouse, and partridge (1)
Cl. difficile: Pseudomembranous colitis in humans and ileocecitis in laboratory animals (2) on antibiotic regimes
Cl. spiroforme: Spontaneous and antibiotic-induced enterotoxemia in rabbits

Laboratory Procedures

Direct Examination for Clostridia

Smears are prepared from the suspected site of the infection for Gram and fluorescent antibody staining. Clostridial infections yield large, gram-positive rods, the number and size of which may be quite variable. The size and shape of the organism and the position of any spores present may provide valuable pointers to the identity of the species involved. Commercially available fluorescein-labeled antisera can be used to identify several species directly.

Isolation Procedures

General

The investigator concerned with the isolation of clostridia from animal tissues is usually presented with material from a dead animal. Animals may have been dead for varying periods of time before discovery, with the result that varying degrees of postmortem change may have occurred. At the death of an animal the normal barriers of infection are broken down and invasion of the tissues by organisms present in the intestine occurs. *Clostridium perfringens* type A and *Cl. septicum* are frequently recovered from the intestine of normal animals and, as these organisms grow rapidly on the nutrient media commonly used for the isolation of anaerobes, they may rapidly outgrow the specific pathogen. The investigator may thus be faced with isolating the casual organism from a mixture of contaminants. Techniques for the isolation of clostridia from animal tissues must take account of this factor, and the following general principles should be observed:

1. The tissues to be cultured must be carefully chosen.
2. The growth medium used must be adequate for the growth of the pathogen.
3. The tissue should be smeared directly on to the surface of the appropriate plates and should not be cultured in an intermediate fluid medium. Fluid media tend to distort the flora by selecting the faster-growing organisms, which are not necessarily the specific pathogen.
4. Stiff (3%) agar or other antispreading devices should be used in order to minimize the spread of motile strains.
5. Different species of clostridia have different nutritional requirements and a variety of culture media are essential for satisfactory differentiation.
6. Different species of clostridia differ in their sensitivity to oxygen and particular attention may have to be paid to the speed with which manipulations are undertaken and the oxidation–reduction potential of the culture medium.

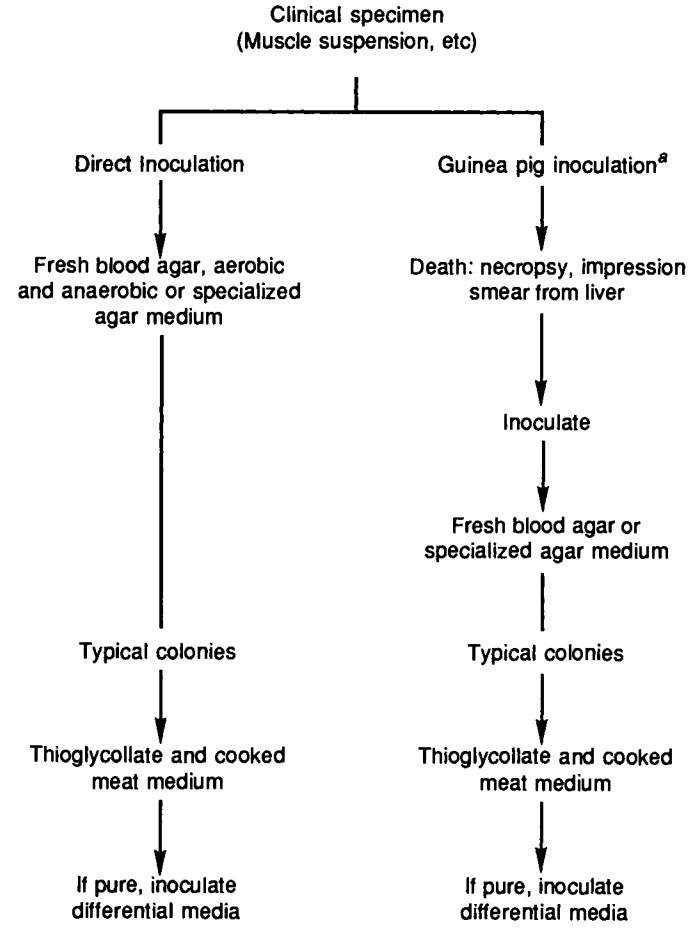
The growth of certain clostridia, such as *Cl. novyii* types B, C, and D, is only achieved under very strict anaerobic conditions. The reputation of the genus *Clostridium* for being particularly difficult to grow and isolate is due to the neglect of these criteria.

Direct Cultivation

A general procedure is outlined in Table 19-1.

Using the criteria outlined above, the piece of tissue is streaked onto freshly poured plates. The range of media used to isolate the various

Table 19-1
A Procedure for the Isolation of Clostridia



[a] Young adult guinea pigs are preferred.

clostridia is outlined in Appendix B. In general, blood agar plates are perfectly satisfactory for the majority of clostridia. Specialized media are used where indicated. Individual colonies can be transferred to thioglycollate broth (with glucose) or cooked meat medium and used to inoculate differential media required for the tests referred to in Tables 19-2 and 19-3. Liquid media are immersed in a boiling water bath for 10 min and inoculated immediately on cooling to 37°C.

Table 19-2
Reactions of Clostridia on Half-Antitoxin[a] Lactose Egg Yolk Milk Agar[b]

Organism	Diffuse lecithinase C opacity		Restricted opacity, pearly layer (lipase)	Lactose fermentation	Proteolysis
	Produced	Inhibited			
Cl. perfringens A–E	+	+	–	+	–
Cl. bifermentans/ sordellii	+	+	–	+	+
	+	+	–	–	+
Cl. botulinum					
A	–	–	+	–	+
B	–	–	+	–	+ or –
C	–	–		–	–
D	–	–	+	–	–
E	–	–	+	–	–
F	–	–	+	–	+ or –
G	–	–	+	–	+
Cl. sporogenes	–	–	+	–	+
Cl. novyi					
A	+	+[c]	+	–	–
B	+	–[d]	–	–	–
C	–		–	–	–
D	+	–[d]	–	–	–
Cl. septicum	–		–	+	–
Cl. chauvoei	–		–	+	–
Cl. tetani	–		–	–	–

[a]Mixture of *Cl. perfringens* type A and *Cl. novyi* type A antitoxic sera.
[b]Modified from Willis and Hobbs (6).
[c]Note that the restricted lipase opacity is obscured on the nonantitoxin half of the plate by the lecithinase C opacity.
[d]Inhibited by *Cl. novyi* type B antiserum.

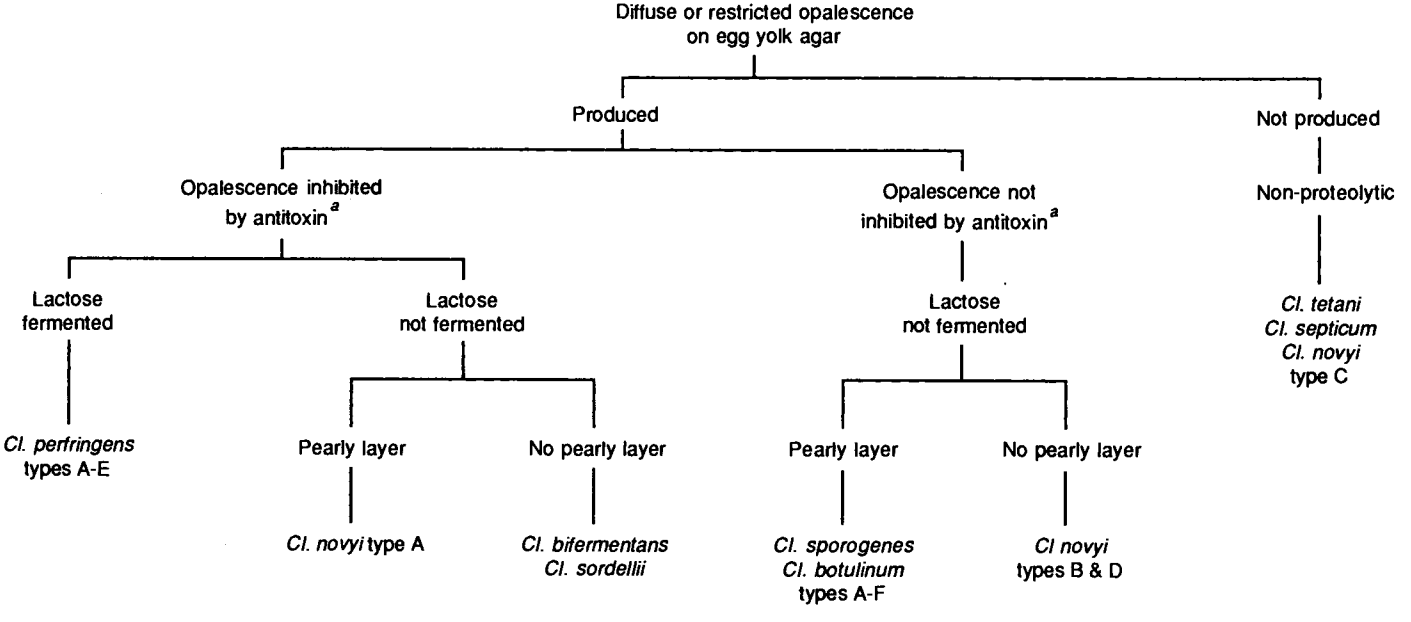
The following procedure has been described for *Cl. colinum* (3,4): Primary isolation of this fastidious organism has been made in tryptose-phosphate glucose broth with 8% horse plasma and in the yolk sac of 5- to 8-day-old embryonated chicken eggs. After several passages in these media or in thioglycollate broth with 3%–10% horse serum, the organism can be grown on blood agar like other fastidious anaerobes.

Guinea Pig Inoculation

Because *Cl. chauvoei, Cl. septicum, Cl. novyi,* and *Cl. sordellii* can be identified in clinical materials by the fluorescent antibody procedure described below, it is seldom necessary to inoculate guinea pigs.

A piece of tissue, carefully selected as outlined above (approximately 10 g), is placed in a sterile mortar containing 15–20 ml of broth. Mince the tissue finely with sterile scissors and press the tissue fluid with the pestle. Pour off the supernatant after the tissue fragments have settled. Two young adult guinea pigs are inoculated intramuscularly

Table 19-3
A Scheme for the Identification of Some Clostridia Using Lactose Egg Yolk Milk Half-Antitoxin[a] Plates[b] (From Willis, 1977)



[a]A mixture of *Cl. perfrigens* type A and *Cl. novyi* type A antisera
[b]Instead of using the complex egg yolk medium, may use separate tests: half--antitoxin egg yolk agar, milk (or casein) agar and a lactose fementation test.

in the rear leg with either 0.5 or 1.0 ml of the supernatant. The pathogenicity of the clostridia can be enhanced if equal amounts of calcium chloride solution (5–10%) and supernatant are injected into the muscles.

When the guinea pigs show signs of infection, or immediately after death, impression smears are made from the liver or muscle for microscope examination and agar plates are inoculated with suitably infected tissue. Typical colonies can be transferred into thioglycollate and cooked meat medium for subsequent identification.

Cultural Characteristics

Provided adequate precautions have been taken, colonies of clostridia should be apparent after 24 hr. In the case of more fastidious species, incubation may have to be extended to 48 hr and even 72 hr. The aerobic and anaerobic plates should be compared and Gram-stained smears made from colonies unique to the anaerobic plate. The presence of large gram-positive rods suggests clostridia. Spores may or may not be present and are usually aids in provisionally identifying the organism. Colony morphology is usually characteristic and may be of the rough or smooth variety. The edges may be smooth or rhizoidal.

The following properties are extremely valuable in the early recognition of species of clostridia: (1) reactions of various species on egg yolk medium; (2) proteolysis in terms of digestion of proteins such as serum and milk; and (3) fermentation.

The reactions of various anaerobes on egg yolk media (5) are shown in Table 19-2. It can be seen that five species of clostridia produce opalescence in egg yolk medium. *Clostridium perfringens, Cl. bifermentans/sordellii,* and *Cl. novyi* Types A, B, and D produce opalescent changes due to the production of lecithinases [Fig. 19-1(top)], which are inhibited by specific antitoxic sera. *Clostridium botulinum, Cl. sporogenes,* and *Cl. novyi* type A produce, in addition to a restricted opalescence not inhibited by specific antitoxic sera, a pearly layer or iridescent film that covers the colonies and in some cases extends beyond their edge onto the surface of the medium [Fig. 19-1(bottom)]. These are due to lipolysis.

Lactose egg yolk milk agar medium, described by Willis and Hobbs (6), provides information not only on the production of lecithinases and lipases, but also on lactose fermentation and proteolytic activity as evidenced by the digestion of milk. It also enables a presumptive diagnosis of some anaerobes to be made from a single plate culture (6) (see Table 19-3).

Regarding fermentation, the most important fermentable substances for clostridia are glucose, maltose, lactose, and sucrose, together with

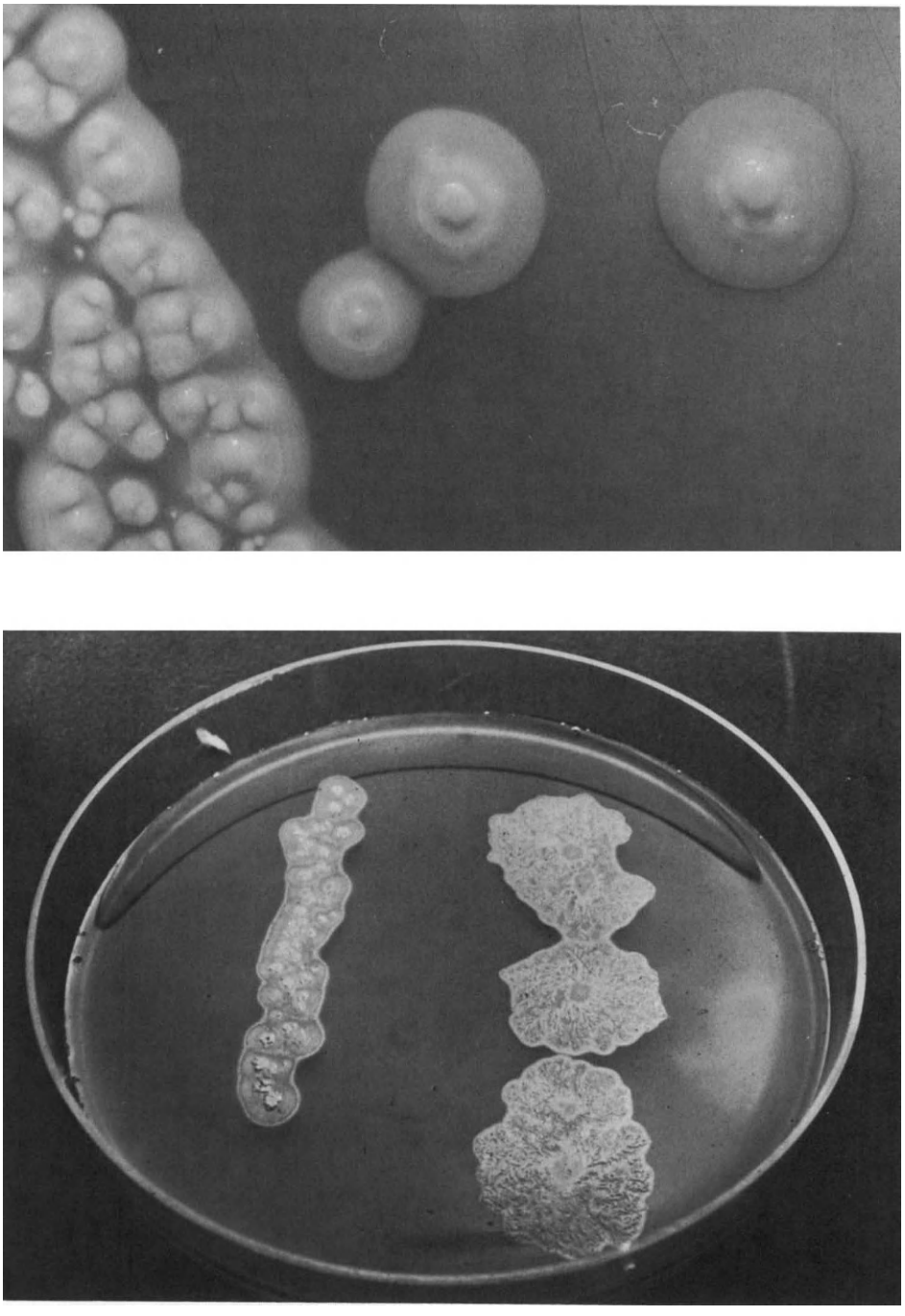


Figure 19-1. *Top:* Colonies of *Cl. perfringens* grown on lecthinase agar plates showing zones of opacity surrounding the colonies. *Bottom:* Colonies of *Cl. botulinum* type E showing pearly layer.

Table 19-4
Some Differential Characteristics of Important Species of *Clostridium*[a]

Species	Nitrate	Indole	Acid production					Urease
			Glucose	Maltose	Lactose	Salicin	Sucrose	
Cl. perfringens	+	−	+	+	+	V	+	−
Cl. septicum	+	−	+	+	+	+	−	−
Cl. chauvoei	+	−	+	+	+	(+)	+	−
Cl. novyi A	−	−	+	+	−	−	−	−
Cl. novyi B	+	−	+	V	−	−	−	−
Cl. haemolyticum	−	+	+	−	−	−	−	−
Cl. sordellii	−	+	+	+	−	−	−	+
Cl. bifermentans	−	+	+	+	−	+	−	−
Cl. botulinum	−	−	+	V	−	V	V	−
Cl. tetani	−	+	−	−	−	−	−	−
Cl. sporogenes	−	−	+	+	−	V	V	−
Cl. colinum	−	−	+	+	−	+	+	−

[a]Symbols: +, positive; −, negative; V, variable.

mannitol and salicin. Production of indole, reduction of nitrate, and urease production are also useful criteria (see Table 19-4).

The identification of fermentation products by gas chromotography has also been found to be helpful in identification. (7).

Identification

Identification by Antisera

Antisera against *Cl. chauvoei, Cl. septicum, Cl. novyi* (types A and B), and *Cl. sordellii* are available commercially (Burroughs Wellcome Company, Research Triangle Park, North Carolina) for the identification of these species by passive protection tests. The procedures given below are those recommended by the producer.

1. Determine the approximate lethal does of a thioglycollate broth culture of the strain to be identified by inoculating intramuscularly pairs of guinea pigs with a series of doses ranging from 0.1 ml to 1 ml.
2. Inoculate pairs of guinea pigs subcutaneously with 1 ml of each of the specific sera. Twenty-four hours later, inoculate intramuscularly each of these guinea pigs and two control guinea pigs with a lethal amount of culture. Protection afforded by the serum is specific.

Identification of *Cl. chauvoei, Cl. septicum, Cl. novyi,* and *Cl. sordellii* by Means of Specific Fluorescein-Labeled Antisera

Labeled antisera for these four clostridia are available commercially (Burroughs Wellcome Company, Research Triangle Park, North Carolina). The directions given by the producer are as follows:

1. Smear a portion of the suspected lesion onto a microscope slide and leave for a few moments until reasonably air dry.
2. Fix by immersing in reagent grade anhydrous acetone for 10 min.
3. Place one drop of a mixture of equal parts of the two sera on the smear and spread evenly.
4. Leave in a moist chamber for 30 min at room temperature. A large petri dish containing moistened filter paper is quite adequate.
5. Rapidly wash off gross excess of uncombined reagents with buffered saline (0.15 *M* sodium phosphate in physiological saline, pH 7.1) and finally leave for a total of at least 10 min in several changes of buffered saline.
6. Blot gently with clean absorbent paper.
7. Mount in buffered glycerin consisting of nine parts of glycerin to one part of buffered saline.
8. The slide is then examined with a conventional fluorescence microscope employing the filters routinely used in bacterial fluorescence microscopy. For further information see Chapter 36. Organisms which fluoresce apple green are *Cl. septicum, Cl. chauvoei, Cl. novyi,* or *Cl. sordellii,* depending on which antiserum was used. The rhodamine FA stain used earlier is no longer used for *Cl. chauvoei.*

Individual Clostridia

Clostridium tetani

Cl. tetani grows readily on blood agar and, if 3% agar is used, separate colonies with a rhizoidal edge can be obtained (Fig. 19-2 top). The organisms produce spherical terminal spores and the typical drumstick appearance is seen in films (Fig 19-2 bottom). Injection of culture or culture filtrate into small animals produces a characteristic paralysis.

Cl. septicum

Clostridium septicum grows readily on blood agar and, because of its tendency to spread, the agar concentration is increased in order to

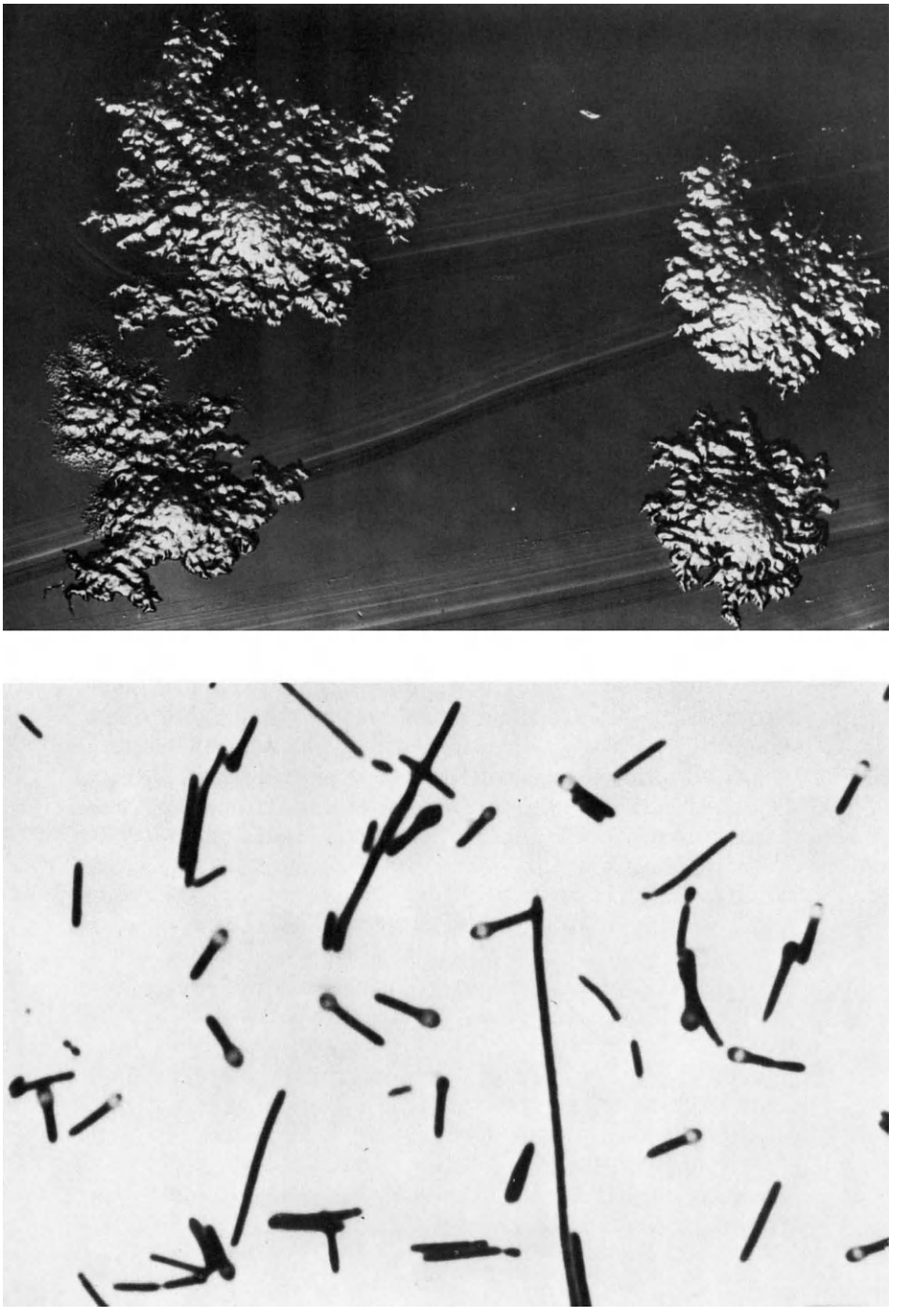


Figure 19-2. *Top:* Typical rhizoidal colony of *Cl. tetani* on 3% blood agar, ×8. *Bottom:* Typical drumstick appearance of *Cl. tetani*; Gram-stained smear showing terminal spherical spores swelling the sporangium, ×2500.

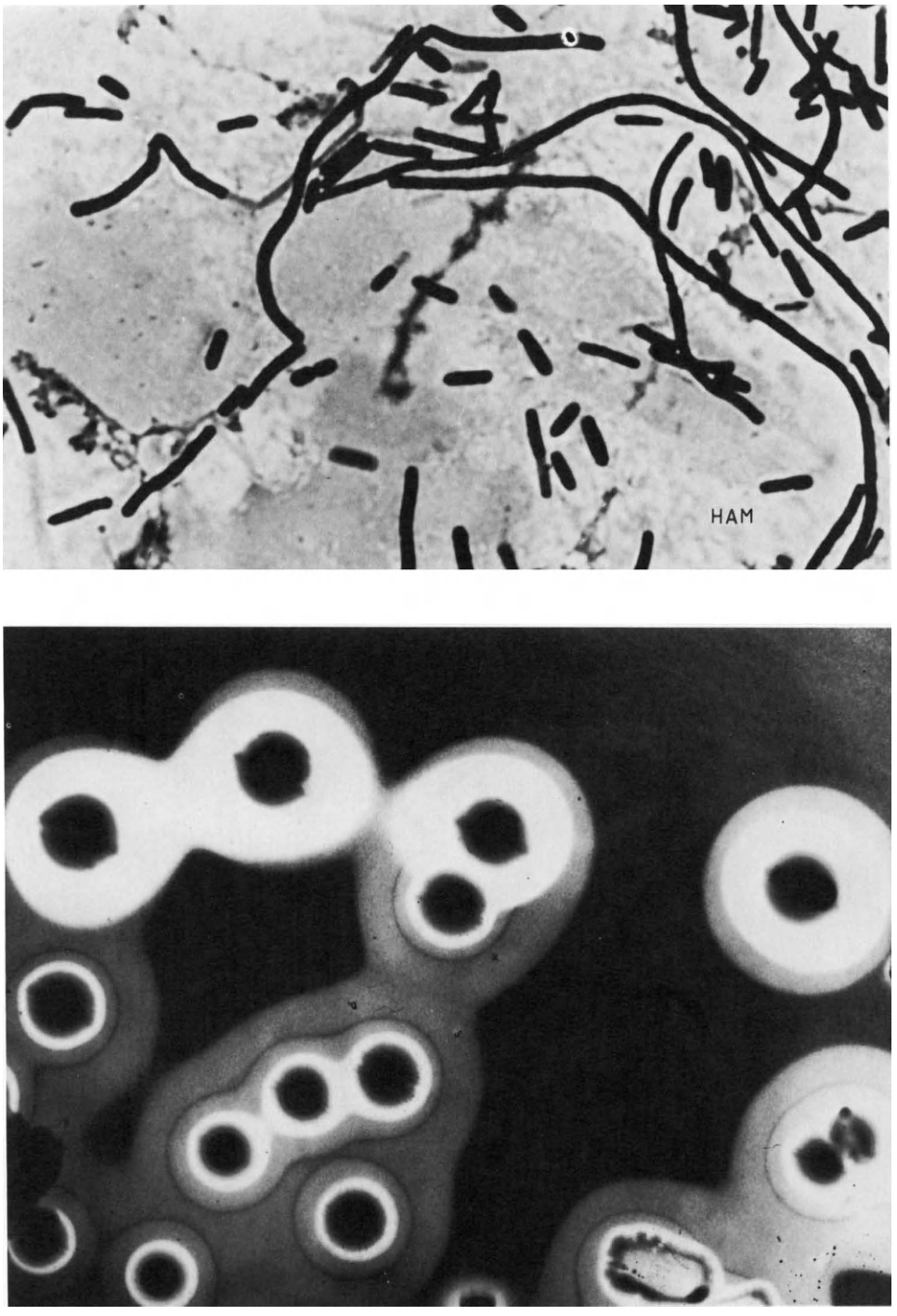


Figure 19-3. *Top:* Long forms of *Cl. septicum* seen in guinea pig liver impression smear. Gram-stain, ×2250 (H. A. McAllister). *Bottom:* Colonies of *Cl. perfringens* type C after 24-hr incubation on blood agar. Note zone of hemolysis caused by toxins of the organism.

obtain isolated colonies. Colonies of *Cl. septicum* are usually irregular with a rhizoidal edge, but smooth, round colonies are produced by some strains. Morphologically, the organism has characteristically filamentous forms (Fig. 19-3 top) and produces oval subterminal spores. It can be differentiated from *Cl. chauvoei* on the basis of antiserum neutralization tests, fluorescent staining, and fermentation of salicin but not sucrose.

Cl. chauvoei

Clostridium chauvoei grows poorly on blood agar, but good growth may be obtained by supplementing the medium with liver extract. Identification of the organism is greatly facilitated by incorporating sheep blood into the agar, resulting in colonies that are surrounded by a wide zone of hemolysis. It produces oval subterminal spores together with numerous pleomorphic forms. Unlike *Cl. septicum*, it ferments sucrose and rarely salicin.

Cl. perfringens

This organism grows well on blood agar, producing smooth, round, glistening colonies. Colonies are surrounded by a zone of hemolysis, which may be quite complex due to the actions of the various toxins. Theta toxin produces a clear zone of hemolyses. On the other hand, the hemolysis produced by α toxin is only partial (Fig. 19-3 bottom). The organisms are short gram-positive rods and spores are rarely seen.

The organism is divided into five types, A–E, based on the extracellular production of four major lethal necrotic toxins: α, β, ϵ, ι (Table 19-5). The principal toxins and other soluble factors of the different types of *Cl. perfringens* have been summarized by Brooks *et al.* (8). Toxins can be identified both in culture and in intestinal contents.

Table 19-5
Clostridium perfringens Types

	Toxins			
Type	α	β	ϵ	ι
A	+	–	–	–
B	+	+	+	–
C	+	+	–	–
D	+	–	+	–
E	+	–	–	+

Laboratory Diagnosis of Enterotoxemia

Specimen

Fresh contents from the small intestine

Direct Examination

Gram-stain smears from the mucosa of the small intestine, and take note of the character and number of gram-positive rods. There are few bacteria in the healthy jejunum and ileum.

Test of Intestinal Contents or Cultures for Toxicity

The intestinal contents should be collected as soon after death as possible. If the contents are overly mucinous, they should be diluted with an equal amount of sterile physiological saline. Shake well, then centrifuge to separate out the bacteria and particulate material. Draw off the supernatant. Filtration is often difficult and can be omitted. Penicillin and streptomycin can be added to suppress bacteria. Young cultures in cooked meat medium are handled similarly. Trypsinization as described below should be carried out with cultures, but is not usually required for intestinal contents.

Each of three mice is inoculated intravenously in the tail vein with 0.3 ml (0.4 ml if contents have been diluted) of the supernatant. Two to three times as much toxin is required to kill mice by the intraperitoneal route. If toxin is present in significant amounts, deaths usually take place within 10 hr. Deaths occurring within 5 min are usually attributed to shock. Identification of the toxin type is carried out as described below if the contents have been found toxic.

Mice are easily restrained for intravenous inoculation by pulling the tail through a hole in the metal top of a cylindrical mailing carton. The mouse is allowed to enter the carton, and the lid is screwed on. Suitable mouse holders are available commercially.

Identification of Types of *Cl. perfringens*

The types can be determined by toxin–antitoxin neutralization tests performed either intradermally in depilated albino guinea pigs or intravenously in mice. The mouse test is less informative and more liable to nonspecific effects. As the neutralization procedure is rather involved, the laboratory may wish to submit a culture of the *Cl. perfringens*, if available, to a reference laboratory for type identification.

Neutralization Tests in the Skin of Guinea Pigs

For this test 0.5 ml of suspected toxin is mixed with 0.2 ml of nutrient broth and 0.1 ml of antiserum to give a total volume of 0.8 ml. When more than one antiserum is required in the mixture, the amount of nutrient broth is reduced so that the total volume is still 0.8 ml. The composition of the mixtures required is shown in Table 19-6 (supplied by Burroughs Welcome Co., Research Triangle Park, North Carolina).

Table 19-6
Neutralization Reactions for *Cl. perfringens* Identification

Mixtures	Reactions with *Cl. welchii Type*[a]				
	A	B	C	D	E
Filtrate (untreated)					
Broth	+	+	+	±	+
Type A serum (anti-α)	−	+	+	±[b]	±
Types A + C sera (anti-α + anti−β)	−	±[b]	−	±[b]	±
Types A + C + D (anti-α + anti-β + anti-ϵ)	−	−	−	−	±
Broth	±	+	±[c]	+	+
Type A serum (anti-α)	−	+	±[c]	−	+
Filtrate (trypsinized)					
Types A + D sera (anti-α + anti-ϵ)	−	±[c]	±[c]	−	+
Types A + C + D sera (anti-α + anti-β + anti-ϵ)	−	−	−	−	−
Types A + E sera (anti-α + anti-ι)[d]	−	+	±[c]	+	−
Diagnostic toxins identified	α	β + ϵ	β	ϵ	ι

[a] −, No reaction; +, necrotic lesion.
[b] Nearly always −, as ϵ toxin in protoxin form.
[c] Nearly always −, as β toxin destroyed by trypsin
[d] Usually omitted, as ι toxin seldom present

Similar mixtures are prepared using trypsinized filtrate, that is, filtrate treated with 1% trypsin powder (Difco 1:250) for 1 hr at 37°C. The trypsin activates ϵ protoxin and destroys β toxin. The mixtures are left at room temperature for 30 min; then 0.2 ml is injected intradermally into guinea pigs. It is convenient to inject four mixtures on one flank and four mixtures on the other.

Reactions are read at 24 and 48 hr. If the guinea pig dies, excess ϵ toxin may be present and the test should be repeated with filtrate diluted one in five with broth.

Serum Neutralization Tests in Mice

Mixtures are prepared as for the skin tests in guinea pigs, and 0.3-ml quantities are injected intravenously into pairs of mice. The results are read for up to 3 days, and the interpretation is similar to that given above, except that death or survival are used as indicators of the presence or absence of the diagnostic toxins. If toxin is present in appreciable amounts, deaths usually take place within 10 hr.

The tests in mice may also be carried out according to the following schedule.

Test Fluid (ml)	Type Serum (ml)	Mouse Dose (ml)	No. of Mice	Group
0.9	A:0.3	0.4	2	Serum
0.9	B:0.3	0.4	2	Serum
0.9	C:0.3	0.4	2	Serum
0.9	D:0.3	0.4	2	Serum
0.9	E:0.3	0.4	2	Serum
0.9	None	0.4	2	Control

The tests are read over a 3-day period and interpreted as follows:

Type A antitoxin neutralizes only the homologous toxin.
Type B antitoxin neutralizes the toxins of types A, B, C, and D.
Type C antitoxin neutralizes the toxins of types A and C.
Type D antitoxin neutralizes the toxins of types A and D.
Type E antitoxin neutralizes the toxins of types A and E.

Readers are referred to Sterne and Batty (9) for additional information on the toxins of *Cl. perfringens* and other pathogenic clostridia.

Some strains of *Cl. perfringens* type A produce an enterotoxin while sporulating. It has been suggested that this toxin, a cause of human food poisoning, may be responsible for diarrhea in animals, particularly horses and rabbits. Procedures for the identification and production of this enterotoxin have been described by Dowell *et al.* (10).

Cl. novyi

Clostridium novyi is divided into four types based on the production of the extracellular toxins. The distribution of the two main lethal, necrotic toxins is shown in Table 19-7. Unlike the other types of *Cl. novyi*, *Cl. novyi* type A can grow on normal blood agar and produces

Table 19-7
Clostridium novyi Types

	Toxins	
Type	α	β
A	+	−
B	+	+
C	−	−
D	−	+

large irregular colonies with a rhizoidal edge surrounded by a large zone of hemolysis (Fig. 19-4). The organisms are large gram-positive rods with oval to cylindrical subterminal spores (Fig. 19-4b) not swelling the sporangium.

Types B, C, and D are extremely demanding, both in their nutritional requirements and in their tolerance of oxygen. In our hands, consistent, luxuriant growth on plates can only be obtained by meticulously following the methods of preparing and inoculating the plates described below.

The medium of Moore should be used. The medium should be prepared freshly and plates preincubated in a H_2/CO_2 (95:5 v/v) mixture prior to inoculation. Provided that such preincubated plates are quickly streaked and reestablished under anaerobic conditions, good growth ensues. If there is a lag of more than 15 min before streaking, subsequent growth is reduced or even prevented. Colonies are smaller than those of *Cl. novyi* type A and usually rhizoidal in nature (Fig. 19-5a). The success of this medium is largely attributable to the presence of cysteine and dithiothreitol. Under the usual conditions of medium preparation, pouring, and inoculation, oxidation of the cysteine in the medium proceeds rapidly so that cysteine is likely to be largely inactivated by the time the cultures are set up. Dithiothreitol protects the free thiol group of cysteine from oxidation, thus preserving its reducing activity.

Cl. sordellii

Clostridium sordellii grows well on stiff agar and produces irregular colonies. Colonies lose their translucent appearance and become white on aging due to the production of spores. The organisms are largely gram-positive rods with cylindrical spores not swelling the sporangium. The organism can be differentiated from the closely related *Cl. bifermentans* by production of urease.

Cl. botulinum

Clostridium botulinum is divisible into a number of types, A–G, based on the production of specific lethal toxins. Intoxication results from the consumption of foodstuffs containing toxins as a result of growth of the organism.

Types A, B, F, and G are responsible for classical botulism in humans, type E for human botulism from marine sources, and types C and D for botulism in animals. Types A, B, F and G consist of rods with oval subterminal spores swelling the sporagium and produce

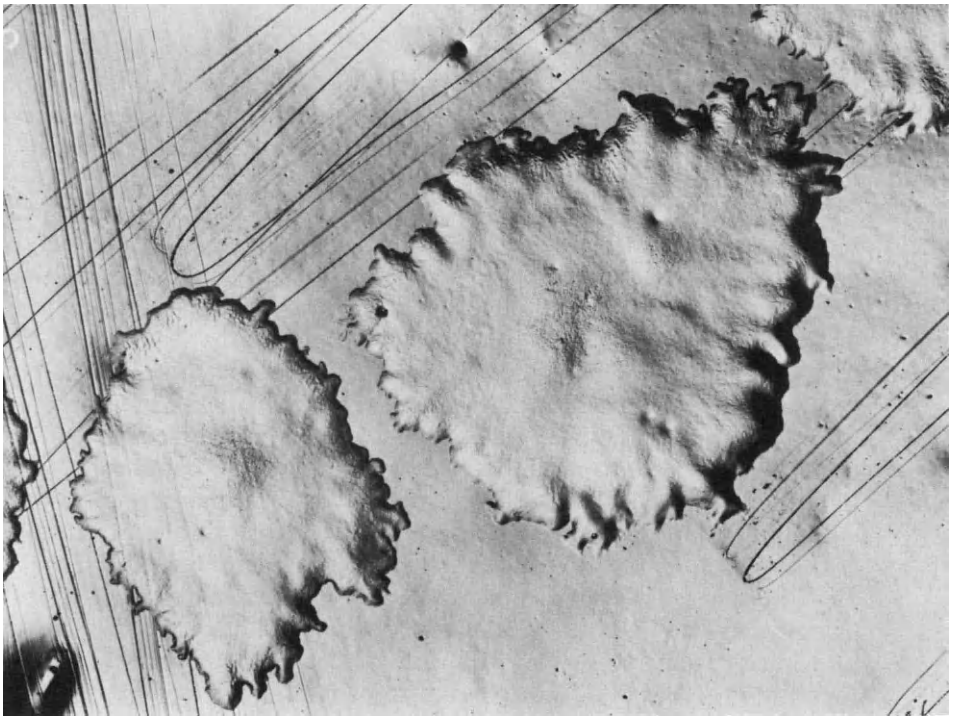


Figure 19-4a. Large irregular colonies of *Cl. novyi* type A on blood agar.

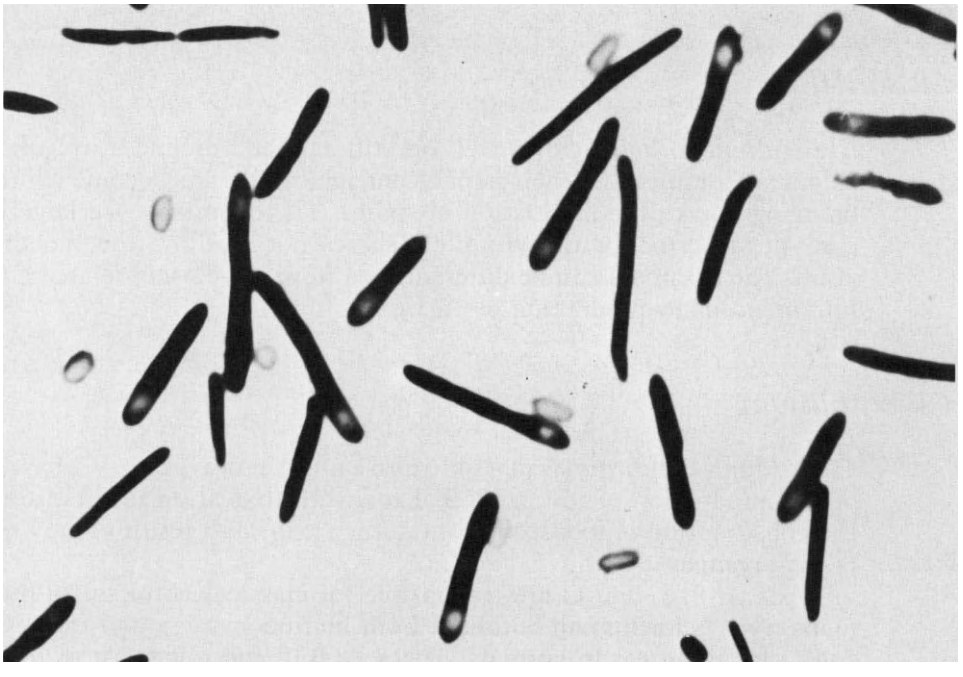


Figure 19-4b Gram stain of *Cl. novyi* species showing large gram-positive rods with oval to cylindrical subterminal spores not swelling the sporangium, ×2500.

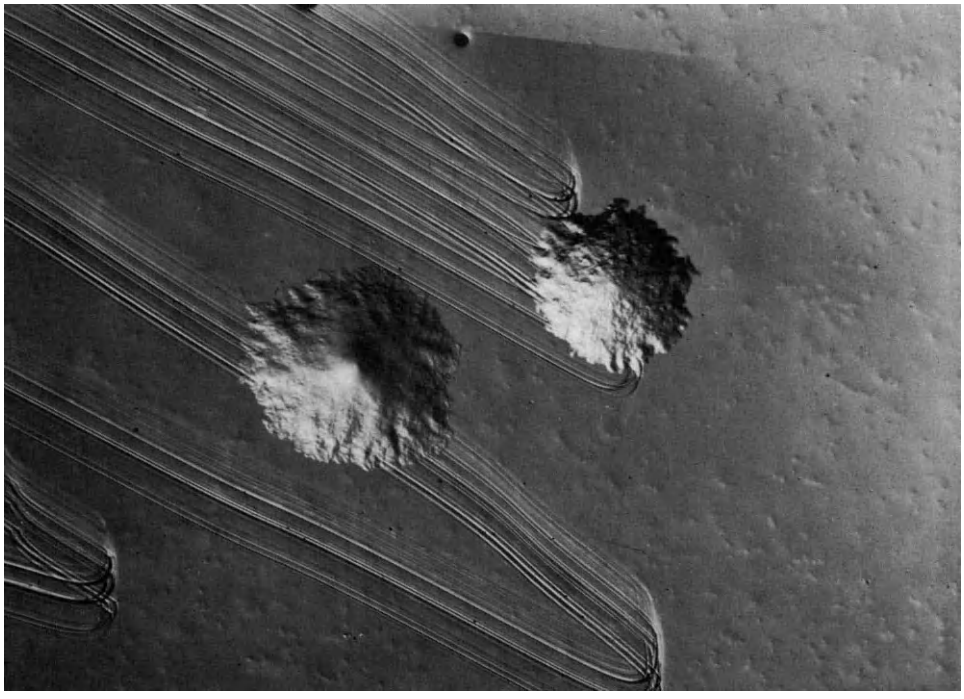


Figure 19-5a Colonies of *Cl. novyi* type B on Moore's medium, ×12.

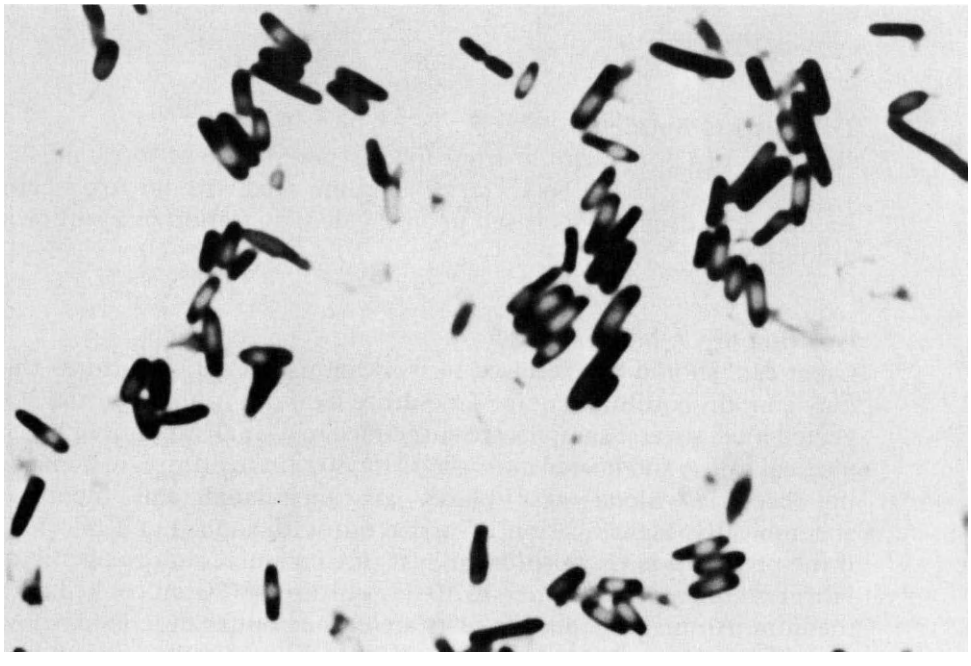


Figure 19-5b Gram-stain of *Cl. botulinum* type D showing large gram-positive rods with oval to cylindrical spores not swelling the sporangium, ×2500.

round umbonate colonies with a granular periphery and irregular margin. With the exception of some strains of type B, all are strongly proteolytic. *Clostridium botulinum* types C, D, and E resemble each other morphologically and culturally. They consist of large rods with oval to cylindrical terminal spores not swelling the sporagium (Fig. 19-5), and they produce irregular colonies with a granular surface and rhizoidal margin. All are nonproteolytic. In manuals of classification, *Cl. botulinum* is often reserved for types C, D, and E, while the name *Cl. parabotulinum* is used to distinguish the proteolytic types.

The distribution of types of *Cl. botulinum* in relation to some cases and outbreaks of botulism in different animal species is summarized below:

Type	Principal hosts	Toxin source
A	Humans, chickens (limberneck), and mink	Canned vegetables and fruits, meat and fish
B	Humans, horses, cattle and mink	Prepared meat, especially pork
C	Wild birds (aquatic), chickens (limberneck)	Rotting vegetation, toxin concentrated in fly larvae
	Cattle, horses	Forage poisoning
	Mink	Meat products
D	Cattle (lamziekte)	Carrion, bones
E	Humans	Uncooked fish and other marine products
F	Humans	Liver paste

Diagnosis of Botulism

Recovery of *Cl. botulinum* from food is not sufficient to establish a diagnosis of botulism. Food can be contaminated without toxin being produced. A diagnosis is based upon the demonstration of a sufficient amount of toxin.

Isolation of *Cl. botulinum*

Great care should be exercised in working with food or cultures that may contain botulinus toxin. To culture *Cl. botulinum* from the suspected food, several samples are suspended in a small amount of physiological saline and heated at 65–80°C for 30 min to eliminate nonsporing bacteria. Blood agar plates are inoculated and incubated anaerobically. Identification is carried out with the aid of Table 19-3. If the organism is *Cl. botulinum*, tests are conducted for toxigenicity. Filtrates are prepared from 5- to 10-day cultures (30°C) in cooked meat medium. Animal inoculation tests are carried out as described below, the culture filtrate being substituted for the filtrate prepared from food or other materials.

Demonstration and Identification of Toxin

The following may be examined for the presence of toxin: samples of food or anything else that might have been eaten; serum and urine from affected animals; and cecal contents and feces from an affected animal.

Although the demonstration of botulinus toxin is within the capability of most laboratories, it may not be feasible to carry out the rather involved neutralization procedures described below. If the latter is the case, the help of a reference laboratory should be sought.

Food is macerated in physiological saline and left to soak overnight if time permits. The suspension is then centrifuged until a clear supernatant is obtained. The supernatant may be sterilized by Seitz or other filtration, but this is not usually necessary. If a membrane filter is used, a porosity of 0.45 μm will remove bacteria. Without filtration, one must eliminate bacterial infection as a possible cause of deaths. If there is sufficient supernatant, treat nine parts of the test fluid (pH 5.5–6.6) with one part of trypsin solution (1% trypsin; Difco 1:250). The mixture is incubated at 37°C for 45 min. Cecal fluid is handled in a similar fashion.

Adult guinea pigs are each given 2 ml intraperitoneally. At the same time, a control group is given 2 ml of the heated extract (10 min at 100°C) intraperitoneally. Guinea pigs in the antitoxin groups receive a mixture of unheated extract and antitoxins of known type. Two or three guinea pigs are usually used for each group. Antitoxin is available from several sources.[1] It is usually administered in a ratio of one volume of antitoxin to four volumes of test fluid.

Mice may be used instead of guinea pigs. The intraperitoneal dose for the former is 1 ml. The presence within 5 days of flaccid paralysis followed by death in unprotected animals suggests the presence of botulinal toxin. Feces from animals suspected of having botulism may be examined for toxin as described above.

Proof that the food produced the intoxication can be obtained by feeding normal animals of the same species with the actual food.

Botulism in wild ducks has been diagnosed by the inoculation of the serum of affected ducks into mice. One milliliter of serum is inoculated intraperitoneally into each mouse. One group of mice is given serum only, and if toxicity is demonstrated serum is administered simultaneously inoculated with the different antitoxins. The effectiveness of this test is dependent upon a high level of toxin in the affected animal.

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[1]Biologics Reagents Section, Laboratory Branch, U.S. Public Health Service, Communicable Disease Center, Atlanta, Georgia. Pasteur Institute, Paris, France. Types A & B, Burroughs Wellcome Co., Research Triangle Park, North Carolina.

Cl. spiriforme

There has been much interest in both spontaneous and antibiotic-induced enterotoxemia in rabbits (11–13). The disease is characterized by explosive outbreaks of fatal disease in breeding colonies with no previous signs of clinical illness (14,15). A toxin neutralized by *Cl. perfringens* type E antitoxin (i) was detected in the intestines of dead animals (16). Although it was concluded that *Cl. perfringens* type E may have been involved in the etiology of the disease, no isolations were made. More recently, it has been shown that a helically coiled anaerobic gram-positive sporeforming bacillus identified as *Cl. spiriforme* can be isolated from the cecal contents of all rabbits with spontaneous diarrhea (17). *Clostridium spiriforme* was shown to produce a toxin *in vitro* that was lethal to mice, caused dermonecrosis in guinea pigs, and was neutralized by *Cl. perfringens* type E ι antitoxin.

Cl. difficile

This organism is present in the normal bowel flora and is selectively enhanced following administration of antibiotics such as clindamycin (18), which alter the normal bowel flora by selectively killing species of *Bacterioides*. The organism has been shown to produce toxins which are cytotoxic and enterotoxic.

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Listeria and *Erysipelothrix*

G. R. Carter

Species of these genera are similar in a number of respects. The organisms are slender, gram-positive, nonsporing, facultatively anaerobic, fermentative rods. *Erysipelothrix rhusiopathiae* and *Listeria monocytogenes* are the important pathogenic species; however, additional species of *Listeria*, all but one of little disease significance, have been described and are referred to below. In 1987, Takahashi *et al.* described species *E. tonsillarum* as a nonpathogenic commensal recovered from the tonsils of healthy swine (1). Both *E. rhusiopathiae* and *L. monocytogenes* are capable of survival in the soil and existing in the animal in commensal and carrier states.

Listeria

The taxonomy of the genus *Listeria* and organisms closely related to *Listeria* is somewhat confused. The species listed below, in addition to *L. monocytogenes*, are those recognized by *Bergey's Manual* (1) and recent publications.

L. ivanovii

This is the only species other than *L. monocytogenes* that is frankly pathogenic for animals including humans. It is pathogenic for mice experimentally, and for pregnant sheep, in which it may cause abortion. There is a carrier state in healthy animals and humans. Little is yet known about the extent of infections due to this species.

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L. innocua

This species (2) has been isolated from soil, plants, and human and animal feces.

L. welshimeri

This organism has been recovered from soil and decaying vegetation.

L. seeligeri

This organism has been recovered from soil, vegetation, and animal feces.

L. murrayi (Murrayi murrayi)

Its natural habitat is probably soil and vegetation. Retention of the genus name *Listeria* for this and the following species has been proposed (3).

L. grayi (Murrayi grayi)

Its natural habitat is probably soil and vegetation.

Other Genera

One report claims that *L. denitrificans* does not belong in the genus *Listeria* and should be transferred to a new genus, *Jonesia* (4). It has only rarely been isolated and its natural habitat is not known. It is pathogenic for rats and mice experimentally.

The taxonomic position of isolates referred to as *L. bulgaricus* by Rocourt *et al.* has not been established (5).

Listeria monocytogenes

Pathogenicity

Infections occur in a wide range of animals. Two forms of the disease are seen, the neural and the visceral. The neural form, characterized by microabscesses in the brain stem and meningitis, occurs most com-

monly in cattle, sheep, and goats and occasionally in horses, dogs, and humans.

The visceral form of the disease, with liver necrosis, is seen in rabbits, guinea pigs, chinchillas, swine, ferrets, raccoons, calves, lambs, and other animals. An epizootic form occurs in chickens and turkeys, characterized by necrotic foci of the liver and pericardium.

The organism is an important cause of abortion in cows and ewes, and in such abortions, it can be recovered from the aborted fetuses. On occasion, *L. monocytogenes* is shed in the milk of infected cows, goats, and ewes.

Pregnant women may develop a generalized infection that resembles influenza. The organism can cross the placenta, resulting in abortion and systemic disease and/or meningitis in the newborn. Infections characterized by meningitis or septicemia are encountered in immunocompromised hosts. Occasional human infections have been traced to improperly pasteurized dairy products.

Isolation Procedures

Blood agar and blood agar containing 0.05% potassium tellurite (which inhibits many gram-negative species) are used if listeriosis is suspected. In the visceral form, material is seeded directly onto the solid medium. In the neural form, the medulla and a portion of the cord are cut into small pieces by means of sterile scissors and placed in broth. It is advisable to sample various parts of the medulla. A 10–20% suspension is prepared in a tissue grinder, with mortar and pestle, or with a blender. After inoculation of media, the suspension is stored in the refrigerator for possible future examinations. These are especially indicated if histopathological sections of the brain suggest listeria infection. If listeria are not recovered initially, the suspension should be examined at the end of the first, third, sixth, and twelfth weeks. The organism can be more readily recovered, that is, with less storage time, from the ovine brain than from the bovine brain.

Cultural Characteristics

Listeria grow as small, round colonies with a narrow zone of β-hemolysis. The latter may be seen around the colony or only underneath. Both smooth and rough colonies are seen. Stained smears reveal small gram-positive rods (Fig. 20-1). Primary cultures are frequently pleomorphic and can be mistaken for diphtheroids (corynebacteria), diplococci, and streptococci. The organisms are easily decolorized and as a consequence can be mistaken for gram-negative rods.

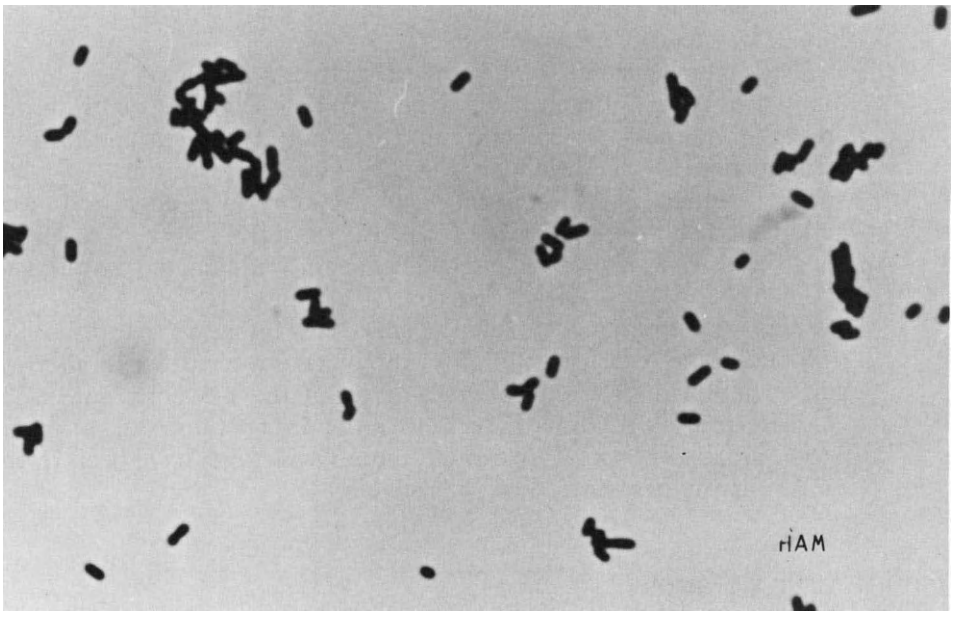

Figure 20-1. *Listeria monocytogenes* from a blood agar plate culture. Gram stain, ×2250 (H. A. McAllister).

Motility is an important differential characteristic of *L. monocytogenes*. A characteristic end-over-end tumbling motility is seen in broth cultures at room temperature (about 25°C). In semisolid agar an umbrella-shaped pattern of growth is seen after overnight incubation at 25°C.

Identification

Some important features that differentiate *L. monocytogenes* from *E. rhusiopathiae* are listed in Table 20-1. Additional characteristics that distinguish *L. monocytogenes* are the following:

- Fermentation: glucose, maltose, levulose, salicin, and trehalose
- Irregular fermentation: arabinose, sorbitol, sucrose, galactose, and glycerol
- Not fermented: mannitol, dulcitol, inositol, inulin, and raffinose
- The oxidase test is negative.
- Nitrate is not reduced.
- Catalase is produced.
- MR and VP tests are usually positive.

Table 20-1
Differentiation of *Listeria* spp. and *Erysipelothrix*

Species	Umbrella motility (25°C)	β-Hemolysis	CAMP Test		Acid production		
			S. aureus	*R. equi*	Mannitol	Rhamnose	Xylose
L. monocytogenes	+	+	+	+	−	+	−
L. ivanovii	+	+	−	+	−	−	+
L. innocua	+	−	−	−	−	v	−
L. welshimeri	+	−	−	−	−	v	+
L. seeligeri	+	+	+	−	−	−	+
L. murrayi	+	−	−	−	+	v	−
L. grayi	+	−	−	−	+	−	−
E. rhusiopathiae	−	−	−	−	−	−	−

- Esculin is hydrolyzed.
- Indole is not produced.
- Gelatin is not liquefied.
- Urease is not produced and starch is not hydrolyzed.

Because *Listeria monocytogenes* causes different diseases than *E. rhusiopathiae* and is thus usually isolated from different clinical specimens, their considerable similarity is not usually a problem in the laboratory. Unlike *Listeria* spp., *E. rhusiopathiae* is catalase negative.

Identification of the other species of *Listeria* listed earlier is based upon the characteristics listed in Table 20-1.

As indicated in Table 20-1 the CAMP test is helpful in the identification of species of *Listeria*. This test is described in detail in Appendix B using a *Staphylococcus aureus* streak. With *Listeria*, streaks of *S. aureus* and *Rhodococcus equi* are used. The suspected *Listeria* is inoculated at right angles and close to the *S. aureus* and the *R. equi* streaks. After overnight incubation the plates are examined for augmentation of hemolysis of the suspected *Listeria* near the staphylococcal and rhodococcal streaks. The CAMP effect is not seen with *E. rhusiopathiae*.

There are reports of the successful employment of the fluorescent antibody staining procedure in the identification of listeria in smears and in fixed sections of tissues (6). Fluorescent antibody may be used for the presumptive identification of *L. monocytogenes* from cultures.

Animal Inoculation

Ordinarily, animal inoculation is not required for the identification of *L. monocytogenes*. *Listeria ivanovii* and *L. monocytogenes* are the only species that are pathogenic when inoculated into mice. *Listeria ivanovii*, unlike *L. monocytogenes*, is negative in the Anton test described below.

The following applies to *L. monocytogenes* although the mouse pathogenicity may also apply to *L. ivanovii*. Mice, rabbits, and guinea pigs are susceptible to experimental infection if a considerable number of organisms are used. Several mice are inoculated subcutaneously with graded doses (maximum 0.5 ml) of a 24-hr broth culture. Deaths usually occur 3–10 days postinoculation, and necropsy reveals many necrotic foci in the spleen and liver.

If a small amount of a broth culture is placed into the conjuctiva of the rabbit or guinea pig, or swabbed on the everted lid, a severe keratoconjunctivitis develops within 24–36 hr, followed by opacity of the cornea. This is called the Anton Test.

Serology

Tests of sera for antibody in suspected listeriosis are not useful or practical. Four serotypes and several subtypes of *L. monocytogenes* have

been recognized for some time. More than a dozen serotypes or serovars have been identified based upon different O and H antigens (7). Serotyping is carried out by some reference laboratories.

Erysipelothrix rhusiopathiae

Synonym: *Erysipelothrix insidiosa*

The organism is present in the soil and manure associated with swine. In this habitat, particularly alkaline soil, it survives for long periods and proliferates. Many pigs carry the organism and it can frequently be recovered from feces and tonsils.

The name *E. tonsilarum* has been proposed for serotype 7 of *E. rhusiopathiae* based on the lack of mouse pathogenicity and on DNA homology studies (7). This proposed new species was isolated from the tonsils of healthy pigs and is morphologically and biochemically indistinguishable from *E. rhusiopathiae.*

Pathogenicity

Swine show a septicemic form, a chronic form with arthritis, endocarditis, or a skin manifestation called diamond skin disease. Sheep and cattle show chronic polyarthritis. Turkeys, chickens, geese, pheasants and other avian species have severe infections often involving large numbers. The acute, septicemic disease occurs most frequently in turkeys. In dogs there are a number of reports of valvular endocarditis. Cattle, horses and other species demonstrate infrequent infections of varying severity. Humans have localized infections called "erysipeloid" usually involving hands and fingers; systemic infections occur but are rare. Dolphins show septicemia and urticaria.

Isolation Procedures

In suspected erysipelas infection, materials are inoculated onto blood agar. Blood agar containing sodium azide (see Appendix B) may be employed to depress contaminants and used to recover *E. rhusiopathiae* from manure and soil.

Although *E. rhusiopathiae* grows aerobically, primary growth is accelerated if plates are placed in the candle jar or 10% carbon dioxide.

Cultural Characteristics

Smooth colonies are small and round; rough colonies are larger and have irregular borders. The latter are more common from chronic infections. Growth is sparse after 24 hr of incubation but readily apparent after 48 hr. A zone of α-hemolysis is usually seen around young colonies, followed by some clearing of the zone on further incubation. Smears from smooth colonies disclose slender gram-positive rods indistinguishable from those of *L. monocytogenes* (Fig. 20-2). Rough colonies yield highly pleomorphic and filamentous forms. Like *L. monocytogenes, E. rhusiopathiae* may sometimes be gram-variable and appear almost gram-negative.

Identification

This is based upon features listed in Table 20-1 and upon the characteristics listed below:

- Fermentation reactions are variable: Lactose, glucose, levulose, and dextrin are frequently fermented; some acid production may be

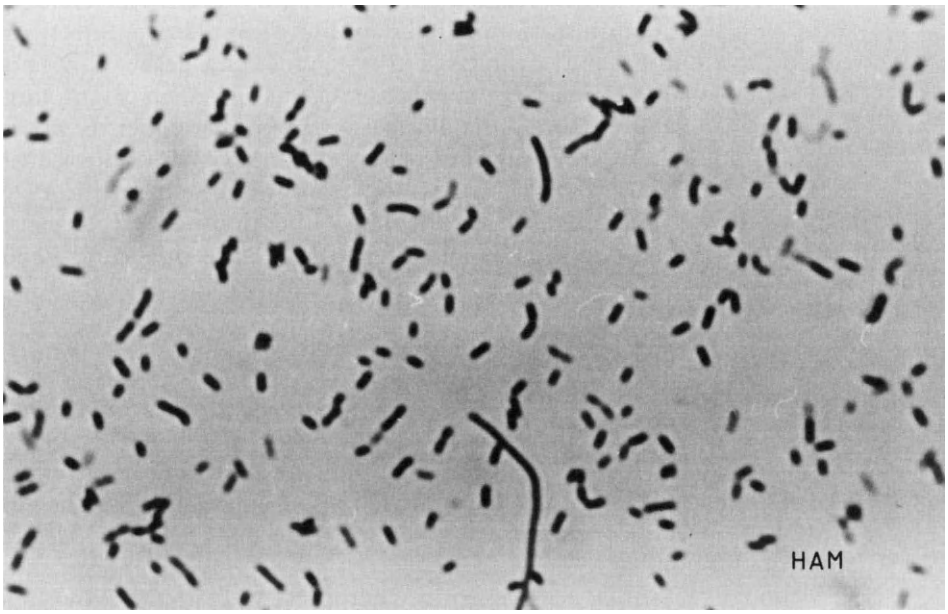

Figure 20-2. *Erysipelothrix rhusiopathiae* from a blood agar plate culture. Gram stain, ×2440 (H. A. McAllister).

noted in sucrose, arabinose, dulcitol, mannitol, xylose, galactose, and fructose; litmus milk may be acidified.
- Esculin is not hydrolyzed.
- Hydrogen sulfide production and nitrate reduction are variable; oxidase, catalase, and indole are not produced; and gelatin is not liquefied.
- "Test tube brush" growth along stab line in gelatin incubated at 20°C for 3–5 days.

Most strains of *E. rhusiopathiae* affect triple sugar iron agar and Kligler's iron agar in a characteristic manner. Growth is sparse, with yellowing of slant and butt and the production of hydrogen sulfide along the stab line.

Fluorescein-labeled antibody for the identification of this organism has not been available commercially.

The new species *E. tonsillarum,* which apparently is identical to avirulent strains of serotype 7 *E. rhusiopathiae,* can only be distinguished from the latter by DNA homology values (1).

Animal Inoculation

Mice and pigeons are susceptible, dying within 4 days after intraperitoneal inoculation of 0.1–0.5 ml of broth culture.

Mice infected with 0.1 ml of broth culture can be protected by the inoculation of 0.3 ml of commercial antierysipelas serum. This protection test can be used to confirm identification.

Serology

Serologic tests are rarely used now to detect swine with chronic infections. At least 20 serotypes of *E. rhusiopathiae* have been identified. Two major serotypes account for 70–80% of infections (8). Serotyping is carried out in some research and reference laboratories.

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21

Corynebacterium

G. R. Carter

Members of this genus are gram-positive, small, pleomorphic, nonsporing rods. All are nonmotile (except some plant pathogens), usually facultatively anaerobic, and fermentative. The group contains a miscellany of species, some of which will ultimately, on the basis of chemotaxonomic studies, be assigned to other genera. The names given in *Bergey's Manual* (1) and the *International Journal of Systematic Bacteriology* will be used.

These small, pleomorphic rods display varying degrees of metachromatic staining; *C. diphtheriae* is outstanding in this regard. Many corynebacteria or diphtheroids occur widely in nature and as commensals on the skin and mucous membranes of animals. They are usually of little or no pathogenic significance when recovered from clinical specimens. However, some are reported to cause opportunistic infections in humans. These miscellaneous diphtheroids are more frequently recovered from tissues taken from animals some hours after death. Ordinarily they are not identified as to species.

Pathogenicity

Corynebacterium pyogenes

This important organism, which is catalase negative, did not belong in the genus *Corynebacterium* and has been transferred to the genus

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Actinomyces. It is now *Actinomyces pyogenes* and discussed in Chapter 22.

C. equi

This organism is now named *Rhodococcus equi* and is discussed in Chapter 22.

C. renale

It causes cystitis and pyelonephritis in cows, and kidney abscesses in swine. Cystitis and pyelonephritis due to *C. renale* have been reported infrequently in bitches. Enzootic posthitis in castrated male sheep has been attributed to it (2).

C. cystitidis

Although immunologically related to *C. renale* it is considered a distinct species (3). It causes cystitis and pyelonephritis in cows.

C. pilosum

This organism is also closely related to *C. renale* (3) and is considered another cause of cystitis and pyelonephritis in cows.

C. pseudotuberculosis (C. ovis)

This facultative intracellular parasite causes caseous lymphadenitis in sheep; occasional abortion in ewes; arthritis and bursitis in lambs; ulcerative lymphangitis in equine species; and "chest" abscesses in horses, with or without habronemiasis. Infrequent infections have been reported in camels, goats, cattle, deer, laboratory mice, and other animal species (4).

C. suis

This organism is now named *Eubacterium suis* and is discussed in Chapter 15.

C. kutscheri (C. murium)

This organism causes a disease of mice and rats characterized by caseopurulent foci in the lungs, lymph nodes, and less frequently the liver, kidneys, and subcutis (5).

Other Corynebacteria

These are listed after Identification below.

Isolation Procedures

Gram-stained smears of pus and affected tissues will indicate the presence of corynebacteria. Their pleomorphism may lead to their being confused with streptococci, staphylococci, and listeria. Identification requires isolation.

Material is streaked on blood agar and incubated aerobically at 37°C. After inoculation of plates, swabs are placed in tubes of brain–heart infusion semisolid medium, which are also incubated at 37°C.

Guinea Pig Inoculation

Intravenous inoculation of *C. pseudotuberculosis* produces abscesses in the lungs and liver, with death in 4–10 days. Orchitis is produced in the male guinea pig as a result of intraabdominal inoculation of organisms.

Cultural Characteristics

The cultural characteristics of the most important species are described below. The microscopic morphologic differences among the different species are not significant. Gram-stained smears from colonies disclose small, gram-positive, pleomorphic rods that may form "palisades" and "chinese letters". (Figs. 21-1, 21-2).

C. renale

In 24 hr, small dewdroplike colonies appear. These become yellow and opaque in 48 hr. In broth, a fine powdery sediment is noted on the side and bottom of the tube.

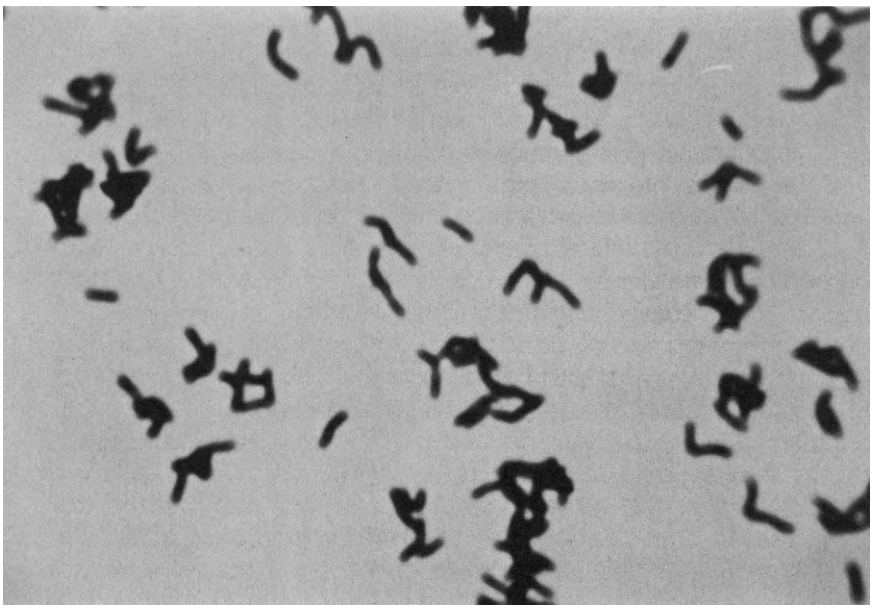

Figure 21-1. A Gram-stained smear of *Corynebacterium kutscheri* displaying "chinese letters" arrangement of cells.

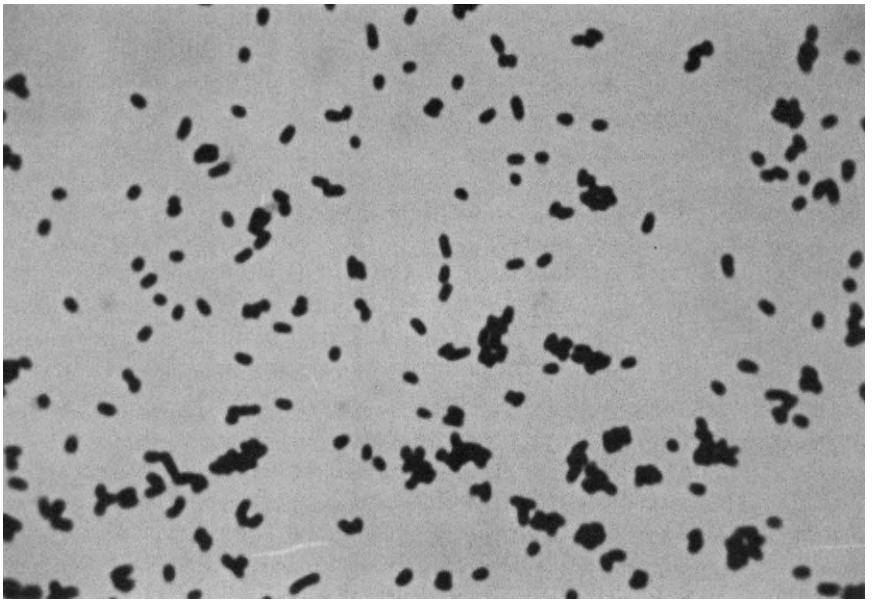

Figure 21-2. A Gram-stained smear of *Corynebacterium pseudotuberculosis.*

C. cystitidis

Small, white, circular, semitranslucent whitish colonies are apparent after 48 hr of incubation. It grows at 41.5°C.

C. pilosum

Colonies are about 1 mm in diameter after 24 hr of incubation. They are round, pale yellow, and opaque. No growth at 41.5°C.

C. pseudotuberculosis

Growth is sparse initially. Small, translucent colonies enlarge in 3–4 days to take on a cream to orange color, becoming opaque, dry, and crumbly. They are β-hemolytic. A coarse, granular sediment is produced in broth.

C. kutscheri

Yellow-white glistening colonies 1–4 mm in diameter are apparent after 2 days incubation on blood agar.

Identification

The various corynebacteria are definitively identified according to the criteria presented in Table 21-1. The three closely related species *C. renale, C. cystitidis,* and *C. pilosum* are differentiated according to the characteristics in Table 21-2.

Biberstein *et al.* (6) have described two types of *C. pseudotuberculosis.* The one that was nitrate negative was usually recovered from sheep and goats, while the nitrate positive variety was usually isolated from cattle and horses.

Fraser (7) has described CAMP-like tests to aid in the identification of *Rhodococcus equi, Actinomyces pyogenes, C. renale, C. pseudotuberculosis,* and *C. haemolyticum.*

Other Species

For the identification of these less important species, consult Table 21-1.

Table 21-1
Differential Characteristics of Some Corynebacteria[a]

	β-Hemolysis	Urease	Nitrate red.	Gelatin liq.	Acid				
					Glucose	Maltose	Sucrose	Mannitol	Xylose
C. renale	−	+	−	−	+	−	−	−	−
C. pseudotuberculosis	+	+	−/+	−	+	+	−	−	−
C. kutscheri	−/+	+	+	−	+	+	+	−	−
C. bovis	−	−	−	−	+	−	−	−	−
C. minutissimum	−	−	−	−	+	+	−/+	−	−
C. xerosis	−	−	+	−	+	V	+	−	−
C. ulcerans	+	+	−	−/+	+	+	−	−	−
C. striatum	−	−	+	−	+	−	+	−	−
C. flavescens	NA	−	−	−	+	−	−	NA	−
C. haemolyticum[b]	+	−	−	−	+	+	−/+	−	−
C. diphtheriae	−/+	−	+	−	+	+	−	−	−

[a] −/+, negative and positive strains; V, variable; NA, information not available.
[b] Catalase negative; other corynebacteria tested are positive

Table 21-2
Differentiation of *C. renale*, *C. pilosum*, and *C. cystitidis*[a]

Characteristic	*C. renale*	*C. pilosum*	*C. cystitidis*
Colony color	Yellow	Yellow	Whitish
Growth in broth at pH 5.4	+	−	−
Acid from			
Xylose	−	−	+
Starch	−	+	+
Nitrate reduction	−	+	−
Casein digestion	+	−	−
Hydrolysis of Tween 80	−	−	+
Original designation	*C. renale* type I	*C. renale* type II	*C. renale* type III

[a]Adapted from Yanagawa and Honda (3).

C. bovis

This organism, which apparently does not belong in the genus *Corynebacterium* (1), is recovered frequently from cows' milk. It is usually considered nonpathogenic but is thought by some to be capable of producing occasional udder infections. It is a slender, nonmotile rod that grows well on enriched media. Colonies on agar are circular, gray, slightly raised, and dry. Colonies are more frequently found in the fatty areas on plates streaked with milk because of their requirement for oleic acid (8). Other differential features are listed in Table 21-1.

C. diphtheriae

It is an important pathogen of humans. Infections in animals have been reported but do not appear to have been well substantiated.

C. minutissimum

This organism causes erythrasma, an infection of the stratum corneum of humans. It has been associated with an acute, moist inflammation of the interdigital space, "scald" in young lambs, with scabs on the brisket and docking wounds, and on the skin of the udder of normal cattle (9). The lesions in which this organism is found are distinctive in that they have a coral-red fluorescence in ultraviolet light. It is claimed that Mueller–Hinton agar is especially suitable for the demonstration of the fluorescence (10).

C. xerosis

This human, nonpathogenic species also produces fluorescence.

C. ulcerans

This is not an official species. It is an occasional cause of mastitis in cows.

C. striatum

This organism was recovered from the milk of cows with mastitis (1).

C. flavescens

It has been isolated occasionally from dairy products (1,11).

C. haemolyticum

Roberts (12) reported the isolation of this species, which occurs in humans, from a case of ovine pneumonia. *Corynebacterium haemolyticum* was recently transferred to the genus *Arcanobacterium* and named *A. haemolyticum* (13).

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22

Actinomyces, Nocardia, Streptomyces, Dermatophilus, and *Rhodococcus*

J. F. Prescott

This chapter describes some of the irregular, nonsporeforming gram-positive rods that are commonly encountered in diagnostic laboratories, either as aerobic or as facultatively anaerobic isolates. *Nocardia* and *Rhodococcus* are closely related to the genera *Mycobacterium* and *Corynebacterium* but differ distinctly from *Actinomyces* in cell wall chemical characteristics and percentages of guanine and cytosine. Aerobic and aerotolerant *Arachnia* and *Propionibacterium* may be isolated rarely from clinical specimens and are not considered further. Uncommon, irregular, gram-positive nonsporing rods may sometimes be difficult to identify in nonspecialist veterinary laboratories but detailed descriptions are available (1).

Actinomyces

Actinomyces are gram-positive, diphtheroidal or branching filamentous rods, 0.2–1.0 μm in diameter. Short rods, which may show clubbed ends, are common and may occur singly, in diphtheroidal pairs, in short chains, or in clusters. *Actinomyces pyogenes* are commonly coccobacillary in an aerobic atmosphere. *Actinomyces* are facultatively anaerobic, with most species being preferentially anaerobic although some species grow well in air; 10% CO_2 improves growth of aerotolerant species and is required for aerotolerant strains of preferentially anaerobic species (Table 22-1). *Actinomyces* are thus fermentative in metabolism whereas *Nocardia* are oxidative. Colonies my be either rough and dry or soft and mucoid, with transitional forms being common.

Actinomyces cause chronic infections of soft and hard tissues, often

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Table 22-1
Identification of Actinomyces Species[a]

Species	Catalase	Urease	Gelatin hydrolysis	Nitrate reduction	Fermentation of				Aerotolerance
					Mannitol	Salicin	Rattinose	Xylose	
A. bovis	–	–	–	–	–	–	–	–	M or An
A. israelii	–	–	–	V	V	+	+	+	M or An
A. odontolyticus	–	–	–	+	–	V	–	V	M or An
A. howellii	+				–	–	+	+	F
A. naeslundi	–	+	–	+	–	V	+	V	F
A. viscosus	+	V	–	V	–	V	+	–	F
A. pyogenes	–	–	+	–	–	–	–	V	F
A. hordeovulneris	+	–		–	–		W	+	M or An

[a]Key: V, variable; W, weak postive; M, carboxyphilic; An, anaerobic; F, grows aerobically and anaerobically.

with the formation of sulfur granules. The presence of sulfur granules, while striking, is not diagnostic of *Actinomyces* infections but also occurs in *Nocardia* or *Streptomyces* infections. The organisms may be hard to demonstrate outside these bodies.

It is generally taught that *Actinomyces* are not moderately acid-fast, while *Nocardia* are; exceptions to this rule are not uncommon.

Actinomyces infections are often associated with the presence of foreign bodies, whereas *Nocardia* infections are seen in immunocompromised hosts or as iatrogenic infections.

Actinomyces bovis

Pathogenicity

This organism is the cause of actinomycosis (lumpy jaw), a chronic suppurative osteomyelitis of the mandible of young cattle. Such infections also occasionally occur in swine, horses, dogs, and humans. On occasion soft tissues are involved, and these rarely are generalized infections. In horses the organism was at one time common in fistulous withers and poll evil, associated synergistically with *Brucella abortus* infection or with damage due to ill-fitting harnesses in draft animals. In lumpy jaw other bacteria may be present such as *Actinobacillus actinomycetemcomitans* and a variety of nonsporing anaerobic bacteria.

Direct Examination

The best specimen for direct examination in a case of suspected lumpy jaw is the sulfur granule. This should be crushed and a Gram-stained smear made to demonstrate the delicate, branching gram-positive filaments.

Isolation Procedures

Aerobic and anaerobic cultures are made, and the plates incubated for 2–5 days. A plain Sabouraud agar plate should also be inoculated and incubated at room temperature and 37°C. *Actinomyces* species grow very poorly, if at all, on Sabouraud's agar, a clear distinction from the excellent growth seen with *Nocardia* and *Streptomyces*. Liquid thioglycollate media (Chapter 15) must also be inoculated with sulfur granules. Details on anaerobic culture methods are given in Chapter 15. The aerobic plates should be incubated in an atmosphere of 10% CO_2.

Cultural Characteristics

Actinomyces bovis (naeslundii, odontolyticus, and *israelii)* grow well anaerobically but grow poorly, if at all, in an air with 10% CO_2. They do not grow aerobically. Colonies are white, rough, and nodular, 2–3 mm in diameter; smooth and rough forms are common. The colonies

adhere tenaciously to solid media and are removed with difficulty. Gram-stained smears from growth on solid or in fluid media show gram-positive, slightly branched filaments or short hyphae. On subculture the organisms may become diphtheroidal or coccobacillary. Growth usually occurs within 2–4 days but plates should be incubated for 7 days, and thioglycollate broth for 2 weeks.

Identification

Presumptive identification can be made on the basis of finding sulfur granules consisting of gram-positive branching filaments associated with a suppurative, proliferative osteomyelitis. Recovery of an organism with the colonial features of *A. bovis* is usually considered definitive. Identification requires that the organism possess the biochemical properties shown in Table 22-1. Fluorescent antibody identification is most useful but is available only in certain laboratories.

Actinomyces viscosus

Pathogenicity

Two forms of canine actinomycosis have been seen (2). The less common is the localized granulomatous abscess involving mainly the skin and subcutis. The other form involves principally the thoracic or abdominal cavities. Pyothorax with granulomatous lesions of thoracic tissues and accumulation of pleural and pericardial fluid containing soft, gray-white granules is characteristic of this deep form; it is a serious infection which requires long-term treatment (3). The organism can be found rarely in similar infections in cats and other species.

Direct Examination

Gram-stained and modified acid-fast stained smears are made of purulent material and, whenever possible, from sulfur granules. In the absence of sulfur granules it is hard to demonstrate the non-acid-fast, gram-positive filaments. Diphtheroidal forms may sometimes predominate. The sulfur granules in canine actinomycosis are usually soft.

Isolation Procedures

Materials, particularly sulfur granules, are inoculated onto blood agar plates and incubated in air with 5–10% CO_2. Discernible colonies are seen after 2–4 days incubation at 37° CO_2. The organism will grow anaerobically but grows better in air. It is a facultative anaerobe.

Cultural Characteristics

Two colony forms are seen: One is a smooth, entire, convex, glistening, mucoid or soft colony composed of dipththeroidal forms, and the

other consists of rough, irregular, heaped, granular, and slightly dry colonies, which yield branching filaments. Both forms are usually seen, but one may predominate.

Identification
The organism is distinguished from other *Actinomyces* in that it grows well aerobically; it is readily distinguished from *Nocardia* or *Streptomyces* by its failure to grow on Sabouraud dextrose agar. Its distinguishing biochemical properties are shown in Table 22-1.

Actinomyces pyogenes

Previously described as *Corynebacterium pyogenes* (4).

Pathogenicity
It is commonly isolated from purulent conditions in cattle, sheep, and swine, occasionally goats, and is the major opportunist pathogen of cattle, recovered from wound infections, pneumonias, genital tract and udder infections, and sites of bacteremic spread including aborted fetuses.

Direct Examination
On Gram stain they are often pleomorphic and commonly with clusters of gram-positive, club-shaped, short rods but may appear coccobacillary or as short, branching rods.

Isolation Procedures
It grows in air in 24–48 hr on blood agar at 37°C, 10% CO_2 improves growth, and grows well anaerobically.

Cultural Characteristics
Aerobic cultures are usually dry, white, 1-mm colonies at 48 hr, growth easily missed at 24 hr; colonies sometimes smooth or mixed rough and smooth. Narrow 0.5–1-mm zone of clear hemolysis; clarity increases with time.

Identification
Microscopic appearance highly pleomorphic, as described above. Varies from small "chains of cocci" to short, branching rods but is usually a mixture of coccobacillary forms with some club-shaped rods in clusters. Proteolysis on milk agar is a simple identifying step, combined with negative catalase test and the characteristic colonial appearance.

Actinomyces hordeovulneris

Pathogenicity
Isolated from abscesses, serositis, or recurrent localized infections in dogs, often associated with migrating grass awns ("foxtails") of the grass genus *Hordeum* (5). The infection is common in California.

Direct Examination
Pleomorphic rods and branching filaments, 0.5–1.0 μm diameter

Isolation Procedures
Grows on blood agar incubated with increased 10% CO_2 (e.g., candle jar) or anaerobically, but not in air.

Cultural Characteristics
Two millimeters after 72 hr, colonies white, adhere to agar, molar toothed but tend to shift to conical, domed, less adherent forms. Nonhemolytic, but some strains produce weak hemolysis after 7 days.

Identification
Growth in media greatly enhanced by 10–20% fetal calf serum; weak to moderate catalase activity; other properties as shown in Table 22-1

Other *Actinomyces* Species

The distinguishing characteristics of other well-recognized *Actinomyces* species are shown in Table 22-1; these are uncommon isolates in lower animals. *Actinomyces howellii* has been isolated from dental plaque in cattle. An organism designated *A. suis* has been isolated from granules in udder actinomycosis in swine (6), but its significance is unknown.

Nocardia, Actinomadura, and *Streptomyces*

These species are obligate aerobic saprophytes, found in soil. A few species are rare opportunist pathogens, causing disease in cases in which predisposing factors such as immunity or normal body defence mechanisms are grossly impaired. Colonies vary from heaped, waxy, and variably pigmented to dense, white mycelial, and moldlike (5). All generally grow in 3–5 days at 37°C and are catalase positive. Of the three genera *Nocardia* are most commonly encountered, but *Actinomadura* and *Streptomyces* may be cultured from mycetomas in animals in tropical areas. Care should be taken to distinguish these genera

from rapidly growing *Mycobacterium* species; the latter differ by the presence of rods rather than of fragmenting mycelium, by relatively poor Gram-staining, and by strong acid-fastness (7). They differ from *Actinomyces* by their aerobic rather than facultative anaerobic character, among many other properties.

Nocardia

The pathogenic *Nocardia* are often acid-fast (or partially so), gram-positive, branching filamentous rods that break up into bacillary or coccoid forms. They are strictly aerobic and catalase positive. The species of importance is *N. asteroides.* Details on the identification of *Nocardia* are available (7).

Nocardia asteroides

Pathogenicity

Nocardiosis is usually a chronic, progressive disease characterized by suppurating, granulomatous lesions. In cattle, the organism produces acute and chronic mastitis with granulomatous lesions and draining sinus tracts (8).

In dogs, cats, and other animals, localized subcutaneous lesions or lymph node involvement, or both, are seen. Generalized nocardiosis characterized by pneumonia and the accumulation of large quantities of red fluid in the thoracic or abdominal cavity, or in both, occurs not infrequently in dogs. The fluid in these cavities is serosanguinous and infrequently contains small (<1 mm) sulfurlike granules. Care must be taken to distinguish any 'Nocardia' isolated from *A. viscosus.*

Direct Examination

Granules, if present, are examined as described earlier. Gram-stained smears of pus or crushed granules reveal gram-positive branching filaments, with or without clubs (Fig. 22-1). The modified acid-fast stain often shows the retention of some carbolfuchsin, but this is not a reliable characteristic of *Nocardia.* Pus containing the characteristic elements is inoculated onto several blood slants or plates and Sabouraud dextrose agar without inhibitors. Incubate at room temperature and at 37°C for up to a week.

Cultural Characteristics

Growth is evident in 4–5 days, and colonies are irregularly folded, raised, and smooth or granular. The color varies form white through yellow to deep orange. Gram-positive, partially acid-fast mycelial filaments, which break up into bacillary forms, are evident under oil immersion. The presence of mycelial elements distinguishes nocardia

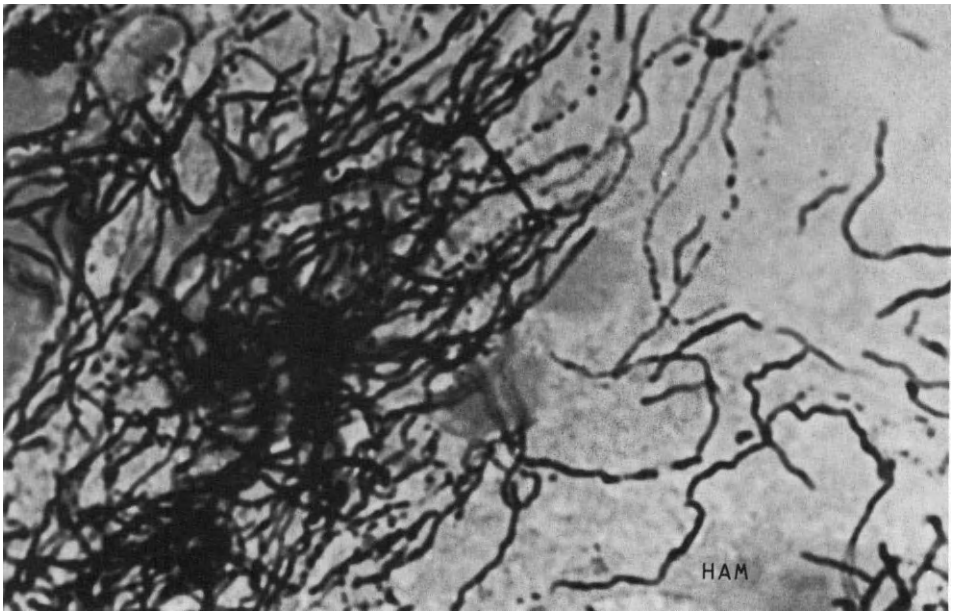

Figure 22-1. *Nocardia asteroides* in smear of pus. Gram stain, ×2250 (H. A. McAllister).

from saprophytic and atypical mycobacteria. The mycelial forms of the nocardia can be readily seen in slide cultures on Sabouraud dextrose agar. The species grows well at 45°C.

Identification

In animals, a highly presumptive identification of *N. asteroides* infection is based on pathology, demonstration of typical organisms, and colonial, cultural, and morphological characteristics. The species can be distinguished from less common *Nocardia* species, *Streptomyces,* and *Actinomadura* on the basis of properties shown in Table 22-2. Four serotypes of *N. asteroides* have been described in animal isolates (9).

Antibiotic Susceptibility

Until 1977 there was no satisfactory method for accurately determining the antimicrobial susceptibility of *Nocardia.* A study of *in vitro* susceptibility has shown that all *N. asteroides* tested were sensitive to sulfixazole, trimethoprim-sulfamethazole, doxycycline, and minocycline and most to ampicillin; about half were resistant to tetracycline (10).

Table 22-2
Identification of Selected Aerobic Actinomycetes[a,b]

Test	*Actinomadura madurae*	*Nocardia asteroides*	*N. brasiliensis*	*N. caviae*	*N. dassonvillei*	*Streptomyces griseus*	*S. somaliensis*
Acid-fastness	–	V	V	V	–	–	–
Decomposition of							
Casein	+	–	+	–	+	+	+
Hypoxanthine	+	–	+	+	+	+	–
Tyrosine	+	–	+	–	+	+	+
Urea	–	+	+	+	V	+	–
Xanthine	–	–	–	+	+	+	–
Resistance to lysozyme	–	+	+	+	–	–	–
Diaminopimelic acid[c]	m	m	m	m	m	l	l

[a]From Brown, J. M. (12).
[b]+, positive reaction; –, negative reaction; V, variable reaction.
[c]Isomer (meso, levo) of diaminopimelic acid in hydrolysates.

Other *Nocardia*

Nocardia species other than *N. asteroides* are uncommon opportunist pathogens but have been recovered on occasion (11). *Nocardia farcinica*, the cause of bovine farcy in tropical cattle, is thought to be a complex made up of either *N. asteroides* or *Mycobacterium* species (7). The original isolates made in the late nineteenth century by Nocard have long been lost.

Streptomyces and *Actinomadura*

These are very rare pathogens of animals and are most likely to be found as contaminating bacteria on agar plates. Like *Nocardia* they grow well on Sabouraud's dextrose agar; details on their differentiation are shown in Table 22-2 and given elsewhere (7,12).

Dermatophilus

The only pathogenic species is *Dermatophilus congolensis*. The one species contained in this genus is an obligate parasite or pathogen of a number of animal species and humans. It differs from the other organisms discussed in this chapter by its production of motile zoospores and by the peculiar way in which the hyphae or filaments segment.

Dermatophilus congolensis

The diseases listed below are caused by organisms that are sufficiently alike that they have been given one species name, *D. congolensis*.

Pathogenicity

The disease caused by this organism is called rain-scald, streptothricosis, or dermatophilosis. In sheep, the infection is sometimes referred to as mycotic dermatitis. Dermatophilosis is a skin infection of cattle, horses, and goats affecting small areas and characterized by the formation of crusts and a tendency to spread over large areas of the body. Removal of scabs leaves moist depressed areas. Infections have been reported rarely in humans, cats, and dogs. In sheep, three forms of the disease are described: (1) dermatitis of the wool-covered areas, referred to as *lumpy wool*; (2) dermatitis of the hairy parts of the face and also of the scrotum; and (3) dermatitis of the lower leg and foot, referred to as *strawberry foot rot*. In temperate climates the disease is a mild, often inapparent infection (13); in tropical countries it may be a cause of severe morbidity and mortality.

Direct Examination

Stained smears are made from the scabs in all of the infections. Prior to preparing the smears, it is essential to soften the scabs in distilled water. Short lengths of narrow, branching, and divided hyphae are seen as well as numerous gram-positive cocci (Fig. 22-2). Smears can also be made from the serous exudate after removal of scabs. In Giemsa-stained smears (methyl alcohol fixed), the hyphae and cocci stain deep purple while the epithelial cells are light blue and the nuclei of leukocytes are dark blue. If the organism can be demonstrated in smears, it can usually be cultured. It is generally sufficient for a diagnosis to show the unique appearance in Giemsa-stained smears of the thickened, branching filaments dividing both longitudinally and transversely.

Isolation Procedures

The organism grows well on blood agar. Plates are inoculated from clean serous exudate or from the lower aspect of moistened scabs. The organism is not fastidious and grows well on unenriched media such as tryptose agar. The organism also grows well in various broth media. There is no growth on Sabouraud dextrose agar.

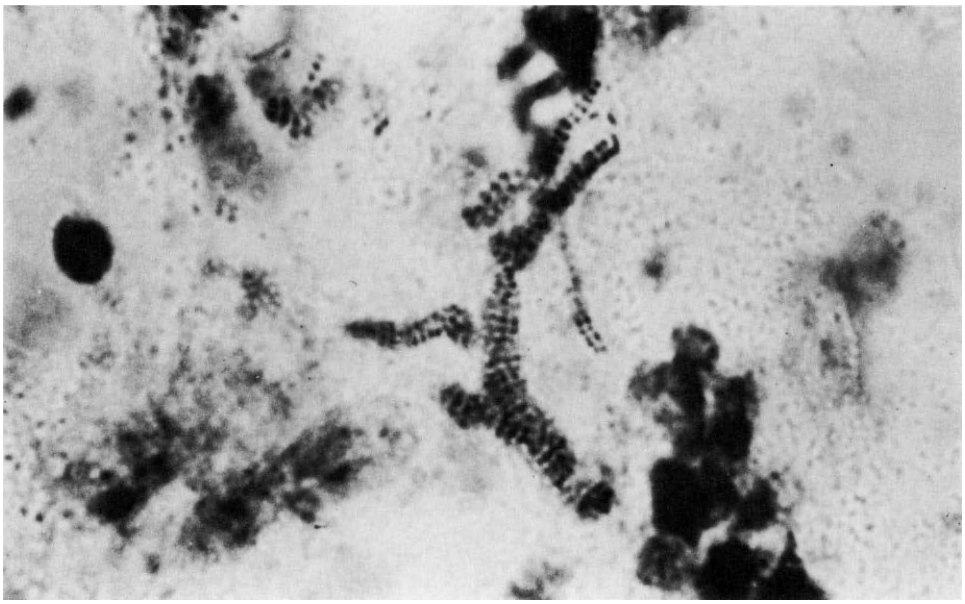

Figure 22-2. *Dermatophilus congolensis* in a smear from a horse. Giemsa stain.

Cultural Characteristics

Pinpoint colonies surrounded by small zones of β-hemolysis are evident after 24 hr of incubation at 37°C. After incubation for 3–4 days, colonies are considerably larger (Fig. 22-3). They may be wrinkled or smooth, convex, and vary in color from grayish white to bright orange.

Identification

The characteristic segmenting appearance seen in tissue is often not seen in Gram-stained smears from cultures; these may at times show cocci only but usually show gram-positive branched filamentous organisms (Fig. 22-4). Motile zoospores can be shown after growth in tryptose broth. Definitive identification is made on the unique appearance in tissue, or on the following tests: catalase (positive); urease (positive); glucose, fructose, maltose are all (positive); indole (negative); gelatin (positive); sucrose, salicin, xylose are all (negative).

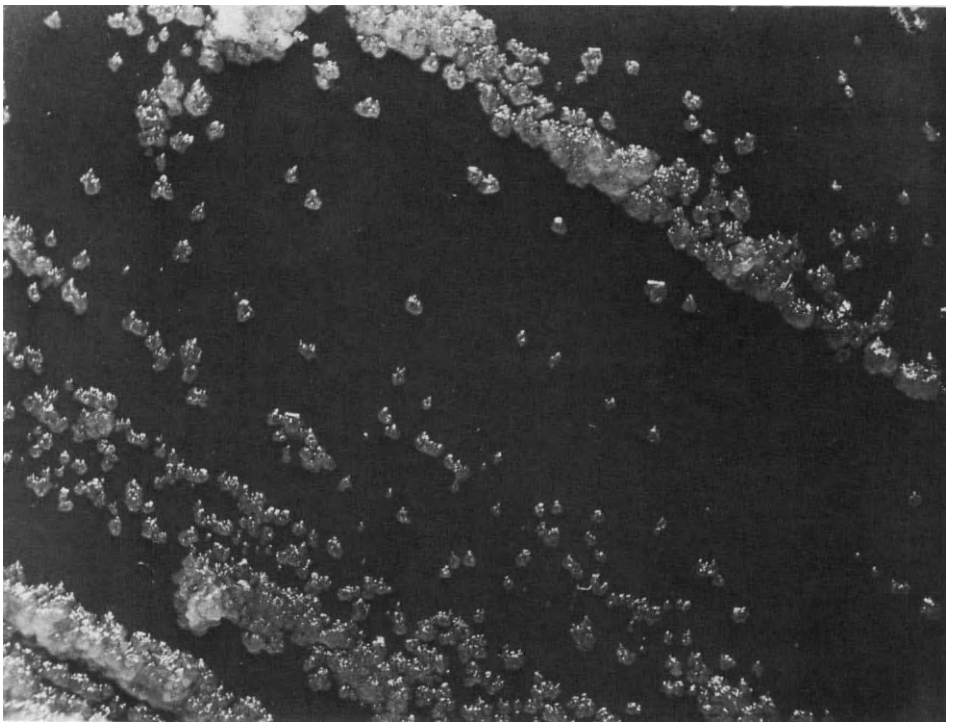

Figure 22-3. Young colonies of *Dermatophilus congolensis* on blood agar, ×8.4. From M. A. Gordon, *J. Bacteriol*, 88:509, 1964.

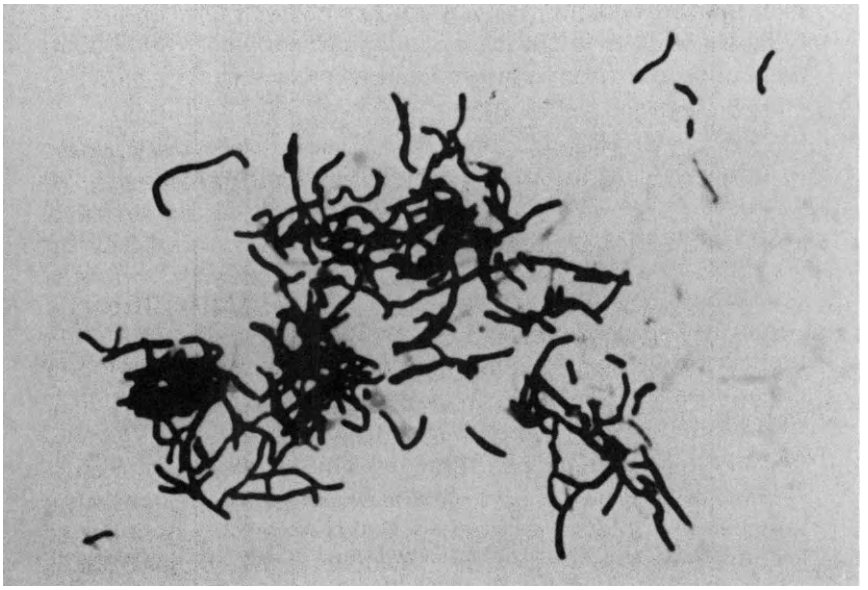

Figure 22-4. *Dermatophilus congolensis* from a blood agar plate culture. Gram stain, ×1900.

Antibiotic Susceptibility
Many drugs inhibit the growth of *D. congolensis in vitro*. These include penicillin, tetracyclines, aminoglycosides, erythromycin, and chloramphenicol. Penicillin–streptomycin is a favored combination.

Rhodococcus equi

Until recently described as *Corynebacterium equi* (14).

Pathogenicity
This is a common cause of bronchopneumonia in foals—rarely in adult horses—cervical lymphadenitis of swine, occasional isolations from lungs and lymph nodes of other animals and humans, especially immunosuppressed individuals. Common in soil in the presence of herbivore manure.

Direct Examination
It commonly appears coccoid on direct examination of clinical material; sometimes rod-shaped, rarely see hints of branching.

Isolation Procedures
It grows well on blood agar incubated aerobically at 37°C; selective media described for isolation from feces or soil (15).

Cultural Characteristics
The organism grows in 48 hr as smooth, mucoid, translucent, tear drop colonies, 3–5 mm in diameter. On some media (e. g., glucose–yeast extract) colonies may be orange to reddish, particularly with age, but on blood agar they are translucent to gray-whitish. Colonial variation in morphology may be marked (16).

Identification
Organisms are 0.5–1.0 × 1.0–2.0 μm coccoid rods. Generally nonreactive in standard tests; catalase positive, urease positive, fails to ferment carbohydrates. A simple identifying characteristic is the invariable production of *equi* factors, diffusing partial hemolysins that enhance the hemolysis of the phospholipase D of *Corynebacterium pseudotuberculosis* or the β-hemolysin of *Staphylococcus aureus*. The effect is most dramatic with *C. pseudotuberculosis* and is shown by streaking the *R. equi* to be identified at right angles to a streaked culture of *C. pseudotuberculosis* and incubating for 24–48 hr (17). The APIZYM® enzyme assay system has been reported to produce rapid and reliable identification of *R. equi* (18).

Rhodococcus sputi

Rhodococcus sputi, a species isolated from human patients with chronic pulmonary disease, has been recovered from mesenteric lymphadenitis in swine (19).

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23

Mycobacterium

Charles O. Thoen

The genus *Mycobacterium* includes acid-fast bacteria that are known pathogens, along with numerous saprophytes. The latter are ubiquitous in the environment and occur in soil and water; some are present in the gastrointestinal tract of some humans and animals. It is the responsibility of the laboratory to isolate and identify mycobacteria from specimens of live animals or tissues collected at necropsy. It is necessary to differentiate those organisms that cause contagious or infectious disease from saprophytes (4,5,28). The latter may produce disease in certain situations and are considered opportunists. For example, some otherwise saprophytic mycobacteria may cause infection when introduced into wounds in humans or animals receiving immunosuppressive therapy. Since the saprophytes have staining properties similar to the pathogenic types, a microbiologist working in the diagnostic laboratory must be prepared to differentiate them by bacteriologic procedures.

Tubercle bacilli are the most important clinically significant mycobacteria. *Mycobacterium bovis, M. avium* complex (MAIS complex), and *M. tuberculosis* have been recognized for nearly a century and continue to cause disease in animals, resulting in serious economic losses. Remarkable progress has been achieved toward eliminating bovine tuberculosis in cattle in the United States, yet outbreaks of disease still occur (11). The importance of tuberculosis in exotic captive and wild animals has been emphasized by the widespread occurrence of disease in various species maintained in zoos, animal parks, and primate colonies (42).

Numerous host mechanisms have been associated with the susceptibility and development of disease in animals exposed to virulent tubercle bacilli (29). Although mycobacteria initially encounter normal

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humoral components and granulocytes (neutrophils), the activities of activated mononuclear macrophages are considered to be most important in protecting the host against *M. bovis*. Macrophages are involved in processing mycobacterial antigens and in presenting antigens to T lymphocytes, which are considered a key recognition unit in the immune response to mycobacteria. The subsequent interaction of lymphocytes with specific antigens stimulates the release of soluble substances (lymphokines) that attract, activate, and increase the number of mononuclear cells at the site of infection.

Deficiencies of T-lymphocyte function and mononuclear cell dysfunction have been associated with certain opportunistic mycobacterial diseases (2,3). This abnormality may be mediated by an imbalance of the metabolic products of arachidonic acid, because the lymphocyte responses to specific purified protein derivatives from *M. avium* complex improved in cultures containing indomethacin (29). A similar suppression of lymphocyte responses has been observed when mononuclear cells were treated with tuberculosis plasma and mycobacterial arabinogalactan.

The capacity of tubercle bacilli to produce progressive disease may be related to certain complex lipids present in the cell wall, such as cord factor, sulfur-containing glycolipids (sulfatides), or strongly acidic lipids (29). However, it appears that the effect of these components alone or together on phagosome–lysosome fusion cannot account for virulence. Available information indicates a combination of toxic lipids and factors released by virulent *M. bovis* may cause disruption of the phagosome, interference with phagolysosome formation, release of hydrolytic enzymes from the attached lysosomes, or inactivation of the lysosomal enzymes released into the cytoplasmic vacuole.

Microscopic Examination

Preliminary examination of tissues suspected of being tuberculous should include the preparation of suitably stained smears. The demonstration of acid-fast bacilli by microscopic examination of lesions is important because cultures of material with positive smears will usually grow mycobacteria. In contrast, a negative smear only means that no acid-fast bacilli are seen; it does not mean that no acid-fast bacilli are present. The significance of microscopic findings on smears of specimens in which tuberculosis is suspected should be discussed with veterinarians and epidemiologists responsible for conducting follow-up investigations on exposed animals.

An identifiable smear can be made on a new slide from scrapings of the cut surface of tissue. The smear should be air dried and fixed by flaming for 1–2 sec. The Kinyoun modification of the Ziehl–Neelsen

stain is recommended because no heat is required (34). The stained smears are observed with an ordinary light microscope for the presence of acid-fast bacilli, which appear as red coccoidal or bacillary cells 1–3 μm in length occurring singly or in clumps. An alternate method utilizing fluorescein dyes such as auramine and rhodamine has been described (24). These slides must be observed using an ultraviolet light with a microscope equipped with special filters. Acid-fast bacilli are seen as short rods. Those stained with auramine appear yellow, while those stained with rhodamine appear red.

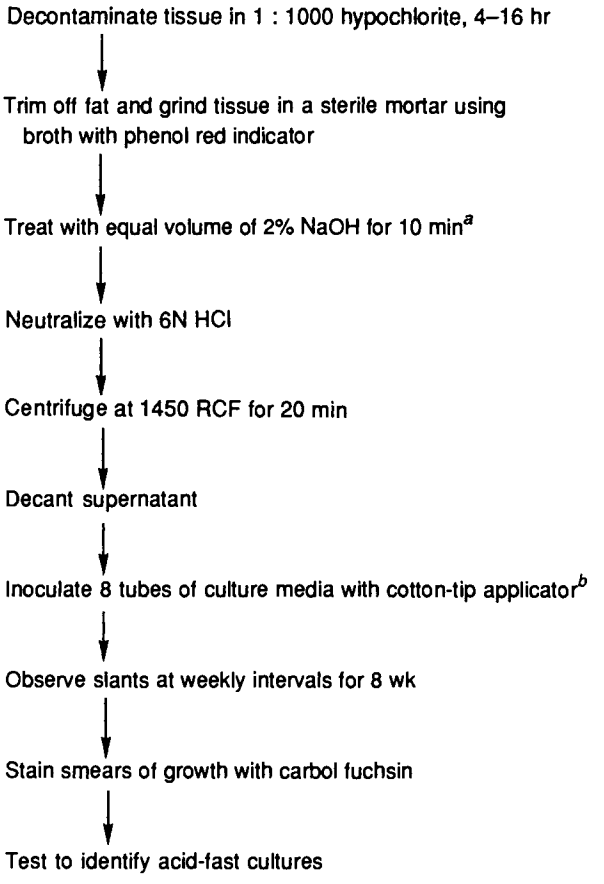
Isolation

Appropriately stained smears provide information on the presence of acid-fast bacilli; however, it is important to remember that a specific diagnosis of tuberculosis requires the isolation and identification of the organism from exudates, body discharges, or from lesions. The procedures for isolating mycobacteria are different from the methods used for culturing other bacteria because specimens must be treated to kill contaminants. Acid-fast bacteria are relatively resistant to alkali and to acids that have been used for this purpose. Most pathogenic mycobacteria of clinical significance grow very slowly; therefore, isolation and final identification may require several weeks. The mycobacteria that cause disease in animals may also infect humans; therefore, laboratories where mycobacteriologic examinations are conducted should be equipped with biologic safety cabinets with separate air exhaust filters. Moreover, routine tuberculin skin tests and/or radiologic examinations should be available for tuberculin-negative employees working in the laboratory and for animal caretakers exposed to infected animals.

Specimens for bacteriologic examination should be collected aseptically and immediately submitted to the laboratory for processing. In instances where specimens must be transported by mail, the specimens should be placed in sodium borate (saturated solution) to minimize the growth of bacterial contaminants (6,44).

A flow diagram of a procedure for isolating mycobacteria is shown in Table 23-1. When excessive contamination is present, it may be necessary to increase the time interval for treatment with 2% sodium hydroxide solution from 10 to 20–30 min to obtain suitable cultures (41). The treated tissue suspensions are used to inoculate various kinds of culture mediums (18,43). Inoculated media should be incubated at 37°C in a slanted position so that the inoculum covers the surface. The cultures should be placed in a tray at about a 45° angle for 1 week and then transferred to a vertical position. The cultures should be observed for the appearance of growth at weekly intervals for 8 weeks. When

Table 23-1
Procedure for Isolating Mycobacteria



From *Vet Microbiol*, 8th ed. 1978. Courtesy of Iowa State University Press.

[a]Urine, milk, and body fluids may be treated with equal volume of 2% NaOH and neutralized with HCl; media may be inoculated with supernatant.

[b]One slant each of Lowenstein-Jensen, Lowenstein-Jensen with glycerol, Egg Yolk Agar (EYA). EYA with glycerol and malachite green, EYA with glycerol, malachite green and mycobactin (2 mg/liter), Middlebrook 7H10 with pyruvate (4.1 g/liter), and two slants of Stonebrinks medium with pyruvate (5 g/1.2 liter).

Mycobacterium paratuberculosis is suspected, the incubation period should be increased to 20 weeks. When growth is detected, smears should be made of colonies and stained by the Ziehl–Neelsen method to determine whether they are acid-fast. A magnifying lens is useful in examining medium slants for the presence of young colonies. Colony morphology is an important characteristic of certain mycobacteria; however, it is not always reliable. Although variations occur, *M. bovis*

colonies usually appear mammilate with a raised center, *M. tuberculosis* colonies have a crumbled-bread appearance, and *M. avium* colonies are often doughnut-shaped. It should be emphasized that colonies of some saprophytes may closely resemble these pathogens; therefore, additional laboratory tests are necessary for their identification.

Identification

The specific identification of acid-fast bacilli involves the use of biochemical and drug susceptibility tests. Characteristics for identifying certain pathogenic mycobacteria are shown in Table 23-2. The details for supplemental procedures used in identifying mycobacteria will not be included here because they have been described in readily available microbiology textbooks (24,46). When problems are encountered in conducting *in vitro* tests for identification, it may be necessary to obtain assistance from a reference laboratory that routinely conducts the tests.

Seroagglutination tests are of value in identifying strains of *M. avium* complex, which includes certain slowly growing mycobacteria previously called *M. avium–M. intracellulare* (Battey bacilli) (24,25). Currently, 30 serotypes of *M. avium* are recognized (24). Serologic tests may be conducted using antisera prepared in rabbits or mice for representative strains (31,44).

Animal pathogenicity tests may also be employed in identifying mycobacteria; however, these procedures are not routinely used because they are expensive and time-consuming. Animal tests are useful in the identification of unusual strains that have aberrant drug or biochemical characteristics. In instances in which mixed cultures present problems, guinea pigs, rabbits, and/or chickens may be inoculated (Table 23-3). Animal inoculation may also be of value when small numbers of organisms are present or when excessive contamination is not controlled by the decontamination procedure.

Clinically Significant Mycobacteria

Some reliable characteristics are included for each of the species.

Mycobacterium bovis (the bovine tubercle bacillus)

This fastidious organism causes tuberculosis in cattle, swine, and cats. It has also been associated with outbreaks of tuberculosis in nonhuman primates and certain exotic hoofed animals (42). Growth appears after 3–8 weeks of incubation at 37°C. Growth in Proskauer and Beck liquid medium (P & B) with 5% serum (P & B) is granular in appear-

Table 23-2
In Vitro Tests for Identifying Some Clinically Significant Mycobacteria[a]

Mycobacterium	Growth rate			Niacin	Nitrate reduction	Cord formation	Thiophen-2-carboxylic acid-hydrazide	NaCl tolerance	Tween 80 hydrolysis	Chromogenicity	Arylsulfatase at 3 days	Glycerol inhibition
	43°C	37°C	31°C									
M. tuberculosis		M		+	+	+	+	–	–	–	–	+
M. bovis		M		–	–	+	–	–	–	–	–	–
M. avium[b]	M	M	M	–	–	–	+	–	–	–	–	+
M. kansasii		M	S	–	+	V	+	–	+	+	–	+
M. marinum			S	–	–	–	+	–	+	+	–	+
M. scrofulaceum		M	S	–	–	–	+	–	–	+	–	+
M. xenopi	S	S		–	–	–	+	–	–	+	V	+
M. fortuitum		R	R	–	+	V	+	+	–	–	+	+
M. chelonei		R	R	V	–	–	–	–	–	–	+	+

[a]Symbols: –, Negative: absence or inhibition; +, positive: production or growth; V, variable; R, rapid (1–6 days); M, moderate (6–14 days); S, slow (more than 14 days).
[b]Includes strains previously identified as *M. intracellulare* (12). *Mycobacterium avium* serotypes 1 and 2 grow best at 43°C; some strains of serotypes 3 through 25 grow best at 22–30°C.

Table 23-3
Pathogenicity of *Mycobacterium bovis*, *M. tuberculosis*, and *M. avium* in Laboratory Animals

Animal	Organism		
	M. bovis	*M. tuberculosis*	*M. avium*
Guinea pig	+	+	−
Rabbit	+	−	+[a]
Chicken	−	−	+

[a]Yersin-type reaction (1).

ance, and strains form short cords. The organism fails to produce niacin or reduce nitrate and is inhibited by thiopen-2-carboxylic acid hydrazide (TCH).

Mycobacterium tuberculosis

This organism (the human tubercle bacillus) causes pulmonary disease in humans, monkeys, baboons, and certain hoofed animals; it has also been isolated from dogs and parrots (15,35). Buff-colored, raised, rough colonies usually appear after 2–4 weeks of incubation at 37°C. Growth in P & B is flocculent, and most strains form serpentine cords. The organism produces niacin, reduces nitrates, and growth is not inhibited by TCH. Most strains of *M. tuberculosis* are inhibited by *p*-aminosalicylic acid (PAS), isonicotinic acid hydrazide (INH), streptomycin, and ethambutol; however, drug-resistant strains have been isolated from human patients. (46).

Mycobacterium avium–M. intracellulare complex

Currently, there are more than 30 different serotypes in the M. avium–intracellulare complex (MAIS complex), including some organisms previously called "Battey bacilli." The isolation of these organisms from patients with acquired immune deficiency syndrome (AIDS) has stimulated an increased interest in their source(s) (48). *Mycobacterium avium* complex is the most common cause of tuberculosis in swine (21,32). However, the serotypes isolated from pigs in different geographical areas of the United States vary (38). Serotypes 1 and 2, commonly isolated from birds, are usually pathogenic for chickens, whereas serotypes 4–30, which have been isolated from humans and other animals, fail to produce progressive disease in chickens (14,33,39). Colonies usually appear in 2–3 weeks incubation at 37°C; they are raised, rounded, smooth, buff or slightly yellow color. Growth in P & B is turbid; cords are not formed. The organisms fail to produce niacin, to reduce nitrates, or to hydrolyze Tween 80.

Mycobacterium kansasii

The organism has been isolated from pulmonary and extrapulmonary lesions in humans (13); it has been isolated from lymph nodes of cattle, swine, and certain exotic animals (34). The importance of this organism in inducing tuberculin-skin reactivity to mammalian tuberculin has been discussed (12). Growth appears in 2–4 weeks of incubation at 37°C. The colonies are colorless to buff color when incubated in the dark but are photochromogenic; that is, the colonies develop yellow color when exposed to light. The microorganism does not produce niacin, but it does reduce nitrates and hydrolyze Tween 80. Some strains form cords and produce carotene crystals.

Mycobacterium marinum

The organism causes swimming pool granuloma in humans; isolations have been made from cold-blooded animals (25). Growth appears in 3–6 weeks of incubation at 30°C. The organism, like *M. kansasii*, is photochromogenic but may be differentiated by its failure to reduce nitrate. Tween 80 is hydrolyzed. Cords and carotene crystals are not formed, and niacin is not produced.

Mycobacterium scrofulaceum

The organism has been isolated from cervical lymph nodes of children and from lymph nodes of pigs (32,46). Growth appears in 2–3 weeks of incubation at 37°C as raised, rounded, yellow to orange colonies in the dark or in light. The organism does not produce niacin, reduce nitrates, or hydrolyze Tween 80.

Mycobacterium xenopi

A few reports are available on isolations of this organism from animals (34). Yellowish, rounded, smooth colonies usually appear in 3–5 weeks incubation at 42–45°C. The organism does not produce niacin, reduce nitrates, or hydrolyze Tween 80. On cornmeal agar, the colonies have a characteristic matted appearance with radiating filaments that may be observed with low-power magnification ($\times 7$) after 2 weeks incubation.

Mycobacterium fortuitum

This organism causes pulmonary disease in humans, thoracic granulomas in dogs, skin granulomas in cats, and mastitis in cattle. The microorganism is often resistant to chemotherapeutic agents used for treating tuberculosis (24). Growth appears in 2–6 days of incubation at 25–37°C as raised, rough, buff-colored colonies. Some strains from loose cords. The cultures fail to produce niacin but do not reduce nitrates and produce arylsulfatase. Growth is observed in media containing 5% sodium chloride.

Mycobacterium chelonei

This mycobacterium has been isolated from injection abscesses and from patients with valvular endocarditis (24); it has also been cultured from lesions in animals (34). It is similar to *M. fortuitum,* but it fails to reduce nitrates. No growth is observed in 5% sodium chloride.

Mycobacterium lepraemurium

The rat leprosy bacillus has not been cultivated on solid media used routinely for cultivating other mycobacteria. However, growth of this organism has been observed in liquid medium enriched with cytochrome *c* and α-ketoglutarate (22). This organism has been suggested as the etiologic agent of a leprosylike disease in cats (17).

Mycobacterium leprae

The leprosy bacillus was first observed by Hansen in Norway; therefore, infection with this organism is sometimes called Hansen's disease. *Mycobacterium leprae* is considered the cause of a naturally occurring disease in armadillos (45). Definitive information is not available on the growth of *M. leprae* in cell-free culture medium; however, the organism will multiply in the footpads of mice.

Mycobacterium paratuberculosis

The Johne's bacillus was first identified in 1895 by Johne and Frothingham while studying chronic dysentery in cattle. The organism has been isolated from cattle, sheeep, goats, swine, and exotic animals in

the United States and Canada (2,10,28,30,32,36,42,47). *Mycobacterium paratuberculosis* is mycobactin-dependent and grows very slowly at 37°C incubation (1,7,19,20). Growth usually appears at 4–10 weeks. The colonies are colorless to white and translucent. Because a prolonged interval is required to isolate this mycobacterium, a presumptive diagnosis may be obtained by preparing appropriately stained smears of biopsies of mucosa of the rectum (34). On microscopic examinations, acid-fast bacilli are readily observed in clumps within macrophages (27).

Cultures for *M. paratuberculosis* may be made on mycobactin-enriched medium and medium without mycobactin after the specimens (intestinal mucosa or feces) are treated with benzalkonium chloride (Zephiran) (44). A portion of intestine 3–4 cm anterior to the ileocecal valve and 3–4 cm posterior with associated lymph nodes should be collected on necropsy, washed to remove fecal material, and submitted to the laboratory frozen. The tissues may be ground in 3% trypsin and the sediment decontaminated in 0.1% Zephiran for 2–16 hr. Fecal specimens (about 1 oz) collected from the rectum of animals suspected of having Johne's disease should be placed in appropriately identified ointment containers. No refrigeration is required. The sample should be transported to the laboratory and processed within 24–36 hr to minimize contamination. An aliquot of the feces is suspended in sterile water and processed with 0.3% Zephiran for 16–20 hr. More recently, cetylpyridinium chloride (0.5–1%) has been used in some laboratories for isolating mycobacteria including *M. paratuberculosis* (26). Herrold's egg yolk agar slants are inoculated with treated suspensions (three tubes with mycobactin and one without mycobactin). Inoculated media should be incubated at 37°C and examined at periodic intervals for 20 weeks. Cultures are identified by growth rate and mycobactin dependency. It should be noted that when large numbers of fecal samples are collected at one time, it is possible to store specimens at −70°C prior to processing in the laboratory.

Measures for controlling and eliminating Johne's disease in cattle have been based on the diagnosis and elimation of infected cattle shedding the organism in the feces (8,16). The success of controlling Johne's disease has been limited by the lack of a rapid diagnostic test. Further investigations are needed to determine the practical value of serologic tests in detecting animals with subclinical Johne's disease. New developments in biotechnology should provide antigens with improved specificity (9). The importance of developing a rapid, simplified diagnostic test has stimulated an interest in enzyme-linked immunosorbent assays (ELISA) for detection of animals exposed to *M. paratuberculosis* and other mycobacteria (1,37,40). Production of immunoglobulin class or subclass specific conjugates for use in ELISA could improve the sensitivity and specificity of ELISA for identification of

diseased animals (28). The importance of vaccine containing killed *M. paratuberculosis* suspended in oil on development and persistance of humoral and cellular responses needs additional investigation. Genetic engineering techniques may be utilized to produce improved vaccines that induce minimal immunologic responses.

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24

Miscellaneous Bacteria and Prototheca

G. R. Carter

Included in this chapter are a number of bacteria that are not included with other major pathogenic categories. Some cause infrequent infections in the lower animals and others are recovered occasionally from clinical materials. Also included are brief discussions of some potentially pathogenic and toxic algae. The latter, although not bacteria, are recovered occasionally in the diagnostic bacteriology laboratory.

Miscellaneous Bacteria

Chromobacterium

Bergey's Manual lists two species of this genus, *Chromobacterium violaceum* and *C. fluviatile.* The former species *C. levidum* is now recognized to be *Janthinobacterium lividum* (1). Both *Chromobacterium* spp. occur in soil and water, but only *C. violaceum* has been incriminated in disease. Both species are facultative anaerobes and produce pale violet colonies on ordinary media. *Chromobacterium fluviatile* grows best at 25°C while *C. violaceum* has an optimum temperature of 30–35°C. Additional criteria for the identification and differentiation of these species are given in *Bergey's Manual* (1).

Chromobacterium violaceum

Pathogenicity

Most infections occur in tropical and subtropical countries. It is an infrequent cause of suppurative pneumonia in swine and cattle.

Among the human infections reported are urinary tract infections, localized abscesses, and systemic infections with localized abscesses.

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Isolation and Cultivation
This species grows well on ordinary unenriched media, including MacConkey's agar, but it is usually isolated on blood agar. On this medium, colonies are black to purple, smooth, and shiny, attaining a size of about 3 mm in diameter after incubation at 37°C for 24 hr. A violet to purple pigment is produced when the organism is grown in broth.

Identification
A short to medium, motile, gram-negative rod, producing violet to purple colonies would strongly suggest *C. violaceum*. See *Bergey's Manual* (1) for definitive identification. Some important characteristics are

- Acid reaction in TSI butt
- Indole is negative; some nonpigmented strains are positive.
- Nitrates are reduced by most strains.
- Litmus milk is peptonized.
- H_2S is not produced.
- MR and VP tests are negative.
- Catalase is almost always positive.
- Glucose is fermented; sucrose variable; maltose, xylose, mannitol and lactose are negative.
- Arginine dihydrolase is produced.
- Lysine and ornithine decarboxylases are not produced.

Streptobacillus moniliformis

This is an extremely pleomorphic gram-negative rod that may have rounded or pointed ends and appear as long, curved and looped filaments. It may, on initial isolation, produce L-type colonies. In smears from clinical materials, the organisms are usually seen as fairly uniform rods with some short filaments. Giemsa or Wayson stain may be more suitable than the Gram method for demonstrating the organism in clinical materials.

Pathogenicity
This organism is a normal inhabitant of the throat and nasopharynx of rats, both wild and laboratory varieties. Some cats may also harbor it. It is associated with myocoplasmas in pneumonic lesions in rats and is considered to be the cause of the disease of mice characterized by diverse manifestations, including septicemia, septic arthritis, hepatitis, and lymphadenitis. Isolations have been made from the middle ears of mice and rats. Outbreaks of *S. moniliformis* infection attributed to rat bites have been reported in turkeys (2). Clinical signs and lesions

were associated with the joints. This species has also been recovered from guinea pigs with cervical abscesses (3).

The infection in human beings is usually called rat-bite fever and is characterized by septicemia and polyarthritis. The disease acquired from drinking rodent-contaminated milk has been called Haverhill fever. Some infections have occurred with no history of a rodent bite. Treatment with penicillin or broad spectrum antibiotics is effective if initiated early.

Isolation Procedures

Blood or other clinical materials can be inoculated directly into thioglycollate broth or BHI semisolid medium containing 10–20% horse serum. Other material is inoculated onto serum or blood agar. Plates should be incubated in air containing 5% CO_2. Mohamed *et al.* (2) incubated their plates in a candle jar. Rogosa (4) has described special solid and fluid media for isolation of the organism.

Cultural and Morphologic Characteristics

Growth in thioglycollate broth or BHI semisolid medium is characterized by the formation of small, fluffy colonies referred to as fluffballs. Colonies are removed with a Pasteur pipette, and smears are stained by the Giemsa or Gram procedure.

On solid media incubated aerobically, small discrete colonies develop within 2–3 days. They are glistening, smooth, colorless to grayish, and irregularly round. Minute L-type colonies may be seen under low power or with a dissecting microscope. Giemsa-stained smears of these minute colonies reveal a variety of forms resembling somewhat those of the pleuropneumonialike organisms. They are best examined *in situ* by Dienes's staining procedure (see Appendix A). Gram-stained smears from the large colonies on solid media and from the fluffballs disclose a remarkable variety of morphological elements including small, slender rods and abundant curved filaments with numerous moniliform (necklacelike) swellings.

Identification

This is generally based upon cultural and morphologic characteristics, source, and associated disease if present.

Two of the turkey isolates of Mohamed *et al.* (2), when incubated in carbohydrate broths enriched with 15% horse serum, fermented maltose, dulcitol, sorbitol, lactose, arabinose, sucrose, and dextrose; trehalose and mannitol were not fermented, and the results with salacin were variable. For additional information on the identification of *S. moniliformis,* readers are referred to the discussion by Rogosa (4).

Bacillus piliformis

This interesting gram-negative organism, which is motile and produces a characteristic spore, has not been cultivated in artificial media. It clearly does not belong in the genus *Bacillus*.

Pathogenicity

Natural Tyzzer's disease has been observed in mice, rats, gerbils, guinea pigs, rabbits, dogs, cats, foals, calves, muskrats, foxes, and monkeys. The epidemic form is a major cause of losses in laboratory mice, particularly those subjected to various stresses such as thymectomy, irradiation, and cortisone treatment. The disease in mouse colonies may be endemic and present in a latent or subclinical state. The principal lesion seen in the acute disease consists of diffusely distributed pale gray necrotic foci in the liver. Lesions may also be present in the small intestine, cecum, and colon.

Direct Examination

Smears are made from the necrotic foci and stained with Giemsa stain. Long, slender organisms staining bluish-purple are seen in the cytoplasm of hepatic cells. Very long, thin, and tortuous filaments are sometimes seen, as well as shorter bacillary forms and occasional forms with subterminal swellings (Fig. 24-1). The latter resemble the

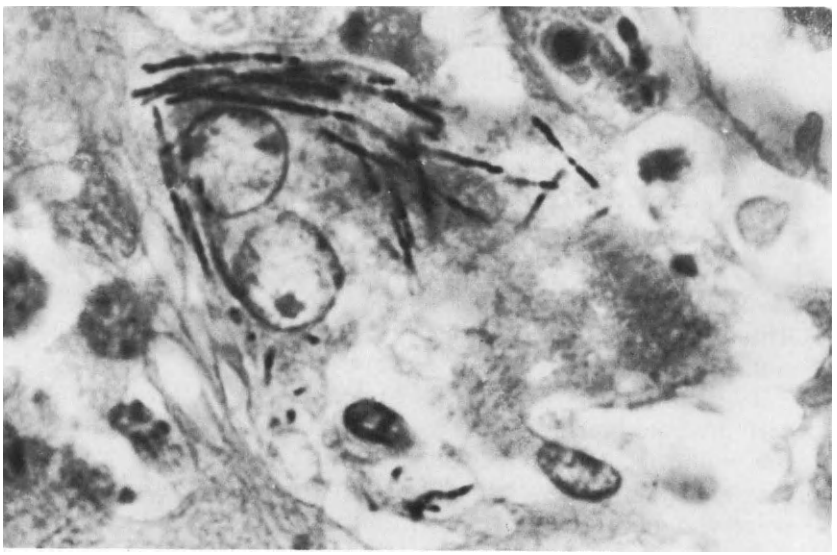

Figure 24-1. *Bacillus piliformis* in hepatic cells. Gomori methenamine–silver nitrate and HE method, ×2000. (Courtesy of Doctor D. Fujiwara.)

moniliform structures that are characteristic of *Streptobacillus moniliformis*. Spores about 1 μm in diameter are seen infrequently in smears and tissue sections (5). Bacillary forms are frequently tapered, and beading is often noted. The motility of *B. piliformis*, due to peritrichous flagella, can be seen with phase-contrast microscopy.

Tyzzer's disease is occasionally diagnosed after routine examination of histologic sections of liver stained by the Warthin–Starry method or other stains used for bacteria. Filamentous rods are seen lying in bundles arranged randomly in the cytoplasm of hepatic cells at the border of necrotic and normal tissue (6).

Isolation Procedures

Methods for the reliable cultivation of *B. piliformis* in artifical media have not been developed. The organism has been cultivated successfully in the yolk sac of embryonated chicken eggs (7). The following procedure has been employed successfully (8): A 10% suspension of affected liver is prepared in brain–heart infusion broth with the aid of a Ten Broeck grinder. After centrifugation in a clinical centrifuge at 2000 rpm for 10 min, 0.2 ml of supernatant is injected into the yolk sac of each of six eggs. The embryos usually die between the fourth and seventh day postinoculation.

Giemsa-stained smears from the embryonic liver and yolk sac disclose the highly pleomorphic and characteristic forms of *B. piliformis*. The viability of organisms can be preserved for a number of months if the yolk material is frozen and stored at −70°F.

Identification

A diagnosis of Tyzzer's disease is based upon the demonstration of the typical organisms with the varied pleomorphic forms in Giemsa-stained smears from the necrotic foci in the liver. Cultivation in the yolk sac of the embryonated egg is confirmatory. Finding typical organisms in stained sections of the characteristic liver lesions is also diagnostic.

The indirect immunofluorescence technique has been used for the identification of *B. piliformis* (9).

Lactobacillus

Lactobacilli are rarely pathogenic. They are occasionally isolated from clinical specimens.

Occurrence

Associated with dairy products, normal flora of the mouth, vagina, and intestinal tract

Characteristics
Long, slender, gram-positive rods often in chains; aerobic and facultatively anaerobic; fermentative, and grow best at a pH near 6. They are catalase negative and nonmotile, which aids in distinguishing them. Lactobacilli can be confused with *Corynebacteria, Listeria,* and nonsporulating *Clostridium perfringens.*

Kurthia

Species of this genus are soil saprophytes that are occasionally recovered from clinical materials but are rarely pathogenic.

Characteristics
Large, gram-positive, motile rods that resemble *Bacillus* spp. but do not produce spores. They are facultatively anaerobic, catalase positive, and oxidase negative; acid is not produced from carbohydrate breakdown.

Oerskovia

Species of this genus are soil saprophytes that occur occasionally in clinical materials but are rarely involved in infections.

Characteristics
They are actinomycetes that are seen as branching gram-positive, filamentous forms that fragment into coccoid forms after extended incubation. Most strains produce a yellow pigment. They are easily confused with some corynebacteria, from which they can be usually differentiated by the fact that *Oerskovia* hydrolyze esculin and are motile (10).

Groups DF-2

Organisms of this group occur as commensals in the mouth and nasopharnyx of dogs, for which they appear to have little pathogenicity. Human infections, particularly in individuals with underlying disease or splenectomies, have resulted from dog bites (11).

Some of the important characteristics of this group, according to Weaver *et al.* (11) are as follows:

- Small, gram-negative, long thin rods in clinical materials
- Nonmotile and facultatively anaerobic

- Grow on blood agar in a candle jar at 35–37°C
- Colonies convex, smooth and round
- No growth on TSI agar or O-F glucose medium
- No growth on MacConkey agar
- Fermentative if serum added to carbohydrate broth
- Catalase and oxidase positive
- Urease negative
- Nitrate not reduced
- Arginine dehydrolase reaction is usually negative

As a result of a recent study (12) which included DNA analysis, two new species, *Capnocytophaga canimorsus* and *C. cynodegmi*, have been proposed for DF-2 and DF-2-like bacteria, respectively. The DF-2-like strains differed from the DF-2 strains in producing acid from sucrose, raffinose, inulin, and melibiose. The DF-2 strains produced acid from lactose, maltose, and, usually, D-glucose. In other respects they were the same.

Simonsiella

This gram-negative bacterium, which occurs as part of the normal oral flora of humans and animals, is motile and nonsporing. These nonpathogenic saprophytic organisms have been reported from dogs, cats, horses, pigs, cattle, sheep, guinea pigs, goats, and fowls.

Its characteristic morphology, which makes possible its recognition in stained smears from oral mucous membranes, is described as follows (13): "Cells 0.4–0.7 by 2–4 μm, closely apposed to form filaments with free faces of terminal cells, rounded. Filaments are flat, not cylindrical, and divide into hormogonia-like units" (Fig. 24.2). Two species, *S. muelleri* and *S. crassa*, have been described.

They can be readily isolated on blood agar from oral mucous membranes. Colonies are 1–3 mm in diameter, smooth, convex, translucent after 3–4 days incubation, and usually β-hemolytic. Identification is usually based upon the characteristic morphology of the organism in Gram-stained smears from typical colonies. Detailed descriptions and differentiation of the two species are provided by Steed-Glaister (14).

Prototheca

Species of this genus are microscopic, colorless, achlorophylic algae of the family Chlorellaceae. They occur widely in nature and are occasionally recovered from clinical specimens. There are a number of reports of human infections, bovine mastitis (15), and infections in dogs

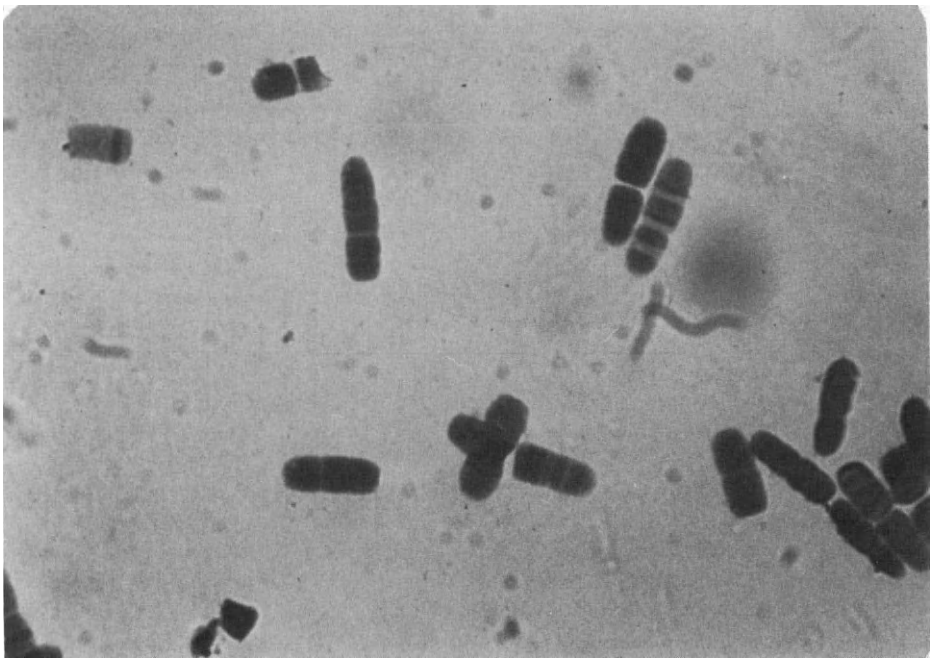

Figure 24-2. Gram-stained smear of *Simonsiella* (originally identified as *Caryophanon*) from the canine gingiva, × ~1600. (From D. A. Saphir and G. R. Carter, *J. Clin Microbiol*, 3:344, 1976.)

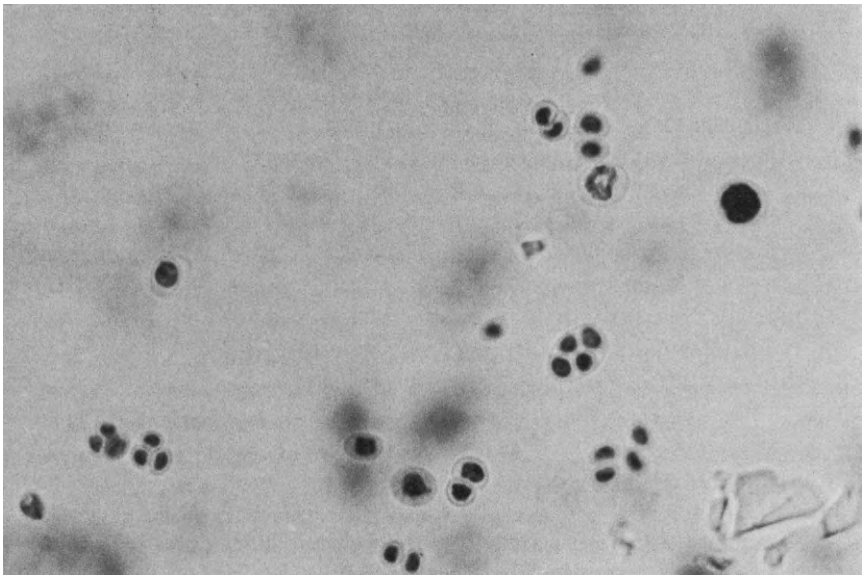

Figure 24-3. *Prototheca* from milk of a cow with mastitis. Note endospores. Lactophenol cotton blue, ×300 (Paul C. Watkins).

and cats due to these algae. Migaki *et al.* (16) have reviewed the literature on canine protothecosis, which usually begins in the gastrointestinal tract and progresses to systemic involvement. Myriads of protothecal organisms in different stages of development can be seen in tissues examined histologically. Organisms can also be demonstrated in scrapings and smears with Giemsa or Wright's stain.

Small colonies resembling *Cryptococcus* are produced on Sabouraud agar (25°C) and blood agar (37°C) in 24 hr. They do not have a capsule and are hyaline and globose to oval in form, with width and length as great as 13–16 μm. As many as eight or more characteristic endospores are produced by internal segmentation (Fig. 24-3). Presumptive identification of *Prototheca* is based upon the characteristic morphology of the organisms as seen in smears, scrapings, histological sections, and cultures. The API 20C system (see Chapter 36) has been used to identify species. Five species of *Prototheca* have been identified with fluorescent antibody reagents (17).

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25

Rickettsiae and Chlamydiae

Johannes Storz

Rickettsiae and chlamydiae are host-cell-dependent, obligate intracellular, prokaryotic organisms pathogenic for humans and animals. These infectious agents are currently classified in the orders Rickettsiales and Chlamydiales. Laboratory diagnostic methods for detecting these infections in animals as well as approaches to cultivate and identify these agents are presented. Additional laboratory methods including staining methods, media, and inoculating procedures are described in Appendix D.

Order Rickettsiales

This order contains the following families: Rickettsiaceae, Hemobartonellaceae, and Anaplasmataceae (34).

Family Rickettsiaceae

The majority of the members of this family are rod-shaped, coccoid, or pleomorphic. Spherical forms have a diameter of 0.2–0.7 μm, while the rods measure 0.3–0.6 μm in width and 0.8–2.0 μm in length. They are gram-negative, possess typical bacterial cell walls containing muramic acid, and multiply by binary fission only inside eukaryotic cells. They parasitize the gut cells of arthropods, which transmit the infection to animals. Capillary endothelial cells of infected animals are parasitized. The organisms grow to high titers in the endothelial cells lining the yolk sac of developing chicken embryos. Some also are adapted to growth in insect or animal cell cultures. These agents retain basic

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fuchsin when stained by the method of Gimenez, a modification of the procedure of Macchiavello (see Appendix D). Growth is inhibited significantly by chlortetracycline, oxytetracycline, chloramphenicol, and erythromycin but to a lesser extent by penicillin and streptomycin. These agents are rapidly inactivated at 56°C, and they are rather unstable outside host cells when separated from host component, except in diluent containing proteins or in Bovarnick's buffer consisting of phosphate-buffered sucrose and glutamate. Included in the Rickettsiaceae are the genera *Rickettsia, Rochalimaea, Cowdria, Ehrlichia, Neorickettsia,* and *Coxiella* (23,24,34).

Rickettsia rickettsii

Pathogenicity

This infectious agent is maintained in rodents and other feral animals and is transmitted to humans by ticks (*Dermatocentor, Amblyomma, Rhipicephalus*), causing Rocky Mountain spotted fever. It multiplies in the cells of the small peripheral blood vessels and induces thrombosis and extravasation. Dogs are susceptible to natural infection by the brown dog tick and develop mild illness with fever, loss of appetite, and lassitude. Some strains cause severe disease and death (2).

Detection

The organisms can be demonstrated in ticks by immunofluorescence. Isolation from blood and tissues of infected animals is rarely accomplished. Antibodies against *R. rickettsii* are detected by the Weil–Felix reaction, by indirect immunofluorescence with cell culture- or yolk sac-propagated antigen, or by indirect hemagglutination (10).

Cowdria ruminantium

Pathogenicity

Heartwater, caused by *Cowdria ruminantium,* is an acute, noncontagious, tick-borne disease of domestic and wild ruminants. It has been known to occur principally in Africa but was confirmed as an animal disease in the Caribbean island of Guadeloupe. Different species of the *Amblyomma* ticks, known as three-host ticks, harbor and transmit this infection. The primary sites of *C. ruminantium* multiplication in infected animals are the regional lymph nodes and spleen, which are infectious 3 days before infectivity is present in the blood (13,17).

Exfoliative Cytological Detection

The cowdrial organisms are found singly or in colonies of varying sizes in the cytoplasm of reticular cells, neutrophilic leukocytes, and vascular endothelium. Impression smears for diagnostic attempts are made from hippocampus, the cerebral cortex, spinal cord, and the intima of large veins. Demonstration of typical cytoplasmic inclusions in endo-

thelial cells of Giemsa- or immunofluorescently stained preparations indicates infection. The disease can be confirmed in live animals by demonstrating the cowdrial inclusions in smears made from needle biopsy specimen of the cerebral cortex or in cultured neutrophilic leukocytes (13).

Animal Inoculation

Inoculate blood or homogenized spleen into susceptible sheep. Some isolates of *C. ruminantium* will, but others will not, multiply in mice after intraperitoneal inoculation with infectious material. The organism will survive for weeks in infected mice (13).

Serology

An indirect fluorescent antibody test with antigen from primary neutrophil cultures was developed (27). Strong cross-reactions were found between *C. ruminantium* and *Ehrlichia equi* and *E. canis* (13).

Ehrlichial Infections

Ehrlichial organisms are pathogenic for dogs, horses, cattle, sheep, and humans. Reticuloendothelial cells including, characteristically, circulating white blood cells but not erythrocytes are infected. The organisms grow in the cytoplasm of infected cells (Fig. 25-1). One or multiple compact inclusions form, and they contain individual ehrlichial forms. The inclusions are termed *morulae* because of their berrylike

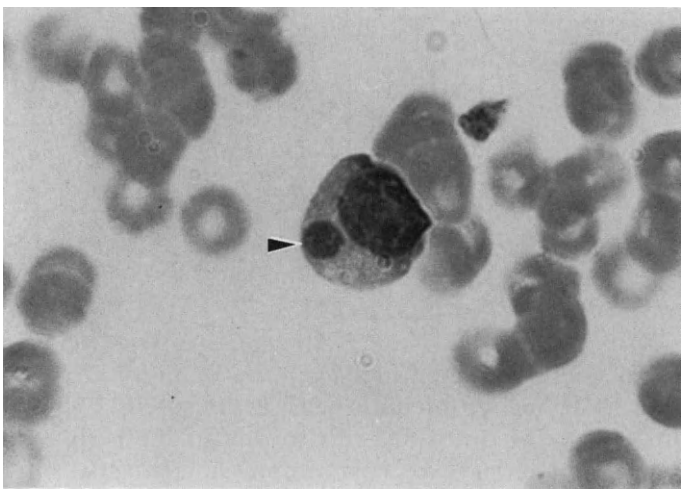

Figure 25-1. Lymphocyte containing inclusion of *Ehrlichia canis* in cytoplasm. From a dog 12 days after inoculation, ×1800. (Courtesy of Dr. K. A. Gossett, School of Veterinary Medicine, Louisiana State University.)

appearance, but single organisms may be scattered in the cytoplasm (23). Ehrlichial infections were recently identified as the causes of several newly recognized, distinct diseases (4–6,8,16).

Ehrlichiosis in Dogs

Pathogenicity

Two different species, *Ehrlichia canis* and *E. platys*, affect dogs. Virulent strains of *E. canis* parasitize lymphocytes and monocytes, while a milder strain multiplies in leukocytes and eosinophils. In contrast, inclusions induced by *E. platys*, a newly identified species, are found only in platelets. Canine ehrlichiosis induced by *E. canis* is also known as tropical canine pancytopenia, and *E. platys* is the cause of canine infectious cyclic thrombocytopenia (4,6).

Infections of dogs with *E. canis* are characterized by a long course of recurrent fever, nasal discharge, bleeding tendencies, weight loss, and depression. Gross lesions consist of hemorrhages in subcutaneous tissues and major organs, generalized lymphadenopathy, and edema of the limbs (4,6). Perivascular accumulations of lymphoreticular cells and plasma cells are prominent in meninges, kidneys, and the lymphopoetic tissues. Cases terminating fatally are frequently complicated with babesiosis or hemobartonellosis. The brown dog tick, *Rhipicephalus sanguineous*, was proven to transmit the infection. This disease is diagnosed with increasing frequency in dogs of the United States and is known to affect dogs in Africa, the Mediterranean basin, the West Indies and South America, Asia, and throughout the Orient (4,6).

Infections of dogs with *E. platys* causes a milder form of ehrlichiosis characterized by cyclic episodes of ehrlichemia and thrombocytopenia, both recurring within periods of 1–2 weeks following experimental infections. Hemorrhage is not a manifestation of this infection even though thrombocytopenia may be severe. Characteristically, ehrlichial inclusions are found in platelets. Dogs infected with *E. platys* develop antibodies reactive in the indirect FA test against *E. platys* but not against *E. canis* (6). The antigenic relationship of the two canine ehrlichial species has not been clearly defined.

Hemocytological Examination

The optimal time for finding ehrlichiae in lymphocytes and monocytes in Giemsa-stained blood smears or buffy coat preparations is around 13 days after infection with *E. canis* (Fig. 25-1). The ehrlichial inclusions are at times difficult to find in the peripheral blood. It is advisable at necropsy to prepare impression smears from organs, especially the lungs. *Ehrlichia canis* infects endothelial cells of pulmonary vessels, reticuloendothelial cells of alveolar septal walls, and fixed macrophages of lungs, liver, spleen, and kidneys (7). Direct immunofluorescence increased sensitivity in detecting ehrlichial inclusions in

buffy coat smears and preparations of other tissue (3). Dogs infected with *E. platys* had inclusions in platelets 7 and 9 days after exposure, but few were found during the period of the most pronounced thrombocytopenia (6).

Ehrlichial Infections of Ruminants

Pathogenicity

Several clinical disease entities were recently found to be associated with ehrlichial infections of ruminants (11,16). Bovine petechial fever or "Ondiri disease" was described in Kenya. The causative agent was present in leukocytes, and it was tentatively classified as *E. ondiri* (11). "Grazing fever" was differentiated in Switzerland as a significant disease of cattle occurring after they are put on pastures. Cytological and therapeutic evidence identified *E. phagocytophila* as the cause. The cattle develop high fever and their milk production declines. Ehrlichial inclusions are present in leukocytes. *Ixodes* ticks are suspected of transmitting the infection in Switzerland (16).

Tick-borne fever of sheep as observed in Africa and in Scotland is caused by *E. phagocytophilia,* parasitizing leukocytes. An ehrlichial infection of sheep in Algeria principally involved monocytes, and the organism was named *E. ovis* (23). All these ehrlichial agents have similar morphology, and those from cattle were transmitted successfully to sheep. Cultivation of any of them has not been reported. Interestingly, *E. phagocytophilia* infection of sheep increased susceptibility to superinfection with the louping-ill virus. The concurrent dual infections cause severe disease with pronounced depression, dysentery, and death (19).

Hemocytological Examination

The diagnosis of these ehrlichial infections of ruminants currently is made through hemocytological evaluations with the demonstration of characteristic inclusions in leukocytes or lymphocytes and monocytes (11,16).

Ehrlichial Infections of Horses

Pathogenicity

Two different ehrlichial species cause infections and diseases in horses: *E. equi* and *E. risticii* (5,8,9). The disease induced by *E. equi* is called equine ehrlichiosis and is clinically characterized by fever, depression, partial anorexia, petechiation, icterus, limb edema, ataxia, and reluctance to move. It is a seasonal disease of horses observed in California during late fall, winter, and spring. Cases were diagnosed in Colorado, Illinois, Florida, Washington, and New Jersey. Leukocytes are the principal targets of infection among the circulating white blood cells (12).

Ehrlichia risticii is a newly established species of ehrlichial organisms isolated by Ristic and his co-workers and proven to be the cause of the disease now referred to as equine monocytic ehrlichiosis (8,9). Initially, it attracted attention as "Potomac horse fever," identifying the location of the first recognized cases in Virginia and Maryland, and it was also referred to as equine ehrlichial colitis. This disease was diagnosed in horses of the eastern United States including Pennsylvania, New Jersey, Ohio, New York, Connecticut, and west in Idaho (8,21). A seasonal pattern from late spring to early fall with peaks in July and August is characteristic. Affected horses develop anorexia, depression, fever, leukopenia, and dehydration resulting from profuse watery diarrhea. Ehrlichial organisms were detected in cells of the large colon, small colon, jejunum, and cecum (20). Laminitis is an additional complication. About 30% of the affected horses die.

Hemocytological Examination

A conclusive diagnosis of equine ehrlichiosis can be made when characteristic cytoplasmic inclusion bodies in neutrophilic leukocytes are seen in Giemsa- or Wright-stained blood smears.

Cultivation of Ehrlichiae

Blood monocyte cultures are prepared in 25–cm^2 tissue culture flasks with medium 199 supplemented with 1% L-glutamine and 20% heat-inactivated serum of normal horses. Macrophage cultures (Fig. 25-2) also support growth of equine and canine ehrlichiae (31). These insights lead to the observation that the continuous murine macrophage cell line P388D1 is susceptible to infection with *E. canis*, *E. equi*, and *E. risticii*. The cells are incubated at 37°C in an atmosphere of 5% CO_2 in air. The medium is supplemented with 10% fetal calf serum and 1% L-glutamine and adjusted with sodium bicarbonate to a pH of 7.4 (22). Other ehrlichiae probably could be cultured in this or similar systems.

Tests for Ehrlichial Antibodies

The indirect immunofluorescence test was developed to detect antibodies to *E. canis*, *E. platys*, and *E. risticii* (3,22). Preparations of murine macrophage cells with 60–80% infection are used as antigens. Fluorescein-conjugated equine or canine anti-immunoglobulin G raised in rabbits is used to trace the bound primary antibody. Antibodies are detected 7–10 days after exposure, reach a maximal level at 14–20 days, and decline in titer at about 50 days. These test results correlated well with clinical findings and pathogenetic events of experimental and natural infections. An enzyme-linked immunosorbent assay (ELISA) with *E. risticii* antigen prepared from Sephacryl S-100-purified organisms propagated in P388DI mouse macrophage cells was developed. Anti-IgG and anti-IgM conjugates were used. Results correlated well with the IFA test findings (18).

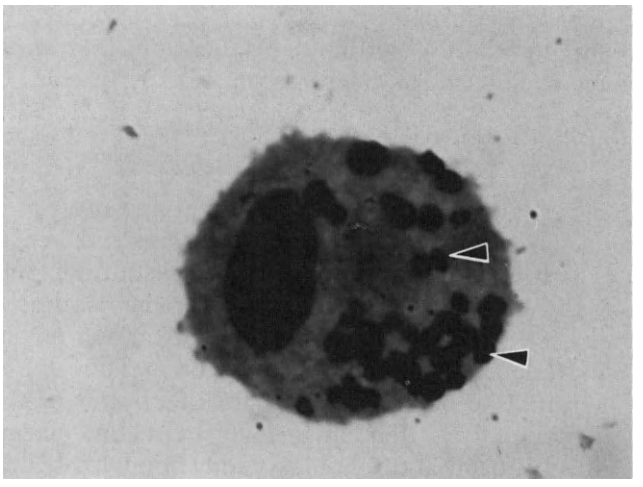

Figure 25-2. Cultured canine peritoneal macrophage 8 days after infection with *Ehrlichia canis*, × 1000. (Courtesy of Dr. E. H. Stephenson, Walter Reed Army Institute of Research, Washington, D.C.)

Neorickettsia helminthoeca and the Elokomin Fluke Fever Agent

Pathogenicity

This organism is the cause of the salmon poisoning disease complex of dogs. It occurs when dogs consume raw salmon and trout that carry the rickettsia-infected metacercariae of the intestinal fluke *Nanophyetus salmincola* (1).

The incubation period is usually 5–7 days. The disease, which is characterized by high fever, depression, and severe diarrhea, can terminate fatally in 7–10 days. These dogs have enlargement and necrosis of all lymph nodes, particularly those of the intestinal tract, which may have hemorrhagic inflammation (1).

Parasitological and Cytological Examination

Laboratory diagnosis is based on demonstration of the characteristic fluke eggs in the feces of affected dogs. The rickettsiae are found in cells of smears of fluid aspirated from mandibular lymph nodes. Various lymph nodes may contain white foci of necrosis, and smears from these disclose small (about 0.3 μm), coccoid or coccobacillary intracytoplasmic bodies. They stain purple with Giemsa and are red or blue after staining with the methods of Gimenez or Macchiavello (34).

The elementary bodies of a similar rickettsia, the elokomin fluke fever (EFF) agent, are also seen in smear of lymph nodes and resemble *N. helminthoeca*. This organism, along with *N. helminthoeca*, occurs in the canine disease, but the EFF agent alone can infect dogs, foxes, coyotes, racoons, and ferrets.

Serological Procedures
Complement fixation, serum neutralization, and animal protection tests have been used for diagnosis on an experimental basis.

Coxiella burnetii
Pathogenicity
Coxiella burnetii causes query or Q-fever and multiplies only in animal cells. The infection cycles between ticks and free-living vertebrates. It is indirectly transmitted to domestic animals by ticks through contact with their heavily infected excreta, facilitated through the agent's high degree of resistance to chemical and physical influences (34). Tick-independent cycles of infection can develop within populations of animals, especially cattle. The infection in cattle, sheep, goats, and other animals is frequently inapparent, but placental infections with multiplication in trophoblasts and abundant shedding at birth occur. This infection may lead to abortions in small ruminants and abortions or other infertility problems in cattle. Infection of the mammary glands of cows leads to intermittent shedding in the milk. What makes this infection important is that infected animals transmit the disease to human subjects through milk, or through abundant shedding during parturition, or through contaminated wool under conditions creating infectious, coxiella-laden dust. Clinical signs associated with coxiella-induced diseases are not specific enough for an accurate diagnosis (29,34).

Exfoliative Cytology and Histology
The direct visualization of *C. burnetii* in cells of infected tissues by Gimenez- or Giemsa-staining, or by analysis through immunofluorescent methods, is relatively rapid and specific (29).

Isolation Procedures
Cultivation of *C. burnetii* can be readily accomplished by inoculation of 5–7-day-old developing chicken embryos via the yolk sac route (34). This agent also multiplies in several types of cultured cells, where it can establish persistent infections. Isolation of this pathogen from clinical specimens by culture in chicken embryos, or the inoculation of samples into guinea pigs or mice, and subsequent proof of seroconversion, are time-consuming and only done in specialized laboratories.

Serological Procedures
A tentative diagnosis of human and bovine Q fever in most cases has to be confirmed by serological procedures. Standard tests were complement fixation (CF) and microagglutination (34). Higher sensitivity was achieved with the enzyme-linked immunosorbent assay (ELISA) in which commercial CF antigens were used. Early stages of *C. burnetii*

infection can be diagnosed by ELISA with a single serum sample by demonstrating specific IgM before appearance of CF-reactive IgG antibodies. Simultaneous presence of IgM and IgG indicates recent infection. Cattle vaccinated with a commercial phase II vaccine could be distinguished from naturally infected cattle by employing conjugates specific for bovine IgG_1 and IgG_2 because vaccination induced predominantly IgG_2 antibodies (29).

Family Hemobartonellaceae

Hemobartonella felis

Pathogenicity

Hemobartonella felis causes an acute or chronic disease of cats. The causative organisms are found on the surfaces of erythrocytes and occasionally free in the plasma. The number of red blood cells affected varies with the severity of the infection. Infected cats may form antibodies to their own erythrocytes, resulting in hemolytic anemia. The modes of natural transmission have not been established. A significant proportion of the cat population may have clinically inapparent infection (24).

Hemocytological Examination

The organisms are found in various numbers on the surface of erythrocytes of peripheral blood or bone marrow and occasionally free in the plasma. *Hemobartonella felis* consists of small, coccoid, rodlike or ring-shaped organisms with diameters of 0.2–1.0 μm for coccoid forms and a length of 3.0 μm for the rod forms.

Family Anaplasmataceae

Anaplasma marginale

Pathogenicity

This family includes *Anaplasma marginale,* which parasitizes erythrocytes of cattle or related ruminants and induces anemia. The host range of this organism is limited to ruminants and does not include laboratory animals. The relatively nonvirulent *A. centrale* occurs in Africa with *A. marginale* and can be differentiated by the more central location in the erythrocytes. Numerous species of ticks are biological vectors *(Boophilus, Rhipicephalus, Dermatocenter, Hyalomma,* and *Ixodes)* and transmit the infection. Biting insects such as tabanids are effective mechanical vectors (21,25).

Hemocytological Examination
The organism can be found as marginal bodies in erythrocytes of infected cattle in Giemsa-stained blood smears. Up to 50% of the erythrocytes may be parasitized. Prolonged cases with extensive red blood cell destruction may have few anaplasma bodies in the blood.

Serological Procedures
Common serological techniques for the detection of antibodies to *A. marginale* include card agglutination, the CF test, direct and indirect immunofluorescence, capillary tube or latex agglutination, and ELISA. An ELISA with improved sensitivity and standardized solid phase coating was recently described and is based on sodium dodecyl sulfate-treated anaplasma antigen from infected bovine erythrocytes (21,35).

Order Chlamydiales

Characteristics

A monogeneric family, Chlamydiaceae, is described; three species are recognized in the genus *Chlamydia*. *Chlamydia trachomatis*, *C. pneumoniae*, and *C. psittaci* are differentiated from each other by stable characteristics. Chlamydiae associated with trachoma, inclusion conjunctivitis, lymphogranuloma venereum, and other genital tract infections of humans belong to the species *C. trachomatis*. The newly recognized *C. pneumoniae* causes respiratory infections and pneumonia in humans. Chlamydial agents that cause infections in various domestic and wild mammals belong to the species *C. psittaci*, which comprises a large collection of chlamydiae that differ antigenically and culturally (14, 32, 33).

Chlamydiae are obligate intracellular parasites that multiply in the cytoplasm of animal cells and form membrane-bound cytoplasmic inclusions (Figs. 25-3, 25-4). During multiplication, they go through a complicated developmental cycle. The morphology of the organism changes sequentially from small, infectious elementary bodies to larger, reticulate bodies that reach a diameter of 1.0μm, divide by binary fission, and reorganize into highly stable, infectious elementary bodies with a diameter of 0.2–0.25 μm. The reticulate bodies are extremely fragile extracellularly and are not infectious. All chlamydial agents share group- or genus-specific antigens that are heat stable and consist of a lipoprotein–carbohydrate complex. These organisms cannot generate high-energy compounds. Chlamydiae are sensitive to antibiotics such as chloramphenicol, tetracycline, cycloserine, and penicillin. Chlamydial strains of the species *C. trachomatis* are inhibited by sulfonamides.

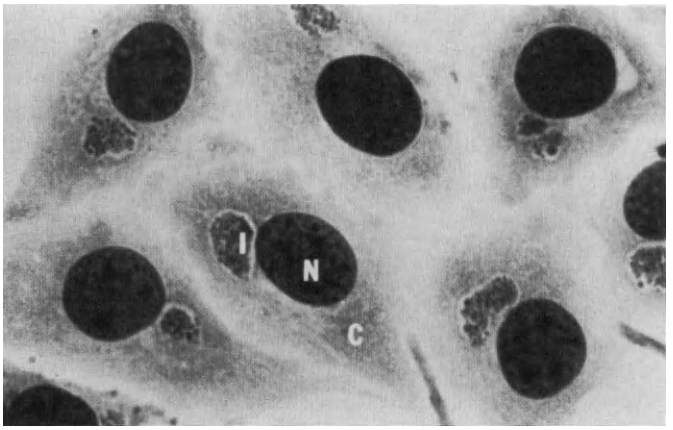

Figure 25-3. Mouse L cells infected with a bovine chlamydial strain. Notice early inclusion (I) in cytoplasm (C) of L cell. Nucleus (N) is prominent. ×900, Giemsa stain.

Pathogenicity

Chlamydiae of the species *C. psittaci* infect a wide range of animals, as well as humans, through heterologous chains of transmission. Epithelial cells of mucous membranes are infected, but infections may generalize after penetration of mucous membranes when endothelial and other cells become infected. Chlamydial infections of animals may lead to the following disease syndromes: intestinal infection and diarrhea, pneumonia, placental infections and abortion, genital

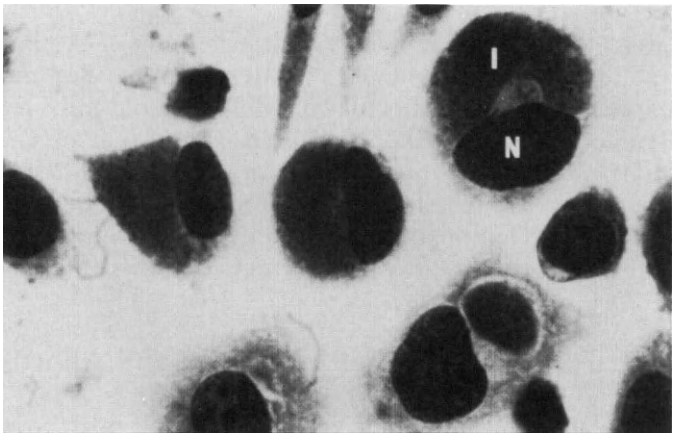

Figure 25-4. Late inclusions (I) in cytoplasm of mouse L cells infected with chlamydial strain of feline pneumonitis. N, nucleus. ×900, Giemsa stain.

infections, mastitis, polyarthritis–polyserositis, encephalitis, and conjunctivitis. Avian chlamydial infections may lead to pneumonia an airsacculitis, pericarditis, conjunctivitis, and encephalitis, as well as intestinal infections and diarrhea. Clinically inapparent intestinal infections usually lead to prolonged fecal shedding of chlamydiae (29,30).

Intestinal Infection

The frequently overlooked intestinal chlamydial infections may cause primary diarrhea in young animals, initiate pathogenetic events in other chlamydial diseases, and play a role in perpetuating and spreading this infection. Chlamydiae were isolated from feces or diarrhea fluid of calves, sheep, goats, swine, and dogs, as well as domestic poultry and many bird species (14).

Detection
Isolation of chlamydiae in cell cultures or developing chicken embryos is required. The isolation methods are described in Appendix E.

Respiratory Infections and Pneumonia

Chlamydial pneumonia is found worldwide in laboratory mice, cats, calves, foals, lambs, goats, swine, and rabbits, as well as humans. This infection is expressed as rhinitis and conjuntivitis, tracheitis, and interstitial bronchopneumonia (32,33).

Detection
Isolation of chlamydiae from nasal secretions or specimens from affected lungs, preferably in cell cultures, or in developing chicken embryos, or by intranasal inoculation of 3-week-old mice free of chlamydial infections, should be attempted. Evaluation of Giemsa-stained exfoliative cytological preparations of scraping from the conjunctivae or respiratory tissues of affected animals gives reliable and fast diagnostic results. Demonstration of circulating or secretory antibodies in the IIFA test or the ELISA gives evidence of this infection (15).

Placental and Fetal Chlamydial Infections and Abortions

Placental and fetal infections with chlamydiae, with ensuing abortion or birth of weak offspring, are now recognized as a significant cause of reproductive failure in sheep, goats, cattle, and other domestic animals. During a chlamydemic phase in pregnant subjects, the placental

junction is breached, and infection is established in chorionic epithelial cells, which develop large cytoplasmic inclusions. Local spread involves cotyledons and pericotyledonary tissue with focal necrosis. Fetuses also become infected (32,33).

Detection

Exfoliative cytological examination of affected placental tissue after Gimenez staining is a reliable tool to detect chlamydial elementary bodies singly or in clusters. Fluorescent antibody (FA) techniques make this approach even more specific (Fig. 25-5). Enzyme immune assays to detect chlamydial antigens should be employed for objective spectrophotometric evaluation. Isolation of chlamydiae from placental and fetal tissues by cell culture or chicken embryo techniques was accomplished by many workers, but this approach is time-consuming and not sensitive enough, because chlamydial infectivity in specimens from aborted placentas and fetuses may be inactivated by the time samples reach the laboratory. An antibody rise following abortion links this infection causatively. Optimal methods for detecting the unique antibody response of pregnant animals experiencing chlamydia-induced abortions are the IIFA test or the ELISA (Fig. 25-6).

Urogenital Infection and Seminal Transmission

Chlamydial agents were isolated from semen and genital tissues of rams and bulls, as well as guinea pig boars affected with breeding disorders, in different parts of the world. Poor semen quality, orchitis, and seminal vesiculitis are associated with this infection (32,33).

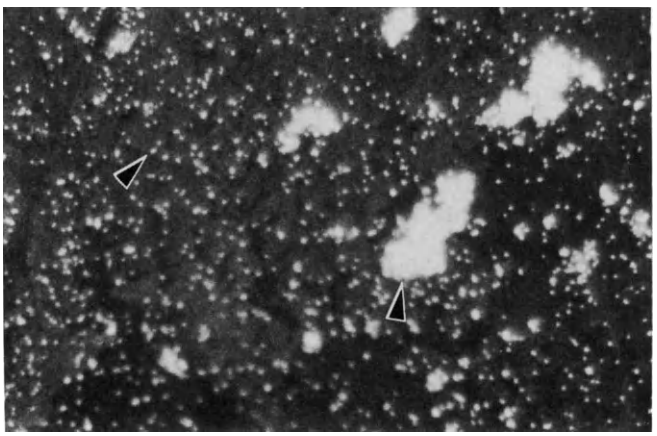

Figure 25-5. Chlamydial elementary bodies as seen after indirect immunofluorescent staining, ×1800.

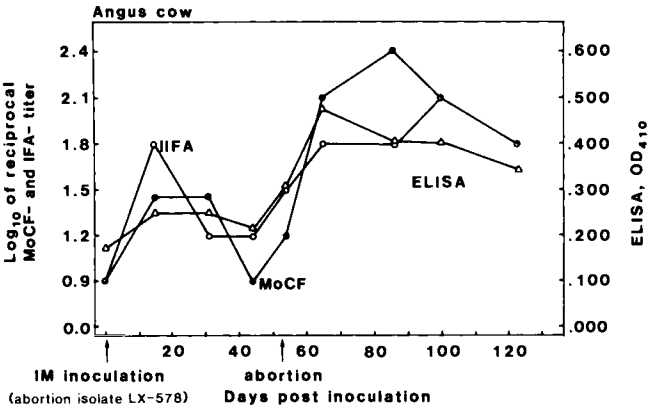

Figure 25-6. Chlamydia-specific antibody response of a cow experiencing chlamydial infection leading to abortion. IIFA, indirect inclusion fluorescent antibody test; MoCF, modified complement fixation test.

Detection

Isolation of chlamydiae from semen or genital tissues is currently required to diagnose this infection. The presence of secretory antibodies in semen has not been evaluated sufficiently for diagnostic purposes.

Polyarthritis–Polyserositis and Sporadic Bovine Encephalitis

Some chlamydiae induce systemic infection and have a propensity for synovial tissue of lambs, calves and young cattle, swine, and foals. This infection leads to polyarthritis of lambs and to polyarthritis with fibrinous serositis and pericarditis in calves. Young cattle with this infection may develop encephalitis in sporadic cases. The chlamydial strains causing this type of infection in cattle and sheep differ biologically and antigenically from chlamydiae associated with abortions and are distinguished as immunotype 2.

Detection

Isolation of chlamydiae from affected joints is required for a specific diagnosis and for differentiation from other arthropathogenic infections. Exfoliative cytologic examination of synovial fluids and affected serosal membranes also reveals this infection through the presence of free elementary bodies or inclusions. The serological tests mentioned detect rising antibody responses to this infection during the acute phase of disease (32,33).

Conjunctivitis and Ocular Disease

Conjunctivitis and keratoconjunctivitis are caused by strains of *C. psittaci* in cats, lambs, calves, piglets, and guinea pigs. Ocular involvement has long been recognized as a clinical sign of chlamydiosis in pigeons, ducks, and geese. Chlamydiae infect conjunctival cells, leading to exfoliation, hyperemia, conjunctival inflammation with pannus formation, and keratitis as well as mucopurulent ocular discharge (32,33).

Detection

Exfoliative cytological examination of conjunctival scrapings from subjects with conjunctivitis gives a clear indication of this infection if conjunctival and monocytic cells contain chlamydial inclusions after Giemsa or FA staining (Fig. 25-7). Elementary bodies can be detected in the background of such smears. Chlamydiae can be isolated by the methods described from conjunctival scrapings that contain follicular contents. Secretory antibodies in lacrimal fluids detected by the IMIF test have diagnostic significance in inclusion conjunctivitis of guinea pigs.

Avian Chlamydiosis

Avian chlamydiosis is an infectious disease of domesticated and wild birds caused by *C. psittaci*. The disease induced by *C. psittaci* in human beings and psittacine birds was previously called *psittacosis* or "parrot fever," whereas the infection involving nonpsittacine birds and humans was called *ornithosis*. Birds are the most frequent source of human infection, but *C. psittaci* infection can also be transmitted to humans by cats and other mammals. *C. psittaci* is often harbored in the intestinal tracts, spleen, and kidneys of birds appearing clinically normal, but these birds shed the agent in the feces over long time periods. Avian chlamydiosis is a systemic chlamydial infection leading to pneumonia and airsacculitis, pericarditis, enteritis with diarrhea, and conjunctivitis (32,33).

The disease in humans begins with inhalation of infectious dust and leads to pharyngitis and pneumonia with chlamydemia and systemic involvement. Coughing, headache, fever, and malaise are prominent clinical signs.

Special Caution

Chlamydia-infected live and dead birds shed large quantities of chlamydiae and may generate dangerous aerosols through infectious dust.

Great care should be exercised in handling birds suspected of having chlamydiosis. Dead birds should be dipped in effective disinfectant before necropsies are done (33).

Detection

Exfoliative cytological examination of impression smears of affected serosal surfaces, exudates of body cavities, or lung, liver, and spleen reveals chlamydial elementary bodies free or within cytoplasmic inclusions of infected cells. Isolation of the agent from tissue or fecal samples can be accomplished by the methods described. Cell culture methods are more efficient. An amplified ELISA employing monoclonal antibodies to capture common chlamydial antigens is equally sensitive and less time consuming. The CF test can be used for most avian sera, but the indirect CF test is used for sera from chicken and turkeys to detect chlamydial antibodies in infected flocks (33). ELISA or IIFA are the current choices for monitoring the antibody response.

Exfoliative Cytological and Histological Techniques

Exfoliative cytology can be a powerful tool for diagnosing chlamydial infections of birds with airsacculitis and pericarditis and of mammals with conjunctivitis, polyarthritis, placentitis, pneumonia, or peritonitis (32). Samples of conjunctiva are collected by scraping the affected surface with an edged tool to collect conjunctival and inflammatory cells. Synovial or peritoneal fluids are applied directly onto slides, or the cells in these fluids are harvested, washed, and applied to slides. These cytological preparations may be fixed with methanol, but cells are best preserved by Bouin or Zenker fixing solution. Specimens of this type are stained by the Giemsa or Gimenez method. Cytoplasmic chlamydial inclusions in infected cells are the diagnostic indicators (Fig. 25-7). The types of inflammatory and other cells present and the occurrence of single or connected groups of conjunctival cells are also diagnostic considerations. Chlamydial inclusions must not be confused with pigment granules or artifacts. These staining methods also reveal chlamydial infections in cultured cells by differentiating between inclusions and other cytological features.

The Gimenez or the Machiavello procedures are the staining methods for detecting chlamydial elementary bodies in yolk-sac impression smears, in touch preparations of peritoneal surfaces, spleen, and liver of infected birds and mice, and in exfoliative cytological analysis of chlamydia-infected placentas. Elementary bodies, single or in aggregates, stand out as bright red dots. Microscopic demonstration of elementary bodies in impression smears of infected placentas provides firm proof of infection. Bacteria having diverse properties are also

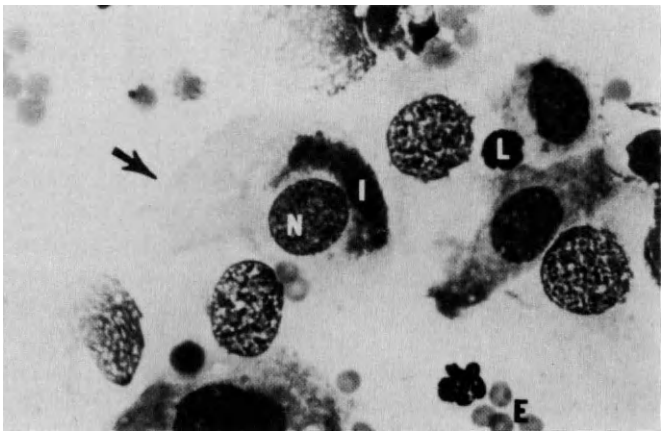

Figure 25-7. Exfoliative cytological preparation of conjunctiva from chylamydia-infected lamb. The arrow points towards an infected conjunctival cell with late chlamydial inclusion (I) and nucleus (N). L, leukocyte, E, erythrocyte. ×690, Giemsa stain.

stained and readily seen. Cryostat sections of placental tissues from the margins of lesions fixed with paraformaldehyde and Gimenez-stained may give more information, since elementary bodies in heavily infected trophoblastic cells stand out as red chlamydial colonies.

Histological examination of specimens from intestinal, placental, lung, or joint infections may locate chlamydia-infected cells, but success of this technique depends on using sections with a thickness of 4 μm or less. Tissues embedded in plastic give best results. Both Giemsa and Gimenez staining reveal inclusions in infected cells of these tissue preparations. Washing sections in ammonia water before Gimenez staining enhances the contrasting red of the elementary bodies in inclusions.

Fluorescent antibody techniques bring immunological specificity to all histological and cytological diagnostic procedures. The indirect FA method is more flexible, once established, for a given system. The immunofluorescence preparation may be restained by Giemsa or Gimenez methods to relocate inclusions.

Isolation and Identification of Chlamydiae from Clinical Specimens

Cell culture methods for isolating chlamydiae from diagnostic samples are effective. The advantages of cell culture methods are the high sensitivity when enhancing methods are used, the short time period

required until results can be evaluated (days instead of weeks), and the reduced danger of loss of the culture because of bacterial contamination (30). Chlamydia-infected cells can be detected even in cell cultures contaminated with bacteria.

The infectivity-enhancing method of choice is centrifugation of the inoculum onto the cell monolayer. This procedure should be carried out at 37°C. Cycloheximide (2 μg/ml) is useful for most strains of *C. psittaci* isolated from animals; however, strains associated with polyarthritis, encephalitis, and conjunctivitis of ruminants generate aberrant forms in the developmental cycle and produce fewer infectious particles in the presence of cycloheximide.

To trace infection of cultured cells in isolation procedures, the cells grown on cover slips are fixed in Bouin's fluid or methanol 40–60 hr after inoculation, stained by the Giemsa or Gimenez method, and examined microscopically for chlamydial inclusions in the cytoplasm. Parallel cultures are kept for subpassages. If subpassages are made, as depicted in Figure 25-8, these cultures are treated with sound to disrupt infected cells to liberate chlamydial elementary bodies. At least three subpassages at 2–3-day intervals should be made before a sample is considered free of chlamydiae.

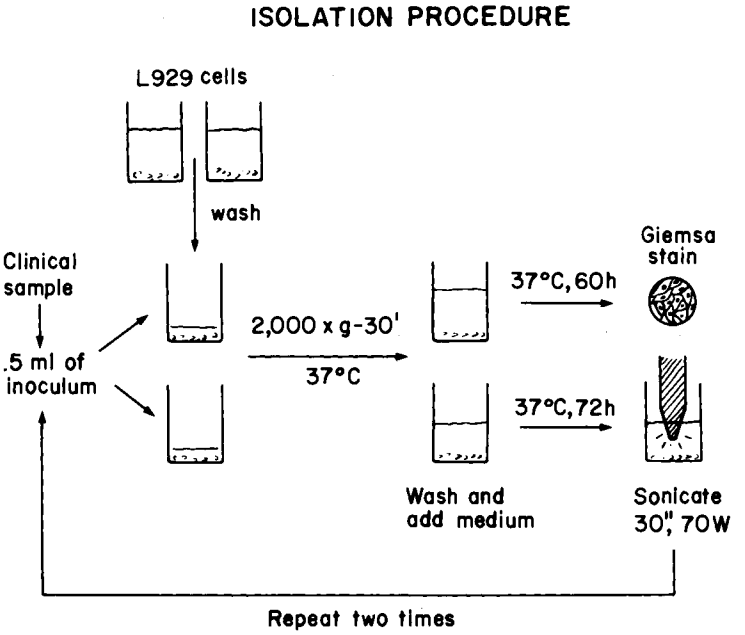

Figure 25-8. Illustration of procedure for isolation of chlamydiae by the use of cell cultures.

The chicken embryo technique for isolating chlamydiae from animals has been quite successful. This method may remain useful for laboratories not equipped to use the more sophisticated cell culture methods. Sometimes, several blind passages requiring weeks have to be made before results can be assessed. The 7-day-old chicken embryos inoculated by the yolk-sac route must be candled daily. Embryos that die within 3 days of inoculation are tested for sterility, then discarded; the average time of death of all dying chicken embryos is recorded. The pathological changes in yolk-sac membranes and chicken embryos are evaluated. Chlamydial infection is verified by the presence of elementary bodies in Gimenez-stained impressions of washed pieces of infected yolk-sac membranes. Chicken embryos should be tested for bacterial, mycoplasmal, or viral infections in the course of chlamydial isolation procedures.

Some other indicator hosts used to cultivate rickettsial and chlamydial infections are summarized in Table 25-1. Intranasal or intraperitoneal inoculation of 3-week-old mice yields chlamydial isolates from avian chlamydiosis. Chlamydiae from cattle, sheep, and goats usually do not readily multiply in mouse tissues during primary isolation steps. This host has an advantage for isolating chlamydiae from diarrhea or fecal specimens of birds because low levels of bacterial contamination are eliminated by the mouse. The naturally occurring

Table 25-1
Systems for the Isolation and Cultivation of Some Rickettsial and Chlamydial Agents

Agent	Host system	Route of inoculation
Rickettsia rickettsii	Cell cultures	—
	Chicken embryos	Yolk sac
	Guinea pigs	Intraperitoneal
Cowdria ruminantium	Cultured leukocytes	—
	Mice	Intraperitoneal
Ehrlichia canis	Monocyte or macrophage cultures	—
Ehrlichia platys	P388D1 (mouse macrophage cell line)	—
Ehrlichia equi	—	—
Ehrlichia risticii	P388Di, canine tumor cells	—
Neorickettsia helminthoeca	Dog monocyte cultures	—
Coxiella burnettii	Cell cultures	—
	Guinea pigs	Intraperitoneal
Hemobartonella felis	Cats	Oral or parenteral
Anaplasma marginale	Deer, sheep	Parenteral
Chlamydia psittaci	Cell cultures (HeLa, Vero, or L cells)	Several enhancing methods
	Chicken embryos	Yolk sac
	Mice	Intranasal, intraperitoneal
	Guinea pig	Intraperitoneal

pneumonic chlamydial infection of mice may confuse isolation results unless mice are free of this infection.

Similarly, guinea pigs may have naturally occurring chlamydial infections, but they have been used to isolate chlamydiae from fecal specimens of cattle and sheep. Elimination of contaminating bacteria after intraperitoneal inoculation of guinea pigs can be considered an advantage of this method; however, isolation of chlamydiae in cultured cells or chicken embryos after careful bacterial decontamination is better.

An important step in isolating chlamydiae from heavily contaminated specimens such as feces, placental tissues, or semen is elimination of contaminating bacteria. Streptomycin (500 μg/ml), vancomycin (75 μg/ml), gentamicin (50 μg/ml), and mycostatin (500 units/ml) suppress bacterial contamination in cell cultures or chicken embryos used for chlamydial propagation. These antibiotics do not interfere with chlamydial multiplication. Differential centrifugation at 2000 *g* for 30 min at 4°C is most effective. The aim is to leave chlamydiae in suspension but to centrifuge out all larger, contaminating bacteria. Repeated cycles of differential centrifugation may be required with transferring only the top components of the supernatant fluid because of the motile bacteria in fecal specimens. This decontamination scheme is illustrated in Figure 25-9.

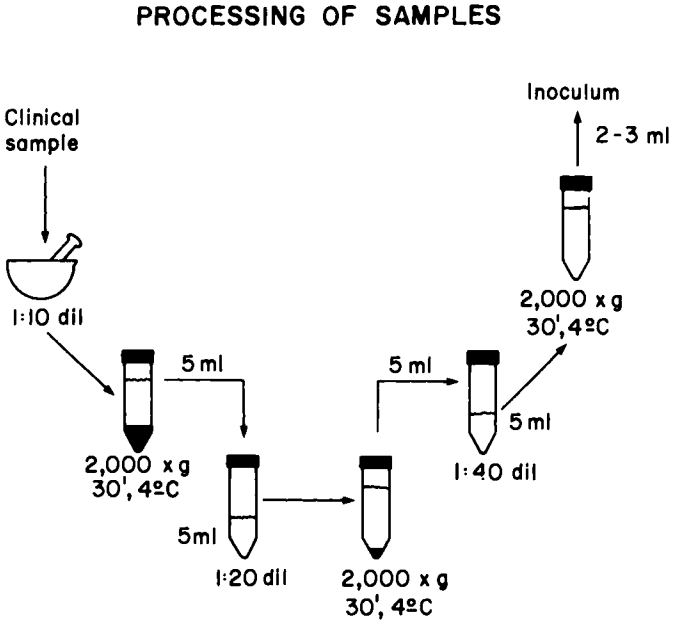

Figure 25-9. Processing of contaminated samples for chlamydial isolation.

Serological Procedures to Detect Chlamydial Antibodies

The serological test most widely used in the past to detect chlamydial antibodies in animal and human infections was the CF test. Active systemic chlamydial infections induce titers that have diagnostic significance if fourfold or higher antibody increases develop that are related to a disease episode. Since guinea pigs have naturally occurring chlamydial infections, guinea pig complement must be checked for freedom from chlamydial antibodies (32,33). The indirect CF test or a modification of the direct test must be used with sera from turkeys and chickens because their sera do not fix guinea pig complement. Similarly, supplementation of guinea pig complement with fresh bovine serum free of chlamydial antibodies enhances the sensitivity of the CF test for bovine antibodies. The CF test is virtually replaced by modern enzyme-linked immune assays.

ELISA is a rapid and sensitive serological technique for diagnosing chlamydiosis in birds. Soluble genus-specific antigens were employed but particulate antigens of partially purified elementary bodies can be used and have species and genus reactivities. Antibodies are detected 1–2 weeks after infection, and they are detectable for much longer by ELISA than by CF (26,28).

The antibody responses of pregnant cows and ewes experiencing chlamydial infections leading to placental and fetal manifestations and abortions were recently analyzed with the ELISA and the IIFA test (15). The responses are characteristic and diagnostically indicative. After inoculation of pregnant ewes or cows, an initial mild rise of antibodies is observed. The titers decline to low levels before abortion or parturition, provided these events occur later than 4 weeks after inoculation or exposure. Delivery of chlamydia-infected dead or live fetuses with infected placentas stimulates a rapid rise in antibody titers that reach maximum levels 14–21 days after termination of pregnancy (see Fig. 25-6). Accordingly, paired serum samples taken at the time of abortion and 2–3 weeks later have a significant increase in titer if the abortion resulted from chlamydial infection. Partially purified elementary bodies are used to sensitize the test plates so that the predominant chlamydial antigens reactive in these tests are proteinaceous antigens of species, type, and strain specificity. (H + L)-chain-specific conjugates are used to detect all immunoglobulin isotypes. Interestingly, the chlamydia-specific antibody fraction of bovine serum contains high levels of noncomplement-binding IgG_2 when tested with the corresponding immunoglobulin subclass-specific conjugates (29). The previously employed standard CF test was genus-specific and relatively insensitive, because bovine IgG_2 antibodies do not fix guinea pig complement unless supplemented with bovine complement at concentrations of 5% fresh bovine serum. The ELISA can be set up with single serum

dilutions of 1:100 if strictly quantitative evaluation and appropriate internal negative and positive controls are used. Fetuses may have elevated levels of immunoglobins that are not reactive in the CF test, but they react with chlamydial antigens in the double immunodiffusion test or ELISA.

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26

Avian Mycoplasmas

Harry W. Yoder, Jr.

Avian Mycoplasmas* are primarily associated with respiratory diseases of chickens and turkeys, although synovitis is sometimes significant. They are the smallest free-living bacterial organisms, having no rigid cell wall but possessing a limiting membrane that is antigenic. Their reproductive cycle is complex but variable, frequently with elementary bodies often arising from a budding-type process. The mycoplasmas are fastidious organisms that require a protein-base medium enriched with various sterols.

Freundt (1) published the characteristics of the most important avian mycoplasmas in the Eighth Edition of *Bergey's Manual of Determinative Bacteriology.* The family Mycoplasmataceae was placed under the order Mycoplasmatales. It contains the genus *Mycoplasma,* which includes numerous species of significance in animals and humans. The family Acholeplasmataceae contains the common saprophyte *Acholeplasma laidlawii,* which does not require added sterols for growth and readily multiplies at room temperature.

The possible relationship of avian *Mycoplasma* to so-called L-phase organisms of various bacteria is still controversial. However, most workers consider *Mycoplasma* to be true bacteria, with some possible L-forms present in media due to the inhibition of other bacterial vegetative forms by the common addition of bacterial inhibitors (penicillin and thallous acetate) to *Mycoplasma* media.

Approximately 20 serotypes of *Mycoplasma* have been characterized

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from avian sources (2, 3, 4, 5, 6, 7). Those serotypes representing significant pathogens have been given genus and species designations, as have a few of lesser importance (8). Thus, it has become necessary to discuss the entire group under the broad designation of avian mycoplasmas, with more complete treatises presented for only the most significant members: *Mycoplasma gallisepticum, M. meleagridis, M. synoviae,* and *M. iowae.*

A summary concerning the major aspects of *Mycoplasma* associated with poultry is presented in Table 26-1.

Mycoplasma gallisepticum

Pathogenicity

Mycoplasma gallisepticum infection is commonly designated as chronic respiratory disease (CRD) of chickens and infectious sinusitis of turkeys. It has (rarely) caused infection in pheasants and quail. It is characterized by respiratory rales, coughing, nasal discharge, and frequently in turkeys by a sinusitis. The clinical manifestations are usually slow to develop, and the disease has a long course. Air sac disease designates a severe airsacculitis that is the result of *M. gallisepticum* infection complicated by some respiratory virus infection and also usually *Escherichia coli.*

Mycoplasma gallisepticum can be spread by direct contact, indirect contact with contaminated objects, apparently by air transmission to a limited extent, and via egg transmission. Voluntary control of *M. gallisepticum* in chickens and turkeys is a common procedure throughout most of the world based on selection of serologically negative breeder flocks to avoid egg transmission of the infection to progeny flocks.

Isolation Procedures

Mycoplasmas are fastidious organisms that require a protein-base medium enriched with 10–15% serum or serum factors. No single medium formulation has been accepted as suitable for the various avian mycoplasmas, and mediums for antigen production are frequently further modified. See "Mediums for Growth" in Appendix E.

Since *Mycoplasma* infections tend to be respiratory and involve most birds in a flock, culturing 5 or 10 tracheal swabs is often sufficient. If airsacculitis is present, culturing air sac swabs may be done directly into media, or from a suspension prepared with a mortar and pestle using a small amount of nutrient broth. Sinus exudate may usually be aspirated with a syringe and needle.

Table 26-1
Characteristics of Avian Mycoplasmas

Serotype	Genus and species	HA	Ferments glucose	Requires Serum	Requires NAD	Arginine decarboxylation	Pathogenicity	
							Chickens	Turkeys
A	*Mycoplasma gallisepticum*	+	+	+	−	−	+	+
B	*M. gallinarum*	−	−	+	−	+	−	−
CO	*M. pullorum*	−	+	+	−	−	−	−
DKP	*M. gallinaceum*	−	+	+	−	−	−	−
EG	*M. iners*	−	−	+	−	+	−	−
F	*M. gallopavonis*	−	+	+	−	−	−	−
H	*M. meleagridis*	−[a]	−	+	−	+	−	+
IJNQR	*M. iowae*	+	+	+	−	+	−	+
L	*M. columbinasale*	−	−	+	−	+	−	−
S	*M. synoviae*	+	+	+	+	−	+	+
	Acholeplasma laidlawii	−	+	−	−	−	−	−
	M. anatis	−	+	+	−	−	(+ ducks)	−

[a]Most isolates are HA negative but may be HA positive.

Once specimens have been collected and the broth medium inoculated, the tubes should be incubated at 37°C for at least 14 days before they are discarded as negative. Cultures should be transferred daily whenever evidence of growth (increased turbidity) or change in pH is observed. Always transfer a generous amount with a pipette, such as 1 ml into fresh tubes of 5–10 ml of medium. If there is no evidence of growth by day 4 or 5 incubation, 1 drop from a pipette should be transferred to a plate of mycoplasma agar medium streaked with a soft wire loop. Several cultures can be streaked on a single plate. Sometimes agar medium can be streaked directly with swab specimens, but initial broth passage tends to give better results and fewer cultures are lost because of gross bacterial contamination.

Inoculated agar plates should be incubated for 3–5 days at 37°C in a moist container. Maintenance of a moist atmosphere is very important. The addition of 5% carbon dioxide or the use of a candle jar is sometimes suggested. Some isolates appear to prefer anaerobic conditions for isolation and a few initial passages, but this is rarely provided.

Cultural Characteristics

Colonies are best observed with the aid of approximately ×20 to ×50 magnification through a dissecting microscope employing oblique indirect lighting. Typical colonies are 0.1–1 mm in diameter, with a central, elevated, more dense portion (note colony types as presented in Fig. 26-1). These elevated centers may not be present in initial cultures but tend to be more obvious after two or three passages in media. Colonies may be transferred with a wire loop, but a flame-sterilized scalpel blade is more satisfactory. A small block of agar is most readily excised and transferred into broth or inverted and spread over the surface of a new plate of agar medium. This procedure may be repeated three or four times to select reasonably pure colony cultures. However, there may be *Mycoplasma* cells on the agar surface adjacent to single colonies that are also picked up with the excised agar block.

Observation of coccoid bodies approximately 0.250 μ in diameter in Giemsa-stained colonies or broth culture sediment is suggestive of *Mycoplasma* but rarely proves much.

Cultural and biochemical characteristics of selected avian *Mycoplasma* species are presented in Table 26-1. However, such information is rarely sufficient for serotyping purposes.

Identification

Since avian *Mycoplasma* are classified by serological procedures, no other method actually is a valid substitute. Cultures with somewhat

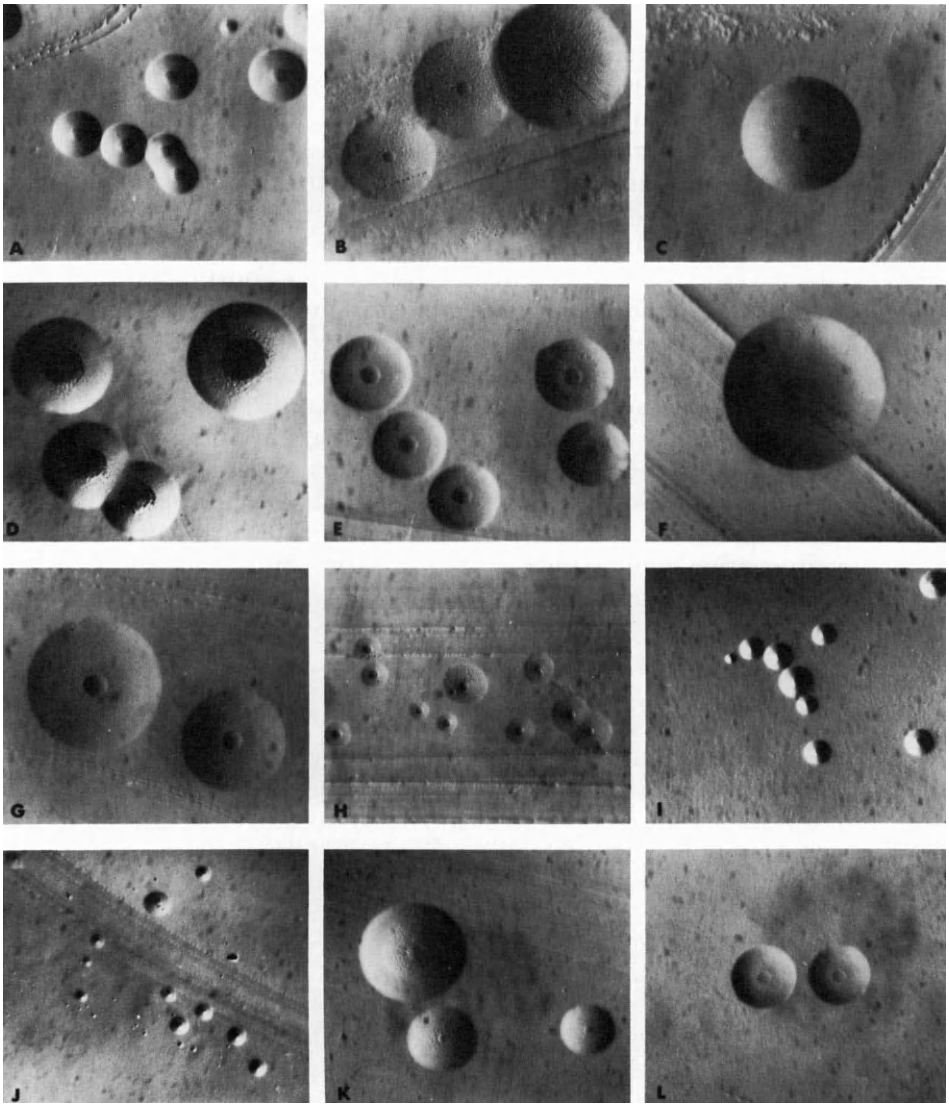

Figure 26-1. Typical colonies of avian mycoplasmas representing stereotypes A through L as indicated. Approximately 48 hr incubation (37°C) on turkey serum agar with 5% yeast autolysate, ×28. From H. W. Yoder and M. S. Hofstad (4).

typical *Mycoplasma* colonies that fail to grow on most ordinary bacteriological media can most rapidly be identified by the flourescent antibody (FA) test applied directly to colonies on an agar surface. High-titer FA conjugate prepared from *M. gallisepticum* antiserum produced in rabbits is used (9).

Preparation of agglutinating or hemagglutinating antigens for use against homologous and heterologous prepared antisera is relatively slow and expensive. Isolates may be typed by the agar gel precipitation (AGP) system employing centrifuged broth culture sediment as antigen after it is frozen and thawed 10 times (10). AGP typing serum can be prepared by inoculating type cultures into the footpads of young chickens and waiting 5–6 weeks to collect the serum.

Serological tests to identify antibodies against *M. gallisepticum* in infected poultry may be used as laboratory diagnostic aids.

The rapid serum plate test employs stained antigens prepared specifically for *M. gallisepticum, M. synoviae, or M. meleagridis* separately. Approximately 0.02 ml of serum is mixed with 0.03 ml of antigen on a glass plate. Rotate the plate for 5 sec, then read the results at 1 and 2 min. Be sure to have the antigen, serum samples, and plate at room temperature. Several obvious reactors or strong suspicious samples indicate possible reactions that should be confirmed by the hemagglutination inhibition (HI) test. HI titers of 1:80 or greater are considered positive, while titers of 1:40 are strongly suspicious when conducted in well-controlled series employing known positive and negative samples. The tests should be run with 2 HI units for turkey sera and 4 HI units for chicken sera. Some judgment is often needed, and repeat testing in 2–3 weeks is frequently suggested before a firm status is indicated.

The tube agglutination test is similar to the plate test in being a simple screening procedure for flocks. Serum dilutions of 1:12.5, 1:25, and 1:50 are frequently tested, and reactions at 1:25 or greater are considered positive. They should be confirmed by the HI test.

Experimental Animals

Inoculation of avian tissues or exudates containing *M. gallisepticum* into 7-day-old embryonated chicken eggs via the yolk sac route usually results in embryo deaths within 5–7 days. However, one or more yolk passages may be necessary before typical deaths and lesions occur. Dwarfing, generalized edema, liver necrosis, and enlarged spleens are most typical. The frequent presence of other bacterial contaminants makes embryo inoculation studies far less productive than broth culture studies employing high levels of penicillin and thallous acetate.

In general, turkeys appear to be more susceptible than chickens; at least turkeys develop more severe sinusitis, airsacculitis, and tendovaginitis following inoculation of those sites. However, the production of specific antibodies within 3–5 weeks is readily detected employing

standard antigens to test sera of turkeys or chickens inoculated into the tendon sheath areas of the hock and footpad.

Mycoplasma synoviae

Mycoplasma synoviae infection is commonly designated as infectious synovitis, an acute to chronic infectious disease of chickens and turkeys involving primarily the synovial membranes of joints and tendon sheaths, producing an exudative synovitis or tendonvaginitis. However, during recent years *M. synoviae* has less frequently been associated with synovitis but more frequently associated with airsacculitis in chickens and sometimes in turkeys.

This newer clinical manifestation closely resembles the air sac disease (airsacculitis) historically associated with *M. gallisepticum* infection complicated by some respiratory virus disease. It definitely seems to become more evident during the winter months, and like *M. gallisepticum,* it can be spread by direct contact, indirect contact with contaminated objects, apparently by air transmission to a limited extent, and via egg transmission. Voluntary control of *M. synoviae* in poultry is based on selection of serologically negative breeder flocks to avoid egg transmission of the infection to progeny flocks.

Isolation and Identification

Mycoplasma synoviae grows best in Frey's medium (see Appendix E) supplemented with 10–15% normal swine serum. The swine serum should be heat inactivated at 56°C for 30 min. In addition, *M. synoviae* requires the addition of 0.1% reduced nicotinamide adenine dinucleotide (NAD) to broth and agar media. *Mycoplasma synoviae* readily ferments glucose, as does *M. gallisepticum;* thus, evidence of growth in liquid medium can be noted by a change in the phenol red indicator from red to yellow as the medium becomes acid. Note other characteristics recorded in Table 26-1.

Colonies on agar surfaces are very similar to those of *M. gallisepticum.* However, they are readily identifiable by use of the fluorescent antibody (FA) system employing specific *M. synoviae* and *M. gallisepticum* conjugates. Broth culture sediment can be prepared as antigen for typing by the AGP system (10).

Broth cultures may be inoculated into the tendon sheath areas of the hock and footpad of young chickens or turkeys to produce specific antibodies, which can readily be identified by standard plate, HI, and AGP tests. This is a very practical method for culture typing if one can tolerate the 3–5 weeks required for adequate antibody titers to develop.

Mycoplasma meleagridis

Mycoplasma meleagridis infection is very common in turkeys throughout the world. It tends to localize in tracheas and cloacal areas, where it may live for many months. It produces airsacculitis in young turkeys and seems to be associated with decreased hatchability, certain skeletal abnormalities, and poor growth performance. *Mycoplasma meleagridis* can be spread by direct and indirect contact, and apparently by air transmission to a limited extent. However, the major means of transmission is through infected eggs to progeny flocks. Considerable progress has been made to establish breeder flocks free of *M. meleagridis*, and clean parent stock is available commercially.

Isolation and Identification

Mycoplasma meleagridis is rather difficult to cultivate, since it grows rather slowly upon initial isolation and does not ferment glucose nor reduce most tetrazolium salts that might serve as growth indicators. It tends to prefer the surface of agar medium and frequently is aided by the presence of a small amount of broth medium added to the base of agar slants prepared from media best suited for the growth of *M. meleagridis* (note the media descriptions in Appendix E). The inclusion of 10–15% horse serum is especially important.

As noted in Table 26-1, *M. meleagridis* has few cultural characteristics to aid in its identification. Therefore, it is essential to employ the FA test procedure to identify cultures grown on agar surfaces.

Mycoplasma iowae

Mycoplasma iowae infection is rather common in turkeys, and sometimes in chickens. Evidence continues to suggest that *M. iowae* is a potential pathogen [11–13]; capable of causing turkey and chicken embryo stunting and death, as well as exudative airsacculitis in young poults and sometimes leg deformities or tenosynovitis of the digital flexor tendons above or below the hock. *Mycoplasma iowae* is probably spread through eggs from infected flocks to their progeny.

Unfortunately, there are no commercially available sources of test antigens nor culture typing procedures for *M. iowae* at this time.

Other Mycoplasmas

Mycoplasmas of the other serotypes are sometimes encountered during routine culture work, since almost all of them will grow in the

usual media formulation. However, *M. gallinarum, M. iners, M. meleagridis,* and *M. columbinasale* do not ferment glucose. Thus, evidence of growth is not readily apparent. Subculture onto agar medium is necessary. Serotypes represented by *M. pullorum, M. gallinaceum, M. iowae,* and *M. anatis,* as well as *Acholeplasma laidlawii,* do ferment glucose. *Acholeplasma laidlawii* is a true saprophyte that does not require added serum in the medium for growth and can multiply at room temperature. Pigeon isolate are generally nonpathogens.

Specific identification of most of these "other" serotypes is rarely conducted, since specific FA conjugates are mainly available only for *M. gallisepticum, M. synoviae,* and *M. meleagridis.*

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27

Mycoplasmas of Animals

Ole H. V. Stalheim

The mycoplasmas (members of the class Mollicutes, order Mycoplasmatales) are the smallest free-living organisms. Unlike bacteria, they have no cell wall but are bounded by a membrane. This explains their remarkable pleomorphism. In stained smears, they are seen as ring forms, globules, small coccobacilli, of filaments. Although the "fried egg" colony is the hallmark, a high percentage grow with aberrant colonial morphology when first isolated or grow as tiny colonies (T mycoplasmas or ureaplasmas) visible only under the low power microscope (Fig. 27-1). Because most mycoplasmas require sterol for growth, suggestions were made that they be placed in the animal kingdom, but the current tendency is to place them into a sixth division of microbiology as distinct from bacteria, viruses, fungi, protozoa, and the blue-green algae. The nonsterol-requiring organisms are known as acholeplasmas, whereas the family Mycoplasmataceae contains five genera, including pathogens of humans, animals, plants, and insects, and parasitic or free-living mycoplasmas of such diverse environments as the intestine and rumen of the cow, hot springs, and the waters of abandoned coal mines.

The class Mollicutes (Table 27-1) contains more than forty pathogens of humans and animals including the causative agents of three respiratory diseases formerly thought to be due to viruses: primary atypical (virus) pneumonia of humans (*Mycoplasma pneumoniae*), mycoplasmal (virus) pneumonia of swine (*M. hyopneumoniae*), and bovine contagious pleuropneumonia (*M. mycoides* subsp. *mycoides*). Mycoplasmas are mostly host-specific but *M. bovis*, for example, which causes a variety of lesions in cattle, has been isolated from the lungs of pneumonic sheep and from human patients with respiratory disease. The mycoplasmas of bovine origin were classified into eight

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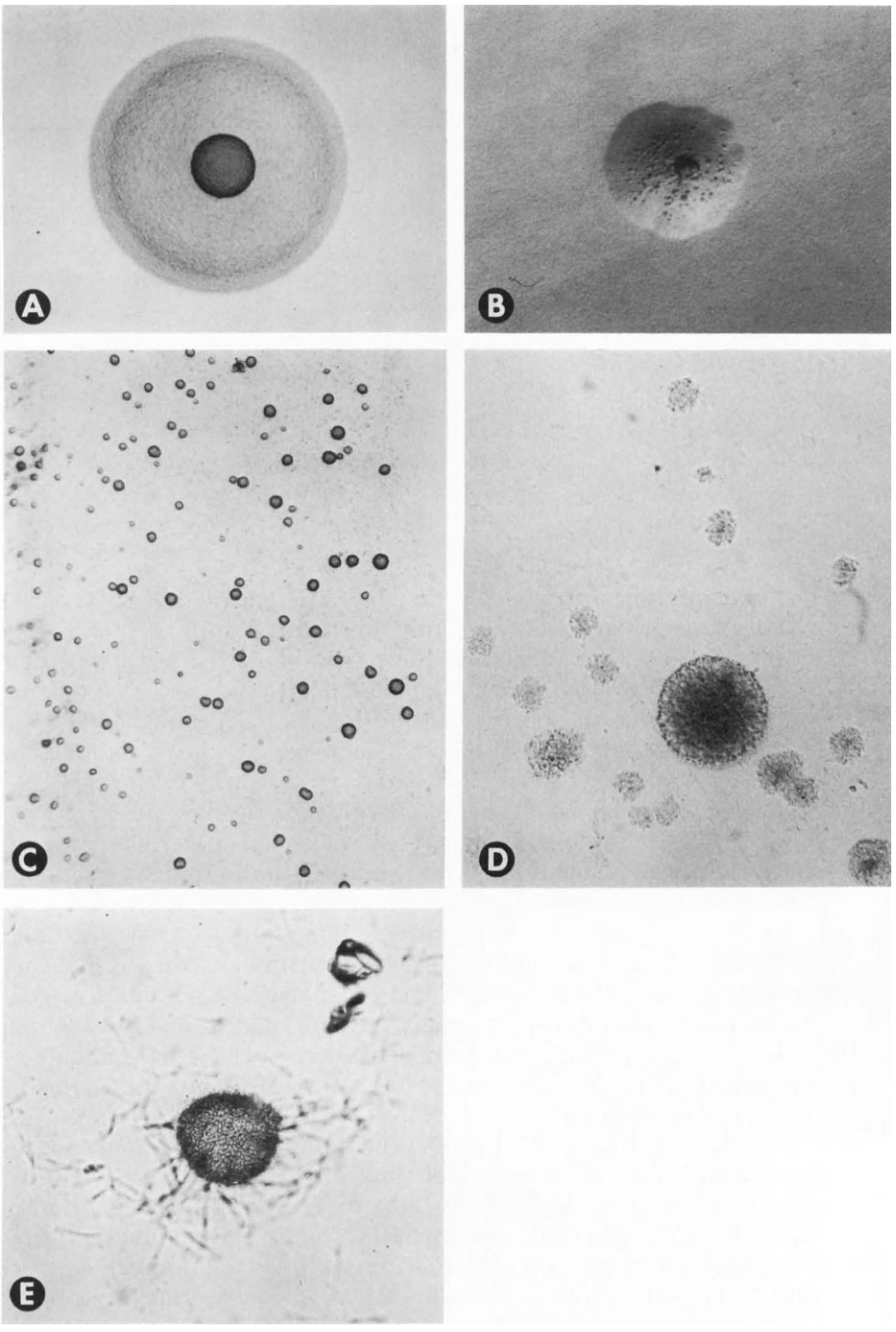

Figure 27-1. Mycoplasmal colonial morphology. *A*, unidentified isolate from eye of a sick goat; *B*, unidentified isolate from aborted bovine fetus; *C* and *D*, *Mycoplasma bovis*, showing small colonies without halos, and granular colonies, recovered from experimentally infected cows (×40); *E*, ureaplasma from bull semen (×200).

groups by Leach (1). Most isolates from sick cattle can be identified by serologic procedures as members of these groups. Several other species have been recovered from the eyes or genital secretions of cattle but their pathogenicity has not been demonstrated.

The ureaplasmas (see Table 27-1) or T mycoplasmas are distinguished by their ability to split urea into ammonia and carbon dioxide. Those of animal origin appear to be antigenically distinct from those associated with nongonococcal urethritis of humans, and they have different nutritional requirements. The role of ureaplasmas in animal diseases appears to be expanding.

At the present time, the acholeplasmas (see Table 27-1) are not known to be pathogenic for animals. Spiroplasmas (motile, helical

Table 27-1
Taxonomy of Class Mollicutes[a]

Class : Mollicutes
- Order I: Mycoplasmatales
  - Family I: Mycoplasmataceae
    - Genus I: *Mycoplasma*
      - Species: *M. pneumoniae* and numerous animal pathogens
      1. Sterol required for growth
      2. Sensitive to digitonin
      3. Genome size: 4.5×10^8 daltons
    - Genus II: *Ureaplasma*
      - Species: *U. urealyticum* of man and animals
      1. Sterol required for growth
      2. Sensitive to digitonin
      3. Genome size: 4.5×10^8 daltons
  - Family II: Acholephasmataceae
    - Genus I: *Acholeplasma*
      - Species: *A. equifetale*
      1. Sterol not required for growth
      2. Insensitive to digitonin
      3. Genome size: 1.0×10^9 daltons
  - Family III: Spiroplasmataceae
    - Genus I: *Spiroplasma*
      1. Sterol required for growth
      2. Sensitive to digitonin
      3. Genome size: 1.0×10^9 daltons
    - Genera of uncertain affiliation:
      - *Anaeroplasma*
        1. Some strains require sterols; some do not
      - *Thermoplasma*
        1. Sterol not required
        2. Genome size: 1.0×10^9 daltons

[a]Reprinted with permission from *Laboratory Diagnosis of Mycoplasmosis in Food Animals*. Courtesy of the American Association of Veterinary Laboratory Diagnosticians, Tucson, Arizona.

mycoplasmas) cause diseases of plants. Because they can cause cataracts in suckling mice and rats and microphthalmia in hamsters (2), they are considered potentially pathogenic for animals.

Strictly anaerobic mycoplasmas (anaeroplasmas) exist in the rumen of cattle and sheep (3). They are antigenically distinct from intestinal mycoplasmas (4).

Many established tissue culture cell lines are now known to be contaminated with mycoplasmas. They were ignored for a long time by laboratory workers since they seemed to have no effect on cell cultures. In recent years, it has become evident that contaminating mycoplasmas are capable of altering the activity of cells and their sensitivity to viruses and drugs. Primary cell cultures are much less apt to be contaminated.

Clinical Manifestations

Clinical observations, laboratory studies, and the results of controlled research have demonstrated causal relationships to disease for several mycoplasmas (Table 27-2). Pathogenic mycoplasmas have a predilection for serous surfaces, such as the thoracic, abdominal, and articular

Table 27-2
Mycoplasmas and Acholeplasmas Associated with Diseases of Animals

Animal species affected	Disease	Organisms
Cattle	Pneumonia	*M. mycoides* var. *mycoides*
		M. bovis
		M. dispar
		Ureaplasmas
	Arthritis	*M. bovis*
		M. bovigenitalium
	Mastitis	*M. bovis*
		M. californicum
	Abortion	*M. bovis*
	Vaginitis	Ureaplasmas
		M. bovigenitalium
	Seminal vesiculitis	*M. bovis*
		M. bovigenitalium
	Uncertain	*M. bovirhinis*
		M. alkalescens
		M. arginini
		A. modicum
		A. laidlawii
		M. bovoculi
		M. verecundum
		M. canadense
		M. alvi

Table 27-2 (*continued*)
Mycoplasmas and Acholeplasmas Associated with Diseases of Animals

Animal species affected	Disease	Organisms
Swine	Pneumonia	*M. hyopneumoniae*
	Arthritis	*M. hyorhinis*
		M. hyosynoviae
	Uncertain	*M. flocculare*
		M. sualvi
		A. axanthum
		A. granularum
		M. hyopharyngis
Sheep and goats	Pneumonia	*M. ovipneumoniae*
	Pneumonia,	*M. mycoides* subsp. *capri*
	arthritis,	*M. mycoides* subsp. *mycoides*
	mastitis	*M. agalactiae*
		M. putrefaciens
	Conjunctivitis	*M. conjunctivae*
	Arthritis, pneumonia, mastitis	*M. capricolum*
	Uncertain	*M. arginini*
		M. oculi
Horses	Uncertain	*A. equigenitalium*
		M. equirhinis
		M. subdolum
		M. felis
		M. arginini[c]
		M. salivarum
		A. equifetale
		A. hippikon
		A. laidlawii
Rats and mice	Pneumonia	*M. pulmonis*
	Arthritis	*M. arthritidis*
	Rolling disease	*M. neurolyticum*
Guinea pigs	Uncertain	*M. caviae*
Dogs	Pneumonia	*M. cynos*
	Uncertain	*M. spumans*
		M. maculosum
		M. edwardii
		M. molare
		M. canis
		M. opalescens
Cats	Pneumonia	*M. felis*
		M. feliminutum
		M. gateae

cavities of cattle and swine and the air sacs of poultry, where they localize and persist protected from antibody and therapeutic agents by the fibrinous tissue reactions that characterize mycoplasmosis. In swine, they cause pneumonia, arthritis, and serositis; in cattle, sheep, and goats, they cause mastitis (5), arthritis, and pneumonia. The

significance of mycoplasmas in diseases of the genital tract is not clear. Under experimental conditions, some species (*M. bovis*) caused severe diseases [seminal vesiculitis of the bull, salpingitis abortion (6) of the cow, for example], whereas other species are considered of potential etiologic importance.

The clinical signs of mycoplasmosis are not distinctive. Mycoplasmal pneumonia cannot be diagnosed by observations of affected animals or by the appearance of lesions at necropsy, except perhaps for the characteristic sequestra of contagious bovine pleuropneumonia.

Swine

The clinical signs of swine mycoplasmosis include pneumonia (caused by *M. hyopneumoniae*), polyserositis, and arthritis in young pigs (*M. hyorhinis*) and arthritis in older pigs associated with *M. hyosynoviae*. Mycoplasmal pneumonia of swine is a chronic disease with a high morbidity and a low mortality characterized by chronic, nonproductive coughing. With stress and secondary bacterial infections, infected pigs may develop clinical signs of pneumonia. They may die, recover, or be stunted. The economic losses from mortality, poor weight gains, and poor feed conversion are high (7).

Cattle

The clinical signs of bovine mycoplasmosis include pneumonia, arthritis, and mastitis; mycoplasmas have also been associated with bovine abortion, conjunctivitis, salpingitis, seminal vesiculitis, and vaginitis. Mycoplasmas (*M. dispar, M. bovis,* and ureaplasmas) caused subclinical infections, and with other bacteria or viruses resulted in severe clinical disease (8).

Mycoplasmal mastitis should be suspected whenever there is an increase in cases of severe purulent mastitis involving more than one quarter (usually all four) with tannish or brownish secretions and marked loss of milk production. The outbreak may be explosive or insidious; affected cows are not septic and continue to eat. The most common cause was *M. bovis;* treatment was not effective and infected cows shed mycoplasmas indefinitely (9). The same mycoplasma, *M. bovis,* has been recovered from cases of severe, intractable arthritis, often following outbreaks of mastitis or respiratory disease

A granular vulvo-vaginitis syndrome in Ontario dairy cattle was associated with ureaplasmas. It was characterized by hyperemia, epithelial cysts, and a purulent vulvar discharge. The morbidity was highest in winter months and had a significant effect on fertility (10). Many

cows are vaginal carriers of large numbers of ureaplasmas without any clinical signs. Clinically normal bulls may carry virulent strains of ureaplasmas, which are transmitted during coitus and induce the vulvitis syndrome.

Sheep and Goats

Mycoplasmas were first isolated from small ruminants in the Middle East with contagious agalactiae. The casual agent, *M. agalactiae,* was isolated and identified from an arthritic goat in California (11).

In 1969, a mycoplasma was isolated from the conjunctival sac of a goat in Connecticut. At first, it was identified as *M. mycoides* subsp. *capri;* further studies revealed it to be *M. mycoides* subsp. *mycoides* (hereinafter referred to as *M. mycoides*). Additional isolations have been made from goats in six states with pneumonia, arthritis, mastitis, and conjunctivitis, but most were from the milk of apparently normal or mastitic goats. In some flocks of goats, *M. mycoides* caused severe morbidity of young animals; in other flocks, the organism persisted in the udder and was shed in the milk but the goats were apparently healthy. Treatment with heavy and repeated doses of antibiotics failed to eradicate the infections. Still another mycoplasma, *M. putrefaciens,* also caused caprine mastitis (11). These organisms have not been isolated from sheep in this country (12). Other mycoplasmas were associated with disease outbreaks of both goats and sheep (Table 27-2). In Kenya, MacOwan and Minette recovered an antigenically distinct strain, F38, from the lungs of goats with contagious caprine pleuropneumonia. It has not been identified in the Americas.

Rats and Mice

Chronic respiratory disease has been a problem in laboratory rats and mice for a long time (see review by Cassell and Hill). In addition to mortality losses, the presence of intercurrent disease undermines the validity of research performed with pneumonic animals. The causative agent of murine respiratory mycoplasmosis (MRM) is *M. pulmonis.* Rats and mice require MRM from their mothers at an early age. The infection is usually subclinical; it may persist for life. Mortality is cumulative over many months; mice are less severely affected than rats. Elimination of *M. pulmonis* from an infected colony of rats or mice is a very difficult undertaking. Spontaneous polyarthritis in laboratory rats is caused by *M. arthritidis.* The disease is usually enzootic with only a few clinical cases. An infection of *M. neurolyticum* in mice is manifested as conjunctivitis. Inoculations into mice of infected tissue, cultures of *M. neurolyticum,* or cell-free filtrates cause rolling disease.

Dogs and Cats

Mycoplasmas were first isolated from dogs with distemper in 1934. They were recovered from the nose, throat, or vagina of 30% of apparently healthy dogs in Japan (13) and from the lung, liver, kidney, and spleen of dogs with respiratory and other diseases, but there is no evidence that mycoplasmas are responsible for any specific disease of dogs. The *Mycoplasma* species of canine origin are *M. spumans, M. maculosum, M. edwardii, M. cynos, M. molare,* and *M. canis.* The latter species was recovered from a woman with respiratory illness similar to an illness in her pet dog. The woman and her three children had close contact with the dog. All four persons and the dog harbored *M. canis* in their throats, and all had serologic reactions to this mycoplasma (14).

Of the mycoplasmas isolated from dogs (Table 27-2), only *M. cynos* was found consistently in the lungs of dogs with distemper. When the culture was given to puppies, it induced focal pneumonia. The organism persisted in the lungs as long as 3 weeks after inoculation; clinical signs were not manifested (15).

Cats harbor *M. gatae, M. arginini,* and *M. felis.* The latter species has been associated with outbreaks of acute conjunctivitis in cats (16), but attempts to induce the disease experimentally have failed or yielded equivocal results. Switzer (17) isolated a mycoplasma from the pneumonic lung of a kitten but could not induce the disease experimentally. Eighty-one strains of *M. arginini* were isolated from a series of 555 cats. When experimentally inoculated into young kittens, *M. arginini* colonized the inoculated sites but clinical disease was not induced (18).

Ureaplasmas were isolated from the throat or vagina of 25 of 36 apparently healthy cats (19). When given by the oral or intraperitoneal route to normal cats, clinical signs were not induced. However, when given by the vaginal route to three pregnant cats, two gave birth to kittens that died in 10 and 12 days, and the third cat aborted 10 days postexposure. Ureaplasmas were recovered from the uterus or vagina of all three cats (20).

Horses

The mycoplasmas most frequently isolated from horses *(M. equirhinis* and *M. felis)* were recovered from clinically normal horses as well as those with respiratory disease. By serologic procedures, specific antibodies were detected in serums from two of six horses that harbored *M. equirhinis* (21). The virulence of mycoplasmas for horses has not been demonstrated. Two unidentified mycoplasmas of equine origin cross-reacted with strains of *M. mycoides* of caprine origin (22).

Laboratory Procedures

The isolation and further cultivation for identification of most mycoplasmas from clinical materials is not difficult if a suitable, well-prepared medium is used. Satisfactory mediums are described in Appendix F. The basic ingredient of each is an infusion of meat that is supplemented with yeast extract, serum, and perhaps deoxyribonucleic acid or nucleotides. The quality of water used in the medium is extremely important. It should equal that used for cell cultures. Some batches of phenol red have been shown to be toxic and have either delayed or inhibited the growth of mycoplasmas. Arginine is inhibitory for *M. bovoculi*. Thallium acetate at a fungistatic concentration is inhibitory to ureaplasma species. Penicillin G and benzyl penicillin may be inhibitory to the growth of *M. dispar*. When either a new bottle or batch of a component is prepared, it should be pretested to determine that it has no inhibitory effect.

Special Techniques

Swine

The specific-pathogen-free (SPF) program of swine health was developed to eliminate mycoplasmal pneumonia of swine (MPS) and other diseases; herds are monitored for continued freedom from MPS and are dropped from the program if it is recognized in the herd. The monitoring procedures include serology, clinical and slaughter inspections, and laboratory attempts to recover *M. hyopneumoniae*. Because the latter is expensive, laborious, and often unsuccessful, it is not routine at most laboratories. The Danish isolation method, which claimed success in virtually all attempts, was recently assessed in Indiana; *M. hyopneumoniae* was isolated from 17 of 20 lungs with uncomplicated lesions of MPS. Samples of lung, including bronchii and advancing edges of atelectic areas, were processed at once (or frozen), homogenized, serially diluted in Friis's medium (10^{-4}), and incubated (37°C) for 3 weeks. One ml of the 10^{-2} dilution was added to 50 ml of medium and passed through a 0.20-μm cellulose filter. Each inoculated membrane was placed in growth medium until the pH changed, rinsed, dried, and examined for colonies of *M. hyopneumoniae* by the indirect immunofluorescence test. The procedure should be useful for the definitive laboratory diagnosis of MPS (23).

European swine harbored a relatively fastidious mycoplasma that grew slowly and formed small colonies (0.5 mm) without halos; it was named *M. flocculare* because of the tendency of some isolates to form small aggregates in liquid medium. Several isolations of *M. flocculare* were recently made from swine in the U.S. by supplementation of Friis's medium with cycloserine (0.5 mg per ml) and *M. hyorhinis*

antiserum (5%) to inhibit the faster-growing *M. hyorhinis* (24). Neither the incidence nor the role of *M. flocculare* in MPS is known.

Ruminant Animals

Special mediums are useful for the isolation of *M. dispar* (M-96 medium), *M. arginini* (add 1% L-arginine, adjust pH to 7.0), the ureaplasmas (modified Hayflick's), and the anaeroplasmas. When first isolated on agar medium, some mycoplasmas display aberrant colonial morphology (see Fig. 27-1). Colonies of *M. dispar* and *M. ovipneumoniae* lack the central downward growth that contributes the "fried egg" appearance regarded as classic for mycoplasmas.

When samples of ovine lung were cultured for mycoplasmas, the most common strains grew slowly in broth, fermented glucose, formed small colonies without halos, and reacted with *M. ovipneumoniae* antiserum (25). Some laboratory diagnosticians reported that the most common mycoplasma isolated from dairy goats was *M. mycoides* (11). Australian workers compared strains of *M. mycoides* from cattle and goats. Strains of caprine origin grew to greater turbidity in broth and formed larger colonies in agar (diameters of 2.2 mm versus 1.0 mm), digested casein, liquefied inspissated serum, and survived longer at 45°C. Most strains from goats were the large-colony (LC) type, whereas strains from cattle were the small-colony type (26). Strains of *M. mycoides* from goats in this country were the LC type, with one exception, but only a few strains have been examined.

Use and Source of Serum

Serum from several species of animals may be used as a supplement for the growth of mycoplasmas. These may be obtained from commercial suppliers or may be processed in the laboratory. The growth of mycoplasmas may be influenced by the species of animal used as the serum source and whether or not the serum was inactivated. Serum and serum fractions are generally utilized at concentrations of 10–20%, depending in part on the animal source of the serum and in part on the mycoplasma that may be in the clinical material. In general, 5–15% is used for maintenance mediums and up to 20% for isolation mediums. Horse serum is considered the best for general use because of its ready availability and economy. Pooled serum is probably best, but each lot should be pretested for the purpose intended (isolation or maintenance). Unsatisfactory serum should not be mixed with acceptable serum but should be discarded. Whether or not fresh serums should be inactivated by heat and/or acid treatment is a choice each laboratory diagnostician must make. For acid inactivation, add 1 *N* HCl until a pH value of 4.2 is attained. Refrigerate the serum overnight, and read-

just the pH to 7.1 with 1 *N* NaOH. Clarify the inactivated serum by centrifugation or filtration. Inactivation may be attained by storage combined with freezing and thawing.

Human serum may be prepared from either human whole blood or human plasma as follows: Add one part of a 2.5% solution of calcium chloride to one part blood or plasma. Remove coagulated material by centrifugation, and adjust the pH to 7.2. Human serum is probably the best supplement for mycoplasmal growth medium. Swine serum may be excellent but varies markedly between animals and batches. The donor animals must be free of mycoplasmas.

Inhibitors

Two inhibitors are frequently added to mycoplasmal growth medium: Thallium acetate at a concentration of 1:2000 to 1:4000 is a fungal inhibitor, and crystalline penicillin G at 250 to 1000 units/ml is a bacterial inhibitor. Amphotericin B (5 μg/ml) is commonly used as a replacement for thallium acetate in ureaplasmal growth medium.

The pH of the medium may significantly alter the isolation of mycoplasmas. Mycoplasma of bovine origin grow well between pH 7.0 and 7.6; others are tolerant of a low pH. Ureaplasmas require a low pH for growth. Some mycoplasmas will survive longer at incubation temperature if the pH of the medium is 8.5

Plastic tubes and petri dishes have replaced glassware in many laboratories. These have been reported as the cause of poor mycoplasmal growth. To prevent such problems, quality control tests could be made when a new batch or lot is received. A simple comparative test between old and new plates or tubes is all that is required.

Quality Control of Medium and Components

Qualitative checks for the acceptability of a new component may be conducted by preparing two lots of medium, one with the old and the other with the new component. For solid medium, inoculate duplicate plates of each medium with a measured amount of inoculum of a titrated culture. The mediums are compared on the basis of rate of growth, colony size, and number of colonies that develop. A similar study should be carried out for liquid medium using a 10-fold titration method. Duplicate titrations for each test medium should be used for such comparisons.

Pretitrated cultures for inoculum in quality control may be prepared as follows. Grow the test species in broth, and dispense 0.1 ml in each well of a disposable microtiter "U" plate. Seal the plate with cellulose

tape, and freeze at −70°C for 14–21 days. Titrate the culture by cutting off two or three wells with sterile scissors. Aseptically remove the covering tape, and place each pellet in a tube containing 0.9 ml of either glucose or arginine broth. Make tenfold dilutions from the initial tube to 10^{-9}. Inoculate duplicate plates of solid mediums from 10^{-4} to 10^{-8} dilution tubes. Incubate the broth and solid medium at 37.5°C. After 4–5 days, count the colonies and determine the average colony count per milliliter. Incubate the broth medium and determine the most probable number of organisms per milliliter. The two figures obtained should be almost equal. If the pretitrated culture is maintained at −70°C, there will be little change from the original in the postfreezing titration result over a 6-month period (27).

Isolation Techniques

To isolate mycoplasmas from sick animals, swabs containing exudates from the eye, nostril, trachea, and the genitalia are placed in tubes of mycoplasmal broth medium. The inoculated tubes should be chilled to 2–3°C and transported to the laboratory as quickly as possible. They should be processed on the day they are collected, if possible, but viability will be maintained for 48–72 hr at 2–3°C. Beyond this time, storage should be at −70°C. After thorough mixing, 0.1 ml is delivered to agar plates and dilutions made into broth mediums.

Cervico-vaginal mucus is aspirated from the vaginal fornix with aseptic precautions, sealed in the collecting rod, and transported to the laboratory. The mucus (0.2 ml) is added to 1.8 ml of broth and mixed thoroughly; 0.1 ml of the mixture is used as the inoculum for agar medium and 0.2 ml for broth medium. Plating the mucus directly on solid medium may increase the recovery rate if only a few mycoplasmas are present.

Preputial material may be collected either on a swab inserted through a sterile tube to the preputial fornix or by flushing the preputial fornix with 10 ml sterile distilled water or broth medium. The fluid is centrifuged (9000 *g* for 15 min) at 5°C in an anglehead rotor. The sediment is resuspended in 1 ml of broth and inoculated into both liquid and solid mediums.

Semen from bulls may be examined for mycoplasma either as whole fresh semen or after being processed by an artificial insemination unit. Whole semen (0.2 ml) is diluted with 1.8 ml of a medium without inhibitors, mixed thoroughly, and inoculated to solid and liquid mediums. Processed semen is added directly to solid medium (0.1 ml) and liquid medium (0.2 ml). Because processed semen contains microbial inhibitors, direct cultures are often negative in low dilutions but positive in higher dilutions. To certify that semen destined for interstate

commerce is free of mycoplasmas, special procedures are being developed.

Fluid materials such as milk, body wastes, fetal fluids, synovial fluids, and fetal stomach contents are inoculated directly into liquid and solid mediums.

Tissues such as lung, liver, spleen, and kidney may be examined in two ways: (a) Cut a block of tissue and streak it directly across the surface of an agar plate. A sterile loop can be used to spread the inoculum. (b) A block of tissue can be suspended in 9 ml of broth medium and homogenized in a Ten Broeck tissue grinder. Both solid and liquid mediums are inoculated. The tissue suspensions should be serially diluted to 10^{-6} or 10^{-7}. Frequently, lower dilutions will be negative for mycoplasmal growth due to a carry-over of inhibitory agents in the tissue, while higher dilutions are positive (27).

For routine attempts to isolate mycoplasmas, inoculate the clinical materials into tubes of usually two liquid mediums (modified Hayflick's with inhibitors and Livingston's modification of Hayflick's medium for ureaplasmas) and on two plates of solidified Hayflick's medium. The addition of semisolid medium (Hayflick's plus 0.15% agar) was reported to increase the rate of isolations. If fastidious organisms such as *M. dispar* are to be isolated, special medium (Gourlay and Leach) must be included.

The inoculated plates are incubated in a humid atmosphere at 37.5°C; one plate should be incubated aerobically, the other in 5% carbon dioxide and 95% nitrogen. After 48 and 96 hr incubation, the plates should be examined using obliquely transmitted light and a stereoscopic microscope at 25–40 magnifications. If the growth is sparse (1–10 colonies/plate), flood the plate with approximately 1.5 ml of sterile broth and incubate for an additional 48–72 hr or longer. If colonies are not detected after 14 days, the plates may be discarded as negative. Tubes of liquid medium are checked daily. When slight turbidity is detected (or when the color changes), transfers are made to an appropriate solid medium. Some workers "blind-passage" from broth to broth at intervals of 7, 14, and 21 days.

Purification of Isolates

Before fresh isolates can be identified, they must be purified by the single colony technique. A plate with discrete colonies is selected for cloning. Individual colonies are removed by cutting out a small block of agar using a sterile scalpel. Transfer the colony to a tube containing 2–3 ml of broth and incubate it for 48 hr or longer. Draw all of the culture into a sterile syringe and express through a Swinney filter (0.45-μm pore size). Dilute the filtrate 1:10 and 1:100 in broth, spread

0.05 ml of the dilutions on plates of solid medium, and incubate. Repeat the colony selection and filtration procedure for at least three cycles. If morphologically distinct colonies are present, pick and clone representative colonies for each type. Because not all colonies grow, start with four or five individual colonies of each morphologic type.

Colony cloning of primary isolates may be performed without filtration. Cut and remove an agar block containing a single colony. Invert it on an agar plate, and push it back and forth across the surface of the agar. After incubation (48–72 hr), pick an isolated colony and repeat the transfer a second time and again for a third time. A single colony picked from the third transfer is considered to be a cloned strain. The only serological test applicable to an uncloned isolate that can be reliably interpreted is the fluorescent antibody (FA) test (27).

Maintenance of Reference Cultures and Isolates

To identify isolates, type cultures and specific hyperimmune antiserums against the type cultures may be obtained from the American Type Culture Collection. Maintenance of reference or type cultures in a pure condition requires constant vigilance. The following suggestions may be useful. Have only one reference culture in use at any one time. If possible, work only in a biohazard or similar type of hood. Sterilize the hood before introducing the culture and container. Chemically sterilize the outside of the culture container. Make all transfers in a sterile manner with a pipette filler; do not put the pipette in your mouth. Transfers of cultures should be performed in a manner that minimizes the formation of aerosols. Because the pathogenicity of mycoplasmas of animal origin for laboratory personnel is unknown, all reasonable precautions should be taken to minimize self-exposure.

Reference cultures of mycoplasma may be stored in the lyophilized condition, or a simple method to preserve reference cultures for up to 4 years is as follows. Cut blocks from solid medium with almost confluent colonial growth and place in a small vial (two per vial). Add approximately 0.2 ml of liquid medium and incubate overnight (37.5°C). Verify the viability of the cultures by testing random samples of the vials, and freeze the balance of the vials at −70°C. Field isolates may be stored in the same manner (27).

Biochemical Tests

Biochemical tests for the characterization of mycoplasmas were recommended by the Subcommittee on the Taxonomy of Mollicutes (28). In many situations, a much less detailed approach will provide significant information useful in identifying mycoplasmas isolated from

animals. Before proceeding with biochemical tests, it is necessary to purify the unknown isolate by cloning and to show that it is in fact a mycoplasma as follows.

Absence of Cell Wall

Electron microscopy is the preferred method to determine the absence of a cell wall, but since this is not practical for most laboratories, a substitute procedure is the examination of a broth culture by phase-contrast or darkfield microscopy to show pleomorphic morphology (small coccoid bodies, ring forms, and fine filaments).

Detection of Bacterial L-forms

One is technically required to complete five consecutive subcultures on medium without antibacterial agents to test for reversion from an L-form to a bacterium. For most diagnostic purposes, this is excessive. If the culture appears to be a mycoplasma and can be characterized biochemically and serologically as a known species, this is rather conclusive evidence.

Colonial Morphology

A stereodissecting microscope with magnification to ×30 is needed for examining mycoplasmal colonies. Greater magnification may be necessary, especially for the study of ureaplasmal colonies. Dienes' staining procedure (29) is also a useful tool. Colony morphology is variable, depending upon the culture medium and the conditions of incubation. Some combination of these should, in most instances, grow the typical "fried egg" colony with the central spot growing down into the medium and a peripheral zone of surface growth. Variations are seen, especially with recent isolates, from all "central spot" to all surface growth.

Sterol Requirement

Dependence on sterol for growth is the criterion for determining the family: Mycoplasmataceae are sterol-dependent, and Acholeplasmataceae are sterol-independent. Procedures for a direct determination of this dependence have been reported. However, sensitivity to digitonin was shown to closely parallel the sterol requirement and is much easier to determine. The procedure is similar to disc growth inhibition with specific antiserum. The discs are saturated with 0.025 ml of a 1.5% (w/v) ethanolic solution of digitonin and allowed to dry. A plate of an agar medium that supports good growth of the culture is inoculated so as to give a uniform population of colonies. Flooding the surface with diluted broth culture and removing the excess fluid works well. After the inoculum has dried, the digitonin disc is placed on the surface and the culture is incubated. The test may be interpreted when colonies can be detected. In order to be considered sensitive, the zone

of growth inhibition around the disc should exceed 5 mm. Mycoplasmas are sensitive, and acholeplasmas are resistant.

Because it is not practical to run a long series of biochemical tests in a busy veterinary diagnostic laboratory, some judgment must be used in the selection of tests that will provide the most information for the time and effort expended (30). Three tests (glucose fermentation, arginine hydrolysis, and urea hydrolysis) are of prime importance. Other tests that have shown some usefulness and are not too difficult to perform are phosphatase activity and film and spot formation.

Glucose Fermentation

Two medium controls are essential: (a) Base medium without the test substrate must be inoculated and incubated along with the test; (b) medium with the test substrate must be incubated without inoculation.

Test Media

Add 10 ml of sterile, heat-inactivated (56°C/30 min) horse serum, 5 ml of yeast extract, 10 ml of 10% (w/v) solution of glucose, and 1 ml of 0.5% (w/v) solution of phenol red to 74 ml of heart infusion broth (Difco). After the pH is adjusted to 7.5, sterilize by filtration, and dispense in 5-ml amounts to screw-capped tubes. Alterations in this medium that may prove helpful with some mycoplasmas are (a) substitution of a different serum supplement, or PPLO serum fraction (Difco) for the horse serum, (b) deletion of the yeast extract, or (c) the addition of bacterial inhibitors. Occasionally it will be necessary to use a more complex base medium. The medium of either Frey or Friis is recommended. Each lot of medium should be checked with cultures known to give positive and negative reactions (30).

Yeast Extract

A suspension of 125 g of Fleischmann's® pure dry yeast, Type 2040 (Standard Brands Inc., New York) in 750 ml of water is placed at 37°C for 20 min and then heated to 95°C for 5 min. After cooling, centrifuge at 1000 *g* for 30 min. Dispense the supernatant fluid in 60-ml volumes and autoclave at 115°C for 5 min. This preparation may be stored at −20°C for 3 months.

Inoculation

Test cultures should be subcultured to adapt them to the medium. The test and appropriate control media are inoculated with 1 ml of 24-hr broth culture.

Incubation

Tests are incubated at 37°C for 2 weeks. Broth medium exposed to increased atmospheric CO_2 during incubation can show false positive reactions.

Interpretation

Tests are examined daily for 1 week and every 2 days for the second week. The pH value of uninoculated control media should not change. To be considered positive, inoculated glucose medium should develop an acid shift that exceeds any acid shift in the inoculated base medium by at least 0.5 pH unit. It is helpful to prepare a set of pH standards in test medium for reference purposes.

Arginine Hydrolysis and Urea Hydrolysis

Use the procedure as for glucose fermentation with the following changes: (a) Replace the glucose in the medium with arginine or urea as the case may be; (b) adjust pH of the medium to 7.0; (c) a positive test is an alkaline shift of 0.5 pH unit.

Phosphatase Activity

The following medium is recommended: sterile, heat-inactivated horse serum, 20 ml; yeast extract, 5.0 ml; 1% (w/v) sodium phenolyphthalein diphosphate solution, 1 ml; and heart infusion agar (Difco), 74.0 ml. Adjust the pH to 7.8. Plates of this medium are inoculated in triplicate with a drop from a 24-hr broth culture. Uninoculated control plates are also incubated at 37°C for 3, 7, and 14 days, respectively. One inoculated and one uninoculated plate are examined on each test day by flooding with 5 *N* NaOH. The appearance of a red color after approximately 0.5 min on the inoculated plate indicates phosphatase activity. Uninoculated plates may turn red but at a much slower rate.

Film and Spots

A plate of agar medium is inoculated with a drop from a 24-hr broth culture and incubated for 2 weeks at 37°C. The plates should be examined at 3–4-day intervals for the appearance of a film over the heavily inoculated area. Examination under a dissecting microscope will help detect the crinkled appearance of the film and the small black dots in the upper layer of the medium. The biochemical reactions to be expected with mycoplasmas are summarized in Table 27-3.

Serologic Tests

Growth Inhibition Tests

The inhibitory effect of homologous antiserum on growth of mycoplasmas is highly specific. Although several methods of application are possible, probably the most common growth inhibition (GI) test is that of Clyde (31). The identification of mycoplasmas by GI tests requires a battery of high-quality antiserums. They must have a known zone of growth inhibitory effect against the homologous species with little or no effect against the heterologous species. Such antiserums are not generally available from commercial sources.

Table 27-3
Biochemical Reactions of Selected Organisms of the Order Mycoplasmatales[a,b]

Organism	Digitorin	Glucose	Arginine	Urea	Phosphatase	Film & spots	Serum digestion
M. mycoides subsp. *mycoides*	S	+	−	−	−	+ or −	−
M. bovigenitalium	S	−	−	−	+	+	−
A. laidlawii	R	+	−	−	−	−	−
M. bovirhinis	S	+	−	−	−	+ or −	+ or −
M. bovis	S	−	−	−	+	+	−
A. modicum	R	+	−	−	−	−	−
M. sp. (group 7)	S	+	−	−	−	−	+
M. alkalescens	S	−	+	−	+	−	−
M. arginini	S	−	+	−	−	−	−
M. dispar	S	+	−	−	−	−	−
M. bovoculi	S	+	−	−	−	−	−
M. canadense	S	−	+	−	+	−	−
M. verecundum	S	−	−	−	ND	+	ND
M. mycoides subsp. *capri*	S	+	−	−	−	−	+
M. agalactiae subsp. *agalactiae*	S	−	−	−	+	+	−
M. conjunctivae	S	+	−	−	−	−	−
A. oculi	R	+	−	−	−	−	−
M. ovipneumoniae	S	+	−	−	−	−	−
M. capricolum	S	+	+	−	+	−	+
M. putrefaciens	S	+	−	−	+	−	−
M. hyopneumoniae	S	+	ND	−	ND	ND	ND
M. flocculare	S	+	−	−	−	−	ND
M. hyorhinis	S	+	−	−	+	−	−
M. hyosynoviae	S	−	+	−	−	+	−
A. granularum	R	+	−	−	−	−	−
M. gallisepticum	S	+	−	−	−	−	−
M. meleagridis	S	−	+	−	+	−	ND
M. synoviae	S	+	ND	−	ND	+	ND
M. iners	S	−	+	−	−	+	−
M. gallinarum	S	−	+	−	−	+	−
M. antis	S	+	−	−	+	+	ND
Ureaplasma sp.	S	−	ND	+	ND	−	−

[b]Key: S, sensitive; R, resistant; +, positive; −, negative; + or −, variable; ND, no data.
[a]Reproduced with permission from Blackburn (30).

Antibody Production

Concentrated suspensions (× 100–200) of washed mycoplasmas are homogenized with an equal volume of Freund's complete adjuvant. Homogenization may be accomplished by ultrasonification or with a hypodermic syringe. The latter is filled and emptied several times using an 18-gauge or smaller needle. This usually produces a stable emulsion. A properly prepared emulsion will not separate for several weeks; however, if separation occurs, repeat the process. To minimize abscess formation, 1000 Units of penicillin and 0.1 mg of streptomycin per milliliter may be added.

If rabbits are used to produce antibody, the toepad injection procedure supplemented with intramuscular (IM) inoculation produces a good antiserum. The first injection of 0.1 ml is given in the one toepad and 0.9 ml IM into the same leg. The following week, the same procedures are used with the other leg; a third injection of 1 ml IM is given 1 week later with two subcutaneous injections of 0.25 ml along the back. A small amount of blood is taken from the marginal ear vein 7–10 days later for testing. Animals that have a low titer or poor spectrum of antibody can be boosted by giving them one or more IV injections of 0.1–0.5 ml of mycoplasma concentrate without adjuvants diluted 1:10 at weekly intervals until the desired level of precipitating antibody is attained. Rabbits may be bled 7–14 days after the last injection. It is possible to get up to 50 ml of blood from the marginal ear vein by rubbing the ear with equal parts of toluene–ethanol mixture and making a lateral cut along the vein. The serum should be stored at −70°C.

Test Procedure

1. Sterile filter paper discs (6-mm diameter) are saturated with antiserum of known potency and specificity. Commercially available discs of the type used for antibiotic sensitivity testing are recommended. They uniformly absorb 0.025 ml of antiserum and have no tendency to stick together. The discs are separated in a sterile Petri dish and loaded with a microdiluter calibrated to deliver 0.025 ml. Saturated discs may be frozen and stored at −20°C in a screw cap vial until used, or the saturated discs may be dried overnight under ultraviolet light; dried discs are easier to work with.
2. Cultures should be cloned to assure purity. It is advisable to determine growth characteristics in the mediums of choice.
3. The agar medium is inoculated with a broth culture in log phase of growth. Usually the inoculum will need to be diluted 1:10 or 1:100 to obtain a suitable population of colonies. They should be numerous but not too crowded to permit normal development. The entire agar surface should be inoculated. This can be done by

flooding the surface and removing the excess fluid or by using a bent glass rod to spread the inoculum. After inoculation, the plates are dried with the lids ajar in a 37°C incubator for 30 min–1 hr.

4. The dried, inoculated plates must be marked to identify the discs that will be used to test for inhibition of growth. The method used is not important, but an area about 2 cm square is needed for each disc. The author finds it convenient to mark the bottom of the plate. Individual discs are removed from storage vials with sterile forceps and placed firmly in the appropriately marked area. Care should be taken to assure that the discs do not slide as they are put in place. The plates are returned to the incubator (37°C, high humidity). Antiserums to be used are determined according to the host from which the culture was isolated. If growth is not inhibited with antiserums against the host-specific strains, the remaining antiserums that are available are used in a second series of tests.
5. Most tests will be complete and ready for reading after 2–5 days of incubation, but if the culture grows slowly, it may take longer. A previously determined growth rate on the medium is helpful information. A clear zone with no colonies or a greatly reduced number of colonies around a disc similar to that observed with known homologous strains is a positive test. Zones of 0.5 mm or less should be ignored. Examination under a microscope ($\times$20–100) will often facilitate interpretation of the results. At times, it will be helpful to stain colonies (a drop of Deines' stain diluted 1:50) to make them easier to view (29).

Complement Fixation Test

One of the advantages of the complement fixation test (CFT) is that many diagnostic laboratories are already using the test for diagnostic purposes, and to use it for the identification of mycoplasma isolates requires only the production of antigens and the availability of known antiserums for serotyping. However, adapting some new isolates to growth in broth medium and the production of antigen that is suitably free of anticomplementary (AC) activity can be very time-consuming.

The procedures for the test are fairly simple if the strain grows readily in broth and if the antigen produced does not have AC activity. Depending on the number of serums to be tested and the density of growth in a given medium, 50–200 ml of broth will usually produce enough antigen for identification tests. The broth is centrifuged (27,000 *g* for 15 min), and the pellet is washed three times by resuspension in and centrifugation out of phosphate-buffered saline (PBS) solution, pH 7.0–7.5. After the final wash, the antigen is resuspended in PBS in 2–5% of the original broth volume. Two rows of doubling dilu-

tions of antigen are made in veronal-buffered diluent (VBD) in tubes or in a microtiter tray. Dilutions of 1:2 through 1:128 are commonly used. An equal volume of VBD containing 2 Units of rabbit antiserum, for example, a 1:160 dilution of an antiserum that titers 1:320 is added to each tube or well in one row, and an equal amount of VBD is added to the other row (antigen AC control). Complement (2–5 CH_{50} Units) is added to both rows, and after incubation overnight at 4°C, sensitized sheep red blood cells are added.

Fixation of complement in one or more tubes past the AC activity of the controls is an indication of homology, but in weak reactions, a block titration against several antiserum dilutions may have to be run to make a decision. The significance of weak reactions is easier to ascertain if an antiserum against medium components is also available and is run against a third row of antigen dilutions. If there is no indication of the fixation being contributed to by a reaction between medium components and antibodies against them, it is an indication of specificity and the weakness of the reaction is due to (a) antigen too diluted, (b) partial homology, or (c) mixture of strains. A new antigen should be prepared and/or the test antigen should be run against other antisera to help determine the reason for the weak reactions.

Metabolic Inhibition Test

As the name implies, this test is essentially a neutralization test in which known antiserum slows the growth of the unknown mycoplasma for a period of time or prevents it altogether. The slowed growth is detected by absence of color change. The given change depends on the substrate of mycoplasmal metabolism and indicator used. The metabolic inhibition test (MIT) is less reliable for the identification of mycoplasmas than for serology. First, the accuracy of the test is highly dependent on the unknown strain being a pure culture. Then, too, precautions must be taken to make sure that the organism is growing well enough in broth to change the indicator within a reasonable time. The test is a simple one in concept, but in practice it often does not work well unless the diagnostic laboratory uses MIT routinely.

The first step of MIT is to determine which of the possible substrates is utilized by the unknown strain. After repeated cloning, the strain is introduced into base medium supplemented with either arginine (1%) adjusted to a pH value of 7.3, or dextrose (0.5%) adjusted to a pH of 7.8. Of course, the simpler the base medium, the better. Heart infusion broth plus fresh yeast extract and horse or other serum is often used as the base medium for this test.

The tetrazolium reduction inhibition (TRI) test can be used with those mycoplasmas that utilize neither arginine nor glucose but that reduce tetrazolium (2,3,5-TTC). The addition of sodium thioglycollate

(0.1%) to the base medium is desirable for obtaining the best results with the TRI test.

Miscellaneous Identification Tests

A growth precipitation test has been reported in which a well containing replicating mycoplasma organisms is surrounded by discs previously soaked with reference antisera. Precipitation lines indicate homology (32). In the radial growth precipitation test (33), the unknown mycoplasma is grown in an agar plate; known antiserums are placed in wells in the agar. Lines of precipitation indicate homology. Another method of identification that has been used considerably is polyacrylamide gel electrophoresis of mycoplasmal whole cell or membrane proteins (34).

Fluorescent Antibody Tests

The previously described tests for the identification of mycoplasmas isolated from animals have been criticized for their failure to adequately detect mixtures of mycoplasmal species. Mixed cultures can be difficult if not impossible to purify. The direct or indirect fluorescent antibody (FA) technique for the staining of mycoplasmal colonies in agar plates is the best method for the recognition of mixed cultures and for identifying casual agents of mycoplasmosis in animals.

The production of antiserums to mycoplasmas and the preparation of immunofluorescent antibody has been described (30). The FA test was performed as follows on colonies in primary isolation plates or on cultures grown in medium. Cultures of mycoplasmas were diluted (usually 1:100 or 1:1000) in phosphate-buffered saline (PBS: 0.015 *M* phosphate; 0.15 *M* NaCl in distilled water), and 0.2 ml was spread on the surface of solidified medium. The inoculated plates were incubated in a humidified environment at 37°C. When distinct, well-isolated colonies appeared, they were flooded with ethyl alcohol (95%) and fixed for 60 min. The alcohol was replaced with PBS, and the plates were stored at 5°C. For the FA test, eight small agar blocks (4–5 mm) containing 1–5 colonies were cut and placed in each of the eight outlined areas on tissue culture slides (*Lab-Tex®, Miles Laboratories, Naperville, Illinois) and treated with eight different conjugated antiserums. Each block was treated with a small drop of diluted (usually 1:20) FA. The slide was incubated at room temperature for 20–30 min in a moist chamber (a 100-mm plastic Petri dish containing saturated filter paper). Then, the plastic chamber was replaced on the slide to provide washing chambers for each of the agar blocks. They were washed in PBS containing merthiolate (1:10,000) until the background fluorescence was reduced to acceptable levels (2–48 hr). The slide without chamber was placed under a dissecting microscope. If the mycoplasmal colonies were in the proper position (up), a cover slip (22

× 40 mm) was applied for observations of fluorescence by incident ultraviolet light at × 125. The degree of fluorescence was rated relative to that of the background: negative where background fluorescence was equal to that of the colony, or +1 to +4 when the colony was clearly brighter than the background (35).

Diagnosis

Complement Fixation Tests

These tests have been used for many years to diagnose contagious bovine pleuropneumonia, but their use to diagnose mycoplasmosis in animals in North America is relatively recent. Workers at Iowa State University (36) have used CFT for the diagnosis of mycoplasmal pneumonia in swine for several years. Experimentally exposed pigs developed CFT titers 3–6 weeks postexposure; they persisted for several months. By means of CFT and slaughter of reactors, herds of swine were freed of the infection. They could be kept free by CFT on all replacements and breeding stock. These reports suggest that the techniques for the eradication of mycoplasmal pneumonia from swine may be at hand.

Antigen Production

The nonspecific reagents for CFT to detect mycoplasmal antibodies are available from commercial sources, but few antigens and control antiserums are available. Production of the antigens consists of (a) production of antigen in any medium to which the antigen strain was adapted; (b) three washes in buffered saline with resuspension of antigen in buffered saline at about 5% of the original broth volume; (c) tests for specific and anticomplementary activity; (d) to suitable suspensions of antigen, add glycerol (25%) and store at −20°C or below until used (37).

If the antigen has anticomplementary activity at dilutions beyond usefulness, one of several treatments may be used in an attempt to make it usable. Treatment with trifluorotrichloroethane (Genetron 113) is sometimes quite effective in reducing anticomplementary effect. One volume of antigen suspension is added to one volume of Genetron 113; the mixture is shaken vigorously for 15 sec and then centrifuged lightly. The aqueous top layer usually contains a large proportion of the original specific antigen, with less of the anticomplementary activity. Another means of fractionating antigen that decreases anticomplementary activity and is usable with some, but by no means all, *Mycoplasma* species is the chloroform–methanol lipid antigen extraction method described by Kenny and Grayston (38). Another procedure that is often used to decrease the anticomplementary effect of CFT antigens is heating at various temperatures up to boiling.

Performance of the Test

The microtechnique method for CFT was modified for use with porcine and bovine serums (37). The modification required for bovine serum was the addition of suitable, fresh, unheated calf serum (5%) to the diluent used for diluting guinea pig serum, the source of complement for the test. Until recently, complement was modified in the same way for use with swine serums. Slavick and Switzer rehydrated lyophilized guinea pig complement in undiluted, fresh, unheated swine serum and obviated the frequently encountered procomplementary effects of swine serums (36).

Indirect Hemagglutination Test

The indirect hemagglutination (IHA) test was adapted to the detection in cattle of antibodies to mycoplasmal species, particularly *M. bovis* and *M. bovi-genitalium* (39). The antigen was stable for at least 7 months at 5°C. The IHA test is sensitive, specific, and reproducible. It may have considerable usefulness for the detection of mycoplasmosis in animals, particularly if regulations should be promulgated concerning mycoplasmosis in animals intended for interstate or international transport or in bulls whose semen is widely disseminated for artificial insemination.

Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) was adapted to demonstrate antibodies to *M. bovis* in the serum of artificially and naturally infected cattle (40). The test is rapid, reproducible, and sensitive; some cross-reactions with other mycoplasmas have been reported. The fluorescent antibody procedure was used to identify *Mycoplasma dispar* and ureaplasmas lining the bronchial epithelium of calves affected with pneumonia (41). The enzyme-linked immunoperoxidase technique was applied to frozen lung and bronchial smears from pigs with MPS (42). In the tissue sections, the reddish brown *M. hyopneumoniae* organisms lined the bronchial epithelium or occurred as pleomorphic spots in the smears. This diagnostic test does not require a fluorescent microscope.

Latex Agglutination

The latex agglutination test uses latex beads sensitized with polysaccharide from culture supernatant in a slide aggulatination test to detect serum antibodies in goats. It is more sensitive than complement fixation and can be performed in the field with undiluted serum or whole blood (43).

Ureaplasmas

Although ureaplasmas were associated with nongonococcal urethritis of humans 30 years ago, their role in calf pneumonia and vaginitis of the cow was established only recently by American (44), English (45), and Canadian workers (10). Their role as pathogens appears to be expanding rapidly, for example, in chickens with chronic respiratory diseases and female monkeys with a high incidence of stillbirths and abortions. Ureaplasmas (so-called because of their urease activity) are common in ruminant animals, dogs, cats, and bull semen.

Cultivation and Biochemical Reaction

Media suitable for mycoplasmas are inadequate for the propagation of ureaplasmas (44). Four requirements must be met, or attempts to isolate and propagate ureaplasmas will be frustrated. First, the pH of the sterile medium should be 6.0 ± 0.5. Second, ureaplasmas are very sensitive to pH values above 7.4, and if exposed to alkaline conditions for a short period of time, they will die. In the laboratory, it is convenient to transfer cultures at the end of a work day and subculture the ureaplasmas again the next morning. Serial 10-fold dilutions of the inoculum should be made to avoid losing the cultures. Third, ureaplasmal growth is enhanced by the addition of urea to the medium even though it may not be absolute growth requirement. The hydrolysis of urea with the production of ammonia is the characteristic biochemical reaction of ureaplasmas. None of the mycoplasmas is known to possess urease activity. Finally, ureaplasmas are sensitive to thallium acetate, and it should not be added to the medium.

Bull semen and milk may contain antibiotics or other substances with mycoplasmacidal and ureaplasmacidal activity. Serial tenfold dilutions of milk, semen, tissue suspensions of pneumonic lungs, or urine should be prepared for primary isolation in liquid and solid medium. A suspension of tissue prepared by using 1 g of tissue to 9 ml of diluent and grinding in a Ten Broeck tissue grinder is suitable for primary inoculation.

It should be emphasized that media employed for the isolation and propagation of *U. urealyticum*, (Shepard's U-9 Broth, A-3 solid medium, and A-5 solid medium) usually are not satisfactory for ureaplasmas of ruminant origin. Modified Hayflick's medium and similar media should be utilized instead (see Appendix F). The A-7 plating medium of Shepard and Lunceford (Gibco), however, is satisfactory (46). To increase the number of successful isolations, three media should be inoculated during primary isolation attempts. Serums from

individual horses vary in growth-promoting properties. They should be batch tested before being used and stored at −20°C until needed. The serum does not need to be heat inactivated.

Optimal growth of ureaplasmas can be obtained on agar in an atmosphere containing 5% carbon dioxide in nitrogen or air. A candle jar or tissue culture incubator provides satisfactory growth conditions; growth is obtained under anaerobic conditions. Colonies can be detected 24–48 hr after inoculation using × 100 magnification. Colonies are very small (about 20–40 μm in diameter); they may develop the characteristic central-nippled appearance of mycoplasmas in 3–4 days if they are not crowded on the agar plate. The volume of agar medium in the plate will also affect the size of ureaplasmal colonies; 6–7 ml of molten agar medium should be added to each 50-mm Petri dish. Dienes's stain will be retained if applied to the colony. Older colonies (5–7 days of age) may show an irregular edge and may be multilobate with a "cauliflower head" appearance. Different isolates from the same species of animal may exhibit slightly different growth and colonial characteristics. Liquid cultures are usually clear. Usually, titers of 10^6–10^7 color changing units (CCU) are obtained routinely, but under optimum growth conditions, 10^9 CCU have been observed.

All ureaplasmal isolates of animal origin have phosphatase activity, do not reduce tetrazolium or methylene blue, and are digitonin sensitive. Ureaplasmas do not ferment carbohydrates and apparently do not possess hexokinase activity.

The optimal temperature for growth of ureaplasmas is 36–37°C. Some isolates will grow at 30°C. Most ureaplasmal cultures become nonviable in 3–4 days at 37°C; most cultures remain viable for several weeks at 5°C. Ureaplasmal cultures were frozen at −20°C and remained viable for a year. At −76°C, they have remained viable with loss of less than 1 $\log_{10}$ titer for over 5 years. Ureaplasmas can be successfully freeze-dried (44).

Urease Stain

For differentiation of ureaplasmas, the urease test developed by Shepard and Howard (47) is the most practical and reliable procedure available. The addition of a mixture of equal parts of 10% urea and 0.8% manganese chloride directly applied to 40-hr-old colonies on solid medium results in an immediate color reaction on the surface of ureaplasmal colonies. The colonies at first become golden brown, then dark brown, and may eventually become black as a result of deposition of manganese on the surface of the colony. All serotypes of ureaplasma react similarly, and to date, the author has not observed a single false positive reaction. The mixture of urea and manganese chloride may be

stored at −20°C until used. A solid medium containing test reagent reacts similarly with the developing ureaplasmal colonies (46).

Serology

The serological identification of serotypes of *U. urealyticum* is not a practical procedure at the present time.

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28

Mycology: Introduction

G. R. Carter

Mycology has been a neglected field in many veterinary diagnostic laboratories. A number of important pathogenic fungi can be identified without difficulty. Those that cannot should be forwarded to a reference mycology laboratory. A duplicate culture of the strain submitted may be kept for study after an identification has been made. By this means, a collection of the more common pathogenic and nonpathogenic fungi can be acquired.

The outline that follows is provided as an aid and is not meant to be a substitute for standard texts. A number of useful texts are listed as supplementary readings. The older ones, although useful, do not include the more recent changes in the names of some fungi.

It should be remembered that a number of fungi producing disease in animals are transmissible to humans. *Special care should be taken to prevent laboratory infections.*

Significance of Fungous Isolations

The great majority of fungi live in the soil or water, or as harmless commensals associated with humans and animals; for example, *Histoplasma capsulatum* and *Coccidioides immitis* occur in soil (geophilic), while *Candida albicans* lives in the alimentary canal. Many of the fungi, such as *Aspergillus* spp., *Rhizopus* spp., and *Geotrichum* are widespread in nature and thus are frequently found in clinical materials. The significance of the isolation will depend on such considerations as (1) demonstration of fungi in tissue sections, (2) presence of clinical disease, (3) presence of pathologic lesions, and (4) repeated isolation of the same fungus. The widespread saprophytic fungi that

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occasionally cause disease, such as *Mucor* spp., *Aspergillus* spp., or *Candida albicans,* are frequently referred to as "opportunistic fungi." Production of disease by these fungi is thought to be related to such factors as "impaired resistance," prolonged steroid or antibiotic therapy, and various stresses, including terminal diseases and metabolic disturbances.

Mycological Examinations

Procedures for a Mycological Examination

1. Direct examination of the clinical material: A small amount of material is added to a drop of 10% potassium hydroxide or lactophenol, and a coverslip is applied. The slide is gently heated to remove air bubbles and promote clarification. Some laboratories prefer 20% potassium hydroxide with glycerol (see Appendix C). Gram (special procedure for clinical material), Giemsa, and Wright's stains are also frequently of value. Direct examinations are almost always negative in histoplasmosis and sporotrichosis. Tissue should be set aside in buffered formalin for histopathologic study, if this was not already done.
2. If there is evidence of a fungous infection, appropriate media are inoculated.
3. Examination of the growth both macroscopically and microscopically should be conducted.
4. Those cultures that cannot be identified with certainty are forwarded to a mycology laboratory.

Material Required for Mycological Procedures

Almost all of the equipment required can be found in the diagnostic bacteriology laboratory. Straight dissecting needles are useful for breaking up colonies of fungi for examination. The inocula used to seed media are generally larger than those used in bacteriological work. Forceps and scalpels with small, sharp blades are especially useful. Bacterial contamination can be reduced by aseptic operations.

The reagents, stains, culture media, and other items required especially for mycological work are described in Appendix C.

Culture Media

The media recommended for isolation of the pathogenic fungi along with the incubation temperatures and duration of incubation are summarized in Table 28-1.

Table 28-1
Media for Isolation,[a] Incubation Temperature,[b] and Usual Length of Incubation for the Pathogenic Fungi

Disease	Isolation media	
	25°C	37°C
Zygomycosis (Mucormycosis)	Sabouraud dextrose agar; chloramphenicol (not cycloheximide) can be used (1–3 days)	
Aspergillosis	Same as for zygomycosis (1–3 days)	
Candidiasis	Sabouraud dextrose agar, Sabouraud C and C agar[c] (1–3 days)	
Dermatophytosis (Ringworm)	Sabouraud C and C agar (2–3 weeks)	
Blastomycosis	Sabouraud C and C agar[d] (2–3 weeks)	Brain–heart infusion agar or blood agar (3–7 days)
Cryptococcosis	Sabouraud dextrose agar; chloramphenicol (not cycloheximide) can be used (1–2 weeks)	
Histoplasmosis	Sabouraud C and C agar (2–4 weeks)	Brain–heart infusion agar or blood agar (2–4 weeks)
Coccidioidomycosis	Sabouraud C and C agar (1–2 weeks)	
Sporotrichosis	Sabouraud C and C agar (7–10 days)	Brain–heart infusion agar (1% blood) or blood agar (7–10 days)
Epizootic lymphangitis	Sabouraud C and C agar (2–8 weeks)	Horse blood or serum agar (2–8 weeks)
Geotrichosis	Sabouraud C and C agar (1–2 weeks)	
Chromomycosis	Sabouraud C and C agar (2–3 weeks)	
Maduromycosis	Sabouraud dextrose agar (2–3 weeks)	

[a]In some instances, depending on source of the specimen and history, it will be advisable to inoculate both kinds of Sabouraud media as well as blood agar.
[b]Usual incubation period.
[c]Cycloheximide and chloramphenicol added (Mycosel, Mycobiotic, etc.).
[d]Some *Trichophyton* spp. require special media (see Chapter 29).

Blood agar, Sabouraud dextrose agar, and Sabouraud C and C agar (Mycosel, Mycobiotic, etc.) are used routinely for primary fungous cultivation. The last-mentioned medium is basically the same as the second except that it contains cycloheximide and chloramphenicol for the suppression of some saprophytic fungi and bacteria, respectively. Brain–heart infusion agar can usually be used instead of blood agar. Media are most useful in Petri dishes; the medium should be thicker than usual, 25–35 ml per plate. It should be kept in mind that media containing cycloheximide should be incubated at room temperature (25–28°C) only.

If one is uncertain as to the probable fungus involved, it is advisable to inoculate each specimen onto both kinds of Sabouraud media, and onto blood and brain–heart infusion agar with 1% blood. The latter two media are incubated at 37°C. The media for isolation, the incubator temperature, and the usual time required for growth are listed in Table 28-1. It is a good practice to incubate all plates for fungous isolation for at least 4 weeks.

Examination of Cultures

After gross characteristics of the cultures are observed, a portion of the colony is teased apart with needles, then transferred to a drop of lactophenol cotton blue. A coverslip is then added. An alterative useful procedure is to prepare a tape mount. This procedure is described in detail in Chapter 29. For verification of the morphologic observations, a slide culture may be prepared. The technique is described by Campbell and associates (1) and in Appendix C.

Difficulty may be experienced in obtaining both growth phases of the dimorphic fungi. Methods are described (1) for the conversion from one phase to another.

Many of the saprophytic fungi can be identified by their characteristic fruiting bodies. Figure 28-1 has been provided to aid in the identification of some of the more common contaminants. Unfortunately, fruiting bodies are not always present.

Classification of Fungi

The classification that follows is based upon that of Copper (2), which was patterned after Alexopoulos and Mims (3).

Fungi are placed in one of the subdivisions listed below (excepting subdivision Deuteromycotina) on the basis of their sexual spores. The sexual state of a fungus is called the teleomorph. The sexual state of

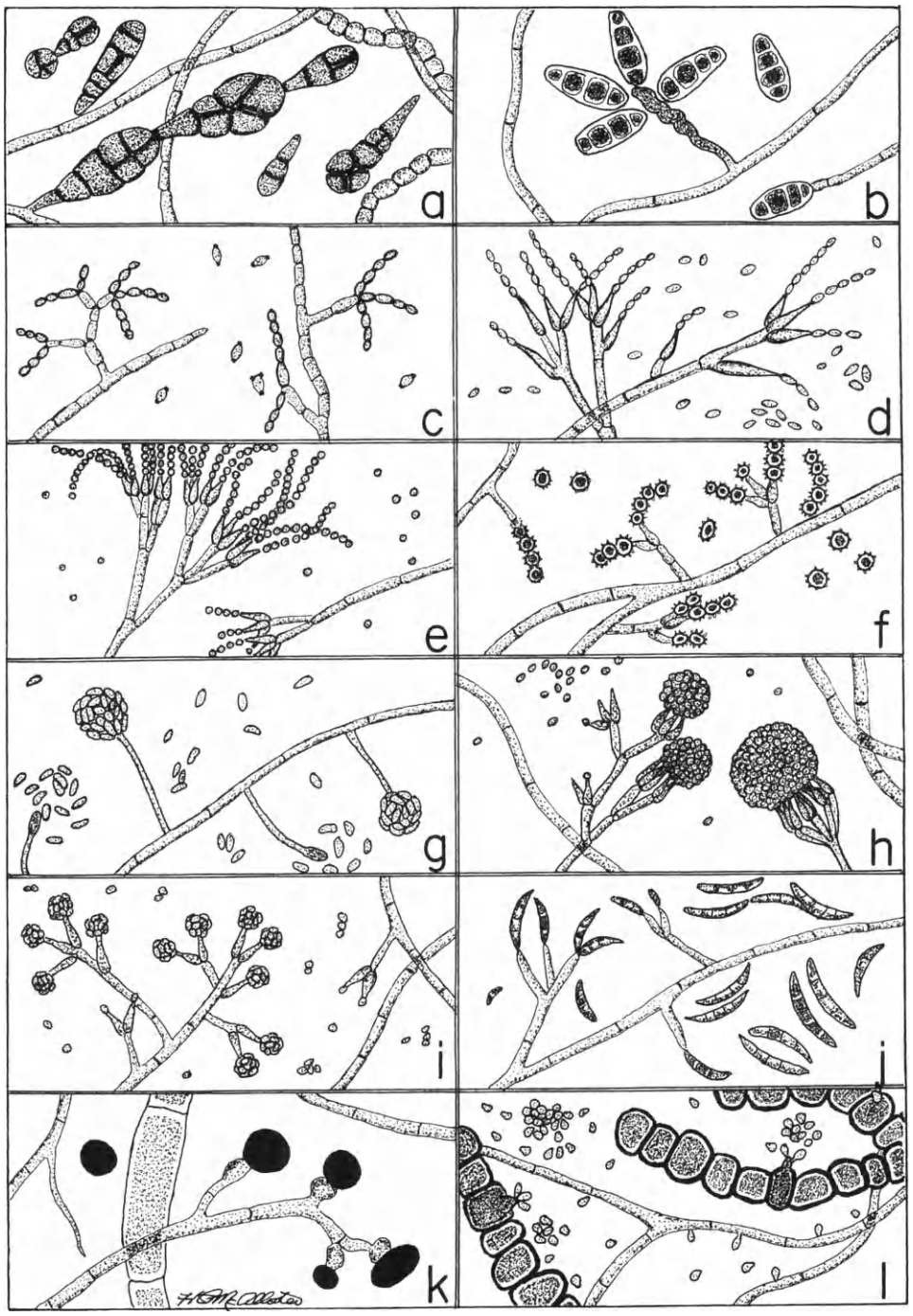

Figure 28-1. Contaminants: a, *Alternaria* sp.; b, *Helminthosporium* sp.; c, *Fonsecaea* sp.; d, *Paecilomyces* sp.; e, *Penicillium* sp.; f, *Scopulariopsis* sp.; g, *Acremonium* sp.; h, *Gliocladium* sp.; i, *Trichoderma* sp.; j, *Fusarium* sp.; k, *Nigrospora* sp.; l, *Exophila* sp. (H. A. McAllister).

fungi in the subclass Deuteromycetes has not yet been discovered. The term anamorph is used to denote the asexual reproductive state.

Kingdom Mycetae (Fungi)
Division Amastigomycota
Subdivision Zygomycotina
Class Zygomycetes
Order Mucorales
Representative genera: *Absidia, Mucor, Rhizopus, Cunninghamella*
Order Entomophthorales
Representative genera: *Basidiobolus, Conidiobolus*
Subdivision Ascomycotina
Class Ascomycetes
Subclass Hemiascomycetidae
Representative genera: *Pichia, Saccharomyces*
Rarely cause disease. Some *Candida* spp. are related to this subclass.
Subclass Plectomycetidae
Order Onygenales
Family Gymnoascaceae
Representative genera: *Nannizzia,* teleomorph of *Microsporum; Arthroderma,* teleomorph of *Trichophyton; Emmonsiella,* teleomorph of *Histoplasma; Ajellomyces,* teleomorph of *Blastomyces*
Order Eurotiales
Family Eurotiaceae
Includes teleomorphs of some *Penicillium* and *Aspergillus* spp.
Subdivision Basidiomycotina
Class Basidiomycetes
Subclass Holobasidiomycetidae
Order Agaricales
Includes poisonous and edible mushrooms
Subclass Teliomycetidae
Order Ustilagenales
Family Ustilagenaceae
Includes *Filobasidiella,* the teleomorph of *Cryptococcus*
Subdivision Deuteromycotina
Form class Deuteromycetes (Imperfect fungi; sexual state not discovered)
Form subclass Blastomycetidae: Imperfect yeasts
Representative genera: *Cryptococcus, Candida*
Form subclass Hyphomycetidae

Form family Moniliaceae
Representative genera: *Coccidioides, Epidermophyton, Sporothrix, Paracoccidioides*
Form family Dermatiaceae
Representative genera: *Phialophora, Exophiala, Fonsecaea, Cladosporium, Wangiella.* These are molds with darkly pigmented hyphae.

Glossary of Mycological Terms

Arthrospore: An asexual spore formed by the disarticulation of the mycelium.
Ascospore: A sexual spore characteristic of the true yeasts and ascomycetes. They are produced in a saclike structure called an ascus. The ascospore results from the fusion of two nuclei.
Ascus: The specialized saclike structure characteristic of the true yeasts in which ascospores (usually eight) are produced.
Blastospore: A spore produced as a result of a budding process along the mycelium or from a single spore.
Chlamydospores: Thick-walled, resistant spores formed by the direct differentiation of hyphae.
Clavate: Club-shaped.
Columella: The persisting, dome-shaped upper portion of the sporangiophore.
Conidium: An asexual spore formed from hyphae by abstriction, budding, or septal division.
Conidiophore: A stalklike branch of the mycelium on which conidia develop either singly or in numbers.
Dematiaceous: Used to denote the dark brown or black fungi.
Dimorphic: Having a yeast form and mycelial form.
Echinulate: This refers to the spiny walls of conidia and conidiophores.
Endogenous: Originating or produced from within.
Endothrix: Arthrospores appear within the hair shaft.
Exogenous: Originating from without.
Favic Chandeliers: These are spherical hyphae that branch with curved and irregular ends, which give an appearance of antlerlike branches.
Geophilic: Denotes fungi whose natural habitat is the soil.
Germ Tube: Tubelike structure produced by germinating spores. They develop into hyphae.
Glabrous: The smooth form.
Hyphae: The filaments that compose the body of the thallus of a fungus.
Macroaleuriospore: This is the larger of the two kinds of conidia that break from the attachment to hyphae by rupture through the cell wall. It is also called a macroconidium.
Macroconidium: A large, sometimes multicellular spore. It is the larger of two types of conidia produced in the same manner by the same fungus.
Microaleuriospore: This is the smaller of the two kinds of conidia that break from the attachment to hyphae by rupture through the cell wall. It is also called a microconidium.
Microconidium: A small, single-celled conidium borne laterally on hyphae.
Mycelium: The mat made up of the intertwining, threadlike hyphae.

Nodes: The points on the stolons from which the rhizoids arise.
Obovate: Inversely ovate, that is, ovate with the narrow end at the base.
Obovoid: Inversely ovoid, that is, ovoid with the narrow end at the base.
Ovate: Egg-shaped.
Phialide: This is a tip cell of a conidiophore (phialophore), which is usually flask-shaped, and from which conidia (phialospores) arise.
Phialophore: A conidiophore which bears phialides.
Pseudohyphae: Filaments constituted by elongated budding cells that have failed to detach.
Pyriform: Pear-shaped.
Racquet Hyphae: Hyphae with terminal swelling of segments giving a shape resembling that of a tennis racquet.
Rhizoid: Rootlike, branched hyphae extending into the medium.
Septate: Having cross walls or septa in the hyphae.
Sporangium: The closed, often spherical structure in which are produced asexual spores by cleavage.
Sterigmata: Specialized structures, short or elongated, borne on a vesicle and producing conidia.
Stolon: A horizontal hypha or runner that sprouts where it touches the substrate. It forms rhizoids in the substrate.
Yeasts: Unicellular fungi that reproduce by asexual budding or by sexually produced ascospores.
Zygospore: A thick-walled, sexual spore of the true fungi that results from the fusion of two similar gametangia.

Serodiagnosis of Fungal Diseases

This area of laboratory diagnosis has received little attention in fungal diseases of animals. This is partly because these diseases are relatively infrequent and the required reagents have not usually been readily available to the veterinary microbiologist. The expense of the reagents has also been a deterrent. Some hospital and public health laboratories will make their serodiagnostic capability available to veterinarians, and some fungal diagnostic reagents are available commercially. Readers are referred to Kaufman (4) for details of procedures and some sources of reagents. The results of these human tests when used in animals may require different interpretations. Attleberger (5) has discussed the use of serologic procedures in the diagnosis of fungal diseases of dogs and cats.

Safety

All fungous cultures and specimens thought to harbor fungi should be considered potentially dangerous. Work with these materials should be carried out in an approved biological safety hood (see Chapter 1).

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29

Dermatophytes and Dermatophytoses[1]

G. R. Carter

The Dermatophytoses

The term *ringworm* is commonly applied to superficial fungal infections involving the keratinized layers of the skin and its appendages (hairs, nails, horns, and feathers) in animals and humans. Ringworm fungi are able to penetrate all layers of the skin, but they are generally restricted to the nonliving cornified portions, especially the stratum corneum (1–3). Invasion of the subcutaneous and deeper body tissues is not a feature of infection by these fungi (1), and for this reason, they tend to be self-limiting and rarely, if ever, lead to death (4). Nonetheless, ringworm can cause severe lesions with concomitant distress in the host, and occasionally significant economic losses may follow (4).

Considering that ringworm is specifically due to a closely related group of mycelial keratinophilic fungi, the dermatophytes, there has been increasing acceptance of the term *dermatophytosis* (3,5–7). The formerly used term dermatomycosis is not recommended as it includes all fungous infections involving the skin.

Origin and Distribution

There are many species of dermatophytes, some of which have only been recovered from soil (3). Traditionally, these organisms have been listed in the class Deuteromycetes (Fungi Imperfecti), but the perfect or ascus-bearing state has been described for some of them (1,3,5,7).

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[1]This chapter is a revision of a chapter prepared by Dr. H. A. McAllister for the Fourth Edition. Much of his material has been retained and his contribution is gratefully acknowledged.

Diagnostic Procedures in Veterinary Bacteriology and Mycology, Fifth Edition

Dermatophytes are categorized as geophilic, zoophilic, and anthropophilic depending on the habitat in which they are most likely to be found. Geophilic dermatophytes inhabit the soil and normally exist as free-living saprophytes. Zoophilic dermatophytes are primarily detected as parasites of animals other than humans, while humans serve as the main host for the anthropophilic types. All three groups nonetheless include species that can cause disease in both animals and humans. Transmission from animal to animal, from animals to humans, from one human to another, and from soil to either animals or humans are all possible.

All dermatophytes may have originated from soil forms, but a significant number appear to have abandoned their saprophytic existence to become parasites (2,3). This adaptive process appears to entail losses in their sexual abilities, especially in the anthropophilic forms. Increasing affinity to the human host is believed to result in the gradual loss of both the sexual state and the ability to produce asexual spores (3). Certain species of dermatophytes have so far defied all efforts to induce a sexual state (3).

The perfect or sexual state of the dermatophytes is useful in the identification of certain species as well as in epidemiologic studies (8). Demonstrating the perfect state requires special mycologic techniques, and clinical microbiologists are mainly concerned with the conidial state seen in isolates from clinical specimens. The nomenclature applicable to the conidial state will therefore be largely adhered to throughout this chapter.

Diagnosticians wishing to induce and study the perfect state of the dermatophytes should consult mycology texts such as those by Rippon (3) or Emmons *et al.* (5) and manuals such as that by Beneke and Rogers (7) for specific laboratory techniques.

Clinical Disease

There are no fundamental differences in the clinical manifestations of infections produced by different dermatophytes; ringworm or dermatophytosis should thus be regarded as a single clinical entity regardless of its causative agent (3). Dermatophytes have adapted to survival in the skin of particular hosts (2,4,5,7,9): *Microsporum nanum* in pigs, *M. canis* in the cat, *M. persicolor* in voles, *Trichophyton rubrum* in man, *T. verrucosum* in cattle, *T. erinacei* in hedgehogs, and *T. mentagrophytes* in rodents. Isolation of such host-adapted dermatophytes may therefore be of no clinical significance, unless there are lesions or there is transmission to susceptible individuals. In the cat, for example, *M. canis* infection is most often subclinical or inconspicuous, and attention is first drawn to the parasite following the development of ring-

worm in another host species after contact with the cat (3). Essentially all rodents carry *T. mentagrophytes* as normal flora with no significant signs of disease (3). When these animals act as vectors in transmitting their specifically adapted dermatophytes to other animal species, the recipients develop the eruptive signs associated with ringworm (2). Human *T. verrucosum* infections are seen most often in farmers who are in contact with cattle (5).

The lesions that develop are dependent not only on the invading agent but also on the host's reactivity to it (2). When the dermatophyte exceeds the limits of a balanced host–parasite relationship or the reactive threshold of the host is reached, clinically recognizable ringworm ensues (2). Since the organism is not usually capable of surviving the resulting inflammatory reaction, it tends to move away peripherally toward normal adjacent skin (2). This process tends to create the classical ringed lesions of alopecia with central healing and peripheral inflammation. Ringworm, however, manifests itself in many ways, ranging from the asymptomatic carrier state to the nodular or tumorous lesions called kerions; the classical ringed lesions may in fact be the exception rather than the rule (2).

The dermatophytes' ability to hydrolyze keratin may cause some damage to the epidermis and the hair follicles, but the mechanism whereby they actually produce disease is through hypersensitivity to their irritants and allergens (2,3,5,8,9). Multiple sterile vesicles known as dermatophytids, or *id* lesions, may appear anywhere in the epidermal cover as part of an allergic reaction to the hematogenous spread of fungal products (3,5,9,10). In humans, they are most often seen in the hands. Id reactions are not seen in most natural infections of animals, however (5). Secondary bacterial invaders such as *Staphylococcus aureus* or *S. intermedius* may further contribute to the development of lesions in dermatophytosis, often generating pustules in the hair follicles.

In dogs and cats, lesions develop most frequently on the head and the extremities, but the infection can become generalized. The disease tends to be more severe in dogs than in cats (3,4). In the horse, lesions are generally dry, raised, scaly, and most abundant in the saddle and girth area as well as in the hindquarters (3,4). These lesions may become small inflamed ulcers with a purulent exudate that tends to glue hairs together (3). Chronic, subclinical infections that cause little or no distress or economic losses are the rule in swine (3,4). Gallinaceous birds, including chickens and turkeys, are afflicted by white, moldy, patchy overgrowths of the comb and wattle; this clinical entity is known as favus (3,4). Thick white crusts often develop, and in severe cases of generalized infections, the base of the feathers is involved (3).

Bovine ringworm is more frequently seen in younger animals, with up to 40% of the calves and yearlings infected, and it is more frequent

in winter than in summer. These facts seem to be accounted for by crowding within buildings, where there is increased contact between animals and with spore-laden debris. Cattle ringworm begins as scattered circular lesions with slight scaling and alopecia. The disease may become stationary and chronic, or the lesions may enlarge into plaques covered by thickened crusts that are firmly attached to the animal. Their removal leaves a weeping, bleeding, erythematous base. Spontaneous healing is the rule, leaving dry, scaly patches with alopecia and scar formation (3). There is no satisfactory topical treatment for ringworm in cattle; good hygiene and sanitation are important for the control of this disease.

Although the septate hyphae of the fungus and arthrospores within hair fragments are often seen in routinely stained hematoxylin–eosin histopathology sections, their presence is best demonstrated utilizing special stains. The periodic acid–Schiff reaction, Gridley's modification thereof, and Gomori's methenamine silver stains are most useful in this respect. The demonstration of dermatophyte-type fungal elements in skin biopsy specimens featuring lesions is a valuable adjunct to culture procedures.

Identification of the Etiologic Agents

Studies of colony characteristics, microscopic morphology, and nutritional requirements make possible the differentiation of dermatophyte species. Large, multicellular spores known as macroconidia or macroaleuriospores are numerous in the genera *Epidermophyton* and *Microsporum* (Fig. 29-1). The size and shape of these structures and the thickness and character of their walls so differ from one species to the other that they constitute an important basis for identification. Unfortunately, macroaleuriospores are few or absent from *Trichophyton* mycelia; although helpful when seen (Fig. 29-2), they cannot be consistently used in the identification of species in the genus. Nutritional requirements and colony characteristics are used instead.

Another criterion useful in the identification of dermatophytes is offered by the arrangement and type of growth of fungal elements along infected hairs. A mosaic of arthrospores outside the hair shaft is said to be *ectothrix* (Fig. 29-3), while arthrospores in roughly parallel chains inside the hair shaft are said to be *endothrix*. *Microsporum* hair infections are generally characterized by a small-spored ectothrix pattern of growth (5). The fungus invades the hair shaft by growing downward in it, and on the surface of the hair it forms a sheath of spores that are 2–3 μm in diameter each (5). Different species of *Trichophyton* produce either endothrix or ectothrix arthrospores. The ectothrix types grow into the hair follicle, surround the shaft, and penetrate it. Growth of hyphae continues both within and on the surface of the shaft, pro-

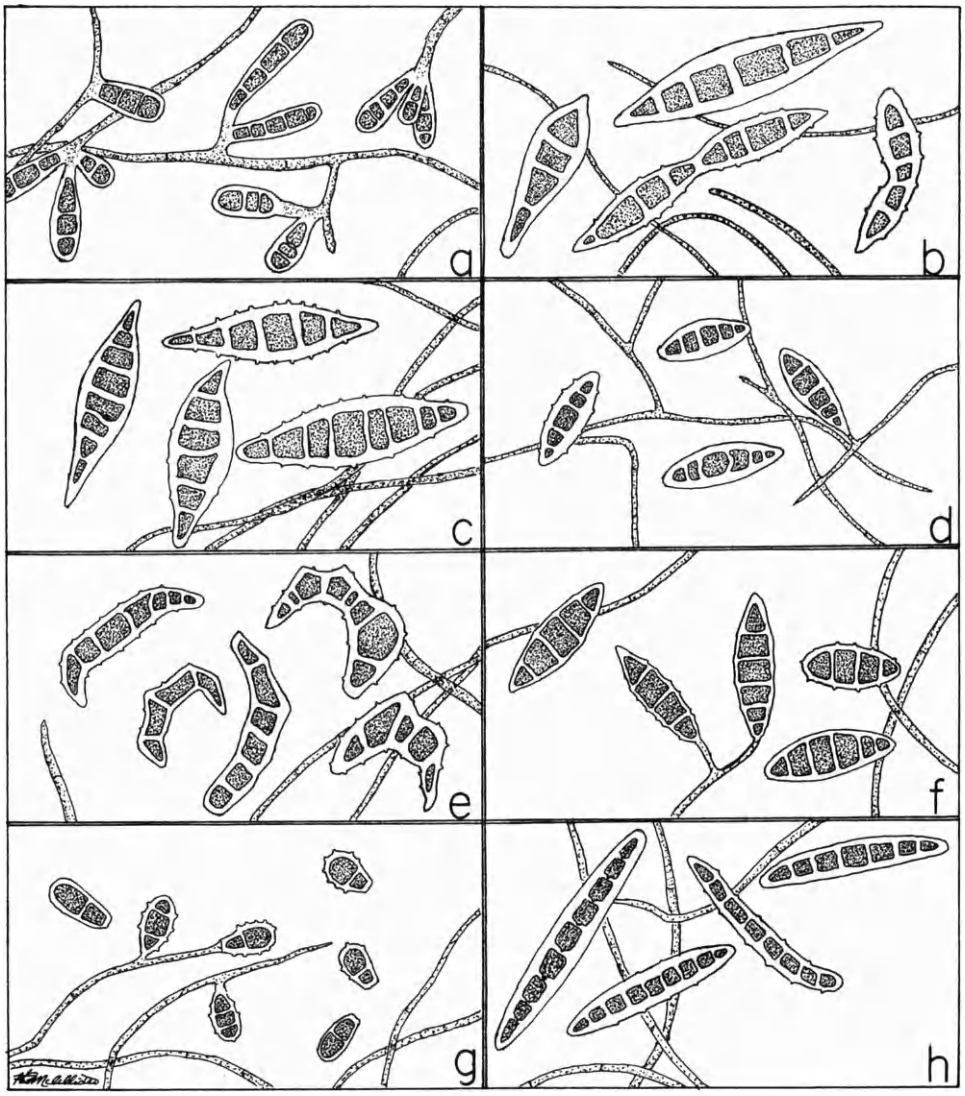

Figure 29-1. Macroconidia: a, *Epidermophyton floccosum*; b, *Microsporum audouinii*; c, *M. canis*; d, *M. cookei*; e, *M. distortum*; f, *M. gypseum*; g, *M. nanum*; h, *M. vanbreuseghemii* (H. A. McAllister).

ducing rows of arthrospores by septation at both sites. There are both small-spored and large-spored types in the genus; the latter feature spores that are 3–5 μm in diameter (5). In endothrix infections, the fungus grows from the epidermis into the hair follicle, penetrates the hair, and extends down into it. After this initial period there is no substantial growth on the external surface of the hair shaft (5).

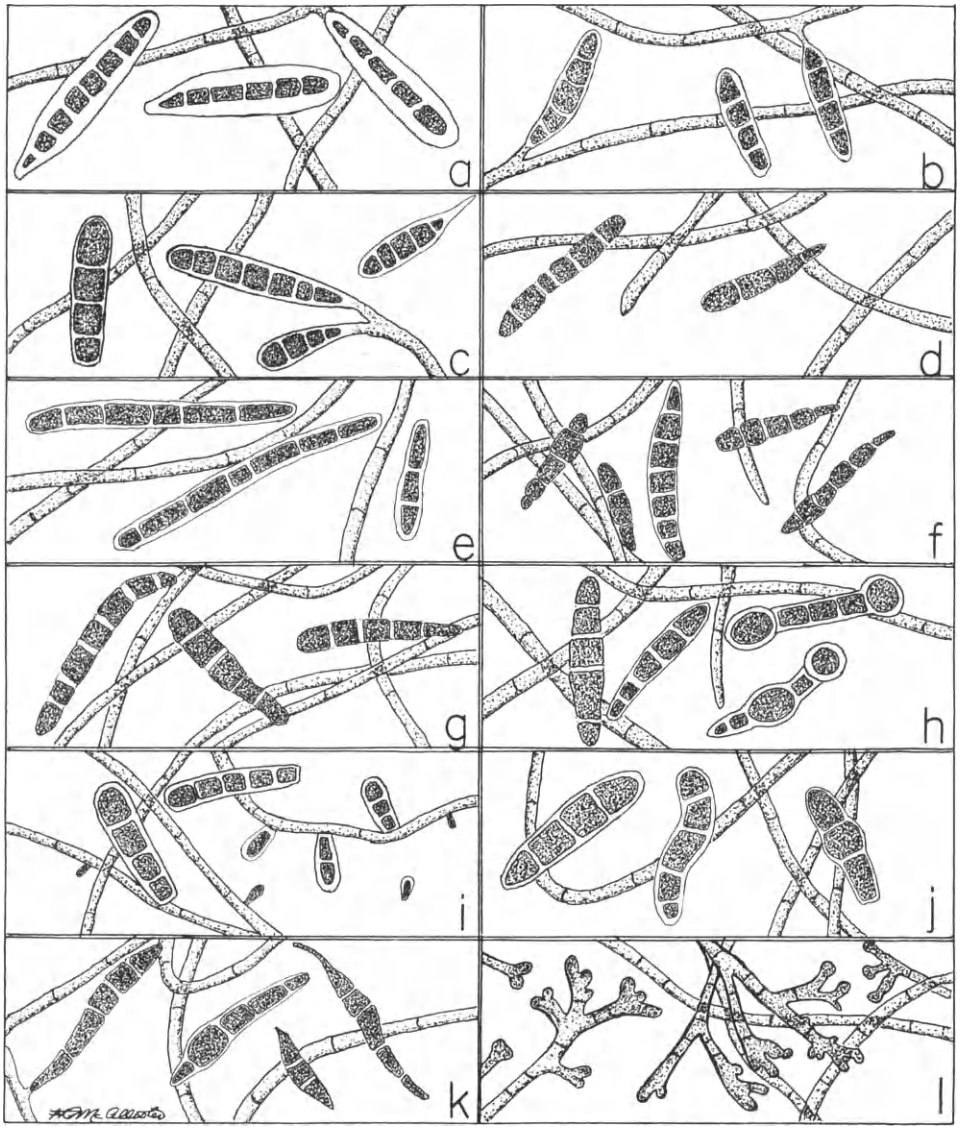

Figure 29-2. Macroconidia: a, *Trichophyton ajelloi;* b, *T. equinum;* c, *T. gallinae (M. gallinae);* d, *T. gourvilii;* e, *T. megninii;* f, *T. mentagrophytes;* g, *T. rubrum;* h, *T. simii;* i, *T. terrestre;* j, *T. tonsurans;* k, *T. verrucosum;* Favic chandeliers: l, *T. schoenleinii* (H. A. McAllister).

Speciation of *Trichophyton* spp. usually requires the expertise of a reference mycology laboratory because a series of test media with various growth factors must be kept on hand. Trichophyton agars #1–7, containing various growth factors, are available commercially (Difco Laboratories) to aid in identification of species. Haley and Calloway

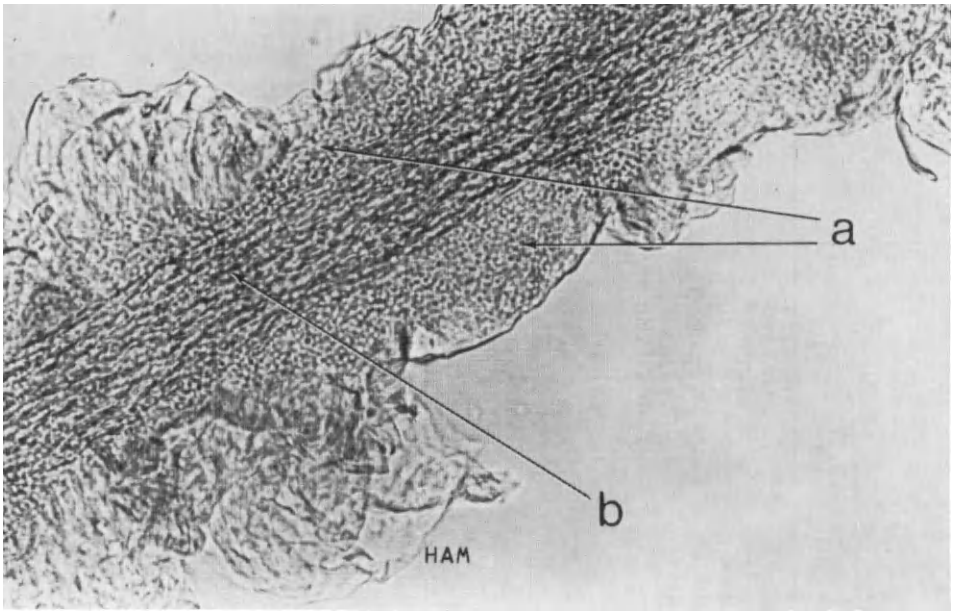

Figure 29-3. Sheath of ectothrix arthrospores around a cat hair: a, arthrospores; b, hair shaft. ×445 (H. A. McAllister).

(11) suggest that clinical laboratories need only use four of the seven agars in the identification of the more important species. A useful differential table employing four agars has been provided by Tilton and McGinnis (10). Differential tables employing Trichophyton agars are also provided by Campbell and Stewart (12). Laboratories with a need to develop expertise in speciating dermatophytes are advised to consult specialized manuals such as those by Al-Doory (13), Beneke and Rogers (7), Haley and Callaway (11), and Rebell and Taplin (8).

Useful identifying characteristics for common dermatophytes of medical importance are provided for the reader's convenience in the figures and in Table 29-1. Many, but not all, dermatophytes should be identifiable down to the species level with the information provided in this chapter. Confirmation by trained mycologists whenever feasible is recommended.

Laboratory Procedures

Preliminary Examination

If possible, examine the patient's lesions in a darkened room using ultraviolet light rays of 3660 Å (Wood's lamp). Since hairs infected by certain *Microsporum* species produce a marked yellowish-green

Table 29-1
Common Dermatophytes of Medical Significance[a]

Species	Stimulatory or required factors	Habitat preference	Hair invasion	Hair fluorescence
E. floccosum	None	Anthropophilic	Hair not invaded	None
M. audouinii	Yeast extract stimulatory	Anthropophilic	Ectothrix	Bright greenish yellow
M. ferrugineum	None	Anthropophilic	Ectothrix	Bright greenish yellow
M. canis	None	Zoophilic	Ectothrix	Bright greenish yellow
M. distortum	None	Zoophilic	Ectothrix	Bright greenish yellow
M. gallinae	Yeast extract and thiamine stimulatory	Zoophilic	Ectothrix	None
M. gypseum	None	Geophilic	Ectothrix	None
M. cookei	None	Geophilic	Hair not invaded	None
M. nanum	None	Zoophilic	Ectothrix (sparse)	None
T. concentricum	Thiamine stimulatory	Anthropophilic	Hair not invaded	None
T. megninii	L-histidine on NH_4NO_3 basal medium	Anthropophilic	Ectothrix	None
T. rubrum	None	Anthropophilic	Ectothrix, hair rarely invaded	None
T. schoenleinii	Generally none, thiamine by some strains	Anthropophilic	Endothrix	None or dull
T. tonsurans	Thiamine greatly stimulatory	Anthropophilic	Large-spored endothrix	None
T. violaceum	Thiamine required	Anthropophilic	Endothrix	None
T. equinum	Nicotinic acid required (except by *T. equinum* var. *autotrophicum*)	Zoophilic	Large-spored ectothrix	None
T. mentagrophytes	None	Zoophilic (except var. *interdigitale*)	Small-spored ectothrix	None or dull
T. verrucosum	Thiamine or both thiamine and inositol	Zoophilic	Large-spored ectothrix	None

[a]The most common zoophilic species are *M. canis* from cat and dog; *M. gallinae* from chickens and turkeys; *M. nanum* from swine; *T. equinum* from horses; *T. mentagrophytes* from rodents; and *T. verrucosum* from cattle. Note special requirements for the isolation of some of the species of medical significance. Refer to text for additional information on all species.

orescence under such light, the precise localization of infected areas may be ascertained. Infections caused by zoophilic dermatophytes such as *M. canis* and *M. distortum* can be detected by this means, but almost all *Trichophyton* species and many *Microsporum* species are not fluorescent in hair.

Specimen Collection

1. Skin: wash the infected area with 70 percent alcohol to remove surface contaminants and collect scrapings from the borders of the lesion.
2. Nails: scrapings or clippings of nails should be collected from spots near the bed of the nail.
3. Hair: remove hairs from the edges of the lesion by plucking them out with tweezers. The basal portions of hairs usually contain the best material.
4. Place skin scrapings, nails, and hair in covered containers. Cardboard specimen boxes, envelopes, or disposable Petri dishes may also be used. It is important to make sure that the hairs and other debris are not spilling out of the container.
5. Skin biopsies: fix the specimen in 10% neutral buffered formalin equal to at least 10 times the tissue volume and allow 24 hr for proper fixation. The volume of formalin can be significantly reduced after fixation for convenience in shipping. Forward the specimen to the laboratory in a small amount of formalin within a spill-proof and crush-proof container. Formalin kills the fungi, so separate specimens are required for culture. Neutral buffered formalin is prepared as follows:

Sodium phosphate, monobasic, anhydrous	4.0 g
Sodium phosphate, dibasic, anhydrous	6.5 g
Distilled water	900.0 ml
37–40% Formaldehyde solution	100.0 ml

Dissolve the reagents in water with the aid of heat. Cool, then add the formaldehyde solution. Unbuffered formalin is acidic and promotes artifactual precipitates in histopathology slides. It is not recommended.

Direct Observation of Clinical Material

Thin nail scrapings, skin scabs, and hairs from suspect cases should be examined after the partial digestion of proteinaceous debris with potassium hydroxide. To do this, some of the material is placed on a microscope slide and 1–2 drops of 10% KOH solution are added. A coverslip is placed over the drops, and then the specimen is allowed to stand for a few minutes with occasional gentle warming (no boiling). Nail scrapings may require several hours for clearing. Microscopic

examination should reveal hyphal fragments and arthrospores in infected material, especially around or within hair shafts. The penetration and clarity of specimens can be improved by the addition of dimethyl sulfoxide (DMSO) to the KOH. A formulation widely used is 20% KOH and 36% DMSO.

A rapid stain described by Swartz and Lamkins (14) greatly enhances visualization of the fungus. It is prepared as follows:

1. Wetting agent: dissolve 0.15 g of sodium benzoate and 0.85 g of dioctyl sodium sulfosuccinate in 100 ml of distilled water.
2. Add together and mix: one part 10% KOH solution, one part wetting agent solution, and two parts permanent blue-black ink.

Staining by this and by comparable alternative mixtures of ink and KOH is less intense than that of lactophenol cotton blue, but the fungi take up the dye selectively and the specimen is cleared with minimal shrinkage (5).

A significant problem with direct observation of clinical material is the presence of large numbers of artifacts in the preparation. Pigment granules (melanosomes) and degenerate keratinocytes within hair shafts are often mistaken for rounded arthrospores, resulting in false positive reports of dermatophyte infection. A network of debris and cholesterol crystals deposited around epidermal cells and known as mosaic can effectively mimic septate hyphae (5). Even experienced workers can have difficulty with artifacts. Moreover, various fungi, such as *Alternaria*, can be present in healthy animal fur as incidental contaminants. For these reasons, culture attempts and the demonstration of fungi and lesions in biopsy specimens are needed to confirm clinical dermatophytosis.

Culture

Whether arthrospores have been detected or not, the materials collected should be inoculated on Sabouraud agar with cycloheximide (Actidione®) and chloramphenicol. Mycobiotic agar (Difco) or Mycosel® agar (BBL) may be used instead. These inhibitory media are very useful in the isolation of dermatophytes from clinical materials heavily contaminated with bacteria and saprophytic fungi, although they sometimes cannot prevent the growth of *Alternaria* species and of other resistant bacteria and fungi.

It should be kept in mind that some dermatophytes (2,3,6–9,11,13,15) have special nutritional requirements (e.g., *T. equinum*, nicotinic acid; *T. megninii*, L-histidine; *T. tonsurans*, thiamine; *T. verrucosum*, thiamine or both inositol and thiamine; *T. violaceum*, thiamine) and that these organisms may fail to grow on ordinary culture media. For this reason, failure to recover a dermatophyte in culture

cannot automatically preclude a diagnosis of dermatophytosis. Appropriate commercially available Trichophyton agars (Difco Laboratories), as listed in the species descriptions, should be on hand in attempts to culture dermatophytes from horses and from cattle.

Some authors (2,5,7) recommend agar slants in cotton-plugged test tubes for fungus cultures. Petri dishes are advised against because spores may escape into the air when the lid is lifted (2). Although this criticism is valid, primary cultures, especially those from primary sources, almost always yield a mixed flora that cannot be effectively resolved into its components when it develops in small tubes and bottles. In a tube, the contaminant fungi can completely overgrow dermatophytes, preventing their detection. In addition, the narrow neck makes it difficult to reach in with forceps to prepare tape mounts.

Isolated colonies of fungi are best obtained by scattering hairs, nails, and scabs throughout the surface of an agar plate and pressing them down gently with forceps or a swab. The size of the inoculum should not be excessive, and about 10 mm should be allowed between individual crusts or hairs. The plates are sealed with masking tape to delay drying and to prevent the escape of spores; they are then incubated at 25°C (except for *T. verrucosum*), lid up, for not less than 2 weeks. The plates must be handled without inverting them, since the maneuver will dislodge spores from the aerial mycelium; the spores fall on the lid and back on the plate, producing additional growth on the agar. Due care and possibly the use of a hood are advisable when opening and closing plates. A good precaution is to place a paper towel soaked in 2% Amphyl® disinfectant (or a similar fungicide) over the work area of the table and to fold and discard it upon completion of the work (2). If growth is fast and the aerial mycelium reaches the lid, then the plate must not be opened at all except under a biologic safety hood.

Dermatophyte test medium (commercial preparations of this medium are available) may be used as a primary culture medium. It can also be used as a differential agar. In the latter case, a suspicious isolate from the original plate is transferred with teasing needles to dermatophyte test medium to confirm identity as a dermatophyte. Such fungi are easily recognizable on this agar because on it they show a white or near-white aerial mycelium and they change the medium's color (due to phenol red) from a dark yellow to red. Unfortunately, this characteristic color change can be produced by some saprophytic fungi, yeasts, and bacteria. These contaminants, however, do not produce a white cottony mycelium.

Final Identification

Plates with colonies of fungi should be examined after the mycelia attain a diameter of 10 mm or more but before individual colonies of different types begin to merge with each other. Colonial characteristics

such as texture, pigment, and rate of growth must be recorded. The mycelium can be examined effectively using a tape mount procedure. Perfectly transparent tape such as Scotch® brand cellophane tape or Scotch® brand double-sided cellophane type is used to mount the hyphal elements with minimal disturbance of the spatial relationships between the reproductive structures.

The procedure is as follows:

1. Place a drop of lactophenol cotton blue fluid (see Appendix C) on a microscope slide and another one on a coverslip; neither drop should be too large.
2. Cut a piece of tape smaller than the coverslip and grasp one corner with forceps, keeping the tape free of dirt and fingerprints.
3. Place the adhesive side of the tape against the fungus and apply gentle pressure with a dissecting needle. Caution: excess pressure will tear off a thick mycelial mass unsuitable for viewing, and spores may be scattered in the hair.
4. Gently transfer the tape to the coverslip. Place a corner against dry glass and the fungus-bearing portion directly over the mounting fluid.
5. Using the dissecting needle, pry the tape loose from the forceps and press the dry corner gently against the glass.
6. Invert the coverslip and place it over the mounting fluid on the microscope slide. Gently press out any air bubbles trapped under the coverslip. Fingernail polish may be used to seal the preparation and prevent quick drying. Note that the only material between the fungus and the microscope objective is the coverslip glass.

Longer-lasting preparations with a more perfect rendition of the spatial relationships are possible with the slide cultures discussed elsewhere in this book, as well as by Beneke and Rogers (7).

When there is doubt as to the identity of a particular isolate it should be sent to a recognized mycology laboratory for definitive identification.

The Dermatophytes

Epidermophyton floccosum

The only widely accepted species in this genus, *E. floccosum* infects only skin and nails, and it is virtually confined to humans (4). Reports of infections in domestic animals are essentially nonexistent (2,7), but

there are some of isolations from mice and from a dog (4,6,7). Since hair is not attacked, there is no fluorescence under Wood's lamp. The perfect state has not been reported. Colonies are usually greenish yellow, olive, or khaki, powdery, with radial furrows and a yellow to tan reverse. Macroaleuriospores are numerous in young cultures, but sterile hyphae may overgrow strains maintained in serial cultures. The macroaleuriospores are two- to six-celled, fingerlike in shape, and arranged in clusters of two, three, or more. Microaleuriospores are absent.

Microsporum amazonicum

This dermatophyte produces distinctive macroaleuriospores. It is rare, having been described only from the apparently normal hairs of certain native Brazilian rats (8). The colonies are fluffy to powdery with an olive gray-buff color. Most of the macroaleuriospores have four cells, but there may be as many as eight; the cell walls and septa are thick, there is an oil-droplet-like inclusion in each cell, and the surface of the spore is echinulate. The sexual state is *Nannizzia borelli.*

M. audouinii

This anthropophilic fungus rarely attacks animals, although isolates have been reported from dogs, cats, guinea pigs, rabbits, gibbons, and a monkey. The correctness of such reports has been questioned (5). The organism produces a yellow-greenish fluorescence in hair, where small ectothrix spores can be demonstrated. The perfect state has not been reported. The colonies are usually light tan to brown with a buff-salmon to orange-brown reverse. Macroaleuriospores are rare, irregular or spindle shaped, with two to nine cells; microaleuriospores are sessile or on short stalks, clavate, and single-celled. Unlike the closely related *M. canis,* this fungus grows poorly on rice. The addition of yeast extract to the medium stimulates the formation of macroaleuriospores by some strains (7).

M. boullardii

Microsporum boullardii is geophilic, and it has been isolated from soil but not from animals. Its macroaleuriospores resemble those of *M. fulvum,* but unlike the latter, it fails to produce ascospores with *Nannizzia fulva* (8).

M. canis

Microsporum canis is generally regarded as a zoophilic dermatophyte, but it often attacks humans. Most human infections are acquired from animals. It is the etiologic agent of roughly 98% of the cases of feline ringworm and about 70% of the cases of canine ringworm in North America (2); in cats, the infection is most often subclinical, whereas in dogs, the disease is more obvious (3,4). The head is the most common site of infection, with areas of alopecia around the nose, eyes, and ears, but the infection can become generalized (3). In addition to humans, cats, and dogs, numerous animals can serve as hosts: bats, canaries, cattle, chimpanzees, chinchilla, donkeys, foxes, gibbons, goats, gorillas, guinea pigs, horses, jaguars, lions, lynx, monkeys, orangutans, pigs, rabbits, sheep, tigers, and others (4). *Microsporum canis* produces a yellow-greenish fluorescence in hair, and it is associated with small ectothrix spores. Its colonies are white to buff in color with a characteristic yellow to orange-brown reverse. The macroaleuriospores are numerous, spindle shaped, with thick walls and 6–15 cells; the microaleuriospores are rare, small, clavate to elongate, and single-celled. The ascomycetous state of *M. canis* is *Nannizzia otae.*

M. cookei

This is a geophilic dermatophyte rarely recovered from animals. It has been isolated in the absence of clinical lesions from a variety of hosts, including baboons, dogs, cats, humans, marsupials, monkeys, rabbits, reptiles, and various wild animals, including rodents (4,6–8). It is not known to invade hair and it produces neither fluorescence nor arthrospores. *Nannizzia cajetani* represents its perfect state. Colonies are powdery yellowish or dark tan with a deep purplish red reverse. The macroaleuriospores are numerous, ellipsoidal, thick-walled, with four–six cells; the microaleuriospores are abundant and obovoid.

M. distortum

Microsporum distortum is a zoophilic dermatophyte that rarely infects humans. It has been isolated from ringworm in dogs, cats, horses, swine, guinea pigs, monkeys, rabbits, and humans (4,6). It produces a yellow-greenish fluorescence in hair, where it is associated with small ectothrix spores. The perfect state has not been reported. Colonies are white to tan, fluffy, with a yellow to tan reverse. The macroaleuriospores are numerous, thick walled, with 6 to 15 cells, bent, and distorted; the microaleuriospores are clavate and sessile.

M. ferrugineum

This is an anthropophilic dermatophyte not yet isolated from animals (7). It produces a yellow-greenish fluorescence in hair, where it forms small ectothrix spores. The perfect state has not been reported. Colonies are waxy, slow growing, deep yellow to orange, with many deep furrows and may be completely lacking in both macro- and microaleuriospores.

M. fulvum

Microsporum fulvum is a geophilic species occasionally involved in dermatophytosis; it has been recovered from humans, pigs, jaguars, lions, and certain types of goats and monkeys (4). Hair invasion is by ectothrix spores without fluorescence (3), and the perfect state is *Nannizzia fulva*. The mycelium resembles that of the *M. gypseum* complex, and in fact, this organism may often have been recorded as *M. gypseum* in the past (4). The colony has a dense downy to floccose chamoislike surface, and it is tawny buff in color with a white periphery. There are large numbers of macroaleuriospores similar to those of *M. gypseum* but longer, more clavate or bullet-shaped, and frequently lateral rather than conspicuously clustered (8). Microaleuriospores are also numerous.

M. gallinae (Synonym: *Trichophyton gallinae*)

This zoophilic dermatophyte rarely attacks humans. It is primarily a cause of ringworm (favus or white comb) in gallinaceous birds such as chickens and turkeys, but it has also been recovered from cats, dogs, laboratory mice, monkeys, pigeons, and quail (4,7).

In mammals, hair invasion is ectothrix without fluorescence. Colonies are heaped, radially folded, with a deep red color that diffuses throughout the medium. Macroaleuriospores may be abundant. They are generally spatulate or slipper-shaped, that is, elongate with a blunt tip, smooth-walled, and with 2–10 cells. Microaleuriospores are pyriform to clavate, single or in clusters, and few in number. The addition of thiamine or yeast extract to the medium may increase sporulation (5,7).

M. gypseum

This is a geophilic dermatophyte that often attacks humans and animals. It has been isolated from baboons, buffaloes, cats, cattle, chickens,

chimpanzees, chinchillas, dogs, fowl, guinea pigs, horses, leopards, monkeys, mice, parrots, rabbits, rats, squirrels, tapirs, tigers, and other species (7). Large ectothrix spores are typical but few in number, and fluorescence is absent or dull. The perfect state is represented by both *Nannizzia incurvata* and *N. gypsea*. The colonies grow fast, producing a flat, powdery, buff to cinnamon brown surface with a pale yellow to tan reverse. Upon microscopic examination, the powder is revealed as virtually solid masses of macroaleuriospores. These are large, rough, ellipsoid, thin walled, ranging from three- to nine-celled types, but preponderantly four- to six-celled. The microaleuriospores are clavate and sessile.

M. nanum

This dermatophyte is a zoophilic species that rarely infects humans, and it has been recovered from the soil only in pigyards. The organism periodically causes ringworm in swine, infecting up to a third of the herd; it is most commonly seen in Yorkshires, but all breeds can be affected (3). There have been no isolations of this fungus in animals other than pigs and humans (4,5,7). The lesions caused by *M. nanum* in swine are usually mild (3). They may cover large areas of the body, but the initial reaction subsides to leave only inconspicuous scaling and discoloration without alopecia or systemic disturbances (3). The disease becomes chronic and subclinical and, once established, there is little tendency for a spontaneous cure (3). Ectothrix spores with little or no fluorescence are produced in hair. The perfect state is *Nannizzia obtusa*. Colonies are white to buff or yellow, cottony, with a red to brown reverse. Characteristic ovoid, clavate, or pear-shaped macroaleuriospores with thin walls and one–three cells are produced in large numbers; microaleuriospores are rarely produced on Sabouraud agar.

M. persicolor

Microsporum persicolor is a zoophilic dermatophyte very rarely isolated from humans. It is a frequent mild pathogen of small wild rodents, particularly bank voles, in which it produces tail lesions (4,5,8). It has also been isolated from field voles, bats, dogs, guinea pigs, shrews, and mice (4). It does not parasitize hair, and its perfect state is *Nannizzia persicolor*. Colonies are flat to gently folded, fluffy, yellowish buff to pale pink, with a reverse that ranges from peach or rose to a deep shade of ochre. Clavate, fusiform, or globose microaleuriospores in clusters similar to those of *T. mentagrophytes* are abundant, while macroaleuriospores are rare, elongate, fusiform to clavate, thin-walled,

and usually six-celled. The organism may be confused with *T. mentagrophytes.*

M. praecox

This a geophilic dermatophyte once isolated from a pustular vesicular lesion in a human wrist (8). No perfect state is known. Colonies are powdery buff with a yellow orange undersurface, similar to *M. gypseum,* but they are longer, narrow and lanceolate in profile, and have six–nine cells.

M. racemosum

Microsporum racemosum is a geophilic species isolated from the soil and from rats (4,8). The perfect state is *Nannizzia racemosa.* Colonies are flat, powdery, cream white, fast-growing, and with a grape-red undersurface. The macroaleuriospores are thin-walled, with 5–10 cells, tapered, and frequently bear a terminal filament. Distinctive club-shaped, mostly stalked microaleuriospores are found in large wandlike clusters.

M. vanbreuseghemii

This dermatophyte is geophilic, and it rarely attacks humans or animals. It has been recovered from a dog, a squirrel, and humans (3–5,8). In infected hair, it produces ectothrix spores and no fluorescence or very poor fluorescence. The perfect state is *Nannizzia grubyia.* Colonies are flat, powdery, creamy yellow to pink, with a colorless or yellow reverse. Numerous long cylindrofusiform, rough, thick-walled macroaleuriospores with 7–10 cells are produced in culture, along with pyriform or obovate microaleuriospores.

Trichophyton ajelloi

Trichophyton ajelloi is a geophilic saprophyte of very low pathogenicity that rarely infects humans or animals. Isolations have been made from baboons, dogs, cattle, horses, marsupials, monkeys, mice, guinea pigs, squirrels, and humans (3,4). It does not attack hair, and the perfect state is *Arthroderma uncinatum.* Colonies are downy, cream to orange-tan, with a colorless, reddish, or bluish-black reverse. The macroaleuriospores are smooth, cylindric, with tapering ends and 5–12 cells. The microaleuriospores are abundant, sessile, and pyriform to ovate.

T. concentricum

This is an anthropophilic species not known to infect animals (7). It does not attack hair, and the perfect state has not been reported. Colonies are white to cream colored, deeply folded, with a cream to brown reverse. No macroaleuriospores or microaleuriospores are produced. The growth of at least half of the isolates is stimulated by the addition of thiamine of the medium (5,7).

T. equinum

Trichophyton equinum is a zoophilic species that rarely attacks humans; it causes ringworm in horses and donkeys, and occasionally, in dogs (4,7). Foals and yearlings are most susceptible, at first developing swellings that can be felt through the hair. These can progress to small inflamed ulcers with an exudate, a condition often known as girth itch. Alopecia develops as the lesions enlarge and turn chronic; crusts may fall off from healed lesions, leaving bald areas with a motheaten appearance. Hair invasion is by large ectothrix spores; there is no fluorescence, and the perfect state has not been reported. Colonies are white to cream-colored with a bright yellow to dark pink or brown reverse. Macroaleuriospores are extremely rare, but variable numbers of thin, elongate to pyriform, stalked microaleuriospores are usually seen. All strains require nicotinic acid (niacin) for growth except those from Australia and New Zealand (3). The organism will grow on commercially available Trichophyton agar #5 (nicotinic acid–casein agar) but not on ordinary Sabouraud agar (13).

T. erinacei

Trichophyton erinacei is a zoophilic dermatophyte primarily associated with ringworm in hedgehogs, but occasionally it has been isolated from mice and rats, dogs, and humans (4,13). Hair invasion is normally ectothrix (8). The perfect state has not been reported. Colonies are flat, powdery, white to ivory white, with a clear yellow diffusing pigment underneath (13). The macroaleuriospores are club-shaped, with fewer cells than those of *T. mentagrophytes*. The microaleuriospores are also club-shaped and produce abundantly on terminal hyphal branches. Forms intermediate in appearance between microaleuriospores and macroaleuriospores are numerous.

T. georgii

This geophilic species is most likely a soil saprophyte, but it has been isolated from the opossum (4). It does not attack hair, and the perfect state is *Arthroderma ciferri*. Its colonies are pale brown with a spotted brown reverse. Microaleuriospores are abundant and variable in size and shape, but no macroaleuriospores are produced.

T. gallinae (see *M. gallinae*)

T. gourvilii

This anthropophilic dermatophyte has not been isolated from infections in animals. Hair invasion is endothrix without fluorescence, and the perfect state has not been reported. Some workers regard it as a soil saprophyte (7). Colonies are folded, heaped, waxy, with a lavender to deep red pigmentation (13). Macro- and microaleuriospores are usually present in very small numbers.

T. megninii

This anthropophilic dermatophyte is rarely isolated from animals, but it has been recovered from dogs, cats, mice, and chickens (4,6). Hair invasion is ectothrix without fluorescence, and the perfect state is not known. Colonies are white to pink with a nondiffusible rose to red pigment on the reverse side (7). L-Histidine is required for growth. The organism grows on commercially available Trichophyton agar #7 (histidine–ammonium nitrate agar). Macroaleuriospores are rare, clavate, 2–10-celled, with thin smooth walls, while pyriform to clavate microaleuriospores are very numerous.

T. mentagrophytes

Trichophyton mentagrophytes is primarily a zoophilic dermatophyte that often attacks humans and may also survive saprophytically in the soil. One variety, *T. mentagrophytes* var. *interdigitale*, is anthropophilic (1). Infections by *T. mentagrophytes* have been reported in a large number of wild and domestic animals, including baboons, buffaloes, cats, cattle, chinchillas, chickens, chimpanzees, dogs, foxes guinea pigs, goats, horses, hedgehogs, kangaroos, kiwis, mice, monkeys,

marsupials, nutrias, opossums, polecats, porcupines, parrots, rabbits, rats, rodents, swine, sheep, squirrels, tapirs, and tigers (4,6–8). *Trichophyton mentagrophytes* is probably the most common dermatophyte.

Hair invasion by *T. mentagrophytes* is usually small-spored ectothrix and without fluorescence, but some strains may be endothrix with a dull fluorescence. The latter (var. *quinckeanum*) have been implicated in mouse favus, which produces numerous white crusty lesions throughout the body. Most rodents, however, carry *T. mentagrophytes* as normal flora without signs of disease (3). Two perfect states are known: *Arthroderma benhamiae* and *A. vanbreuseghemii*. Colonies are powdery or granular, light buff to rose-tan with a buff to deep wine or brown reverse. Macroaleuriospores are 3–5-celled, thin-walled, clavate, and not too abundant. The most consistent microscopic feature is the production of large numbers of microaleuriospores in grapelike clusters, especially in the zoophilic strains.

T. rubrum

Trichophyton rubrum is an anthropophilic species rarely isolated from animals (3). So far, it has been recovered from a baboon, a dog, a cat, cattle, chimpanzee, a rabbit, a guinea pig, sheep, and a mouse (4,6–8). This species has never been isolated from soil (3). It appears to be dependent on humans for dissemination, and no perfect phase is known (3). Hair is rarely invaded, but when it is, ectothrix spores in chains are the rule (3,5,6). There is no fluorescence. Most colonies are white and cottony with a reddish to rose-purple reverse. Macroaleuriospores are absent or rare, but teardrop microaleuriospores lateral to the hyphae or in "pine tree" clusters may be seen.

T. schoenleinii

This anthropophilic dermatophyte is rarely isolated from animals, but it has been recovered from dogs, cats, hedgehogs, mice, cattle, horses, rabbits, and guinea pigs (4,6–8). Hair invasion involves rare endothrix spores without fluorescence or with a dull grayish to yellow fluorescence (5).

Longitudinal tunnels produced within the hair shaft are filled with air bubbles after disintegration of the hyphae (5). No perfect state has been reported. Colonies are slow-growing and waxy with many irregular folds and a yellowish to light brown color. Macroaleuriospores are absent and microaleuriospores rare, but numerous *favic chandeliers* are present; these structures are broadened hyphal tips with short lat-

eral and terminal branches. Favic chandeliers do occur in other *Trichophyton* spp., but they are generally rare (1,6,9). The growth of some isolates is stimulated by thiamine (1).

T. simii

Trichophyton simii may be a soil saprophyte, but it is presently regarded as a zoophilic species that rarely attacks humans. It causes ringworm in poultry and in monkeys; it has also been isolated from chimpanzees, dogs, guinea pigs, horses, rabbits, gerbils, mice, rats, shrews, and squirrels (4,6–8). Hair invasion is both ecto- and endothrix, frequently with a vivid green fluorescence. The perfect state is *Arthroderma simii* (5). Colonies are white to pale buff in color with a colorless to vinaceous reverse. Macroaleuriospores are numerous, cylindrical in shape, with 4–10 cells. Microaleuriospores are rare at first, but they increase in number as the culture ages.

T. soudanense

This anthropophilic species is not known to attack animals (7). Hair invasion is endothrix without fluorescence, and the perfect state is not known. Colonies are slow growing, lemon yellow to apricot-colored, with a yellow to orange reverse. The species produces no macroaleuriospores and variable numbers of ovoid, clavate, or pyriform microaleuriospores (7,13).

T. terrestre

Trichophyton terrestre is a geophilic species of very low pathogenicity. It has been isolated from the hair of animals including badgers, cats, dogs, hedgehogs, horses, mink, moles, monkeys, mice, opossums, polecats, rats, and others as well as humans, but it does not invade hair (4,7). Lesions have not been demonstrated. The perfect states are *Arthroderma insingulare*, *A. lenticularum*, and *A. quadrifidum* (7). Colonies are powdery, velvety, pale lemon to buff, with a yellow or reddish reverse. Clavate to pyriform microaleuriospores are abundant, as well as thin-walled, cigar-shaped, 2–12-celled smooth macroaleuriospores. Traditional forms from micro- to macroaleuriospores are also abundant.

T. tonsurans

This anthropophilic species so far has been isolated from only two animals, a horse and a dog (4,6,7). Hair invasion is large-spored endothrix without fluorescence (5). The perfect state has not been reported. Colonies are folded, velvety to powdery, with considerable color variation. Macroaleuriospores are rare, thin-walled and club-shaped, but microaleuriospores are numerous, attached to the hyphae by short sterigmata. Growth is greatly stimulated by the addition of thiamine to the medium (5,7).

T. vanbreuseghemii

Trichophyton vanbreuseghemii is a geophilic species of doubtful pathogenicity that has so far been isolated only from the dog and the cat (4). Animal infections are rare (7). It has also been implicated in dermatophytosis in the hand of a forestry worker (13). Hair invasion is ectothrix. The perfect state is *Arthroderma gertleri*. Colonies are buff-colored, with the texture of fine glove leather and central folds. The macroaleuriospores are plump, thin walled, cylindrical or club-shaped, with rounded ends, and they appear in small clusters; they are reminiscent of *T. ajelloi* (8). The individual cells of the macroaleuriospores usually separate from each other as they mature. Microaleuriospores are club-shaped and stubby, and they develop mainly along the hyphae.

T. verrucosum

This zoophilic dermatophyte often attacks humans; it has been isolated from buffalo, canaries, cats, cattle, dogs, donkeys, dromedaries, fowl, goats, horses, mules, pigs, sheep, and zebu (4). Presently, *T. verrucosum* is regarded as the primary cause of ringworm in cattle.

Trichophyton verrucosum produces very large ectothrix spores in chains. Infected hair in humans is not fluorescent, but some florescence has been noted in cattle (8). The perfect state has not been reported. Colonies are slow growing, heaped, deeply folded, and white to yellow. The organism may fail to grow in routine media because thiamine is required for growth and many strains also require inositol (7,13). All strains grow on commercially available Trichophyton agar #3 and some also grow on #4 (7,13). Unlike other dermatophytes, the organisms grows best at 37°C (6,9). Macroaleuriospores are rarely seen; they are variable in size and shape with three to five cells and a rattail appearance. Microaleuriospores are tearshaped, abundant only in media enriched with thiamine.

T. violaceum

Trichophyton violaceum is an anthropophilic fungus rarely isolated from animals. It has been reported from buffaloes, horses, cattle, cats, dogs, mice, sheep, a pigeon, and a mule (4,6–8). Hair invasion is endothrix without fluorescence, and the perfect state has not been reported. Colonies are heaped, folded, waxy, slow-growing, and violet in hue, but growth is minimal in the absence of thiamine. The organism grows well on commercial Trichophyton agar #4 (casein–thiamine agar) (7,13). Some pyriform microaleuriospores may develop on thiamine-enriched media. Generally, no macroaleuriospores are seen.

T. yaoundei

This is an anthropophilic dermatophyte also found as a soil saprophyte; no infections in animals have been reported (7). Hair invasion is endothrix without fluorescence, and the perfect state is not known. Colonies are slow growing, white to cream in color initially, changing to a chocolate brown hue as they mature. Macroaleuriospores are not produced; microaleuriospores are rare (13).

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30

Yeasts Causing Infection

John R. Cole, Jr. and G. R. Carter

The yeasts constitute a large group of unicellular fungal organisms. They are placed in the form class Blastomycetes, or in three groups: Ascomycetes, Basidomycetes, or Fungi Imperfecti. A number of species are found as commensals in the gastrointestinal tract of humans and animals. Only a few species have been implicated as causes of disease in animals. A wider variety have been involved in infections in humans, perhaps because of the intensive and prolonged use of antibiotics.

The normal vegetative forms of yeasts are round or oval cells with a diameter usually in the range of 2.5–6 μm. Their mode of reproduction is by budding (blastospores), and some possess capsules. Pseudohyphae and true hyphae are produced by various species. *Candida* spp. produce chlamydospores of different shapes, and *Saccharomyces* produce ascospores.

The principal pathogenic yeasts of animals are *Candida albicans* and *Cryptococcus neoformans*. *Geotrichum candidum* will be discussed in this chapter, although it resembles a mold more than a yeast. Its early colony morphology resembles that of the yeasts.

Yeasts are recovered occasionally from clinical materials. Most often they are contaminants or are derived from the normal flora on mucous membranes, such as *Candida* spp. The kind of disease and/or lesion will usually lead the clinician or pathologist to suspect a yeast infection. Also, the recovery of a potentially pathogenic yeast in nearly pure culture or in large numbers may indicate disease due to a yeast. The repeated demonstration and recovery of a yeast from a lesion provides strong evidence of its significance. In instances of what appear to be contaminating yeasts, efforts at identification are not usually made.

Yeasts will frequently be seen on primary media such as blood agar

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and Sabouraud agar. The colonies are usually small initially and can easily be confused with colonies of micrococci or staphylococci; however, they have a yeasty odor and on further incubation they may display pigmentation, as evidenced by a cream, salmon, or tan appearance. The colonies of *Malassezia* and *Trichosporon* may become membranous after prolonged incubation. Identification of the colonies as those of yeasts is made by demonstration of the typical yeast forms in wet mounts or in Gram-stained smears. Table 30-1 is provided to aid in the identification of the principal genera of yeasts encountered in clinical specimens.

Both carbohydrate fermentation and assimilation tests are used in the identification of yeasts. The former tests can be carried out in conventional carbohydrate broths, and the latter can be performed with carbohydrate discs applied to yeasts in pour plates (1). However, several commercial systems are currently available to facilitate the identification of yeasts recovered from clinical specimens. Three that have been widely used are the API 20 C Strip (Analytab Products, Inc., Plainview, New York), the Minitek yeast carbon assimilation system (BBL Microbiology Systems, Cockeysville, Maryland), and the Uni-Yeast-Tek system (Flow Laboratories, Inc., Roslyn, New York). Additionally, other available systems are Automicrobic System (AMS, Vitek, Hazelwood, Missouri), Autobac (Organon Teknika, Durham, North Carolina), Microdrop Kit (Clinical Sciences, Whippany, New Jersey), Microstix (Ames Company, Elkhart, Indiana), and MS-2 (Abbott Labs, Irving, Texas). All employ carbohydrate assimilation and/or fermentation tests.

Candidiasis—Moniliasis, Thrush, and Candidosis

Cause

Candida albicans.

Pathogenicity

Infections most frequently involve the mucous membranes and are often sufficiently severe to produce diphtheritic membranes. Systemic infections are infrequent.

Chickens, turkeys, and other birds: *Candida albicans* produces infection of the mouth, esophagus, and crop (2,3).

Dogs and cats: *Candida albicans* causes mycotic stomatitis, with white to gray patches on the oral mucosa and enteritis of kittens.

Calves and foals: *Candida albicans* produces infections of the oral and intestinal mucosa; systemic candidiasis has been described in calves on prolonged antibiotic therapy (4).

Table 30-1
Differentiation of Genera of Yeasts Found in Clinical Specimens

Genus	Microscopic morphology	Pseudomycelium	Capsule	Chlamydospores	Urease	Other
Candida	Spherical	+	–	*Candida albicans*, *C. stellatoidea*, *C. tropicalis*[a]	Most negative	*Candida albicans* produces germ tubes
Cryptococcus	Spherical, oval, or elongate	Rudimentary or none	+	–	+	Colonies often very mucoid
Malassezia	Bottle-shaped; monopolar budding	Pseudomycelium rare	–	–	+	Growth stimulated by fatty acids
Rhodotorula	Spherical, oval, or elongate; multilateral budding	–	+	–	+	Orange to red pigment
Saccharomyces	Spherical, oval, cylindrical, or elongate; multilateral budding ascospores, usually four	–	–	–	–	
Torulopsis	Spherical, oval, or elongate; multilateral budding	–	–	–	–	Predominantly non-fermentative (except glucose), non-assimilatory
Trichosporon	Various shapes (barrel-shaped arthrospores)	+ and true hyphae	–	–	Some species +	Resistant to cycloheximide

[a]Chlamydospores produced rarely.

Cattle: *Candida albicans* is a cause of bovine mastitis. Genital infections are rare.

Horses: Genital infections due to candidiasis occur occasionally in the male and female (5).

Swine: *Candida albicans* produces diarrhea, and infections of the lower esophagus and esophagogastric region of the stomach (6). Cutaneous candidiasis has been described in swine (7).

Other Species: *Candida* infections have been reported in rodents, primates, dolphins (8), and other animals. In humans, the mucous membranes of the mouth, tongue, and vulva are more commonly involved than the skin and nails. The oral form with the characteristic white patches is seen frequently in infants. The lungs and bones may be infected, and endocarditis may be seen in the systemic form.

Direct Examination

Yeastlike cells and hyphae can be demonstrated in wet mounts (10% NaOH, 20% KOH, or lactophenol cotton blue) of scrapings from lesions. The thin-walled oval and budding yeast cells are 2–6 μm in diameter.

The examination of tissue sections is of great value in the diagnosis of candidiasis in that the actual invasion of the tissue by the organism can be demonstrated.

Gram-stained smears disclose gram-positive oval and budding yeast cells (Fig. 30-1).

Isolation Procedures

If candidiasis is suspected, the specimen is inoculated onto Sabouraud agar and Sabouraud C and C agar, then incubated at 25°C. It is also advisable to inoculate blood agar and incubate at 37°C.

In 1–3 days, colonies are cream-colored, pasty, and smooth with a yeastlike odor. The colonies resemble those of staphylococci and micrococci.

Identification

Candida spp. produce pseudohyphae and do not possess ascospores. Typical oval and budding yeast cells are seen in wet mounts and in Gram-stained smears. These are of little value in identification. Some differential features of genera of yeastlike organisms are given in Table 30-1.

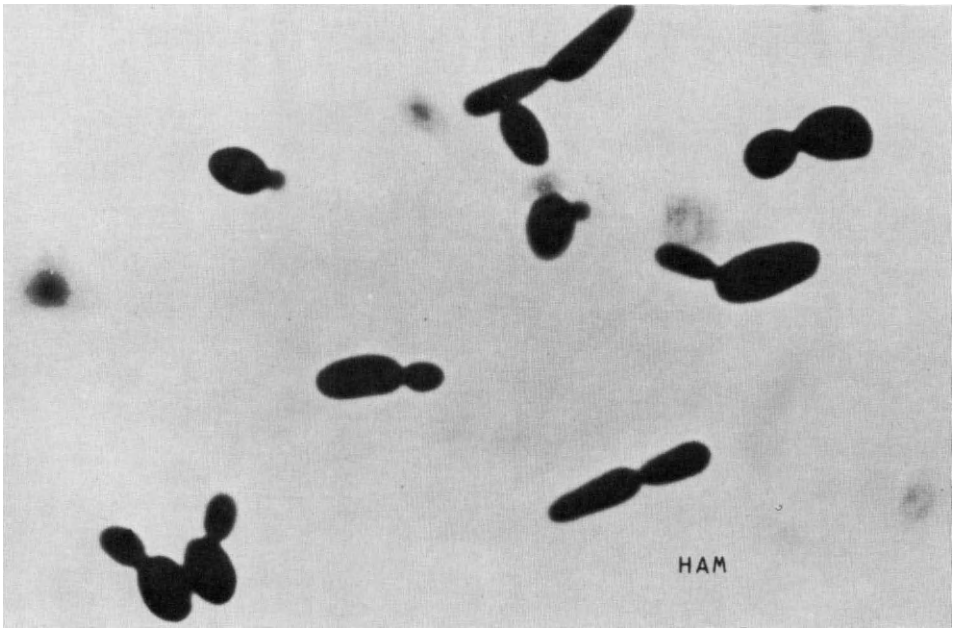

Figure 30-1. *Candida albicans* from Sabouraud dextrose agar. Similar forms are seen in clinical specimens. Gram stain, ×2250 (H. A. McAllister).

Demonstration of the large, round, thick-walled chlamydospores (Fig. 30-2) characteristic of *C. albicans* is essential for identification. These are produced on chlamydospore agar (see Appendix C) and other media, including cornmeal agar with Tween 80. Plates of this medium are inoculated by cutting through the agar along the line of inoculation. The cut in the agar should be at an acute angle to the bottom of the plate to facilitate subsequent microscopic examination. After incubation at 25°C for 2–4 days, the plates are examined under the low-power objective of the microscope. Production of chlamydospores is favored by lowered oxygen tension and, for this reason, they are more apt to be seen below the surface of the medium. The chlamydospores appear blue in the chlamydospore agar as a result of absorption of the trypan blue, whereas the filaments are colorless. It should be kept in mind that *C. tropicalis* and *C. stellatoidea* can produce small numbers of chlamydospores on rare occasions.

The demonstration of germ tubes is another method for identification of *C. albicans*. They may be observed by incubating yeast cells in 0.5 ml of bovine, sheep, rabbit, or human serum for 2–4 hr at 37°C. The tubes appear as small sprouts developing from yeast cells. Alternatively, the tubes may be produced by inoculating a colony onto Levine's eosin–methylene blue agar. On this medium, characteristic germ tubes are produced after incubation for 24 hr at 37°C in a candle jar.

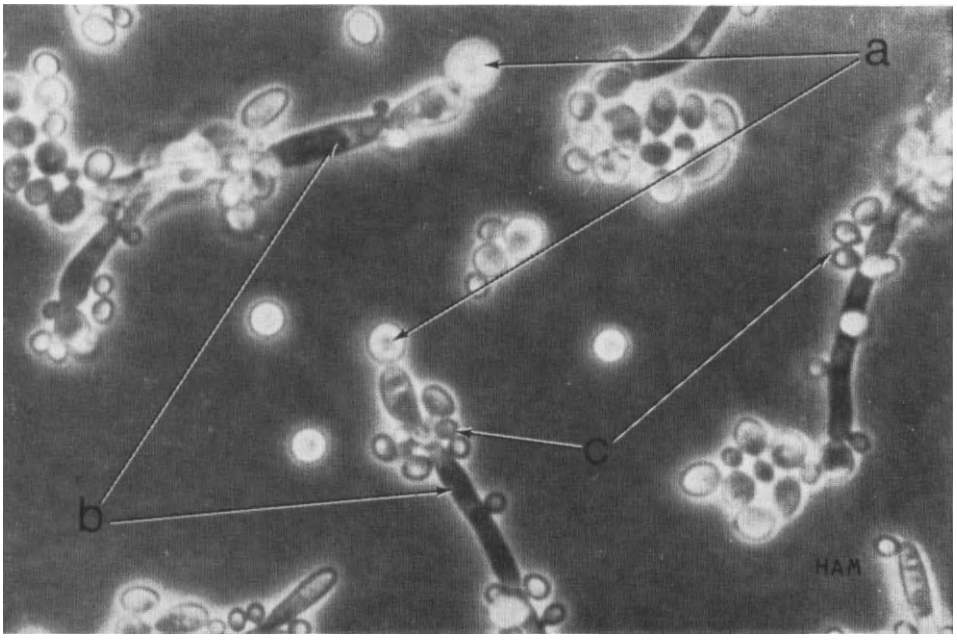

Figure 30-2. *Candida albicans* on chlamydospore agar: a, terminal chlamydospores; b, pseudohyphae; c, blastospores. Darkphase illumination, ×975 (H. A. McAllister).

A small percentage of *C. albicans* cannot be identified by the above procedures. These strains and other species of the genus can only be identified with certainty by means of cultural and biochemical criteria (9) (see Table 30-2).

Animal Inoculation

Strains of *C. albicans* are pathogenic for mice and rabbits. One milliliter of a saline suspension (Brown or McFarland tubes 5 to 6; 24–48-hr culture) administered intravenously to a rabbit or intraperitoneally to a mouse will usually kill within 4–10 days. Numerous small abscesses are found in the swollen kidneys. The typical yeastlike cells can be seen in smears from the lesions (Fig. 30-3). Other *Candida* species do not as a rule kill experimental animals.

Other *Candida* Species

The other *Candida* species are identified by the criteria listed in Table 30-2 and as follows:

Table 30-2
Differentiation of *Candida* Species

Species	Growth 37°C	Pellicle: broth	Chlamydospores	Germ tubes	Fermentation				
					Glucose	Maltose	Sucrose	Lactose	Galactose
C. albicans	+	–	+	+	+	+	–	–	+
C. stellatoidea	+	–	rare	+	+	+	–	–	–
C. tropicalis	+	+	rare	–	+	+	+	–	+
C. pseudotropicalis	+	–	–	–	+	–	+	+	+
C. parapsilosis	+	–	–	–	+	–	–	–	+
C. krusei	+	+	–	–	+	–	–	–	–
C. guilliermondii	+	–	–	–	+	–	+	–	+
C. rugosa	–	–	–	–	–	–	–	–	–

C. tropicalis and *C. pseudotropicalis.* These species are causes of mastitis in cows (10).

C. parapsilosis. Cases of bovine mastitis (11) and abortion in a cow (12) have been attributed to this organism.

C. guilliermondii and *C. krusei.* Gedek (11) recovered these additional species from the milk of cows with mastitis.

C. rugosa. This organism was incriminated as a cause of pyometra in a mare (13) and can also cause bovine mastitis.

Cryptococcosis (Torulosis)

Cause

Cryptococcus neoformans: This yeastlike organism, which is the only pathogenic species of 19 within the genus, occurs widely in nature. There are two varieties, *C. neoformans* variety *neoformans* (serotypes A and D) and *C. neoformans* variety *gattii* (serotypes B and C).

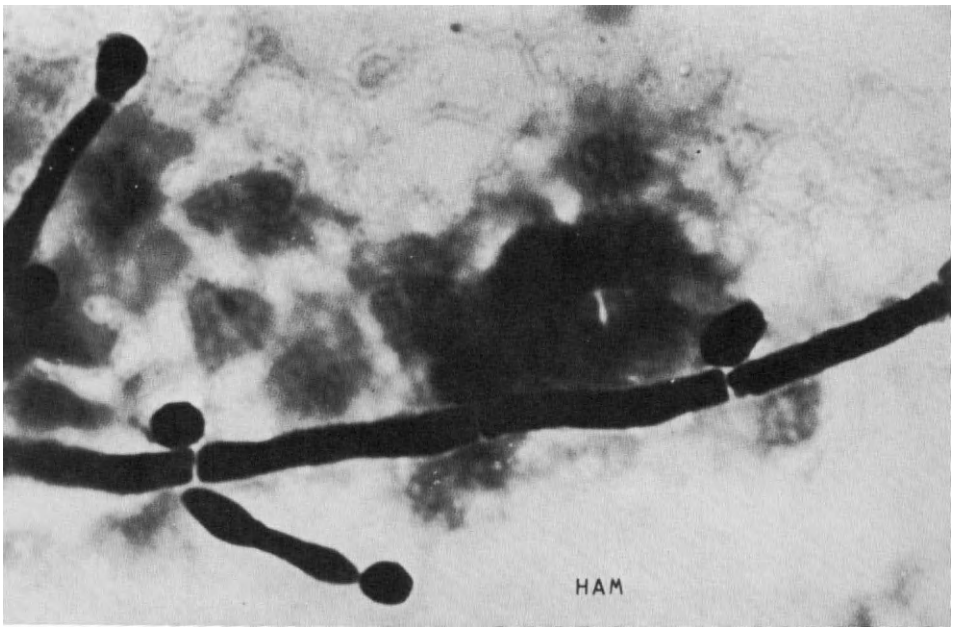

Figure 30-3. *Candida albicans* showing spherical forms and pseudohyphae. Gram-stained kidney impression smear from an experimentally infected mouse, ×2250 (H. A. McAllister).

Pathogenicity

Cryptococcosis is a subacute or chronic infection that frequently involves the central nervous and respiratory systems and the eye.

Dogs and cats: Cryptococcosis is seen most frequently in dogs and less commonly in cats. Infections usually begin in the paranasal sinuses, with later extension to the brain and meninges or lungs. Subcutaneous granulomas are sometimes encountered around the head and feet.

Horses: Infections, which begin usually as nasal granulomas, may extend to the lungs and viscera.

Cattle: Cryptococcosis is a cause of sporadic cases of mastitis.

Other animals: Infections have also been reported in the fox, dolphin, monkey, civet, ferret, guinea pig, cheetah, and various birds. The organism is especially prevalent in pigeon feces.

Direct Examination

A strongly presumptive diagnosis of cryptococcosis can frequently be made by the demonstration of the encapsulated organism in the nasal discharge in the paranasal form and in various clinical specimens such as pus, milk, exudate, and cerebrospinal fluid. The yeast cells are 5–20 μm in diameter. The addition of India ink to wet preparations facilitates demonstration of the characteristic thick capsules (Fig. 30-4).

Isolation Procedures

Blood agar and Sabouraud dextrose agar are inoculated, the former being incubated at 37°C and the latter at 25°C. Cycloheximide inhibits the growth of this organism.

Cultural Characteristics

Growth at 25°C (7–14 days); wrinkled, whitish, granular colonies become slimy, mucoid, and cream to brownish in color on further incubation. No mycelium is present, and the colony flows to the bottom of the slant. At 37°C (7–14 days), colonies are essentially the same as those described above. After shorter incubation periods, colonies resemble those of staphylococci and micrococci.

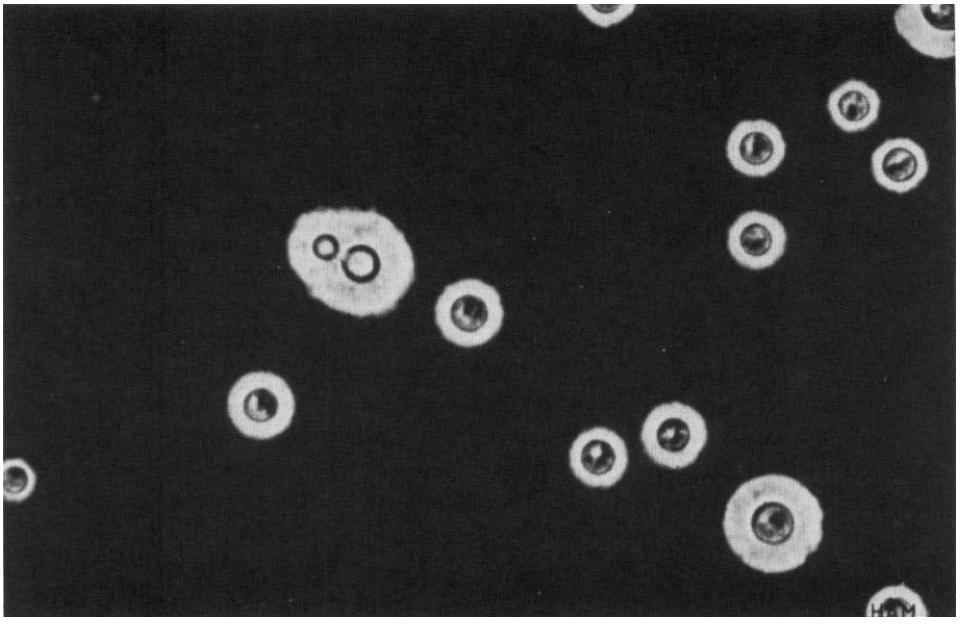

Figure 30-4. Encapsulated yeast cells of *Cryptococcus neoformans* from an experimentally infected mouse brain. India ink preparation, ×975 (H. A. McAllister).

Identification

Budding cells, usually with large capsules, can be seen in wet mounts containing India ink. Some saprophytic strains of *Cryptococcus* spp. will not grow at 37°C.

A presumptive identification of *C. neoformans* is usually based upon the demonstration and recovery of yeast cells with a large capsule from an animal showing clinical signs of cryptococcosis. Because *Rhodotorula* and various species of cryptococci also produce capsules, definitive identification requires additional tests. In addition to the features listed in Table 30-1, differentiation of *C. neoformans* from other species of cryptococci can be accomplished on the basis of (a) ability to grow at 37°C; (b) pathogenicity for mice (see section on Mouse Inoculation); (c) production of brown colonies on birdseed agar (see Appendix C); and (d) typical carbohydrate assimilation pattern (Table 30-3).

Members of the genus *Cryptococcus* produce urease on Christensen's urea agar, while *Candida* spp. do not. Strains of the true yeast *Saccharomyces* can be distinguished from the cryptococci by the presence of ascospores in the former. The ascospores stain well with methylene blue. Species of *Geotrichum* and *Trichosporon* both produce true mycelia.

Table 30-3
Differentiation of *Cryptococcus* Species

Species	Glucose fermentation	Urease	Growth 37°C	Sugar assimilation:					Nitrate assimilation
				Glucose	Maltose	Sucrose	Lactose	Galactose	
C. neoformans	–	+	+	+	+	+	–	+	–
C. albidus	–	+	–	+	+	+	+	+	+
C. albidus var. *diffluens*	–	+	–	+	+	+	–	+	+
C. luteolus	–	+	–	+	+	+	–	+	–
C. laurentii	–	+	– or +	+	+	+	+	+	–
C. terreus	–	+	–	+	+	–	–	+	– or +

The commercial kits referred to earlier in this chapter can be used to identify species of *Cryptococcus*.

Mouse Inoculation

The pathogenic strains of *C. neoformans* kill mice at a dose of 1 ml intraperitoneally or 0.02 ml intracerebrally of a heavy saline suspension prepared from a Sabouraud agar slant.

Pathogenic strains inoculated intraperitoneally may produce lesions in the brain in about 3 weeks, while those injected intracerebrally may develop brain lesions in about 1 week. Those mice that do not die within 2 weeks should be necropsied. Gelatinous masses are found in the abdominal cavity and associated with the brain and lungs. The typical budding, encapsulated organism can be demonstrated and cultured from the material.

Geotrichosis

Cause

Geotrichum candidum: As mentioned previously, this organism is a mold rather than a yeast, but in its early colonial growth, it appears yeastlike. This fungus is found widely in nature, and its isolation is not necessarily significant. Two cultural forms occur. They are referred to as the glabrous, or yeastlike, and the fluffy form. The glabrous form of *G. candidum* is the form usually associated with disease.

Pathogenicity

Infections due to this fungus in animals are rare. They have been reported from pigs, ocelot, horses, cattle, dogs, fowl, and humans. The bronchi, lungs, udder, and the mucous membranes of the alimentary tract are most frequently affected. The disease is usually mild and is characterized by the formation of granulomas that may suppurate.

Direct Examination

Purulent material or scrapings from lesions are examined in wet mounts. The organism appears as rectangular (4–8μm) or large spherical (4–10μm) arthroconidia. They are thick-walled, nonbudding, and in stained smears are strongly gram-positive.

Isolation Procedures

If there is microscopic evidence of *G. candidum*, material is inoculated onto blood agar, which is incubated at 37°C, and onto Sabouraud C and C agar and regular Sabouraud agar, which are incubated at 25°C.

Cultural Characteristics

At 25°C (1–2 weeks) colonies grow fairly rapidly and are membranous with radial furrows and soft with a dry granular surface. At 37°C the fungus does not grow well. The colonies, which appear early, are small, and the mycelium penetrates the subsurface of the medium.

Identification

At both 25°C and 37°C, the mycelium is made up of septate hyphae that fragment, producing chains of rectangular to round arthroconidia (Fig. 30-5)

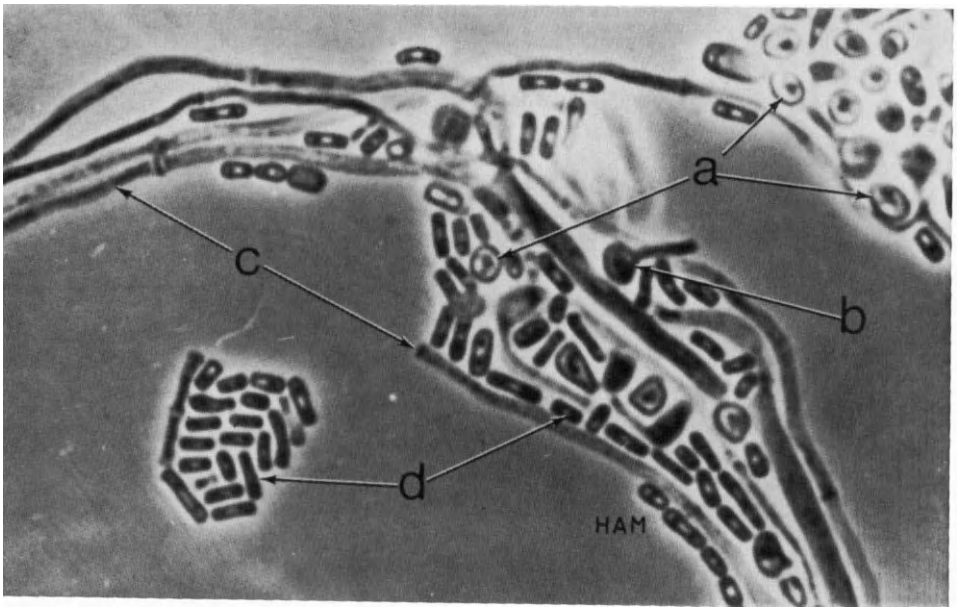

Figure 30-5. Lactophenol cotton blue preparation of *Geotrichum candidum* from a Sabouraud dextrose agar slide culture: a, yeast cells; b, yeast cell with germ tube; c, hyphae; d, rectangular arthroconidia. Darkphase illumination, ×1000 (H. A. McAllister).

Identification is based upon cultural characteristics and the demonstration of the characteristic arthroconidia. This fungus can be distinguished from *Coccidioides immitis* and *Blastomyces dermatitidis* by the fact that these species produce cottony, filamentous colonies at room temperature. *Geotrichum candidum* produces a soft yeastlike colony at this temperature. Also, *G. candidum* does not form blastoconidia, and this characteristic differentiates it from *Trichosporon* spp.

Malassezia (Pityrosporum)

Malassezia is now considered the correct genus name for those lipophilic yeasts that reproduce by unipolar budding. The species *Malassezia furfur* is associated with tinea versicolar, dandruff, seborrhea, and blepharitis in humans.

The yeasts that have been called *Pityrosporum canis* and *P. felis* and similar organisms from other animals have been given the name *M. pachydermatis*. The latter species is thought to occur often as a commensal on the oily areas of the skin and ears of dogs, cats, and probably other animals. It is frequently recovered from the ears of dogs with chronic otitis externa and is thought to have at least a secondary role in the etiology of some cases (14,15).

Malassezia pachydermatis consists of bottle-shaped, small budding cells 1–2 × 2–4 μm. Reproduction is by a process known as bud fission, in which the bud detaches from the mother cell by a septum (Fig. 30-6).

Direct Examination

Wet mounts (10% NaOH) are made from material taken from the affected ear in the inflammatory area. After the mount has cleared, it is examined for clusters of thick-walled, round, and budding forms (3–8 μm in diameter) surrounded by short, straight, and angular mycelial fragments. The short hyphae are generally observed in clinical materials only. Although a presumptive identification can be made on a direct examination, it is advisable to attempt isolation.

Isolation Procedures

Isolations have been made from cases of otitis externa employing Sabouraud agar at 25°C with an incubation period of 2 weeks (16). Growth was increased by the addition of sterile olive or coconut oil to the Sabouraud plates prior to inoculation. Small creamy, punctiform

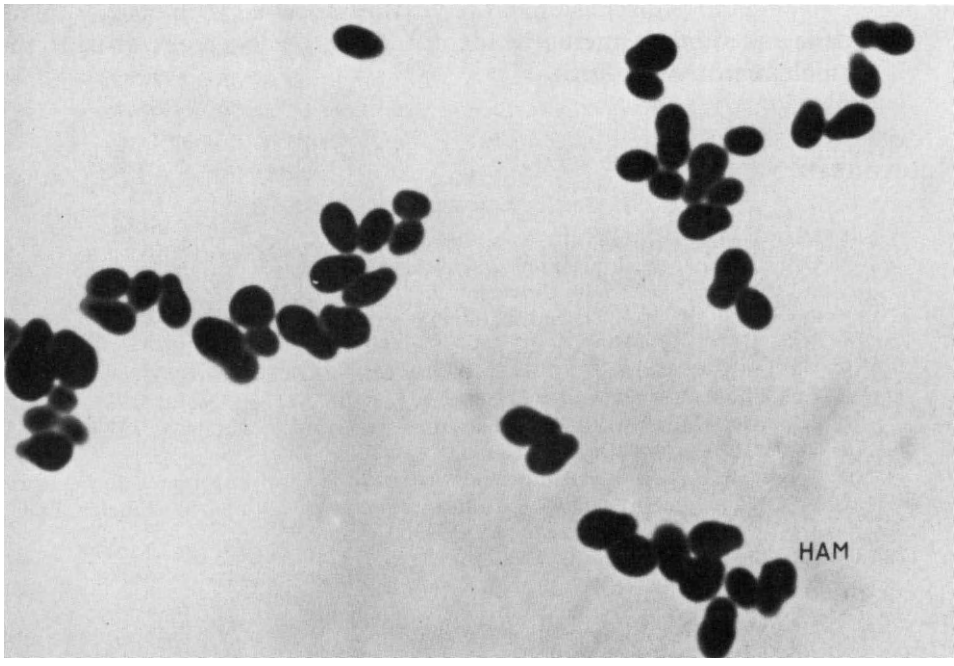

Figure 30-6. *Malassezia pachydermatis (Pityrosporum canis):* Gram-stained smear from an olive oil enriched Sabouraud dextrose agar culture, ×2440 (H. A. McAllister).

colonies give rise with additional incubation to confluent, membranous colonies. On blood agar, discoloration at the inoculation site may be the only evidence of growth.

Additional Yeasts

Torulopsosis is an infrequent disease of humans and has only been reported on several occasions in animals. The species involved in most infections is *Torulopsis glabrata,* which occurs as a commensal in animals and is also found in the soil. It has been reported as causing bovine mastitis and abortion, and systemic infections in dogs and monkeys (17). The genus *Torulopsis* is now considered distinct and not included with *Candida.*

Species of the genera *Rhodotorula* and *Trichosporon* have on occasion been associated with disease in animals. *Trichosporon* has become recognized as an important cause of mycotic mastitis in cattle (18,19). Ainsworth and Austwick (20) have reviewed a number of reports of diseases caused by less well-known yeasts.

For a detailed discussion of the yeasts associated with disease in humans and animals, including identification, readers are referred to the Supplementary Readings.

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31

Fungi Causing Subcutaneous Infections

Thomas J. Inzana and G. R. Carter

Included in this chapter are those fungi that produce infections that principally involve the skin, muscle, bone, and connective tissue. *Sporothrix schenckii* and *Histoplasma farciminosum* are dimorphic fungi. A variety of "monomorphic" fungi that are commonly found in the environment cause the diseases chromoblastomycosis, phaeohyphomycosis, and mycetoma. The remaining disease, rhinosporidiosis, is caused by a fungus that has only recently been cultivated in tissue culture.

Sporotrichosis

Etiologic Agent

Sporothrix schenckii

Pathogenicity

Infections in humans and some animals are characterized by the formation of subcutaneous nodules or granulomas that suppurate, ulcerate, drain, and involve the subcutaneous tissues and adjacent lymphatics. The organisms frequently enter the skin through wounds or by traumatic implantation and are spread via the lymphatics. The fungus is widespread in nature, primarily in soil, wood, and other vegetation. The disease is probably seen most commonly in practice in the horse, in which it is an ascending, lymphocutaneous infection involving the leg. In horses sporotrichosis must be differentiated from epizootic lymphangitis, which is similar in clinical presentation. Involvement of bones and visceral organs with fatal termination has been reported

Diagnostic Procedures in Veterinary Bacteriology and Mycology, Fifth Edition

from humans, swine, horses, donkeys, mules, cattle, fowl, camels, and rodents, but dissemination of the disease is probably most common in dogs.

Direct Examination

In pus and tissue, the organism would be present as single-celled, subglobose to ovoid (cigar-shaped) yeasts and may be present within neutrophils (Fig. 31-1). On histopathological examination an asteroid body may be present with basophilic cells 3–5 μm in diameter and with eosinophilic rays up to 10 μm in diameter. The yeast phase is very difficult to demonstrate in smears and wet mounts of pus and tissue scrapings. Periodic acid–Schiff, Calcofluor White, or direct fluorescent antibody staining can be used to improve the sensitivity of direct examination (1).

Isolation Procedures

Pus is taken aseptically from an unopened lesion and inoculated onto brain–heart infusion agar or blood agar and Sabouraud agar, with and

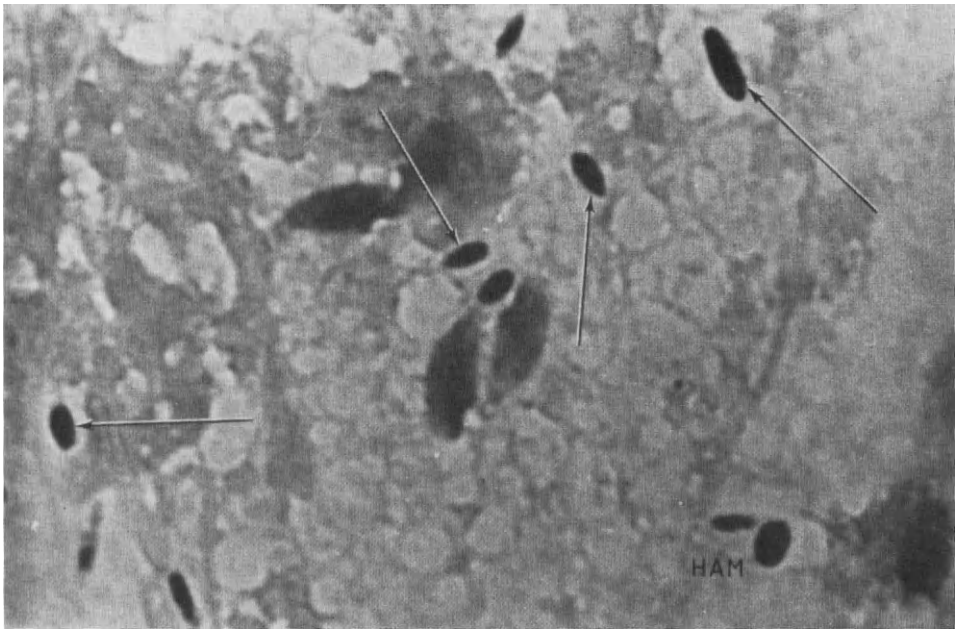

Figure 31-1. *Sporothrix schenckii:* Gram-stained impression smear from experimentally infected mouse testes. The elongated yeast cells are commonly called "cigar bodies." ×2440 (H. A. McAllister).

without cycloheximide. The former two media are incubated at 37°C, and the Sabouraud media at 27–30°C.

Cultural Characteristics

At 27–30°C in 3–5 days the colonies appear early, but the characteristic structures are not evident until the aerial mycelium is produced. Colony appearance may be quite variable. Young colonies are usually cream-colored or white, and later become tan to brown to black. The texture may initially be glabrous or yeastlike and may become leathery, wrinkled, and coarsely tufted. Cornmeal or Czapek's agar may be used to enhance production of conidia.

At 37°C in 3–5 days the colonies that develop are yeastlike, smooth, soft, and cream to tan in color.

Identification

At 27–30°C the mycelium consists of fine, branching septate hyphae that bear pyriform or ovoid microconidia 2–5 μm in diameter. The latter are borne in clusters with floretlike appearance at the end of short conidiophores, or as sessile forms directly on the sides of hyphae (Fig. 31-2). Thick-walled, large chlamydospores may be seen in older cultures. The mold phase may be confused with *Acremonium* species or other fungi.

At 37°C there is little or no mycelium. Colonies are composed of the same elements that occur in pus and tissue. The yeasts are either cigar-shaped (1–4 μm × ~1 μm), spherical, or oval budding cells (2–3 μm) (Fig. 31-3). Some larger pyriform cells (3–5 μm) may be seen.

Laboratory confirmation of *S. schenckii* requires conversion of the mold phase to the yeast phase. This is accomplished by subculturing the mold from Sabouraud agar to brain–heart infusion agar containing 10% blood in 5% CO_2 at 37°C for 3–5 days. Complete conversion of the culture is not required; the demonstration of any yeast cells indicates dimorphism.

Epizootic Lymphangitis

Etiologic Agent

Histoplasma capsulatum var. *farciminosum*: *Histoplasma farciminosum* is not a true *Histoplasma* (2), but is included in this genus because it forms intracellular yeasts in tissues, and the morphology and life cycle are similar to *H. capsulatum*. When cultured, arthroconidia, blastoconidia, and chlamydospores are produced.

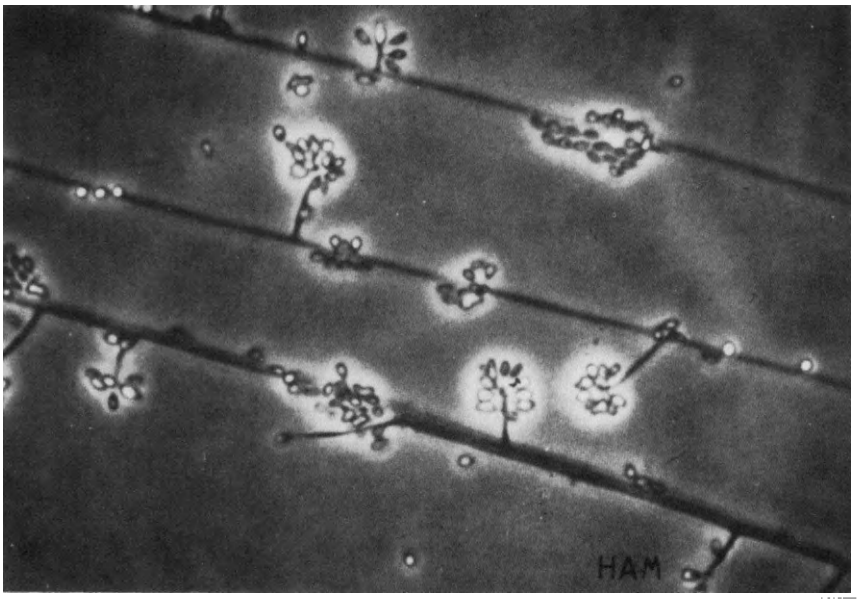

Figure 31-2. *Sporothrix schenckii:* mycelial phase with small conidia in floretlike arrangements. Lactophenol cotton blue preparation from slide culture at 24°C. Darkphase illumination, ×840 (H. A. McAllister).

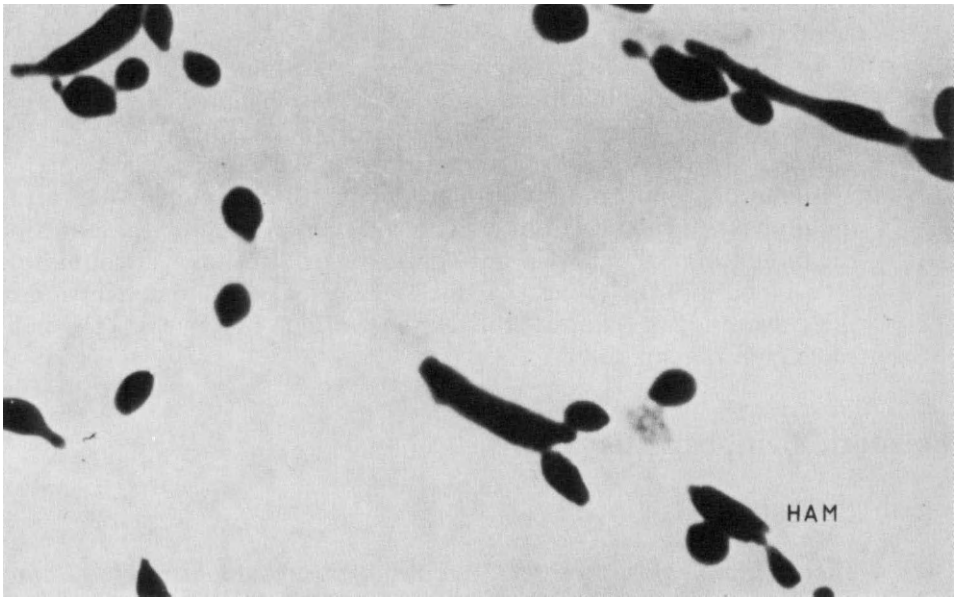

Figure 31-3. *Sporothrix schenckii:* yeast cells from a blood agar plate culture (37°C). Gram stain, ×2250 (H. A. McAllister).

Pathogenicity

Most cases of epizootic lymphangitis are reported from horses (90%), and the remainder from mules and donkeys. The disease is common in Scandinavia, Russia, central and southern Europe, southern Asia, and is particularly prevalent in northern Africa and India. Infections are characterized by nodular, granulomatous, ulcerated lesions of the skin, lymphatics, and subcutaneous tissues. Although the legs and neck are usually affected, the mucous membranes and internal organs may also become involved. A primary pulmonary form of the disease in horses without lymph node involvement has been described (3), as has disseminated disease.

Direct Examination

A diagnosis is usually based upon demonstration of intracellular, yeastlike, globose or pear-shaped, double-contoured cells (2.5–4 μm) in wet mounts of the pus and discharges from lesions. Like other fungi, the organisms are gram-positive. A fluorescent antibody procedure has been used to enhance identification (4).

Isolation Procedures

Material for inoculation should be taken aseptically from unruptured nodules. Cultivation is difficult and slow, and various media have been used. Négre and Boquet (5) used a medium composed of sterile, macerated horse manure, agar, and lymph gland extract. Small colonies were obtained after incubation at 25–30°C for 3–4 weeks. Other workers have had success with Sabouraud agar, which may be used with antibiotics. Bullen (6) obtained primary cultures in Hartley digest agar (with 10% horse serum) incubated at 37°C in 20% CO_2.

Identification

At 37°C in 2–8 weeks the colonies appear as minute gray flakes and are composed of yeastlike cells and some hyphae.

At 25°C in 2–8 weeks the colonies appear as minute gray flakes that later become dry, scaly, and wrinkled like earthworm casts. They are composed of septate mycelia 1–4 μm thick with characteristic, irregular thickening of hyphae. Thick-walled chlamydospores 5–10 μm in diameter, arthroconidia, and blastoconidia may all be present. However, many isolates may not produce conidia.

An enzyme-linked immunosorbent assay has been developed that is specific for *H. farciminosum* (7). In addition, antibodies to H and M antigens can be detected that are variety-specific.

Chromoblastomycosis and Phaeohyphomycosis

These two diseases were previously considered together as one disease, chromomycosis. The infection usually begins in wounds on the feet or legs as ulcerating, nodular lesions and granulomatous lymphadenitis. Chromoblastomycosis is a more specific term referring to traumatic implantation of a dematiaceous fungus into the skin or subcutaneous tissue resulting in verrucous dermatitis. The lesions and satellite lesions remain localized and may persist for many years. The lesions may become large, verricoid, and ulcerated and resemble the head of a cauliflower. Disease typical of chromoblastomycosis has rarely been reported in animals.

Phaeohyphomycosis is a less specific term that refers to a group of infections characterized by dematiaceous, septate hyphae in the tissues, and has been reported from horses (8) and dogs (9). Systemic phaeohyphomycosis may also occur in animals. The same fungi may or may not cause both diseases.

Chromoblastomycosis	Phaeohyphomycosis
Fonsecaea pedrosoi	*Wangiella dermatitidis*
Fonsecaea compacta	*Fonsecaea pedrosoi*
Phialophora verrucosa	*Phialophora verrucosa*
Cladosporium carrionii	*Cladosporium trichoides*
Exophiala jeanselemi	*Exophiala jeanselemi*
	Drechslera species

Direct Examination

Material from granulomatous and ulcerating lesions should be examined in 10% sodium or potassium hydroxide under a coverslip. In pus and granulation tissue the organisms appear as single-celled or clustered, spherical (4–12 μm), thick-walled bodies, with a black or dark brown pigment (sclerotic bodies) (Fig. 31-4). They multiply by cross-wall formation or splitting rather than by budding. These structures are also demonstrable in histopathologic sections. Dematiaceous hyphae (2–6 μm wide) may be seen in skin scrapings, crusts, and aspirates of more superficial specimens. Dematiaceous hyphae, and yeastlike cells or both may be seen in material from lesions of phaeohyphomycosis.

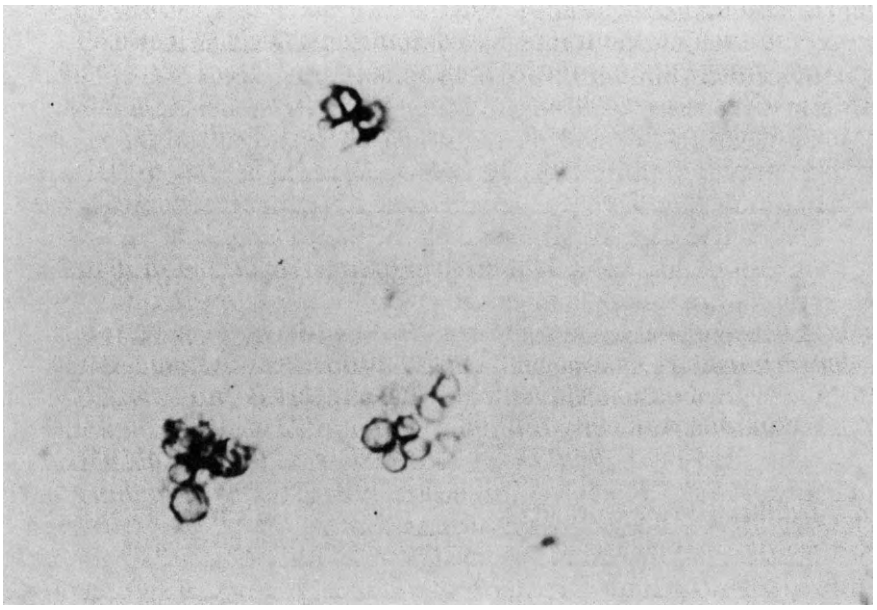

Figure 31-4. *Fonsecaea pedrosoi:* 10% potassium hydroxide preparation of tissue specimen from a case of chromoblastomycosis demonstrating sclerotic brown cells, × ~400. (Courtesy of C. W. Emmons.)

Isolation Procedures

Most of the agents involved in these dematiaceous infections are resistant to cycloheximide and chloramphenicol, although a few are sensitive to these antibiotics. Therefore, specimens should be cultured onto Sabouraud agar with and without antibiotics. The media are incubated at room temperature for at least 6 weeks, as growth and development of conidia is usually slow.

Identification

Identification of these fungi is based on macroscopic and microscopic morphology. Until considerable experience has been acquired in identifying the structures of these dematiaceous fungi, they should be submitted to a reference mycology laboratory for confirmation of genus and species. Detailed descriptions of these fungi are given in standard texts (see Supplementary Readings).

Mycetomas

Mycetomas are localized, deep infections that involve the cutaneous and subcutaneous tissues, fascia, and bone. Following implantation of the agent in a wound, a tumorlike, subcutaneous swelling occurs that eventually ruptures. The lesions consist of abscesses, granulomas, and draining sinuses, occur most frequently on the extremities, but may be found in the nasal mucosa, on the peritoneum, and involve the skin in various locations. A particular characteristic of this disease is the presence of microcolonies and granules in the lesions. The lesions are indistinguishable from those caused by the Actinomycetes. Although many species of fungi have been associated with mycetomas in humans, the disease in animals has been reported infrequently. The cases reported have mostly been from horses, cattle, dogs, and cats (10,11).

Incision of the lesions discloses discrete hyaline or dematiaceous fungal microcolonies embedded in a large mass of granulation tissue (Fig. 31-5). The following species of fungi have been recovered from mycetomas in animals: *Pseudallescheria boydii* (hyaline, rapid-grower); *Curvularia geniculata; Helminthosporium* species; *Cochlio-*

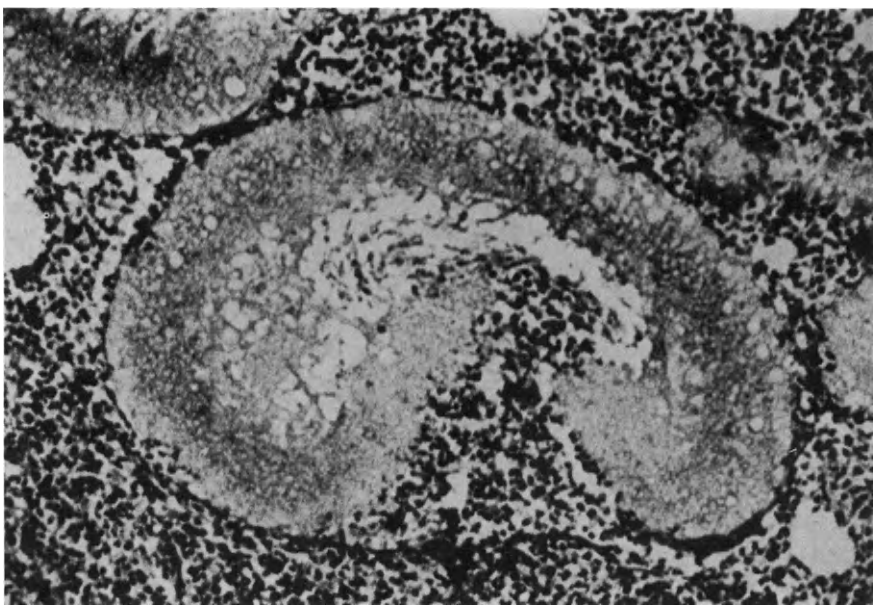

Figure 31-5. *Pseudallescheria boydii:* hematoxylin–eosin-stained section of granulation tissue from a dog with mycetoma. Fungal hyphae are condensed and surrounded by inflammatory cells of the host, × ~250. (Courtesy of Billy C. Ward.)

bolus spicifer; and *Madurella mycetomatis* (dematiaceous and slow growers). *Pseudallescheria boydii* is the most commonly isolated agent of mycetoma and is the sexual stage of this fungus; *Scedosporium apiospermum* is the asexual stage that would most likely be isolated in the laboratory. Additional fungi that have been commonly isolated from humans include *Acremonium* species and *Exophiala jeanselemi.* All of these agents are ubiquitous and common saprophytes.

Direct Examination

Scrapings or biopsy tissues are examined grossly for the characteristic granules, which are small (0.5–3 mm), irregularly shaped, and variously colored. Microscopic examination of these granules reveal broad, intertwined hyphae usually 2–5 μm in width, and swollen cells (15 μm or greater) at the periphery (Fig. 31-6). In contrast, the filaments found in the actinomycotic granules are narrower (0.5–1 μm wide), and may have coccoid and bacillary elements.

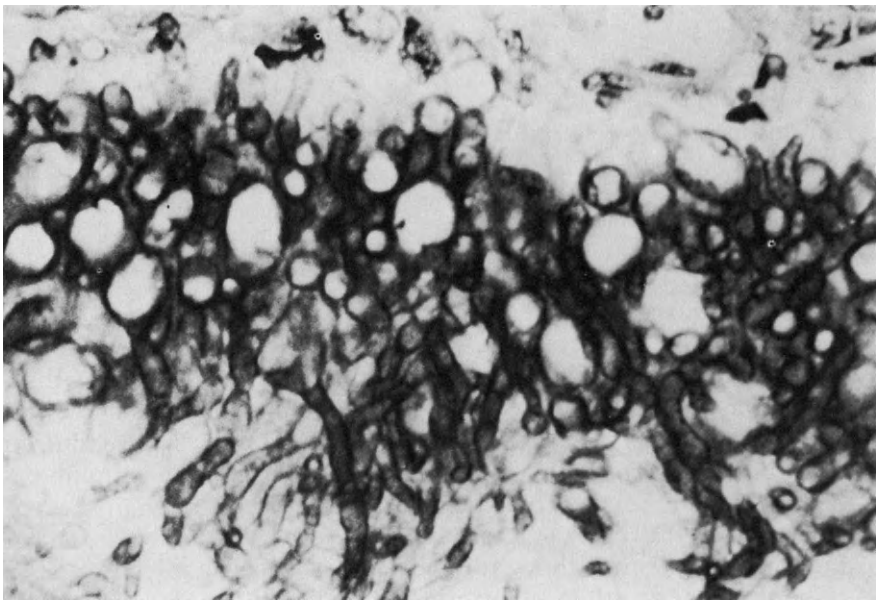

Figure 31-6. *Pseudallescheria boydii:* enlarged view of granulation tissue from Figure 31-5. Area shows the broad, intertwined hyphae (2–5 μm) and swollen cells (≥15 μm), × ~900. (Courtesy of Billy C. Ward.)

Isolation Procedures

Material containing granules may be washed in sterile physiological saline. Contamination can be avoided by obtaining a deep biopsy, or by leaving the specimen overnight in contact with a combination of penicillin (1000 Units/ml) and streptomycin (100 μm/ml), or chloramphenicol (0.05 mg/ml). The fungi of this disease are sensitive to cycloheximide. Several plates of Sabouraud dextrose agar (supplementation with 0.5% yeast extract is helpful) are inoculated and incubated at 25°C. Culture is required to differentiate eumycotic from actinomycotic mycetoma.

Identification

Fungi are identified according to their morphological and physiological characteristics. Detailed descriptions are given in texts (see Supplementary Readings). Cultures may need to be submitted to a reference laboratory for final identification.

Rhinosporidiosis

Etiologic Agent

Rhinosporidium seeberi.

Pathogenicity

Rhinosporidiosis is a disease of cattle, horses, mules, dogs (12), and humans and is characterized by large polyps, tumors, or wartlike lesions on the nasal and ocular mucous membranes. These growths are highly vascularized, sessile or pedunculated and are considered to result from the growth of the spherules and the tissue response to *Rhinosporidium seeberi.* It is of interest that 90% of infections involve the nose of male animals. That the disease may occur in avian species is suggested by the report of a case in a wood duck (13). The natural habitat of the organism is thought to be aqueous because infections appear to be associated with stagnant water. Inhalation of contaminated dust may also be a mode of transmission. The disease occurs mainly in tropical areas and is rare in North America.

Direct Examination

Wet mounts are prepared from tissue taken from the polyps or from nasal discharge. The large sporangia (up to 200–300 μm in diameter) contain thousands of endospores and are readily distinguishable from the smaller spherules of *Coccidioides immitis*.

The sporangia develop from small globose cells, 6–8 μm in diameter. They continue to grow, and when they reach approximately 100 μm, they become sporangia and their contents are transformed into thousands of spores. The sporangia may reach a size of 200–300 μm before the spores are released. The typical sporangia can be readily seen in stained sections of the polyps (Fig. 31-7).

Isolation Procedures

This organism has recently been cultures *in vitro* in a human rectal tumor cell line (14). However, routine culture of this agent in the clinical laboratory is not yet possible.

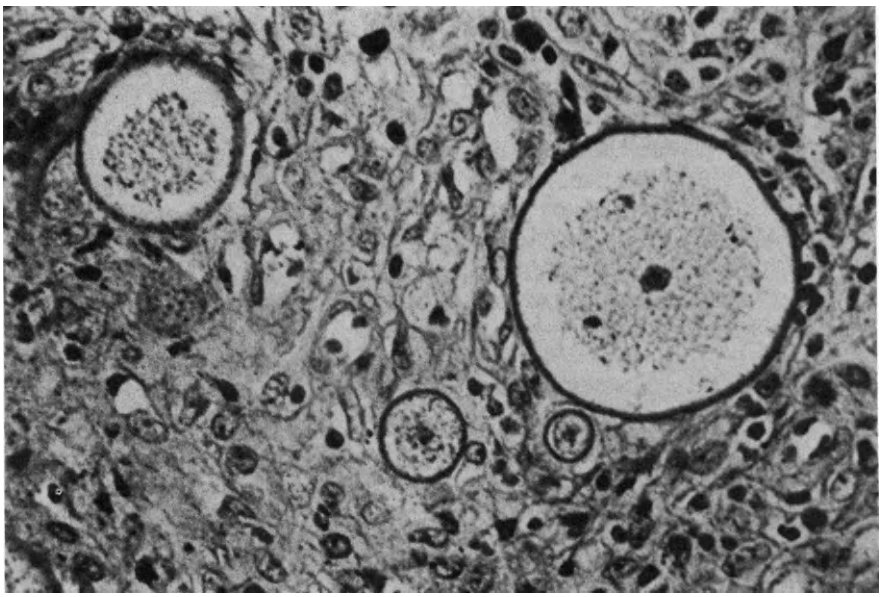

Figure 31-7. *Rhinosporidium seeberi:* hematoxylin–eosin stain of a nasal polyp tissue section. Several sporangia of various sizes are surrounded by host inflammatory cells. Spores are visible within the sporangia, × ~450. (Courtesy of Billy C. Ward.)

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32

Fungi Causing Systemic or Deep Infections

Thomas J. Inzana and G. R. Carter

Included in this section are a variety of fungi that cause infections that may affect the skin, subcutaneous tissues, bone, and one or several organ systems. The most widespread of these infections is histoplasmosis, a disease of the reticuloendothelial system.

Zygomycosis

Zygomycosis includes diseases of the respiratory and gastrointestinal tracts; agents are found in the orders Mucorales and Entomophthorales.

Order Mucorales

Genera in the order Mucorales that have been implicated as causes of animal infections include *Rhizomucor, Absidia, Rhizopus, Mortierella,* and possibly *Mucor.* These organisms occur widely in nature and are characterized by large, aseptate hyphae that form sporangia. The disease results from ingestion or inhalation of a large number of spores. Several species of different genera have been recovered from zygomycosis of horses and livestock. The disease is not contagious.

Most infections of pigs and cattle are caused by *Absidia corymbifera. Mortierella wolfii* is an important cause of abortion (mycotic placentitis) in cattle, which can occasionally be followed in 24–48 hr by acute pneumonia and death (1). *Absidia corymbifera,* occasionally cited as a cause of bovine abortion, was found to be a common contaminant of placentas submitted for microbiological examination (2).

Diagnostic Procedures in Veterinary Bacteriology and Mycology, Fifth Edition

Readers are referred to Rippon (3) for additional information on zygomycosis.

Pathogenicity

Other than exposure to a large number of spores, predisposing factors such as malnutrition, debilitating disease, or overcrowding are usually required for disease to occur. Lesions are granulomatous or ulcerative; the former type is more often generalized. The disease may occur in two forms. The most common form affects the lymph nodes of the respiratory and alimentary tracts. Lymph nodes enlarge with caseating necrosis. The internal organs may also become involved. Cattle and pigs are most frequently affected by this form. In cattle, abortion may also occur. In the second form, ulcerating, granulomatous, and caseating necrosis of the intestinal tract occurs. This form is most severe in young animals and is first recognized by scouring. In acute, fulminating infections the fungi are often found invading blood vessels and causing a necrotizing vasculitis with thrombosis and hemorrhage.

Pigs: Lesions are found in mediastinal and submandibular lymph nodes; embolic "tumors" are seen in the liver and lungs. Zygomycetes are found in gastric ulcers.

Cattle: Lesions are found in the bronchial, mesenteric, and mediastinal lymph nodes and in nasal and abomasal ulcers; abortions are attributed to these fungi.

Horses and other animals: There are several reports of zygomycosis in horses. Infections have also been reported in dogs, mink, guinea pigs, and the mouse. The disease occurs occasionally in fowl and exotic birds.

Direct Examination

Histological examination of tissue sections reveals broad, nonseptate, branching hyphae (10–15 μm in diameter) by hematoxylin–eosin, silver, or periodic acid–schiff stain, or in potassium hydroxide-mounted preparations. Sporangia may sometimes be seen in lung sections.

Isolation Procedures

Blood agar and Sabouraud dextrose agar without cycloheximide are inoculated and incubated at 37°C and 25°C, respectively. The organisms may be difficult to isolate, and diagnosis is usually not made until necropsy. Tissue sections should not be homogenized or processed, but rather, streaked onto and left on the medium. Because these agents are ubiquitous in the environment, repeated isolation of the agent combined with observation of hyphae in the tissue is required for confirming diagnosis.

The zygomycetes are characterized by nonseptate hyphae and the formation of spores by cleavage within a sporangium. Most species are saprophytic. All zygomycetes grow rapidly at both 25 and 37°C on blood and Sabouraud agar. Their growth is inhibited by cycloheximide. Generally speaking, those species capable of causing disease have a greater capacity for growth at 37°C than the strictly saprophytic strains.

Rhizomucor and *Mucor*

Animal infections with *M. circinelloides* have been reported rarely (4). *Mucor pusillus* has been reclassified as *Rhizomucor pusillus. Rhizomucor pusillus* and *R. miehei* are the only recognized pathogenic species of this genus, and both have been reported from animals (3). These fungi produce a thick, gray mycelium, with few or no rhizoids. Sporangiophores are short (less than 3 mm) and bear terminal, black, spherical sporangia. These rapidly growing fungi will fill the petri dish in 4–5 days with an abundant growth of floccose aerial mycelium. It is white at first, then turns a dark brown.

Identification

Structures characteristic of the genus are shown in Figure 32-1. It may be necessary to submit the culture to a reference laboratory for species identification.

Absidia

Absidia corymbifera is the only pathogenic species currently recognized in this genus, and is also the most common agent of zygomycosis from animals. This organism has globose to oval spores 2–4 μm in diameter. The fungus grows rapidly producing a floccose, olive-gray colony that resembles *Rhizopus.* Sporangiophores are long (450 μm) and branch repeatedly. Rhizoids form at swollen areas along the stolon where the stolons contact the medium. The sporangiophores are produced at intervals along the stolon and bear pyriform terminal sporangia.

Identification

Structural elements resemble those of *Rhizopus* spp. Some of the morphological elements that characterize the genus are shown in Figure 32-2. Species identification is best accomplished by a reference laboratory.

Rhizopus

Three species have been reported as pathogenic for animals: *R. arrhizus, R. microsporus,* and *R. rhizopodoformis.* Sporangiophores are

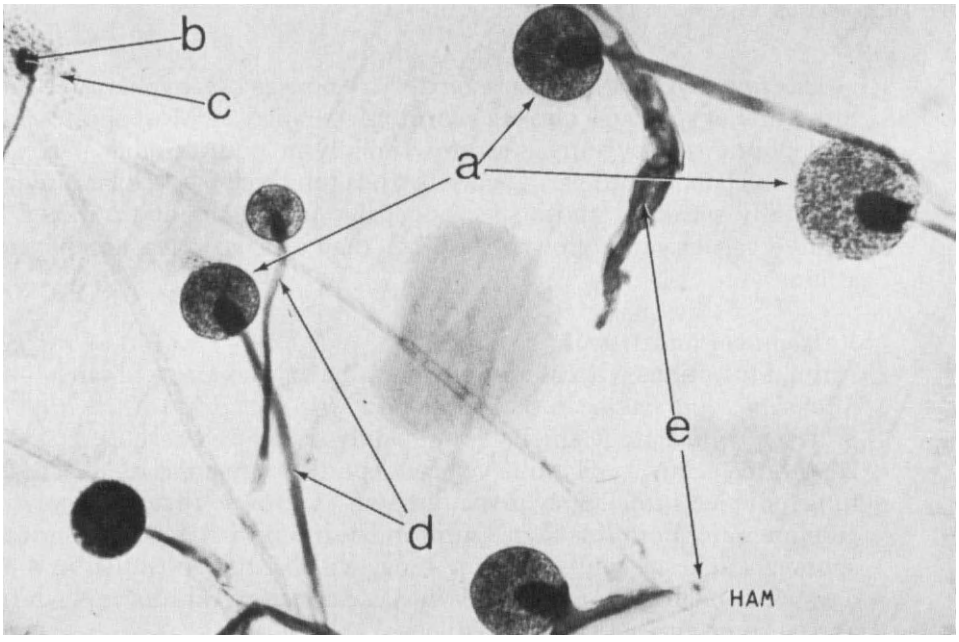

Figure 32-1. *Rhizomucor* sp.: *a*, Mature, spore-filled sporangia; *b*, columella; *c*, spores from ruptured sporangium; *d*, sporangiophores; *e*, nonseptate hyphae. Tape mount, lactophenol cotton blue, ×220 (H. A. McAllister).

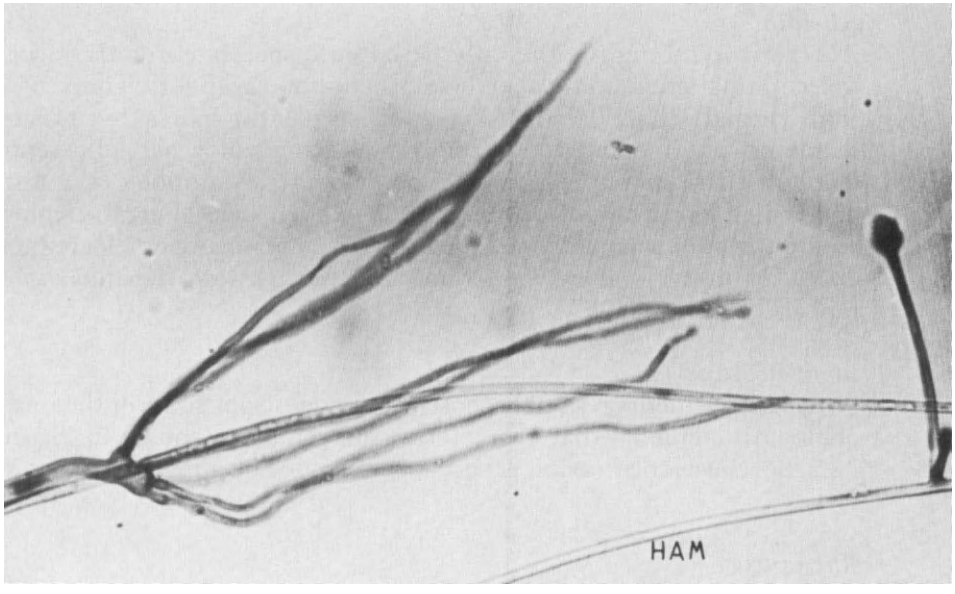

Figure 32-2. *Absidia* sp. showing rhizoids. Slide culture, lactophenol cotton blue, ×250 (H. A. McAllister).

brown and arise singly or in groups from nodes above the rhizoids. Stolons are characteristic of this genus, but the sporangiophores arise only at points of contact with the medium. Rhizoids can usually be seen at the base of the sporangiophores in mycelial mounts (Fig. 32-3).

This fast-growing group will fill the Petri dish in 5 days with an aerial mycelium that is dense, coarse, and woolly. At first their growth is white, then turns brown to black. The sporangia are dark, with colored or hyaline spores.

Mortierella

A characteristic of this genus is the lack of a columella at the junction of the sporangium and sporangiophore. *Mortierella wolfii* and *M. polycephala* have been isolated from animals (3). These fungi grow on blood agar and Sabouraud dextrose agar at 25 and 37°C. However, they do not compete well if there is heavy bacterial growth on blood agar or if grown on mycological agar with fungal contaminants. The colonies on Sabouraud and blood agar are white to grey, velvetlike, dense, and characteristically lobulated (Fig. 32-4), but sterile. The hyphae are hyaline and 2.5–5.0 μm in width. Sporangia are produced on hay and potato–carrot agar. For definitive identification based on the morphology of sporangia and spores, readers are referred to di Menna *et al.* (5) and the Supplementary Readings.

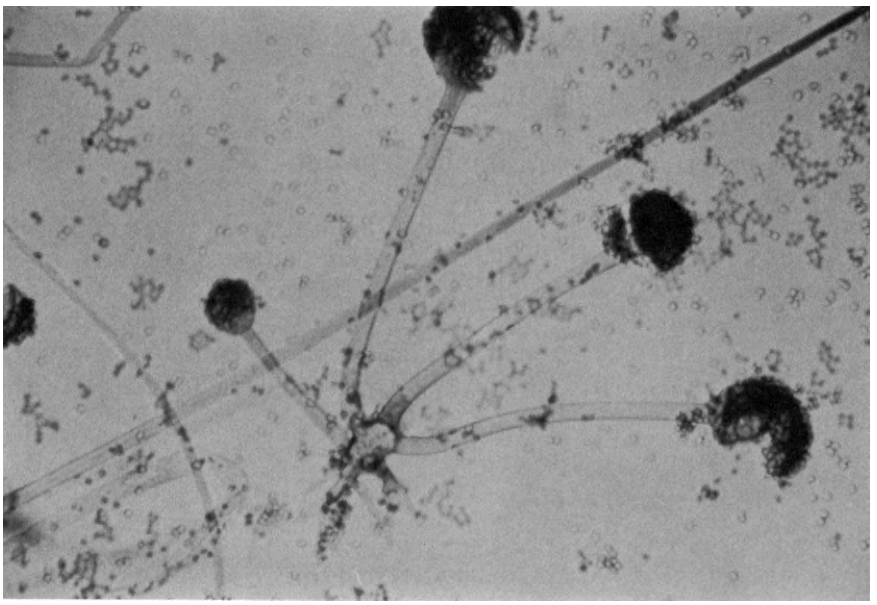

Figure 32-3. *Rhizopus arrhizus:* hyphae are nonseptate and rhizoids occur at the base of the sporangiophores. Several sporangiophores may develop from the same base. Sporangia easily break and release sporangiospores, × ~450. (Courtesy of C. W. Emmons.)

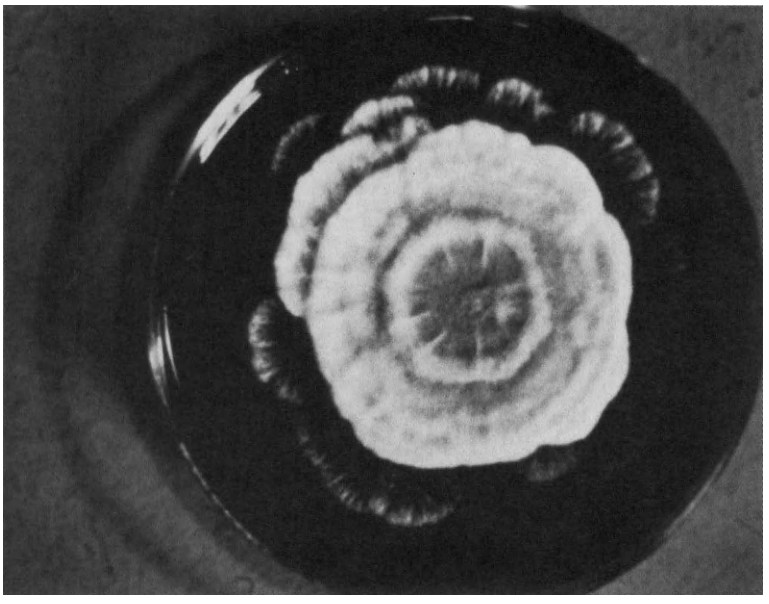

Figure 32-4. *Mortierella wolfii* colony on blood agar, after 72 hr incubation at 37°C.

Order Entomophthorales

The pathogenic fungi in this order cause a zygomycosis that has been referred to as entomophthoromycosis and include the genera *Basidiobolus* and *Conidiobolus*. Animal infections have been caused by *B. ranarum*, *C. coronatus*, and *C. lamprauges* (3). These fungi differ from the Mucorales in that young colonies are flat and waxy, and become white and fuzzy with age. The hyphae are septate, particularly in older cultures, and either conidia or sporangia are produced. *Basidiobolus* produces a unicellular sporangium, whereas *Conidiobolus* produces single-celled spores that appear more like large conidia. Isolates should be sent to a reference laboratory for species identification.

Pythiosis

Pythiosis is a mycosis of horses that is caused by *Pythium insidiosum* (6,7). This agent was previously referred to as *Hyphomyces destruens*, and the disease has been called Florida horse leech because it has been confused with infections due to a nematode larvae of *Habronema*. The true identity of *P. insidiosum* has been classified by successful sporula-

tion of the fungus by de Cock *et al.* (6) using water cultures, and confirmed with antigenic studies by Mendoza *et al.* (7). *Pythium insidiosum* is a Oömycete, rather than a zygomycete. Cultures of the fungus have broad (5–10 μm) hyphae that are branching and sparsely septate.

The infection usually starts at the site of a cut or wound on the hoof or leg. The site may become necrotic with the formation of granulomas and draining tracts. On histological examination masses of hyphae are seen mixed with a variety of inflammatory cells, and form necrotic masses called *kunkers*. The disease is chronic and progressive, but fatal infections have not been reported. A similar disease by the same agent has also been reported in dogs and cattle (3). Isolation and identification procedures for *P. insidiosum* would be similar to those used for the zygomycetes.

Aspergillosis

The most prevalent pathogenic species is *Aspergillus fumigatus*. Other potentially pathogenic species are *A. flavus*, *A. nidulans*, possibly *A. niger*, and *A. terreus*. Isolates of *A. flavus* and *A. parasiticus* can cause aflatoxicosis in swine, cattle, and poultry, which results from ingestion of aflatoxins produced in moldy grain. Many strains of *Aspergillus* occur as contaminants, and therefore, repeated isolation or histological confirmation is required for diagnosis.

Pathogenicity

Fowl: Although the disease is most often reported from domestic fowl and zoo penguins, all wild birds are susceptible. The disease may occur in several forms: (A) a diffuse infection of the air sacs, (B) a diffuse pneumonic form, and (C) a nodular form involving the lungs. The disease may be limited to one organ or become generalized. Brooder pneumonia is an important respiratory form of the disease in chicks and poults.

Cattle: Invasive aspergillosis and mastitis may occur. The most common infection in cattle, however, is mycotic abortion, in which the placenta is primarily infected.

Horses: Infections involving the skin and mucous membranes have been reported, as well as rare cases of abortion and invasive disease. *Aspergillus* spp. appear to be the sole cause of guttural pouch mycosis, a serious disease of stabled horses.

Dogs: Epidemics of aspergillosis have occurred in dogs, resulting in invasion and thrombosis of the vessels of all tissues. Infections of the nasal chambers may also occur.

Pigs, sheep, and other animals: Invasive infections occur occasionally in many animal species.

Humans: Infections are primarily seen in immunocompromised patients or patients receiving antibiotic or antiinflammatory drugs. The disease may be primarily allergic, colonizing (aspergilloma), or invasive. Invasive disease may infect the lungs, skin, nasal sinuses, external ear, bronchi, bones, and meninges.

Direct Examination

Small pieces of tissue or deep scrapings are examined in 10% potassium hydroxide. Calcofluor White (a nonspecific fluorescent stain) can be used to enhance visualization. Short or long pieces of thick, septate, dichotomous hyphae that branch at 45° angles are characteristic. Irregular dilation of hyphae may be seen that resemble yeast cells in cross section. The typical conidial head is only seen in the lung and air sacs where there is access to oxygen.

Isolation Procedures

Material is inoculated onto blood agar or brain–heart infusion agar and onto Sabouraud dextrose agar and incubated at 37 and 25°C, respectively. Streptomycin and penicillin, or gentamicin, can be added to these media to suppress bacterial contaminants.

Cultural Characteristics

Growth is rapid (1–3 days), and colonies are flat and white at first, but later turn grey-green to dark green, folded, and velvety. The morphology is similar at 25 and 37°C.

Identification

The identifying microscopic structure seen at both temperatures is the conidiophore with a large, terminal, flask-shaped (*A. fumigatus*) or globose (*A. flavus*) vesicle. A single row of phialides (flask-shaped hyphae that produce conidia) may be present (*A. fumigatus*), or primary and secondary phialides may be produced from the vesicle (*A. flavus*). Conidia are borne in rows from the terminal phialides (Figs. 32-5 and 32-6).

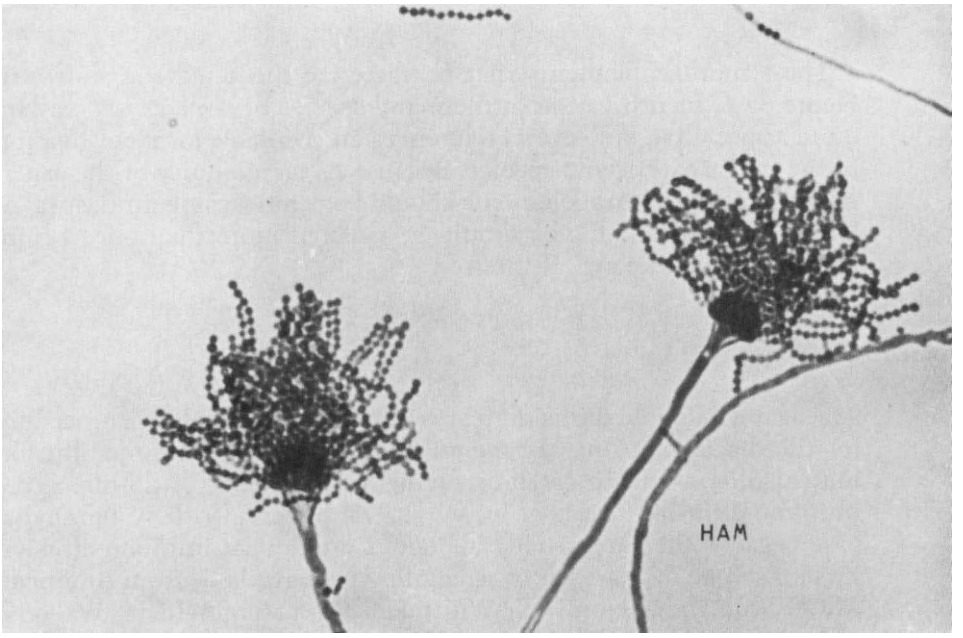

Figure 32-5. *Aspergillus* sp.: slide culture, lactophenol cotton blue, ×445 (H. A. McAllister).

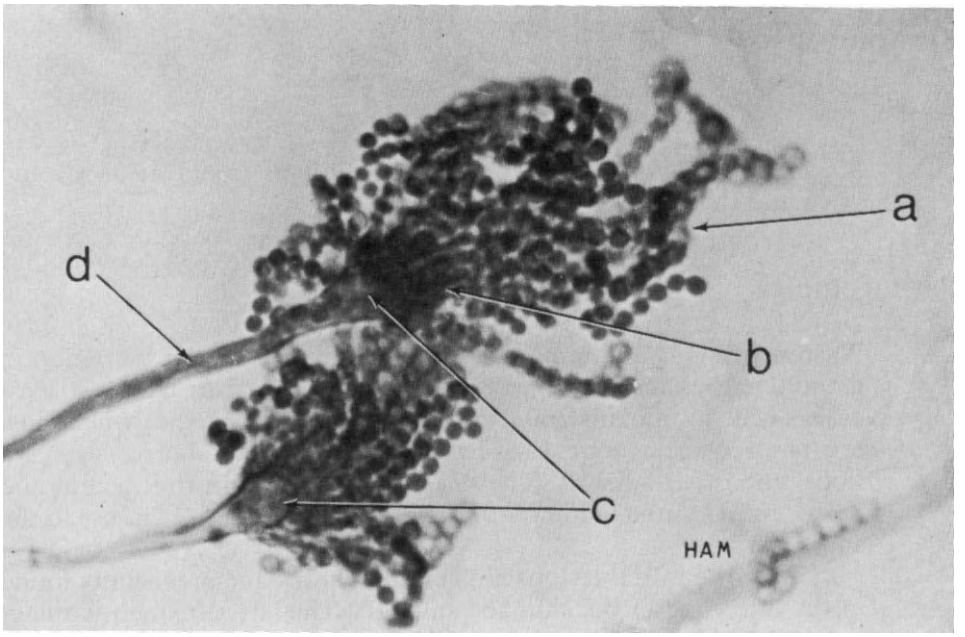

Figure 32-6. *Aspergillus* sp.: *a*, chains of conidia; *b*, sterigmata; *c*, vesicle; *d*, conidophore. Slide culture, lactophenol cotton blue, ×975 (H. A. McAllister).

The structural elements that characterize the genus are shown in Figure 32-6. Identification of the many species of *Aspergillus* may require a specialist, but several references are available for identification of the major pathogenic species. Because of the ubiquity of the aspergilli, typical structural elements should be demonstrable in tissues, or large numbers of colonies should be isolated more than once before pathogenic significance is inferred.

Serology

The agar gel double diffusion test is claimed to be a reliable procedure for the diagnosis of nasal aspergillosis in dogs (8). Immunodiffusion may also be useful in cases involving aspergillomas. In humans, the immuno-diffusion test is of little value in patients with systemic disease because they are usually anergic. Commercial immunodiffusion kits for serologic diagnosis of aspergillosis are available from American Micro Scan, Lexington, KY; Whittaker M. A. Bioproducts, Walkersville, MD; Meridian Diagnostic, Cincinnati, OH; Immuno-Mycologies, Inc., Norman, OK; and Nolan Biological Labs, Atlanta, GA. The comparative sensitivity and specificity of these kits to the Center for Disease Control Fungal ID Reagents has been reported (9).

Blastomycosis

Cause

Blastomyces dermatitidis, a dimorphic fungus (mold at 25°C, and yeast at 37°C)

Pathogenicity

Blastomycosis is a chronic disease characterized by the formation of granulomatous and suppurating nodules with caseating necrosis. Most cases occur in humans and dogs and often occur together when clusters of cases occur. Rare cases have been described in horses, cats, sea lions, and other animals. The disease is endemic in the eastern and midwestern United States, with the highest incidence of disease in the southeast.

In dogs, the initial lesions are granulomatous and are usually found in the lungs and/or the skin and subcutis. The latter frequently ulcerate. In active disease dissemination to the skin, bone, and other body sites frequently occurs. The immune competence of the host determines whether the disease becomes disseminated, often with a fatal

outcome, or takes a more chronic course. In dogs, blastomycosis commonly infects the lungs, skin, bone, urogenital tract, and eyes. In most instances the primary lung infection is subclinical. However, unlike most other fungal infections, once the infection is established the disease is often chronic and progressive and does not resolve without treatment. In humans, infections are more likely to occur in normal hosts than other fungal infections.

Direct Examination

The finding of typical lesions and of organisms in tissue sections or clinical specimens supports the identification. The large, spherical, thick-walled yeasts (5–20 μm in diameter) are readily seen in potassium hydroxide or Calcofluor White wet mounts and have a refractile wall (Fig. 32-7). The yeast can also be demonstrated in tissue sections with histopathological stains, in which the wall remains colorless (Fig. 32-8). A single bud is frequently seen connected to the larger mother cell by a wide base (4–5 μm).

Isolation Procedures

Blood or brain–heart infusion agar and Sabouraud dextrose agar are inoculated. This agent is somewhat sensitive to cycloheximide (especially the yeast phase) and therefore, media with and without antibiotics

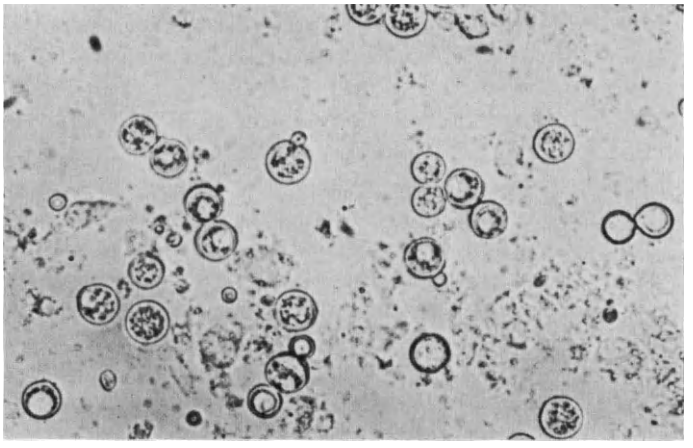

Figure 32-7. Wet mount of exudate from lung of a dog with *Blastomyces dermatitidis* infection. Single and budding spheres with double-contoured, refractile walls, ×410 (F. K. Ramsey and G. R. Carter).

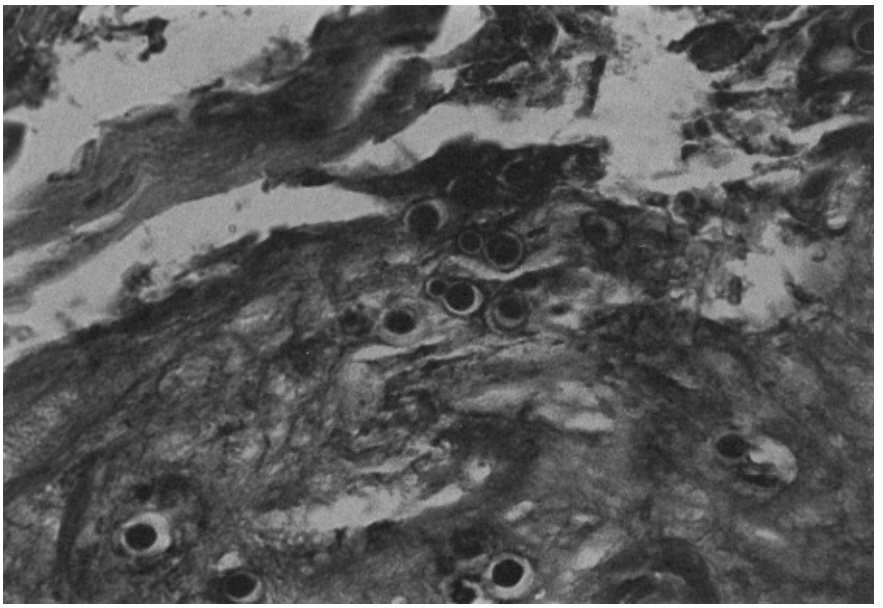

Figure 32-8. *Blastomyces dermatitidis:* periodic acid–Schiff stain of skin from a dog with blastomycosis. Note that the wall of the yeast cells does not stain, ×160.

should be used. The mold phase is isolated after incubation at 25°C, while the yeast phase is isolated at 37°C.

Cultural Characteristics

Growth of some *B. dermatitidis* isolates may begin as early as a few days, while others may require 2 months to grow. For extended periods of incubation, plates should be sealed to prevent drying out. Growth may become apparent on blood agar at 37°C sooner than on other media at 25°C. At 25°C moist, grayish, yeastlike colonies form that produce white, cottony mycelia. As the growth ages, the colonies become tan to dark brown.

At 37°C on blood agar the colonies are more glabrous, feltlike, and folded. "Prickles" radiating from the colonies may be apparent early. With maturation the colonies become creamy, waxy, and wrinkled; they are cream to tan in color.

Identification

Identification is based on cultural and morphological characteristics. At 25°C the conidia are produced by lateral conidiophores, or terminally on septate hyphal branches close to the point of septation (Fig.

32-9). The conidia are small, oval (2–3 μm) or pyriform (3–5 μm). The conidia are similar in appearance to those of *P. boydii*, but dumbbell-shaped or double conidia may be a distinguishing characteristic. Older cultures form chlamydospores (7–15 μm) with thickened walls. Confirmation of the agent requires conversion of the mold to the yeast phase, which can be accomplished by transferring the mold to brain–heart infusion agar containing 10% blood and incubating at 37°C. Any amount of conversion to the yeast phase is indicative of dimorphism.

At 37°C thick-walled, budding yeast cells (7–15 μm in diameter), similar to those seen in tissue sections and exudate (Fig. 32-10), are formed.

Serology

Most serologic tests cross-react with the other dimorphic fungi and therefore have limited usefulness. Skin testing is not useful for diagnosis or prognosis of blastomycosis. An immunodiffusion test and an ELISA test that use the specific "A" antigen has been reported to be reliable in human patients (10). Data is not available on the application of these tests in diagnosing animal infections. Counterimmunoelectrophoresis

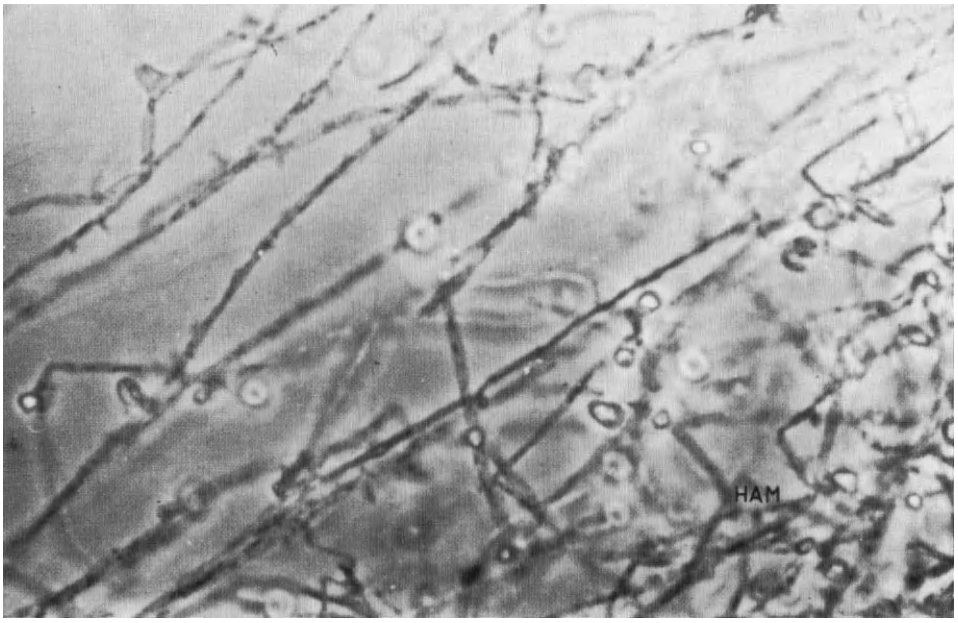

Figure 32-9. *Blastomyces dermatitidis:* mycelial mass showing hyphae and conidia from a Sabouraud dextrose agar slide culture (25°C). Darkphase illumination, lactophenol cotton blue, ×975 (H. A. McAllister).

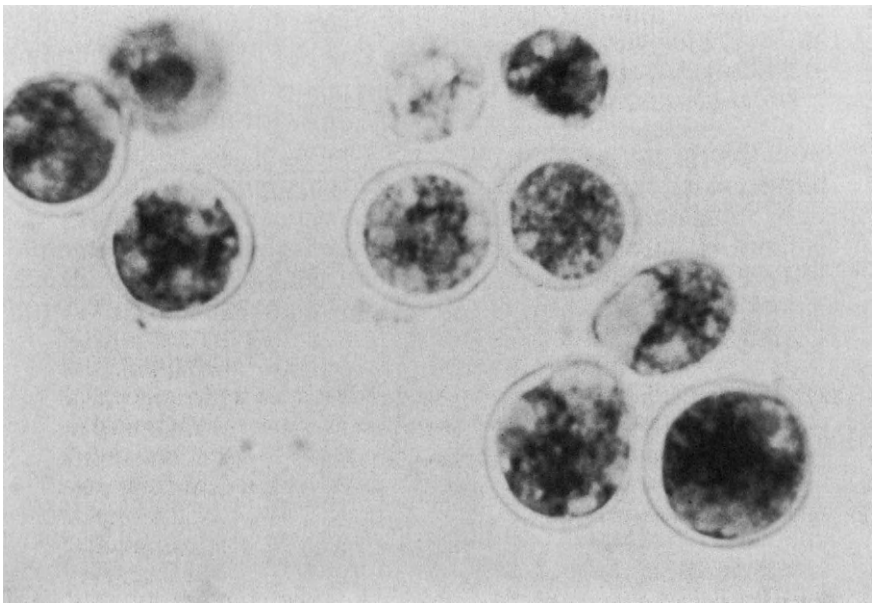

Figure 32-10. *Blastomyces dermatitidis:* yeast stage of the fungus cultured for 2 days on blood agar at 37°C. Note the broad base of the bud and the double-refractile cell wall, × ~1000. (Courtesy of C. W. Emmons.)

using a commercially available antigen has been reported to be reliable for diagnosis of blastomycosis, as well as histoplasmosis, in dogs (11). The loss of antibody during disease may indicate a poor prognosis in dogs. Commercial immunodiffusion kits are available for reaction with the "A" band from the same companies providing kits for *Aspergillus* serology. A fluorescent antibody reagent can be useful for enhancing identification of the yeast in tissue.

Histoplasmosis

Cause

Histoplasma capsulatum, a dimorphic fungus: Histoplasmosis is a granulomatous disease with primary infection of the respiratory tract, which may disseminate to other organ systems via the reticuloendothelial system. This fungus has a worldwide distribution, but is more heavily concentrated in the soil of certain geographic areas. The areas of heaviest concentration in the United States are the northeast, midwest, and south central states. The organism grows best in soils with a high nitrogen content and therefore is found in areas that also have

the highest concentrations of birds and bats. *Histoplasmosis capsulatum* has been readily isolated from soils in bat caves, bird roosts, chicken houses, and silos inhabited by pigeons or other birds.

Pathogenicity

About 95% of infections due to *H. capsulatum* are asymptomatic. Infection usually occurs by inhalation of the conidia, although ingestion of the spores may also result in infection. In most cases the fungus is either killed or local granulomas form and calcify. In rare cases dissemination with fulminant or chronic illness results. Host immunity and the number of conidia inhaled determine which form the infection will take. The disease may range from an acute to chronic or disseminated form. Ulcerations and tuberculosis-type lesions may occur in many organ systems. The primary lesions may be pulmonary or intestinal. Hepatosplenomegaly and lymphadenopathy are commonly associated with the disease. Nodules on the tongue, ocular involvement, and abortion have also been reported. *Histoplasma capsulatum* is a facultative intracellular parasite of macrophages of the reticuloendothelial system, which may actually assist in disseminating the agent. Humans and dogs are particularly susceptible, but the disease has also been reported in cats, cattle, sheep, swine, horses, and wild animals.

Direct Examination

Because *H. capsulatum* is small and rarely found extracellularly, it may be difficult to demonstrate in clinical materials. Smears are made from scrapings of ulcers and from biopsy of lymph nodes and other tissues. Alcohol-fixed tissues are stained by the Giemsa or Wright methods and examined under the oil immersion objective. The organisms occur intracellularly (usually in monocytic cells) as small, round or oval, budding yeasts 2–4 μm diameter. A clear halo is seen around the darker staining central material (Fig. 32-11).

Isolation Procedures

Although *H. capsulatum* may grow on Sabouraud dextrose agar containing chloramphenicol and cycloheximide, blood agar and Sabouraud agar without antibiotics is recommended (3). If a selective medium is needed, a yeast extract agar containing ammonium hydroxide is recommended (12). The preferred incubation temperature is 25°C, because growth is not always obtained at 37°C.

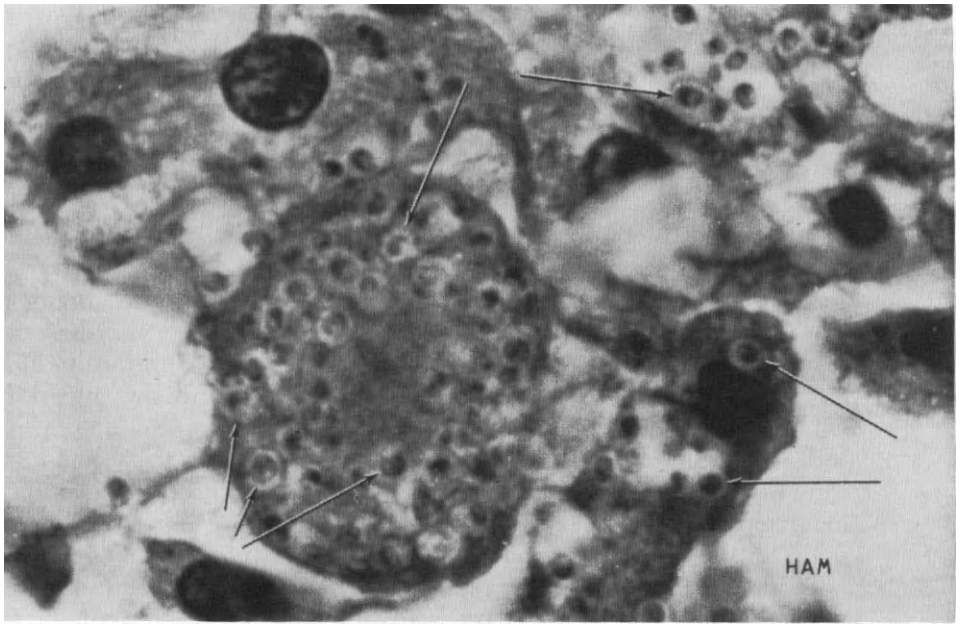

Figure 32-11. *Histoplasma capsulatum:* small intracellular yeastlike cells in a monocyte. Tissue section, hematoxylin–eosin, ×2250 (H. A. McAllister).

Cultural Characteristics

The mold phase is indistinguishable from that of *B. dermatitidis.* Growth may require 2–4 weeks of incubation, and plates should be held for up to 12 weeks. On Sabouraud agar the colonies are cottony, white to cream at first, later becoming tan to brown. On blood agar colonies are more glabrous and usually pink to dark red, becoming white to brown as the mycelium forms.

Identification

At 25°C the mold phase is produced; it may be white or brown. The same culture may produce both morphologic types. Two types of conidia are borne from the separate hyphae on narrow conidiophores: (A) small, smooth, round to pyriform microconidia, either on short lateral branches or attached directly by the base, and (B) large macroconidia (7–18 μm in diameter), that are round, thick-walled, and covered with knoblike or tuberculate projections (Fig. 32-12). Chlamydospores may also form in older cultures. Conversion from the mold to the yeast

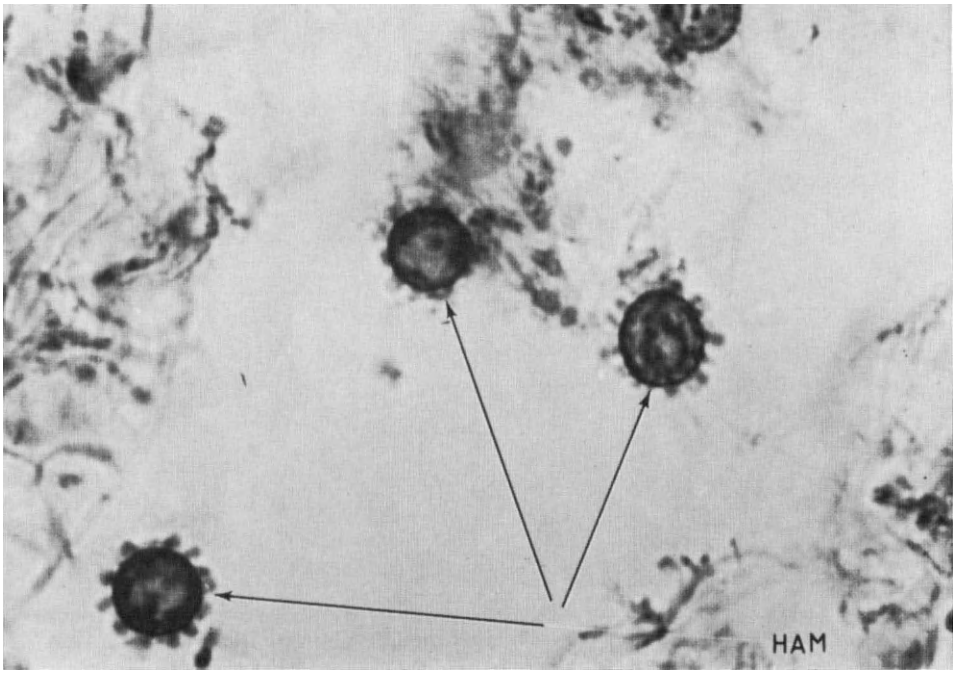

Figure 32-12. *Histoplasma capsulatum:* mycelial phase showing tuberculate macroconidia. Sabouraud dextrose agar slide culture (24°C). Lactophenol cotton blue, ×1140 (H. A. McAllister).

phase is required to confirm identification and can be done by transfer of the mold to blood agar and incubation at 37°C.

At 37°C the colonies are initially glabrous and tenacious and consist of hyphae in the process of converting to yeast cells. Following repeated transfers the yeast becomes white and smooth. Microscopically, oval yeasts 3–4 μm are seen with buds that have a narrow neck (Fig. 32-13).

Mouse Inoculation

The organism can be recovered from grossly contaminated specimens by mouse inoculation. Material is ground in physiological saline containing chloramphenicol (0.05 mg/ml) by means of a tissue grinder, or in a mortar and pestle with sterile alundum. Each of four mice are inoculated intraperitoneally with graded doses, such as 0.2 ml, 0.5 ml, and 1.0 ml. After 2–4 weeks the mice are killed; material from the spleen and liver is inoculated onto media as described above and examined histopathologically.

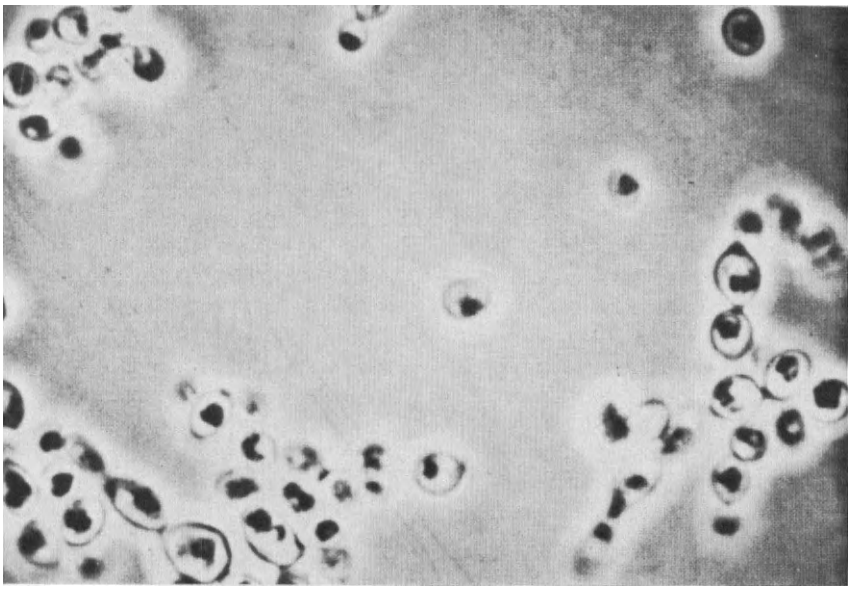

Figure 32-13. *Histoplasma capsulatum:* yeast phase showing yeastlike forms from a culture at 37°C. Lactophenol cotton blue wet mount, darkphase illumination, × 1900.

The mycelial phase may also be converted to the yeast phase by mouse inoculation. Mycelial elements are suspended in gastric mucin (see Appendix C) prior to inoculation.

Serology

The skin test becomes positive following exposure to the organism and probably remains positive for life. Therefore, the skin test is of little use for diagnosing active disease, except that the lack of reactivity may indicate anergy. The complement fixation test is useful for diagnosis of specific antibodies soon after infection, and titers disappear after 9 months. The immunodiffusion test can be used to demonstrate the presence of antibody to the H and M antigens, which indicates active or past infection, respectively. Immunodiffusion tests are commercially available in the same kits as for blastomycosis and aspergillosis. The combination of complement fixation and immunodiffusion provides the most reliable serologic information concerning the diagnosis and prognosis of histoplasmosis. Latex beads coated with histoplasmin can detect antibody early in disease and serve as a useful screening test.

Coccidioidomycosis

Etiologic Agent

Coccidioides immitis, a dimorphic fungus: In most cases infection results in subclinical, or mild to severe, upper respiratory infection that resolves naturally. In rare cases infection may result in an acute or chronic disease that disseminates with a fatal outcome. *Coccidioides immitis* may be the most infectious fungal pathogen, and the disease is not uncommon in laboratory workers who isolate the agent. The fungus occurs widely in the soil of certain arid areas of the southwestern United States, and in Central and South America.

Pathogenicity

Infection results from the inhalation of arthrospores from the soil. Dust storms increase the incidence of infections. Coccidioidomycosis has been reported from humans, dogs, horses, cattle, swine, sheep, cats, sea otters, and wild captive animals. The disease in most animals is minimal and in most cases is localized. The gross lesions in cattle resemble tuberculosis but are usually seen in the bronchial and mediastinal lymph nodes and rarely in the lungs. Disseminated disease occurs regularly only in primates and dogs, in which lesions have been found in lung, brain, liver, spleen, and kidney. Dissemination depends on host resistance and the number of arthrospores to which the animal is exposed. Systemic disease involves the meninges, joints, bones, and subcutaneous tissues with the formation of burrowing abscesses. A suppurating and/or granulomatous reaction in the lesions is characteristic.

Direct Examination

In unstained 10% potassium hydroxide wet mounts, the organism is seen as a nonbudding spherule with a double-refractile wall; it ranges in size from 10–80 μm (Fig. 32-14). These large spherules contain numerous endospores, 2–5 μm in diameter. The large spherules burst, releasing the endospores, and leave empty "ghost" spherules.

Isolation Procedures

Because of the highly infectious nature of this organism, great care must be exercised in handling cultures and infectious materials. Petri dishes should not be used. Growing cultures should be covered with

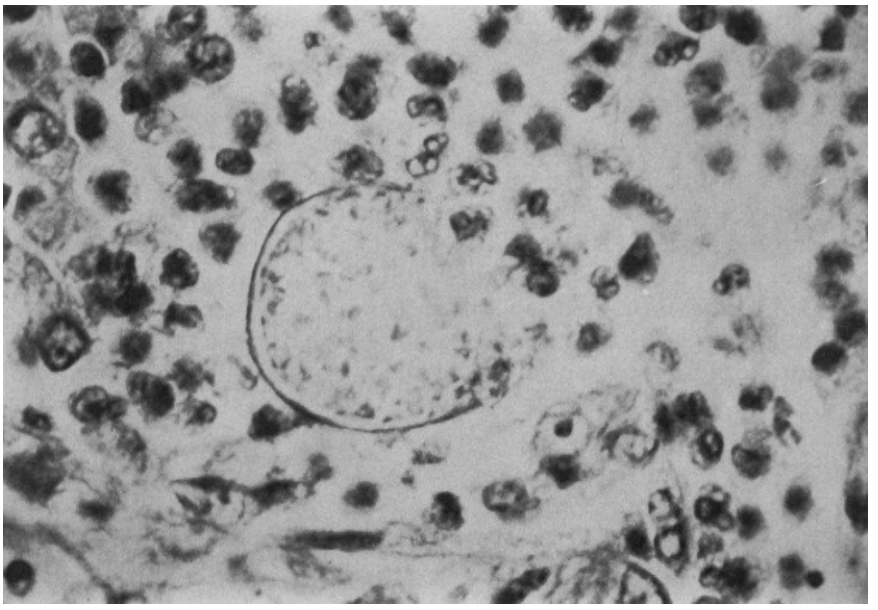

Figure 32-14. *Coccidiodes immitis:* hematoxylin–eosin stain of ruptured spherule in tissue. Endospores are visible within the spherule, which is surrounded by host inflammatory cells, ×675. (Courtesy of Billy C. Ward.)

sterile saline before introducing the inoculating needle to prevent dispersion of arthrospores. Work with the mold phase of this agent and the other dimorphic pathogens should be carried out in a biological safety cabinet, preferably a gloved containment hood.

Clinical material should be inoculated onto blood agar, Sabouraud dextrose agar with chloramphenicol and cycloheximide, and regular Sabouraud agar. The media are incubated at 25°C.

Cultural Characteristics

At 25°C growth usually occurs within 3–4 days and conidia are formed within 10 days. Therefore, *C. immitis* is the fastest growing of the dimorphic pathogens. Colonies are flat, moist, white, and membranous, developing later a coarse, cottony aerial mycelium that turns from white to brown. Cultures incubated at 37°C have similar colonial appearance to those incubated at room temperature.

Only the mycelial form develops on artificial media. The tissue or spherule phase described above can be obtained by transfer to liquid culture at 40°C, or by animal inoculation (3,4) (see below). Due to the hazard in handling this organism and the difficulty of converting it to

the spherule phase, an exoantigen test can be used to conform the identity of the mold. This test requires a standard antiserum, however, and therefore would need to be done at a reference laboratory. Exoantigen tests can also be done for the other dimorphic fungi.

Identification

At 25°C the mycelium consists of narrow septate hyphae, with thicker side branches. The mycelium and hyphae fragment into chains of thick-walled, barrel-shaped arthrospores (2–3 μm long) that alternate with thin-walled, empty (disjunctor) cells (Fig. 32-15).

Serology

Infection with *C. immitis* usually results in strong immunity and protection against reinfection. Serologic tests for coccidioidomycosis are more reliable than for any other mycoses. The coccidioidin skin test is highly useful for diagnosis and prognosis. A negative skin test in the presence of clinical disease is a poor prognostic sign. Other serologic tests include the precipitin test, complement fixation, immunodiffusion,

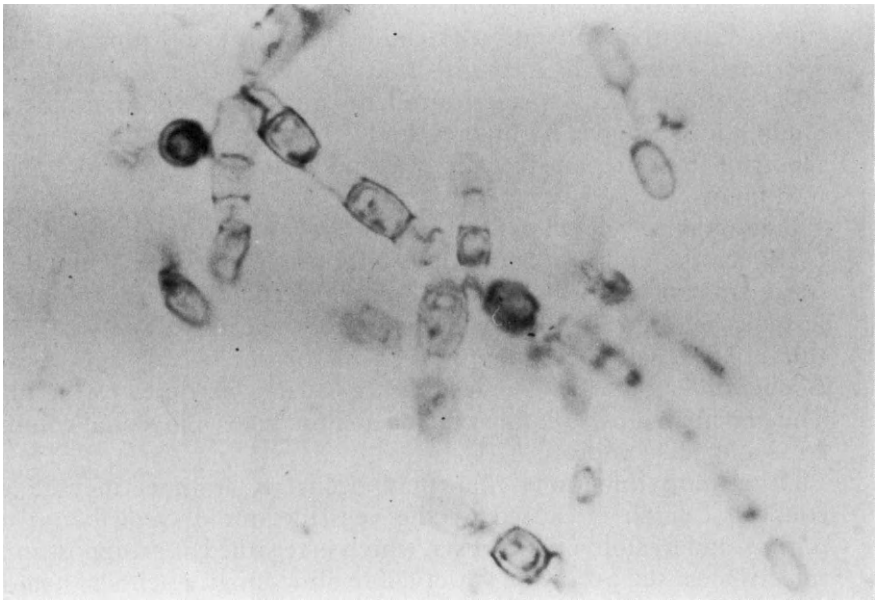

Figure 32-15. *Coccidiodes immitis:* mold phase of thick-walled, barrel-shaped arthrospores alternating with thin-walled disjunctor cells, × ~500. (Courtesy of C. W. Emmons.)

and latex agglutination. In the immunodiffusion test, multiple bands are most often associated with progressive (active) infection, whereas a single band is more often associated with a stable, chronic infection. Immunodiffusion tests for coccidioidomycosis are commercially available from several companies as a kit combined with tests for histoplasmosis, blastomycosis, and aspergillosis.

Animal Inoculation

Saline or broth suspensions of arthrospores from cultures or clinical materials are injected into mice intraperitoneally. Seven to ten days after inoculation, the mice are killed and the peritoneum, lungs, and spleen are examined for nodules or other lesions. Material from the lesions and nodules is stained with lactophenol cotton blue and examined under a coverslip. If the preparation is sufficiently thin, spherules of varying size, including mature ones with endospores, are usually evident.

Rare and Miscellaneous Fungal Infections

Adiaspiromycosis is principally a respiratory infection with adiaconidia (conidia that develop without replication) in the lungs. The infection results from inhalation of conidia from the soil fungus *Chrysosporium (Emmonsia) parvum* or *C. parvum* var. *cresens* (13). The disease affects many species of rodents and other wild mammals, including insectivores, herbivores, and carnivores. There is at least one report of the infection in a dog (14) and several reports of the disease in humans.

Light gray to yellowish focal lesions are found in the lungs of apparently healthy animals. Sometimes only a single nodule is found. The organism produces thick-walled, spherical conidia in granulomatous lesions that are 250–400 μm in diameter; the cell wall may be 70 μm thick. They have been confused in histopathological sections with *Coccidioides immitis*. The lesions are usually restricted to the lungs. The organism grows on most media and produces a mycelial colony at 25°C and adiaconidia at 37°C.

Other rare infections that may occur in animals include geotrichosis, which is caused by the yeastlike mold *Geotrichum candidum*, and hyalohyphomycosis, which is regarded as an opportunistic infection caused by a wide variety of hyaline fungi. Agents of hyalohyphomycosis include but are not limited to *Penicillium, Beauveria, Acremonium, Fusarium, Paecilomyces, Scopulariopsis*, and others (3).

As infections due to these fungi would only rarely be seen in the

veterinary diagnostic laboratory, those desiring detailed information on cultivation and identification of these agents should consult Rippon (3) and other Supplementary Readings.

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33

Laboratory Diagnosis of Infectious Abortion

C. A. Kirkbride

A number of microbial agents have been incriminated as causes of abortion in the principal farm animals. Fetuses and their membranes are frequently submitted to the diagnostic laboratory for microbial examination. The procedures followed in such an examination are essentially similar for the various animals. Because abortion in cattle is of great economic significance, the procedure outlined will deal with this species. Some of the important agents that are implicated in abortions in domestic animals are listed below. The list does not include all of the bacterial species, fungi, protozoa, and viruses that are occasionally isolated from the aborted fetuses of all four species. For a comprehensive discussion of the causes of infectious abortions, readers are referred to the supplementary references.

Bovine: *Brucella abortus*
Campylobacter fetus subsp *fetus*
Campylobacter fetus subsp *venerealis*
Campylobacter jejuni
Leptospira interrogans
Actinomyces (Corynebacterium) pyogenes
Listeria monocytogenes
Fungi: *Aspergillus, Absidia, Mucor,* etc.
Infectious bovine rhinotracheitis virus
Bovine viral diarrhea (BVD) virus
Chlamydia psittaci
Tritrichomonas foetus
Ureaplasma diversum
Equine: Rhinopneumonitis virus
Equine viral arteritis virus

Diagnostic Procedures in Veterinary Bacteriology and Mycology, Fifth Edition

Salmonella abortus-equi
Streptococcus spp.
Klebsiella spp.
Leptospira interrogans
Fungi

Ovine: *Campylobacter fetus* subsp *fetus*
Campylobacter jejuni
Brucella ovis
Coxiella burnetii
Chlamydia psittaci
Listeria monocytogenes
Salmonella spp.
Actinomyces pyogenes
Border disease virus
Blue tongue virus
Toxoplasma gondii

Porcine: *Brucella suis*
Leptospira interrogans
Erysipelothrix rhusiopathiae
Hog cholera virus
Pseudorabies virus
Parvovirus

In addition to the pathogens listed, numerous agents with low virulence that are not generally considered pathogens of adult animals have been associated with abortions. These organisms include *Escherichia coli, Bacillus cereus, Serratia marcescens, Pseudomonas* spp., α-hemolytic streptococci, and *Aeromonas hydrophila.* Some of these organisms are ubiquitous in the environment of livestock and are frequently isolated from abortion specimens. In these instances, a question arises as to whether these organisms should be considered a possible cause of the abortion. If the organism is present in large numbers and nearly pure culture, if lesions compatible with a bacterial infection are present, and if no other significant etiologic agent is found, the organism may be considered to have contributed to the cause of the abortion. It is always possible that the primary infectious agent had been eliminated by the time of abortion or was not discovered by the diagnostic methods used. Bacteria of low virulence may also be present concurrent with a viral infection.

In order to recover the agent from the fetus or its membranes, it is imperative that the laboratory receive fresh or properly preserved specimens. The procedures outlined below refer specifically to the bovine fetus, but they are equally appropriate for other fetuses. The only difference is in the agents being sought.

It is important that tissues be taken for histologic examination.

Some viruses produce characteristic lesions, and the confirmation of a fungus as a cause of abortion depends on the demonstration of fungal elements in lesions.

Examination of the Placenta

1. Remove dirt and debris with running water and spread in a specimen tray.
2. Examine cotyledons and membranes for evidence of necrosis and exudate.
3. Scrape some tissue from a cotyledon with a scalpel blade (choose a necrotic area if it is apparent) and place it on a slide. Add a few drops of 10% KOH and incubate the slide at room temperature 10–15 min. Add a cover slip and examine the preparation microscopically for fungal elements.

   The addition of 0.05% Calcofluor White or Cellufluor to the KOH makes definition of hyphae easier. The preparation is the same as with straight KOH, but the specimen is viewed with a fluorescent microscope. Mycotic elements fluoresce brightly when an exciter filter that transmits the 365-nm group of intense mercury spectral emission lines (Zeiss UG1, G 365, or equivalent) and a barrier filter that transmits visible blue light and longer wave lengths (Zeiss #41, LP420, or equivalent) are used. While counterstaining can be done, it generally is not necessary. A kit with appropriate reagents is available (Fungi-fluor Kit, Polyscience, Inc., Warrington, PA) (5).
4. Prepare smears from lesions and normal cotyledons and stain by the Gram method, Gimenez or Koster's technique, and acid-fast procedures if indicated.
5. If organisms are observed, they will provide guidance in the isolation procedures to be carried out. It is often difficult to isolate the causal organisms on culture media because of contaminants.
6. One or more cotyledons, especially any with lesions, should be fixed in 10% formalin for histologic examination.

Examination of the Fetus

1. Wash the fetus, if necessary, and examine externally for congenital defects, mycotic skin lesions, etc.
2. Open the fetus and examine the internal organs. Fluid from the body cavities or heart blood should be taken for serologic examinations. Take tissues for histologic and microbiologic

examinations. If the fetus is well preserved, brain, heart, lung, liver, spleen, thymus, kidney, and any other tissue containing possible lesions may be included. If postmortem autolysis is extensive, at least lung and liver should be examined histologically. Tissues for virus isolation may be pooled or cultured individually.

3. Puncture the stomach wall with a hypodermic needle and withdraw 2–3 ml of the content into a sterile syringe. The stomach content is generally the best fetal specimen for bacteriologic examination, followed by lung, then liver.
4. Prepare cultures as follows:

Aerobic	Blood agar	37°C
Stomach content, fetal tissues, and placenta		
Stomach content, suspected mycotic skin lesions, cotyledons	Sabouraud's	30°C
Microaerophilic (10% carbon dioxide)	Blood agar	37°C
Stomach contents, fetal tissues, and placenta		

To isolate *Brucella* sp. from contaminated specimens, 6000 Units Polymyxin B®, 25,000 Units Bacitracin®, 100 mg Actidione®, and 20 ml filter-sterilized, brucella-negative bovine serum may be added per liter of tryptose or trypticase soy agar, or 1.25 ml of a 1:1000 solution of ethyl violet or crystal violet may be added to a liter of serum enriched agar. (Biotype 2 *Brucella abortus* is sensitive to all dyes and will not grow in this medium). Inoculated plates of these media should be incubated in an atmosphere containing 10% carbon dioxide.

A special selective medium is of value for the isolation of *Campylobacter* spp. (see Chapter 6). Filtering specimens through a 0.65-μm filter before culturing is an effective means of isolating *Campylobacter* from contaminated specimens.

Although successful fluorescent antibody techniques have been described for such abortifacient pathogens as *Campylobacter fetus, Brucella* spp., and *Listeria monocytogenes,* standardized fluorescent antibody conjugates are not readily available, and diagnosis of these infections is usually done through isolation and identification of the organism. Cold enrichment techniques are not necessary to isolate *L. monocytogenes* from fetal stomach contents or tissues. Darkfield microscopic examination of a drop of fetal calf or lamb stomach content often reveals organisms with the characteristic shape, size, and motility of *Campylobacter* spp. The tentative diagnosis made this way should be confirmed by isolation and identification of the organism. The species and subspecies of *Campylobacter* should always be deter-

mined, because the clinical features of the various species differ greatly in cattle, and vaccines that protect sheep from *C. fetus* alone are ineffective against *C. jejuni.*

Some lamb fetuses aborted because of *Campylobacter* spp. infection have circumscribed necrotic lesions of up to 1.5 cm diameter in their livers. These lesions are not pathognomonic for *Campylobacter* infection. A curved, tapered, flagellated bacterium that grows anaerobically or microaerophilically has been isolated from some lamb fetuses with these lesions. Ovine abortion has been produced experimentally with this bacterium, and the liver lesion occurred in some, but not all, of the aborted fetuses. Information available at this time indicates that this organism is a normal inhabitant of the digestive tract of several species of mammals and that it causes only sporadic abortion. The organism has no official name at this time, but the epithet, *Flexispira rappini,* has been proposed (8).

Leptospirosis

It is generally not practical to attempt isolation of leptospires from aborted bovine fetuses because of the time, effort, and low success rate involved. Leptospires can be isolated from aborted porcine fetuses more easily, but 2–4 weeks are usually required (Chapter 5). Leptospires can be seen frequently by darkfield examination of body cavity fluids of porcine fetuses and, much less commonly, in body cavity fluids of bovine fetuses. Great care must be used in identifying leptospires by direct examination, because many morphologically similar objects are present in body cavity fluid.

Leptospires are more readily isolated from the urine of aborting cows than from aborted bovine fetuses (Chapter 5), and their presence in the urine of a cow that has aborted in the past week or two provides significant diagnostic information.

Leptospires have been demonstrated in fetal tissues by microscopic examination of silver-impregnated tissues (Levaditi stain). Kidney is the preferred tissue. Fluorescent antibody techniques using an incident light microscope have the advantage of more specificity. Kidney smears or cryostat sections may be used. In porcine fetuses, the leptospires tend to retain their morphologic characteristics and may be easily recognizable. In bovine fetuses, the morphologic characteristics are often lost and identification depends upon specific fluorescence. Fluorescent antibody conjugates for detection of leptospires are available from National Animal Disease Center, Ames, Iowa.

Care must be taken in diagnosing leptospiral abortion by serologic methods. Microscopic agglutination titers 1:100 to 1:400 may persist for years following an infection, and up to 50% of the cows infected

with serovar *hardjo* may have microscopic agglutination titers less than 1:100 (4). Vaccination often results in microscopic agglutination titers as high as 1:6400 within 2 weeks. These titers usually decline to less than 1:400 within 8 weeks, but the titers of cattle vaccinated after recovering from an infection often rise above 1:6400 and persist several months above 1:400. Cows that abort from serovar *pomona* infection often have titers greater than 1:12,800 at the time of abortion. Cows that abort because of leptospiral infection have reached or passed their maximum titer at the time of abortion, and one should not expect an increase in leptospiral titer in serum taken after abortion. To determine if leptospiral infection is active in a herd, paired serum samples taken 2–3 weeks apart from a minimum of 10 animals or 10% of the herd should be examined serologically. A fourfold increase in titer in an unvaccinated animal indicates a recent infection.

Virus Infections

For details of the laboratory diagnosis of virus infection, workers are referred to standard texts.

Direct fluorescent antibody examination of cryostat sections of fetal kidney is a highly successful technique for diagnosing abortion caused by the IBR virus (bovine herpesvirus, Group I) (13). Calves aborted because of IBR infection consistently have microscopic lesions of focal hepatic necrosis. In about one-third of the positive cases, IBR virus can be isolated from fetal tissues or placenta using standard cell culture techniques. The identity of the isolated virus is established using specific fluorescent antibody conjugates or immunoelectron microscopy with specific antiserum

The BVD (bovine viral diarrhea) virus and the border disease virus may be detected by direct fluorescent antibody examination of cryostat sections of fetal kidney, spleen, or lung. The border disease virus appears to have an affinity for the thyroid gland, and fluorescent antibody examination of this tissue may be especially rewarding. A negative result is not definitive. The BVD antibody conjugate can be used to detect border disease virus antigen. However, specific border disease virus conjugate may be more effective in some cases. Virus isolation from fetal tissues or placenta using standard cell culture techniques is quite successful. Most BVD viruses associated with abortion are noncytopathic, and their presence in second- or third-passage cell cultures is detected by fluorescent antibody examination using specific BVD conjugate. BVD virus does not consistently produce specific lesions in fetal tissues, and in some cases the virus may be present in the fetus without resulting in abortion. However, its presence in an aborted fetus is indisputable evidence of an active BVD infection in the herd.

The BVD virus is present quite commonly in fetuses aborted because of fungal or bacterial infection.

Porcine parvovirus infection of sows pregnant up to day 56 causes death and resorption of part or all of the embryos, or death and mummification of part or all of the fetuses in a litter. If delivery occurs, generally it is at or after normal term. The most successful method of diagnosis is direct fluorescent antibody examination of cryostat sections of lung from mummified fetuses (9). Parvovirus in fetal tissues may also be identified by specific hemagglutination activity. Parvovirus can be isolated from tissues of many mummified fetuses and some normal fetuses. Fetuses infected after 70 days gestation may produce antibody to parvovirus that can be detected by serum neutralization, indirect fluorescent antibody, or specific hemagglutination inhibition tests run on serum or body fluids.

Pseudorabies (Aujeszky's, porcine herpesvirus) virus infection of pregnant sows often causes fetal death and partial mummification. The virus can sometimes be identified by direct fluorescent antibody examination of fetal lung, liver, or spleen. The virus is readily isolated from fetal tissues and causes herpesvirus-type cytopathic effect on cell cultures. There may be focal necrosis of fetal liver and spleen, and intranuclear inclusion bodies may be present in the margins of these lesions. Serum neutralization titers of 1:2 or greater in unvaccinated aborting sows provides evidence of infection and presumptive evidence of the cause of the abortion.

Gross and microscopic lesions are of value in the diagnosis of equine rhinopneumonitis, and the virus can be readily isolated from fetal lung and liver. The equine arteritis virus can be cultivated in cell cultures of equine kidney or dermis (1).

Numerous other viruses have been associated with livestock abortions, but other than virus isolation techniques, specific diagnostic procedures have not been described.

Standard fluorescent antibody conjugates for IBR, BVD, porcine parvovirus, and pseudorabies virus are available from the National Animal Disease Center, Ames, Iowa.

Chlamydial Abortion

Chlamydiae may be isolated from infected fetal tissues or placenta by inoculation of chick embryos or cell cultures (15). The isolation success rate from bovine fetuses is rather low. Chlamydia-infected ovine and bovine placenta characteristically have necrotic gray-brown cotyledons and a leathery intercotyledonary chorion. Microscopically, there is necrosis of the trophoblasts and inclusions. Examination of smears of these affected cotyledons stained by the Gimenez method

(Appendix D) reveals chlamydial elementary bodies, which appear as tiny red single or aggregated intracellular dots against a blue-green background. Chlamydial elementary bodies can be detected by fluorescent antibody techniques, but standard antichlamydial conjugate is not presently available. Cows aborting due to chlamydial infection characteristically have a significant antibody titer rise 2–3 weeks after termination of pregnancy when examined using the complement fixation test.

Ureaplasma and Mycoplasma Abortion

Ureaplasma diversum can cause granular vulvovaginitis, infertility, and abortion in cattle (10). *Mycoplasma bovis* has caused bovine abortion experimentally and has been isolated from spontaneously aborted calves (14). It appears to be more pathogenic to cattle than are other *Mycoplasma* species. The roles that *Mycoplasma* species, other than *M. bovis*, play in abortion are poorly defined.

Ureaplasma diversum and *M. bovis* cause fetal placentitis and pneumonia. Because *Ureaplasma diversum* and *Mycoplasma* species, other than *M. bovis*, often are present in the lower reproductive tract of normal cows, they may contaminate the fetus as it passes through the birth canal. Therefore, isolation of one of these organisms from an aborted fetus that has no lesions does not offer definite proof that it caused the abortion. Fetal placenta, lung, and stomach contents are the best specimens to culture for *Ureaplasma* and *Mycoplasma* (Chapter 27).

Mycotic Abortion

Fungal infections of fetal calves consistently produce inflammation and necrosis of the placenta, and in about one-third of all cases also produce skin lesions. Occasionally fungi infect fetal lungs, producing bronchopneumonia. Diagnosis is made by histologically demonstrating hyphae associated with inflammation and necrosis. It is often necessary to apply special stains (Gomori's methenamine–silver nitrate, periodic acid–Schiff, or Gridley's) to tissues to make hyphae visible. Fungi often contaminate fetal stomach content and placentas, and isolation of them from these specimens does not necessarily implicate them as the cause of abortion. If a histologic diagnosis of mycotic infection is made, culture may enable identification of the agent(s).

Mycotic abortion occurs rarely in livestock species other than cattle.

Tritrichomonas foetus

Trichomonas infection usually causes bovine abortion before 4 months of gestation. Aborted fetuses are often badly macerated. Trichomonads can be seen by low-power microscopic examination of a drop of fetal stomach content, and the organisms can be cultured in special media (7).

Toxoplasma gondii

Toxoplasma infection is a common cause of ovine and caprine abortion in some areas. Other livestock are affected less commonly. The organisms may be seen by histologic examination in infected cotyledons, and more rarely, in fetal tissues. Isolation of toxoplasma requires mouse inoculation (6). A major proportion of fetal lambs infected with toxoplasma produce antibodies to the infection, and serologic examination of fetal blood or body-cavity fluid is a great aid in diagnosing toxoplasmal abortion. As is true with all fetal serology, the absence of antibody specific for an infection does not totally exclude the possibility that the infection is present. The modified direct agglutination test has proven to be the most sensitive and the easiest to run, and it can be used to test serum from any species without modification (2). At present, however, the antigen is not available commercially. The kits for examining human sera by indirect fluorescent antibody (Gull Laboratories Inc., Salt Lake City, Utah 84107) or enzyme-linked immunosorbent assay (Toxelisa, Whittaker Bioproducts, Walkersville, Maryland 21793) can be modified for use with animal sera. In these case, the slide or plates that come prepared with antigen are used with antiglobulins secured from another source and that are appropriate for the species being tested. Both modified kits have proven successful, but the indirect fluroescent antibody test is easier to run. The latex agglutination test, the indirect hemagglutination test, and the dye test have proven less satisfactory.

Coxiella burnetii

Coxiella burnetii, the rickettsia that causes Q fever in humans, occasionally causes outbreaks of abortion in sheep and goats. The infection causes severe intercotyledonary placentitis with copious amounts of grey or red-brown creamy exudate. The organisms appear as very small (0.2–0.5 μm) coccoid to filamentous forms in placental smears stained with modified Koster's or Gimenez stains. They can be identified by

direct fluorescent antibody examination of smears or cryostat sections of placenta or body organs if specific conjugate is available. The diagnosis can be confirmed by demonstrating seroconversion of mice or guinea pigs inoculated with infected material. Care must be used in handling infected material because of the hazard to human health (12).

Foothill Abortion

Foothill abortion, also called epizootic bovine abortion, is caused by an unidentified infectious agent transmitted by the soft-bodied tick, *Ornithodorus coriaceus*, and the disease is known to occur only where this tick resides. The tick's range is not known precisely, but the foothills surrounding California's central valley, the adjacent areas of Nevada, Oregon, and northern Mexico are known to be infested. An unidentified spirochete has been seen in serum from calves aborted with this disease and in the vector ticks. However, the etiologic role of this agent in the disease has not been definitely established at this time (11). Diagnosis of the infection is based on the history of the geographic location of the victims and the presence of the rather characteristic lesions in the fetus (6).

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34

Bovine Mastitis

Jeffrey L. Watts

Bovine mastitis is the most costly disease confronting the dairy industry. Recent estimates place losses at 2 billion dollars per year in the United States (1). The majority of losses are due to reduced milk production, although milk discarded during and after therapy, increased drug use, increased veterinary services, and premature culling contribute to the losses (1).

The disease is manifested as an inflammation of the udder, usually as a result of microbial infection. Mastitis, or intramammary infection (IMI), can generally be characterized as clinical and subclinical (1). Clinical mastitis, sometimes termed subacute clinical mastitis, is characterized by an abnormal secretion containing clots or flakes. Sudden onset of clinical mastitis accompanied by swelling, hardness, and increased temperature characterizes acute clinical mastitis. Systemic signs such as loss of appetite, fever, dehydration, or depression may also be associated with acute or the more fulminate peracute form.

No apparent changes in the udder or milk are typical with subclinical mastitis, although microorganisms can be isolated by appropriate culture techniques (1). Compostitional changes and increased somatic cell counts (primarily polymorphonuclear leukocytes) usually accompany subclinical mastitis and can be detected by appropriate tests. Chronic mastitis may persist throughout a lactation and may alternate between clinical and subclinical phases (1).

More than 135 different microorganisms have been isolated from bovine IMI (2). However, the majority of infections are caused by staphylococci, streptococci, and gram-negative bacilli. Organisms may be categorized as contagious pathogens (e.g., *Staphylococcus aureus* and *Streptococcus agalactiae*) or as environmental pathogens (e.g., *Streptococcus uberis* and *Enterobacteriaceae*) (1). Coagulase-negative

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staphylococci, previously considered nonpathogenic, colonize bovine teat skin and teat canals, and are classed as skin flora opportunists (3).

The dynamics of udder infection in a dairy herd is comprised of two components: rate and duration. *Rate* is the number of new infections occurring over a given period, whereas *duration* is the length of time an infection persists. A simple plan which reduces both the rate and duration of IMI consists of the following five points: (1) postmilking teat antisepsis (teat dipping), (2) lactational antibiotic therapy, (3) dry period antibiotic therapy, (4) use of functionally adequate milking machines, and (5) culling of chronically infected animals. Adoption of this plan will reduce the level of mastitis over time. Rapid reductions in herd mastitis levels require identification of infected glands and specific pathogens, followed by drug therapy and/or culling of affected cows. Thus, microbiological culturing is the single most valuable tool for developing a specific mastitis control program for a dairy herd.

Routine Procedures

Proper collection of milk samples is essential for accurate diagnosis of IMI. Udders should be cleansed, dried, and several streams of milk forcibly stripped from each quarter. Teats should be scrubbed with a cotton pledget soaked in 70% alcohol and one or two streams of milk collected from each quarter into sterile containers. Samples should be cooled to 5°C immediately and maintained at that temperature until cultured. If culturing cannot be performed within 24 hr, samples can be frozen for periods up to 2 weeks. However, freezing lyses leukocytes, rendering the sample useless for somatic cell count determinations.

Since a wide variety of microorganisms may cause mastitis, a general growth and differential medium such as 5% blood agar is preferred. Most mastitis bacteriology laboratories use bovine (preferably calf) blood. However, ovine blood may be used but hemolytic patterns exhibited by some organisms may differ from those seen on bovine blood agar. Each new lot of blood should be tested for presence of antihemolysins with hemolysin-producing *Staphylococcus aureus* and *Streptococcus agalactiae* strains. Selective media may be inoculated to enhance recovery of certain organisms. Edward's medium (streptococci), mannitol salt agar (staphylococci), eosin methylene blue (EMB), or MacConkey agar (gram-negative bacilli) are examples of selective media.

Visibly abnormal milk samples should be noted and recorded prior to culturing. Samples should then be brought to room temperature and mixed thoroughly. A .01-ml aliquot of milk is transferred from the milk sample to the surface of a blood agar plate using a calibrated plati-

num loop (disposable plastic loops are also available). The milk sample is streaked in a manner to ensure development of well-isolated colonies. Many laboratories divide a blood agar plate into four quadrants, which allows all four quarter samples from a cow to be processed on a single plate. After inoculation, plates are placed in a 35–37°C incubator in an inverted position and examined for growth after 24 and 48 hr.

Samples containing low numbers of bacteria may require additional testing. Recovery may be enhanced in three ways: (1) inoculating a large volume of milk (0.025–0.1 ml) to the surface of an entire blood agar plate, (2) subjecting samples to a freeze–thaw cycle to lyse leukocytes that may contain bacteria, or (3) incubating sample for 18–24 hr to increase microbial populations. It should be cautioned that any contamination will be magnified using the latter procedure. If the sample is very abnormal and no bacteria are recovered, reculture for mycoplasma (Chapter 27).

Identification of Organisms Isolated

Organisms may be presumptively grouped based upon colony characteristics and hemolytic patterns of colonies growing on blood agar. Presumptive grouping should be confirmed by Gram staining before proceeding to preliminary tests.

Gram-Positive Cocci

A catalase test should be performed (see Appendix B) for separation of staphylococci and micrococci (catalase positive) from streptococci (catalase negative). Staphylococci can be separated from micrococci by a variety of methods (see Chapter 16). However, true micrococci are infrequently isolated from bovine IMI. Staphylococci can be segregated into two groups by their ability to clot rabbit plasma. The clumping factor or slide coagulase test (Appendix B) permits rapid screening of large numbers of isolates. All clumping-factor negative isolates should be tested using the tube coagulase test (Appendix B). Three species, *Staphylococcus aureus*, *S. intermedius*, and *S. hyicus*, are coagulase positive. *Staphylococcus aureus* infections respond poorly to antibiotic therapy during lactation. Administration of antibiotic therapy at drying off is the most efficacious method of treatment. Older animals refractory to therapy should be removed from the herd. *Staphylococcus intermedius* is associated with carnivores and is infrequently isolated from infected mammary glands. β-hemolytic, pigmented *S. aureus* strains are easily distinguished from nonhemolytic, nonpigmented *S. hyicus* strains. Differentiation of nonhemolytic *S. aureus* strains from *S. hyicus* requires biochemical testing. Species level identification of coagulase-negative staphylococcal isolates is unnecessary

unless the organisms are isolated from clinical mastitis or high somatic cell count samples. Staphylococcal isolates can be identified using conventional biochemical tests (Chapter 16) or a commercial microbial identification system (4).

Streptococci should be grouped using commercially available coagglutination or latex agglutination tests (5). Streptococci can be further differentiated using the biochemical tests in Table 34-1 (5). *Streptococcus agalactiae* can be presumptively identified with the CAMP test (see Fig. 34-1 and Appendix B). *Streptococcus agalactiae* is primarily spreadduring the milking process with infected udders serving as the infection reservoir. Eradication of this organism from dairy herds is possible through use of strict hygiene and proper antibiotic therapy (6). *Streptococcus uberis* colonizes various body sites including teat skin and has been isolated from bedding material (6). Clean calving areas are important for control of *S. uberis* infections as many infections occur at parturition. Excessive use of water without proper drying of the udder is associated with increased *S. uberis* infections during lactation. *Streptococcus dysgalactiae* is primarily spread during the milking process, but environmental sources are also important. (6). Hygiene and antibiotic therapy readily control this organism. *Enterococcus faecalis* and *Streptococcus equinus ("Streptococcus bovis")* are inhabit-

Table 34-1
Scheme for Identification of Gram-Positive, Catalase-Negative Cocci Isolated from Bovine Mammary Glands

Organism	Lancefield group[a]	Phenotypic characteristics (% positive)[b]						
		Hipp	Esc	BiEs	NaCl	PYR	TRE	SUC
Groupable								
S. agalactiae	B							
S. dysgalactiae	C						100	
S. equi	C						0	
S. canis	G							
E. faecalis	D	95.2	100	100	100	95.2		
S. equinus	D	42.1	100	100	0	0		
S. alactolyticus	D	0	0	0	0	100		
Nongroupable								
S. uberis type I	—	100	100	0	0	100		0
S. uberis type II	—	100	100	0	0	98.5		100
S. saccharolyticus	—	0	100	90	16.7	20		
A. viridans	—	100	100	100	100	83.3		
S. acidomininus	—	80	0	0	0	40		

[a]Determined using commercially available coagglutination or latex agglutination reagents.
[b]Abbreviations for biochemical tests are as follows: Hipp, hippurate hydrolysis; Esc, esculin hydrolysis; BiEs, bile–esculin; NaCl, growth in 6.5% NaCl broth; PYR, pyroglutamic acid; TRE, trehalose, SUC, sucrose.

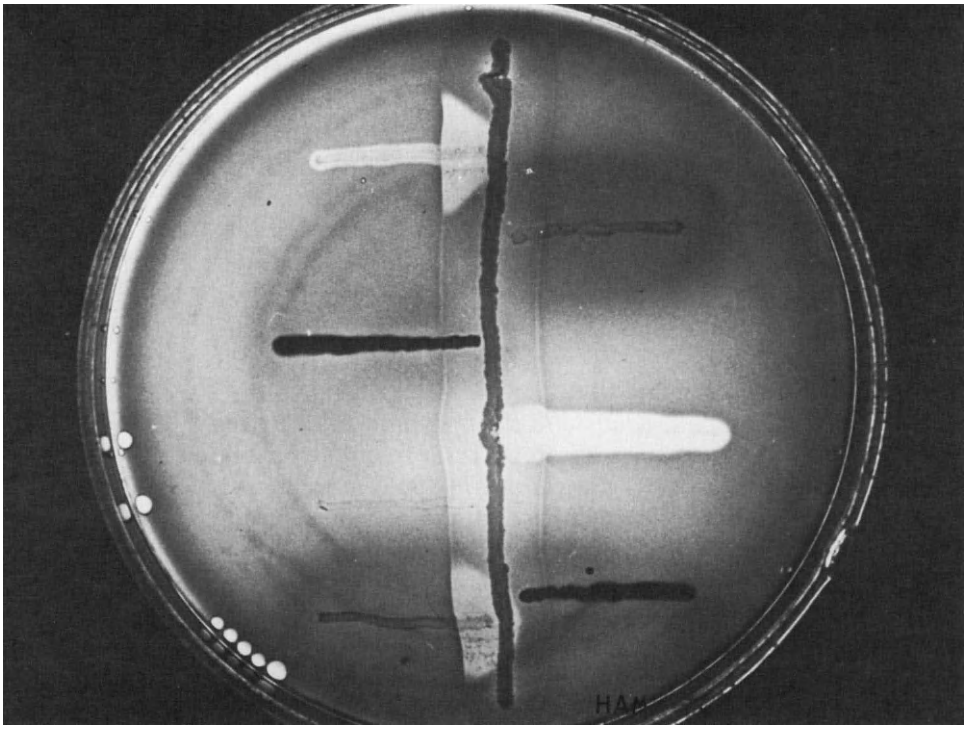

Figure 34-1. CAMP test. Completion of the partial hemolysis along the *Staphylococcus aureus* streak by two cultures of *Streptococcus agalactiae.* The "arrowhead" effect is shown above and below, adjacent to the staphylococcal streak. The other streaks represent other streptococcal species (H. A. McAllister).

ants of the gastrointestinal tract and increased IMI is associated with unsanitary milking or environmental conditions. *Streptococcus canis* (group G streptococci) is a wide-zone β-hemolytic, large colony type streptococcus. These organisms may cause outbreaks of mastitis in individual herds. *Streptococcus canis* can be eliminated from a herd by strict hygiene, antibiotic therapy, and a culling regimen.

Gram-Positive Bacilli

Corynebacterium bovis is a nonsporeforming, short, pleomorphic, gram-positive rod frequently isolated from normal glands. *Corynebacterium bovis* colonizes the teat canal and is controlled by postmilking teat antisepsis. *Corynebacterium bovis* produces small, white, powdery or granular colonies after 48 hr. The organism has a requirement for lipids and tends to grow best in the primary portion of the streak. *Actinomyces pyogenes,* formerly classified as *Corynebacterium pyogenes,* causes an acute, purulent form of mastitis. This organism pro-

duces very small β-hemolytic colonies at 48 hr and may require 72 hr for significant growth. *Peptococcus indolicus* is frequently associated with *A. pyogenes* infections. Biochemical differentiation of gram-positive, nonsporeforming rods is discussed in Chapters 21 and 22.

Bacillus cereus and *B. subtilis* are the most frequently isolated gram-positive, spore-forming, aerobic rods. Mastitis caused by *Bacillus* spp. can be acute and is usually caused by *B. cereus*. Some members of the genus *Bacillus* produce large spreading colonies with hemolytic colonies resembling those of *B. cereus*. *Bacillus subtilis* produces a characteristic "ground-glass" colony. The species can be determined by using techniques described in Chapter 18.

Gram-Negative Bacilli

Escherichia coli, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Serratia marcescens* are enterobacteria frequently isolated from infected mammary glands (1,6). *Klebsiella* spp. are frequently associated with contaminated bedding, particularly sawdust bedding. *Serratia* spp. have been isolated from contaminated teat dips.

Mastitis produced by the enterobacteria is frequently peracute. Prompt attention and supportive therapy is needed for survival of the animal. *Pseudomonas aeruginosa* is the most frequently isolated nonfermenter from bovine mastitis. *Pseudomonas putida* and *P. cepacia* have been isolated from contaminated teat dips. *Pseudomonas* mastitis is often cyclic in nature, alternating between clinical and subclinical phases. As *Pseudomonas* spp. are usually resistant to the commercially available antibiotics, therapy is of little value. The enterobacteria can be differentiated from the pseudomonads by the oxidase test (Chapters 7 and 10). Although conventional tests can be used for identification of the gram-negative bacilli (Chapter 10), many laboratories prefer to use commercial systems (4).

Other Microorganisms

The vast majority of cases of IMI can be attributed to the aforementioned organisms. However, other organisms such as mycoplasmas, fungi (yeasts), mycobacteria, and prototheca may cause IMI in individual herds. A brief discussion of these organisms follows. Specific chapters should be referred to for definitive identification techniques.

Mycoplasma

Recovery of *Mycoplasma* spp. requires the use of Hayflick's or a similar medium. Colonies exhibit a typical "fried egg" morphology after several days incubation. Mycoplasma mastitis is characterized by a sudden onset of clinical signs, often involving all four quarters (6). Antibiotic therapy has little value and culling is necessary to eliminate infected animals from the herd. Strict milking hygiene is necessary

to prevent spread of the organism. Dipping of teats prior to milking (predipping) and mechanical backflushing of teat cup liners are useful techniques to prevent spread of mycoplasmas.

Nocardia

These organisms are soil inhabitants and gain entry to the mammary gland via contaminated treatment equipment and improper teat sanitation prior to treatment. The most common *Nocardia* associated with mastitis is *Nocardia asteroides,* although other *Nocardia* spp. have been isolated from infected mammary glands. Identification to the species level is not necessary. *Nocardia* spp. produce characteristic white to yellow, powdery colonies after 48–72 hr incubation. Colonies are usually embedded in the agar surface. *Nocardia* are not susceptible to currently available intramammary antibiotics. Infected animals should be culled.

Mycobacteria

Mastitis attributed to *Mycobacterium* spp. is usually a consequence of poor treatment techniques or contaminated treatment equipment. Mycobacteria are acid-fast bacilli that produce small white colonies after 5 days. Most mycobacteria isolated from bovine IMI belong to Runyon group IV or the *M. fortuitum* complex. Mycobacteria are resistant to the available antibiotics and removal of infected animals from the herd is required to eliminate the organism.

Fungi (yeasts)

Contaminated antibiotic preparations and treatment equipment are the most common sources of yeasts and fungi. Most yeast infections recover spontaneously. Animals with persistent infections should be removed from the herd. *Candida* spp. are the most commonly isolated yeast, although *Cryptococcus, Pichia, Trichosporon,* and *Saccharomyces* spp. have also been isolated (2).

Prototheca

Prototheca are achlorophyllic algae. They produce small greyish-white colonies on blood agar after 48–72 hr. Two species, *P. wickerhamii* and *P. zopfii,* have been isolated from bovine IMI, but species level identification is not necessary. The microscopic appearance of the organism is distinctive, with large sporangia containing four–eight daughter cells. Contaminated ponds, watering troughs, and rinse water are possible sources of *Prototheca* spp. However, *Prototheca* spp. can survive passage through the gastrointestinal tract and proper manure handling is important in controlling the organism. Infected animals should be culled to remove the reservoir of infection from the herd.

Commercial Systems

Commercial prepackaged microbial identification systems offer several advantages over conventional macrotube methods. They are compact, utilize reduced incubation times, have extended shelf life, minimize misinterpretation of test results, have comparable accuracy to conventional systems, and are of standardized quality. Commercial systems employ one or more of the following test methods: (1) miniaturized conventional methods, (2) chromogenic substrates, and (3) antigen–antibody reactions (4). Commercial systems evaluated with bovine mammary gland isolates are summarized in Table 34-2 (7–14). Selection of a commercial system must be based upon the needs of each individual laboratory and should consider the following: (1) incubation periods, (2) cost of systems (as compared to a similar conventional method), (3) retraining, (4) test system familiarity, and (5) substrate flexibility (4).

Antibiotic Susceptibility Tests

These are performed as described in Chapter 35. A commercially available system (Prompt, 3M Co., St. Paul, MN) may be used for direct preparation of a standardized inoculum from bacterial colonies. This system demonstrated a 95.9% correlation to conventional methods with bovine mammary gland isolates (15). Many laboratories prefer to test the antimicrobial agents currently available in commercial intramammary infusion products such as penicillin, ampicillin (amoxicillin), methicillin (cloxacillin), cephalothin, tetracycline, novobiocin, erythromycin, and streptomycin. *Streptococcus agalactiae* is uniformly susceptible to penicillin and need not be tested. Antimicrobial susceptibility testing is required for *Streptococcus uberis* and enterococci because penicillin resistance is frequently encountered in these organisms. *Staphylococcus aureus* mastitis is characterized by abscess formation and susceptible organisms sequestered within the abscess may be protected from the antibiotic. Thus, *S. aureus* isolates from older, chronically infected animals need not be tested as treatment efficacy is low. However, routine susceptibility testing of *S. aureus* isolates from individual herds allows detection of emerging resistant forms.

Diagnosis

Diagnosis of mastitis is usually based upon isolation of a microorganism coupled with evidence of a host response (i.e., increased milk leukocyte or somatic cell counts). In cases of clinical mastitis, recovery of microorganisms is sufficient for diagnosis. Diagnosis of subclinical mastitis requires isolation of the causative organism in presence of

Table 34-2
Commerical Systems Evaluated with Isolates from Bovine Mammary Glands

System	Cost per isolate ($)	Organism(s) identified	Accuracy[a] level (%)	References
STAPH-Ident Analytab Products, Inc. 200 Express St. Plainview, New York	2.00	Staphylococci	45.1,54,94.3[b],88.1[b]	7–10
STAPH-Trac Analytab Products	2.72	Staphylococci, micrococci	91.2,66.1	8,10
Minitek Gram-Positive Set BBL Microbiology Systems Cockeysville, Maryland	6.61	Staphylococci, micrococci, streptococci	87.7[b] 34.6	11 17
Phadebact Streptococcus Test Remel, Inc. Lenexa, Kansas	3.20	Streptococcal groups A, B, C, G	100	12
Streptex Burroughs-Wellcome Research Triangle Park, North Carolina	4.10	Streptococcal groups A, B, C, D, G	98	12
Rapid Strep Analytab Products	2.72	Streptococci	71.4,88.4	13,18
API 20E Analytab Products	2.90	Gram-negative bacilli	96	14
Rapid Mastitis Test Immucell Corporation 966 Riverside Street Portland, Maine	1.00	*Staphylococcus aureus* *Streptococcus agalactiae*	99.2 100.0	19

[a]Compared to conventional methods.
[b]Requires modification of system.

elevated somatic cell counts (≥200,000 cells/ml). Cells may be estimated indirectly with a screening test such as the California Mastitis Test (CMT). A more precise count can be obtained with the Direct Microscopic Somatic Cell Count (16) or electronic cell counters (Fossomatic or Coulter).

If somatic cell counts are unavailable, serial culturing of milk from infected glands should be conducted. Isolation of a microorganism in pure culture from two consecutive samples taken 24 hr apart is considered confirmatory.

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35

Antimicrobial Agents and Susceptibility Testing

M. M. Chengappa

Introduction

This chapter has two main purposes. One is to provide essential knowledge of antimicrobial susceptibility testing procedures to veterinary practitioners and microbiologists in small diagnostic laboratories. The other purpose is to provide general recommendations for selecting an antimicrobial agent for the pathogen in question. The information provided in this chapter is not exhaustive and deals primarily with methodology and the proper selection of antimicrobial agents. It is imperative that veterinarians and microbiologists be aware of the plethora of information accumulated on the subject in the last 8–10 years. For further information on the methods and theory of the subject, the reader should consult more exhaustive discussions (1, 2, 3).

Since many bacterial isolates show resistance to antimicrobial agents, isolation of an infectious agent from an animal with disease is often not sufficient for determining proper treatment. Also, since susceptibility patterns of bacteria are constantly changing, it is essential for a veterinarian to know the antibiogram of a selected bacterial isolate before the initiation of treatment. The antibiogram depicts the pattern of susceptibility and resistance of a bacterial isolate to various antimicrobial agents.

Since the susceptibility of bacteria to antimicrobial agents cannot be predicted reliably without *in vitro* testing, individual pathogens must be tested against appropriate antimicrobial agents. One important practice that must be emphasized is the performance of *in vitro* susceptibility tests only on pure cultures. Susceptibility testing of mixed bacterial populations will yield erroneous results and such practices should be strongly resisted. The correlation between the *in vivo*

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response to and *in vitro* activity of antimicrobial agents is by no means perfect and is recognized to be affected by multiple host factors including the cellular and humoral defense mechanisms, the site and severity of the infection, the virulence and number of organisms present, and the type and dosage of antimicrobial agents administered (4).

A discrepancy between the *in vitro* and *in vivo* responses due to drug inactivation has been observed with many gram-negative bacteria. False susceptibility results are most likely to occur due to drug inactivation in certain susceptibility testing procedures. Interactions between the cation content of the test medium and the antipseudomonadal effect of aminoglycosides is a problem in relating the *in vivo* and *in vitro* results.

Although some of these discrepancies are strictly methodologically determined, the most important determinants of response to antimicrobial therapy *in vivo* remain the integrity of the host defenses, the site and nature of infections, and the pharmacokinetics of the antimicrobial agents and their penetrations into areas of infections.

Microbiologists can only recommend therapeutic agents based on the results of *in vitro* susceptibility testing of a bacterial isolate. However, the practitioner must make the final choice based on his or her knowledge of all the pertinent facts. These facts include knowledge of the antibiogram of the presumed causal agent, the relationship of the antibiogram of the isolate to that of other members of the same species, pharmacokinetics of the antimicrobial agents, previous knowledge of efficacy in the treatment of infections due to the same species, the nature of the underlying pathological process and its influence on chemotherapy, and the immune status of the animal (1).

The susceptibility testing methods currently in use for both aerobic and anaerobic bacterial isolates are described below. These standardized methods, with little or no modification, are widely used by microbiologists in veterinary and public health laboratories in North America.

Antimicrobial Susceptibility Testing Procedures

Dilution Tests

Dilution tests are of two types: broth dilution and agar dilution (1, 2). These are the most quantitative methods for *in vitro* antimicrobial susceptibility testing. The tests described below are derived from the International Collaborative Study recommendations (5) or from the National Committee for Clinical Laboratory Standards (NCCLS) publication (6).

Broth Dilution Method

For the broth dilution method, decreasing concentrations of antimicrobial agents are prepared in twofold dilutions and placed in tubes or wells of a microtiter tray containing a broth that will support the growth of the test organism. The broth recommended for testing the majority of rapidly growing bacteria is Mueller–Hinton broth supplemented with the cations magnesium and calcium.

A standard inoculum of bacteria (approximately 10^8 CFU/ml for tray test and 10^6 CFU/ml for tube test), as determined by the McFarland nephalometer standards, is added to an equal volume of each concentration of antimicrobial agents. A control tube or well without any antimicrobial agent for each test isolate must be prepared. The inoculated tubes or trays are sealed and incubated at 35°C. If these tubes or trays are not sealed, sufficient humidity in the incubator should be maintained to avoid any evaporation of the medium.

After 16–20 hr of incubation, the tubes or trays are examined from below with a reflective viewer, and the minimal inhibitory concentration (MIC) is then determined. The tubes or trays may also be read visually from the top. The MIC is defined as the lowest concentration of a drug at which the microorganism tested does not show visible growth. This measures the bacteriostatic or bactericidal effect of the antimicrobial agent.

If the concentration of antimicrobial agent represented by the MIC can be achieved in the animal's serum by the recommended route of administration, the bacterium is said to be susceptible. However, if the MIC is above the achievable level or within the toxic level to the host, the bacterial isolate is said to be resistant to the agent.

Agar Dilution Method

The agar dilution test is rarely used in veterinary diagnostic laboratories in the United States. For this method varying concentrations of antimicrobial agents are incorporated in Mueller–Hinton agar plates, one plate for each concentration of agents to be tested. Generally, twofold dilutions of antimicrobial agent are used. To ensure the growth of some fastidious organisms, supplementation of Mueller–Hinton agar with 5% defibrinated ovine, bovine, or equine blood may be necessary. For detailed information on preparation of the medium, the reader is referred to the *Manual of Clinical Microbiology* (1).

The plates containing the dilutions of antimicrobial agents are spot inoculated with a loop calibrated to deliver 1–2 μl. To obtain valid results, the bacterial concentration of the inoculum must be carefully standardized so that each spot should contain approximately 1×10^4 CFU $\pm \frac{1}{2}$ log. A control plate with no antimicrobial agent is also spot-inoculated with the culture. The plates are incubated at 35°C for

16–20 hours in the absence of CO_2. The lowest concentration of antimicrobial agent at which complete inhibition of bacteria occurs is the MIC, with any very fine, barely visible growth being disregarded in this method.

Advantages and Limitations of Dilution Tests

Dilution tests provide accurate MIC values that permit direct comparison between different antimicrobial agents. Information about synergistic and antagonistic responses of two or more antimicrobial agents are provided simultaneously (7, 8). The dilution methods are also the methods of choice for testing anaerobes in many laboratories. With the use of a replicating apparatus, a large number of isolates can be tested using the agar dilution technique. The agar dilution technique permits detection of contamination and microbial heterogeneity readily by observing the nature of bacterial growth on the surface of the plates. The medium in the agar dilution technique may be supplemented with specific nutrients to allow testing of nutritionally fastidious bacteria.

Due to the slight variation in reading the endpoints, the MICs in the agar dilution technique tend to be lower than that of the MICS of the broth dilution technique (5). The following are some of the limitations of dilution tests (9): Greater technical expertise is required for dilution tests as compared to diffusion tests; handling of drug dilutions is prone to error; contamination is difficult to detect in broth dilution techniques; and the MIC may be a less sensitive measure of a resistant variant. In addition, the dilution tests provide only discontinuous, arbitrary endpoints. This may be a problem with drugs with a narrow margin between toxic and therapeutic levels. The dilution tests are relatively time-consuming and costly to perform; therefore they are not suitable for small laboratories operating with limited budgets. One of the major problems in veterinary medicine is the lack of sufficient data on achievable levels of antimicrobial agents in the body fluid and tissues of animal species. Consequently, reporting a set of MIC values for a bacterial isolate may be of limited value.

Diffusion Methods

In diffusion methods, a thin uniform inoculum of the test strain is exposed to a disk of known concentration of antimicrobial agent. In the United States, only one disk potency is recommended for each antimicrobial agent. The antimicrobial agent from the disk gradually diffuses into the agar and creates a concentration gradient of the drug. The susceptibility of the organism to the agent is indicated by a clear zone of inhibition around the disk. The diameter of the zone of inhibi-

tion is directly proportional to the susceptibility of the organism tested. Absence of a zone of inhibition around the drug reservoir indicates complete resistance.

Since the qualitative results of the diffusion tests correlate well with the quantitative results of the dilution tests and the test procedure is simple to perform, the disk diffusion test is widely used throughout the world. Currently, there are two disk diffusion techniques recommended for use. The most common diffusion technique used in the United States is the standard disk diffusion test (5, 10), commonly referred to as the Bauer–Kirby or Kirby–Bauer procedure. For accurate and reproducible results, the Kirby–Bauer procedure should be followed exactly as recommended. The second acceptable method is the agar overlay method of Barry *et al.* (11). This method has been shown to give acceptable zone sizes and satisfactory correlation with MIC values. The direct inoculation of a clinical specimen has been proposed as a quick, alternative method of susceptibility testing (3). The results of this procedure are usually well outside the range of standard antibiograms. The direct test may occasionally be desirable in a clinical emergency situation. Results generated by this method should be considered "preliminary" and should be confirmed later by a standard procedure.

The Bauer–Kirby Procedure

The United States Food and Drug Administration officially introduced this method to veterinary medicine over a decade ago (12). This method has been standardized with Mueller–Hinton agar, which can be supplemented with 5% defibrinated animal blood to promote the growth of fastidious organisms. The addition of blood to the medium has very little effect on the results. However, novobiocin and nafcillin should not be tested on media supplemented with blood.

Mueller–Hinton agar is dispensed into petri plates to yield a uniform depth of 4 mm. For plates with diameters of 100 or 150 mm, the dispensing of 25 or 60 ml, respectively, will provide the desired depth. Prior to use, the plates are stored at 4–8°C for not more than 2 weeks and should be free of moisture on the surface of the medium. The antimicrobial disks should be stored under refrigeration or frozen at −14°C or lower. A working supply of disks should be stored in the refrigerator with an adequate desiccant and be allowed to warm at room temperature before being dispensed. For more information on storage of disks, the reader is advised to review the manufacturer's recommendations.

An inoculum should represent an adequate cross section of the population so that both susceptible and possibly resistant colony types are included. To achieve this, four or five well isolated colonies of the same type from the culture plate are suspended in 4–5 ml of a suitable

broth such as trypticase soy broth or brain–heart infusion broth. The tubes are incubated at 35°C until a visible turbidity appears. The density of the culture is then adjusted to a 0.5 McFarland nephelometer standard with sterile saline or broth. The standard is prepared by adding 0.5 ml. of 1% (11.75 g/liter) $BaCl_2 \cdot 2H_2O$ to 99.5 ml of 1% (0.36 *N*) H_2SO_4. Standard turbidity tubes are available commercially for known bacterial densities. A sterile cotton swab is used to transfer the inoculum onto the plate. Excess fluid is removed by rotating the swab several times against the inside wall of the tube above the fluid level. The plate is inoculated uniformly by streaking the swab over the surface. The streaking is repeated three times and for each time the plate is rotated roughly 60° to ensure even distribution of inoculum. Within 10–15 min after the plates are inoculated, disks are applied either with a dispenser or by hand with forceps. All disks are gently pressed down to ensure complete contact with the agar surface. The plates are incubated at 37°C in an inverted position. Incubation of plates in a CO_2 atmosphere must be avoided if possible, since the CO_2 may alter the surface pH enough to affect the results.

Following 16–18 hr of incubation, the plates are examined and the diameter of the zone of inhibition is measured to the nearest millimeter with a ruler, sliding calipers, template, or electronic instrument. Whenever there is partial inhibition or the outer rim of the zone of inhibition is ragged, the zone to be measured is the outermost rim of the zone which exhibits a homogeneous and complete zone of no growth. For further information on zone variations in the Bauer–Kirby test, the reader is referred to *Manual of Clinical Microbiology* (1) and *Antibiotics in Laboratory Medicine* (3). The zone diameters are expressed as susceptible, intermediate, or resistant according to an interpretive table (Table 35-1). With sulfonamides or sulfonamide–trimethoprim,

Table 35-1
Zone Diameter Interpretive Standards and Approximate MIC Correlates[a,b]

		Zone diameter (mm)			Approximate MIC correlates (μg/ml)	
Antimicrobial agent	Disk potency	R	I	S	R	S
Amikacin	30 μg	≤14	15–16	≥17	≥32	≤12
Ampicillin						
Enterobacteriaceae	10 μg	≤11	12–13	≥14	≥32	≤8
Staphylococcus spp.	10 μg	≤28	—[c]	≥29	β-lact[d]	≤0.25
Haemophilus spp.	10 μg	≤19	—	≥20	≥4	≤2
Nonenterococcal streps.	10 μg	≤21	—	≥30	≥4	≤0.12
L. monocytogenes	10 μg	≤21	—	≥30	≥4	≤0.12
Apramycin	15 μg	≤11	12–14	≥15	—	—
Augmentin[e]						
Staphylococcus spp.	20/10 μg	≤19	—	≥20	—	≤4/2
Haemophilus spp.	20/10 μg	≤19	—	≥20	—	≤4/2
Other organisms	20/10 μg	≤13	14–17	≥18	≥32/16	≤8/4

Table 35-1 (*continued*)
Zone Diameter Interpretive Standards and Approximate MIC Correlates[a,b]

Antimicrobial agent	Disk potency	Zone diameter (mm)			Approximate MIC correlates (μg/ml)	
		R	I	S	R	S
Bacitracin	10 U	≤8	9–12	≥13	—	—
Carbenicillin						
Enterobacteriaceae	100 μg	≤17	18–22	≥23	≥32	≤16
Pseudomonas	100 μg	≤13	14–16	≥17	≥512	≤128
Cephalothin[f]	30 μg	≤14	15–17	≥18	≥32	≤8
Chloramphenicol	30 μg	≤12	13–17	≥18	≥25	≤12.5
Clindamycin	2 μg	≤14	15–16	≥17	≥2	≤1
Colistin	10 μg	≤8	9–10	≥11	≥4	[g]
Erythromycin	15 μg	≤13	14–17	≥18	≥8	≤2
Gentamicin	10 μg	≤12	13–14	≥15	≥8	≤4
Kanamycin	30 μg	≤13	14–17	≥18	≥25	≤6
Methicillin						
Staphylococcus spp.	5 μg	≤9	10–13	≥14	≥16	≤4
Nafcillin[h]						
Staphylococcus spp.	1 μg	≤10	11–12	≥13	≥8	≤2
Nalidixic acid[i]	30 μg	≤13	14–18	≥19	≥32	≤12
Neomycin	30 μg	≤12	13–16	≥17	—	—
Nitrofurantoin[i]	300 μg	≤14	15–16	≥17	≥100	≤25
Novobiocin						
Medium with blood	30 μg	≤14	15–16	≥17	—	—
Medium without blood	30 μg	≤17	18–21	≥22	—	—
Penicillin G						
Staphylococcus spp.	10 U	≤20	21–28	≥29	β-lact[d]	≤0.1
Other organisms	10 U	≤11	12–21	≥22	≥32	≤2
Polymyxin B	300 U	≤8	9–11	≥12	≥50 units	[g]
Spectinomycin	100 μg	≤14	15–17	≥18	—	—
Streptomycin	10 μg	≤11	12–14	≥15	—	—
Sulfonamides	250 or 300 μg	≤12	13–16	≥17	≥350	≤100
Tetracycline[j]	30 μg	≤14	15–18	≥19	≥12	≤4
Ticarcillin	75 μg	≤11	12–14	≥15	≥128	≤64
Tobramycin	10 μg	≤12	13–14	≥15	≥8	≤6
Trimethoprim/ sulfamethoxazole (SXT)	1.25/23.75 μg	≤10	11–15	≥16	≥8/152	≤2/38
Vancomycin	30 μg	≤9	10–11	≥12	—	≤5

[a]R, resistant; I, intermediate; S, susceptible.
[b]Adapted from the fourth edition of *Manual of Clinical Microbiology* (1), second edition of *Antibiotics in Laboratory Medicine* (3), and the 1984 NCCLS publication (14).
[c]—, Not available or not recommended.
[d]Resistant strains produce β-lactamase.
[e]Amoxicillin 20 μg and clavulanic acid 10 μg.
[f]Results can be applied to cefaclor, cefamandole, cefazolin, cefonicid, cefoxitin, ceftazidime, and cefuroxime.
[g]Diffuses poorly in agar, MIC correlate cannot be evaluated from regression analysis.
[h]Results can be applied to oxacillin, cloxacillin, and dicloxacillin.
[i]Apply only to organisms recovered from urinary tract infection.
[j]Results can be applied to chlortetracycline, demeclocycline, doxycycline, methacycline, minocycline, and oxytetracycline.

slight growth (80% inhibition) is disregarded, and the margin of heavy growth is measured. Similarly, a "veil" of swarming *Proteus* sp. is disregarded, and the margin of heavy growth is measured. In a clinical emergency, preliminary results can be obtained within 5–6 hr of incubation, and a final report should follow after a full 16–18 hr of incubation.

The interpretations for the antimicrobial agents in Table 35-1 are those presently recommended by the United States Food and Drug Administration and by the NCCLS. The zones of inhibition are approximately inversely proportional to the MIC values. The MIC values can be compared to concentrations of antimicrobial agent achievable at various body sites as a means of predicting therapeutic efficacy. Taking this into account, it is possible to extrapolate from zones of inhibition determined with the disk diffusion methods to a prediction of therapeutic efficacy. However, a certain degree of imprecision is inherent in this extrapolation. A perfect one-to-one relationship between the MIC values and zones of inhibition rarely exists.

In order to establish a correlation between zone size and the MIC, a "scattergram" is prepared with zone sizes on the X axis and MIC values on the Y axis. The regression line is calculated from the formula of least squares and considers the $\log_2$ of the MIC values as the independent variable and the zone diameter as the dependent variable. The use of regression lines affords quantitative interpretations of the range of MIC from zone diameters (3). Correlation coefficients calculated from linear regression analyses for individual organism-antimicrobial combinations are often as low as 0.70–0.80 (13). This relative lack of correlation can result in interpretive errors.

Interpretive zone standards can be established only when MIC breakpoints have been selected. The MIC upper limit for susceptibility must be lower than the level of drug attainable in the blood or tissues with clinically accepted dosage and route of administration. Strains falling into the intermediate category may be responsive to higher dosages or to levels of the drug achievable in excretory organs (9). The intermediate category provides a buffer zone that minimizes significance of minor variables that cannot be completely controlled.

Agar Overlay Method

For this method, four–five colonies of the same type are selected and a visible turbidity suspension of bacteria is prepared in 0.5 ml of brain–heart infusion broth. The broth is then incubated in a water bath at 35°C for 4–8 hr. A 0.001-ml calibrated loopful of the broth culture is transferred to 9.0 ml of a 1.5% aqueous solution of agar which has been maintained between 45 and 50°C in screw-capped tubes. The seeded agar is thoroughly mixed by gentle inversion and spread evenly over the surface of a 150-mm Petri plate containing Mueller–Hinton agar.

The inoculated plates are allowed to stand for 3–5 min on a flat, level surface before antimicrobial susceptibility disks are dispensed. Preparation of Mueller–Hinton agar plates, application of antimicrobial agent disks, incubation procedures and interpretations of zone diameters are performed as described earlier for the standard Bauer–Kirby procedure.

Sources of Error in Diffusion Methods

Reliability of the results of the diffusion test depends on proper performance of the test. The factors that influence the results of the diffusion tests include the amount of antimicrobial agent in the disk; the agent's diffusibility in an aqueous medium, the pH, cation content, source, depth, age, and composition of the medium; the geometry of disk placement on the medium; the source, storage condition, and age of the disks; the age and turbidity of the inoculum; the way in which the inoculum is spread on the plate; the type of swab used for spreading the inoculum; the temperature, atmosphere, and time of incubation; the method of reading results; reading of test results before the full 16–18 hr of incubation; and inaccurate preparation and maintenance of turbidity standards (2, 9). Excessive delay in the application of the disk after the inoculation of the plates will result in inaccurate antibiograms.

Limitations of Diffusion Methods

The methods are standardized for the testing of rapidly growing organisms, especially for *Enterobacteriaceae,* staphylococci, and most nonfermentative gram-negative rods. In addition, the members of *Streptococcus, Corynebacterium, Pasteurella, Actinobacillus,* and *Haemophilus* genera can be tested provided the agar medium is supplemented with nutrients that permit good growth without seriously affecting the diffusion and potency of the drug. The polymyxins diffuse poorly in agar and the zone diameter is dependent upon the inoculum density (3). Lack of control in the diffusion process and in the size of inoculum can generate a false resistance pattern. Combinations of two antimicrobial agents cannot be tested properly with the disk diffusion techniques. Certain types of resistance are not fully expressed in the disk diffusion methods; consequently, they remain undetected.

Quality Control

In vitro susceptibility test results that correlate well with *in vivo* responses are entirely dependent on quality control measures. Strict adherence to the standard test protocol will alleviate some of the problems. To maintain high standards, susceptibility tests must be

monitored and evaluated using standard quality control strains of bacteria. By testing bacteria with known MIC values and zones of inhibition, microbiologists can uncover faults in techniques or problems with media or reagents. Standard control strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923, ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), and *Streptococcus faecalis* (ATCC 29212) have been recommended by the NCCLS for monitoring the accuracy of susceptibility tests (Table 35-2). The first three strains

Table 35-2
Zone Diameter Limits and MIC Values for Quality Control Strains[a,b]

Antimicrobial agent[c]	Zone diameter limits (mm)			MIC (μg/ml)		
	EC	SA	PA	EC	SA[d]	PA
Amikacin (30 μg)	19–26	20–26	18–26	1–2	2	4–8
Ampicillin (10 μg)	16–22	27–35	—[e]	2	0.5	VR[f]
Augmentin (20/10 μg)	19–25	28–36	—	—	—	—
Bacitracin (10 U)	—	17–22	—	—	—	—
Carbenicillin (100 μg)	23–29	—	18–24	4–8	4	32
Cephalothin (30 μg)	17–22	29–37	—	8	0.12–0.25	VR
Chloramphenicol (30 μg)	21–27	19–26	—	4	4	VR
Clindamycin (2 μg)	—	24–30	—	VR	0.06–0.12	VR
Colistin (10 μg)	11–15	—	—	—	—	—
Erythromycin (15 μg)	—	22–30	—	VR	0.12–0.25	VR
Gentamycin (10 μg)	19–26	19–27	16–21	0.5	0.5–1	2–4
Kanamycin (30 μg)	17–25	19–26	—	2–4	1	VR
Methicillin (5 μg)	—	17–22	—	VR	1–2	VR
Nafcillin (1 μg)	—	16–22	—	—	0.12–0.5	—
Nalidixic Acid (30 μg)	22–28	—	—	2	VR	VR
Neomycin (30 μg)	17–23	18–26	—	—	—	—
Nitrofurantoin (300 μg)	20–25	18–22	—	8	16	VR
Novobiocin (30 μg)	—	22–31	—	—	—	—
Penicillin G (10 U)	—	26–37	—	VR	0.25	VR
Polymyxin B (300 U)	12–16	7–13	11–16	—	—	—
Streptomycin (10 μg)	12–20	14–22	—	—	—	—
Sulfisoxazole (300 μg)	18–26	24–34	—	—	—	—
Tetracycline (30 μg)	18–25	19–28	—	2	0.5	—
Ticarcillin (75 μg)	24–30	—	22–28	2–4	4	16
Tobramycin (10 μg)	18–26	19–29	19–25	0.5	1	1
Trimethoprim/ sulfamethoxazole (1.25/23.75 μg)	24–32	24–32	—	≤9.5/0.5	≤9.5/0.5	—
Vancomycin (30 μg)	—	15–19	—	VR	1	VR

[a]EC, *Escherichia coli* ATCC 25922; SA, *Staphylococcus aureus* ATCC 25923; PA, *Pseudomonas aeruginosa* ATCC 27853.
[b]Adapted from the fourth edition of *Manual of Clinical Microbiology* (1) and the second edition of *Antibiotics in Laboratory Medicine* (3).
[c]Numbers in parentheses indicate disk potency.
[d]*Staphylococcus aureus* ATCC 29213.
[e]—, Not available or not recommended.
[f]VR, very resistant to the drug.

are recommended for routine quality control measures, whereas the fourth strain, *S. faecalis*, is recommended only to test the performance of every new batch of Mueller–Hinton agar plates. Laboratories with a record of satisfactory performance for 30 consecutive days may reduce the frequency of testing the control strains to once a week.

Susceptibility Testing of Anaerobes

The need to conduct susceptibility tests on clinical anaerobic isolates has been a controversial topic. Testing of all anaerobic isolates appears to be unnecessary since many of the isolates have predictable antibiograms to commonly used antimicrobial agents. Clinical significance of anaerobic isolates is frequently questioned by many practitioners in veterinary medicine. This argument is no longer valid. Proper collection of specimens from conditions that are suggestive of anaerobic infections is very important for the determination of significance of the culture results. There is increasing evidence that anaerobes are clinically significant in a variety of clinical conditions. Therefore, the susceptibility testing of anaerobes is just as important as the susceptibility testing of aerobes. Agar diffusion tests are not recommended for anaerobes because of the variable growth rates of anaerobic bacteria. However, several dilution methods have been described in the literature for routine susceptibility testing of clinical anaerobic isolates (1, 3). The methods commonly used include agar dilution tests, macro and microdilution tests, and broth disk tests.

The agar dilution test is the reference method for susceptibility testing of anaerobes. This method is not practicable for routine testing of small numbers of clinical isolates. The macrodilution method is complex and cumbersome and rarely used for routine testing of clinical isolates, whereas the microdilution method is very efficient, less involved, and provides MIC values. The broth disk method has been shown to give results similar to those obtained by the agar dilution and the microdilution methods (3). This method is highly recommended for the average veterinary microbiology laboratory. It is simple to perform and provides accurate results. Unlike the other two tests, results of the broth disk test can easily be translated into determination of a susceptible or resistant category. For methodology, the reader is referred to *Manual of Clinical Microbiology* (1) and *Antibiotics in Laboratory Medicine* (3). The following are the most commonly used antimicrobial disks in veterinary clinical laboratories for anaerobes: ampicillin (10 μg), carbenicillin (100 μg), cephalothin (30 μg), chloramphenicol (30 μg), clindamycin (2 or 10 μg), metronidazole (80 μg), penicillin G (10 U), and tetracycline (30 μg).

Automation in Susceptibility Testing

Automation in the susceptibility testing of veterinary isolates has not developed as rapidly as it has for human isolates. However, considerable effort has been made by veterinary microbiologists to develop automated and semiautomated systems for susceptibility testing. The systems in current use are based on the turbidometric or fluorometric evaluation of bacterial growth in the presence or antimicrobial agents. These systems can yield either a category result or an MIC after a relatively short period of incubation. Most of these systems also contain components for identification of gram-positive and gram-negative microorganisms. In addition, they may contain components for research activities, data management, urine screening, and yeast identification. Some of the systems have been modified by microbiologists and industry, without compromising the quality of the results, to meet individual laboratory needs and requirements. The systems that are commonly used by veterinary microbiologists in the Unites States include: Sensititre (Radiometer/Copenhagen Company), Automicrobic System (Vitek), Dynatech MIC-2000 (Dynatech Laboratories), Autobac (Organon Teknika Corp.), and MS-2 (Abbott).

The results obtained with these systems can be affected by the kind of organism being tested and by the antimicrobial agent being tested. Most of the systems listed here provide results within 3–6 hr. This short incubation period may not be long enough for the production of antimicrobial-modifying enzymes in adequate quantity to render the drug inactive. As a consequence, the organisms would be erroneously reported as susceptible. However, this problem can be minimized or eliminated by changes in the computer programs. In the automated systems, short incubation is also known to yield false susceptibility profiles with heteroresistant staphylococci tested against oxacillin or nafcillin. The heteroresistant staphylococci cultures contain both susceptible and resistant populations. The latter are blamed for the problem as they grow very slowly in the culture medium. Although standard strains are available and recommended for the quality control of automated systems, in most cases they are not helpful. The reader is advised to follow the owner's manual for precise information on quality control.

Selection of Antimicrobial Agents

A battery of antimicrobial agents chosen for routine testing of gram-positive and gram-negative bacteria may vary from laboratory to laboratory. A number of factors are taken into consideration in selecting and using an antimicrobial agent for routine testing. The selection of

the most appropriate antimicrobial agents to test is a decision best made by microbiologists in close consultation with practitioners and pharmaceutical industries. When adding a new antimicrobial disk, the microbiologist may want to conduct a market survey to determine the demand for such inclusion. Feedback from practitioners about new disks is essential for the determination of further inclusion of the disk for routine testing. Antimicrobial agents that should be considered for routine testing of bacteria by veterinary diagnostic laboratories are listed in Table 35-3

Table 35-3
Suggested Antimicrobial Agents for Routine Testing[a]

Antimicrobial agent	Gram-pos	Gram-neg	Anaerobes	Milk[b]	Food animals[c]	Companion animals[c]	Avian pathogens	Urinary tract pathogens
Amikacin	T[d]	T	—[e]	T	T	T	—	—
Ampicillin	T	T	T	T	T	T	—	—
Apramycin	—	T[f]	—	—	T	—	—	—
Augmentin	T[g]	—	—	—	—	T[g]	—	T
Bacitracin	—	—	—	—	—	—	T	—
Carbenicillin	—	T	T	T	—	T	—	T
Cephalothin	T	T	T	T	—	—	—	T
Chloramphenicol[h]	T	T	T	—	—	T	—	T
Clindamycin	T	—	T	—	T	T	T	T
Cloxacillin	T	—	—	T	T	T	—	—
Erythromycin	T	—	—	T	T	T	T	—
Gentamycin	T	T	—	T	T	T	T	T
Kanamycin	T	T	—	—	T	T	—	T
Metronidazole	—	—	T	—	—	—	—	—
Nafcillin	T	—	—	—	—	—	—	—
Nalidixic acid	—	T	—	—	—	T	—	T
Neomycin	—	T	—	T	T	T	T	—
Nitrofurantoin	T	T	—	—	T	T	T	T
Novobiocin	T	—	—	T	—	—	—	—
Penicillin G	T	—	T	T	T	T	T	T
Polymyxin B	T	T	—	—	T	T	—	—
Spectinomycin	T	T	—	—	T	—	—	—
Streptomycin	T	T	—	T	T	—	T	—
Sulfonamides	T	T	—	T	T	T	T	T
Tetracycline	T	T	T	T	T	T	T	T
Ticarcillin	—	T	—	—	—	T	—	—
Tobramycin	—	T	—	T	—	T	—	—
Trimethoprim/ sulfamethoxazole	T	T	—	T	T	T	—	T
Vancomycin	T	—	—	—	—	—	—	—

[a]Adapted in part from the fourth edition of *Manual of Clinical Microbiology* [1].
[b]Drugs currently available for intramammary infusion include ampicillin, cephalothin, cloxacillin, erythromycin, novobiocin, penicillin, streptomycin, and tetracycline. Streptomycin is available only in combination with penicillin for infusion.
[c]May differ from laboratory to laboratory; equine species are included in the companion animal category.
[d]T, Testing suggested or recommended.
[e]—, Testing neither suggested nor recommended.
[f]Recommended primarily for *Escherichia coli* of porcine origin.
[g]Recommended primarily for *Staphylococcus* spp.
[h]Not recommended for food animals.

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36

Rapid Methods of Identification

G. R. Carter and M. M. Chengappa

Introduction

Great progress has been made in the last decade in the development of methods and technologies for the rapid identification of bacteria of medical and veterinary significance. For economic reasons most of the rapid procedures and systems have been developed primarily for public health microbiology laboratories. However, some of the methods, new technologies, and systems can be used in veterinary diagnostic laboratories. That they are not used more in such laboratories, particularly those in developing countries, is mainly because of cost and unavailability.

The ultimate technological goal in the clinical microbiology laboratory is the rapid identification of bacteria by automated means. This is now possible for some bacteria as a result of combining computerization and advanced automation technology. The available semiautomated and automated systems for identification and antimicrobial susceptibility testing will be dealt with briefly below. They are not widely used for identification purposes in veterinary laboratories because of cost and the absence of comprehensive data bases. Automated instruments for antimicrobial susceptibility testing are used in some "large volume" veterinary laboratories. For more detailed information on rapid identification, readers are referred to the discussions by Tilton (1) and the *Manual of Clinical Microbiology* (2).

In addition to the many miniaturized methods at present available, there are some genetic probes and a number of new immunoserologic detection systems in use. The future will see an expansion of the application of these relatively new approaches. The use of some fluorescent

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antibody procedures is now routine in most veterinary diagnostic laboratories.

Some approaches which may have promise for the detection and identification of microorganisms are discussed briefly by Tilton (1). Among them are flow cytometry, nuclear magnetic resonance, Fourier transform infrared spectroscopy, particle concentration fluoroimmunoassay, and circular intensity differential light scattering. Tilton (1) concludes that for the human clinical microbiology laboratory ". . . it is technically feasible for the laboratory to generate same-day results of culture and susceptibility testing on many isolates . . ." When this goal is achieved in the veterinary microbiology laboratory it will indeed be a boon to the veterinary clinician.

Rapid Tests from Primary Plates

There are several rapid tests that may be helpful in the preliminary stages of identification. These can aid in an early presumptive identification that can lead to more rapid diagnosis and treatment.

Gram Stain

The Gram stain, although essential, sometimes gives equivocal results. Older cultures of gram-positive bacteria frequently stain gram-negative or gram-variable. Some *Bacillus* species stain gram-negative. Gram-positive bacteria that lose their cell wall activity for various reasons (e.g., exposure to antimicrobial agents) may stain gram-negative. Some clostridia species frequently stain gram-negative.

The procedures described below have been found useful to distinguish between truly gram-negative and gram-positive bacteria.

KOH Test

A loopful of growth from a colony is emulsified in a drop of 3% KOH on a slide. The suspension is stirred continuously for 60 sec. Gram-negative cell walls lyse, liberating chromosomal DNA, which causes the suspension to become viscid. The stringy DNA can be seen as the loop is drawn from the mixture. Gram-positive cells do not lyse and thus do not result in a viscid mixture.

LANA Test

When a swab impregnated with L-alanine-4-nitroanilide (LANA) is touched to a colony of a gram-negative bacterium, it turns yellow. Although this test and the KOH test are helpful with problem bacteria, they are not foolproof.

Vancomycin Susceptibility

Most gram-positive bacteria are susceptible to vancomycin. This test, along with those just mentioned, may be useful in determining the Gram status of a bacterium. The test for susceptibility is carried out by placing a 5-μg vancomycin disk on a heavily inoculated blood agar plate. A zone of inhibition after overnight incubation usually indicates a gram-positive organism. Some strains of lactobacilli are exceptions.

Most gram-negative bacteria are resistant to vancomycin but susceptible to colistin or polymyxin at 10 μg/ml.

Catalase Test

This rapid, useful slide test is described in Appendix B.

Spot Oxidase Test

This rapid test is described in Appendix B.

Spot Indole Test

This rapid procedure is described in Appendix B.

Spot Urease Test

This rapid test is described in Appendix B.

Among the organisms that are urease positive are *Proteus, Brucella, Citrobacter, Klebsiella, Shigella, Yersinia enterocolitica,* and *Cryptococcus neoformans.*

Miniaturized Methods

Most clinical microbiology laboratories in the United States rely heavily on rapid, miniaturized systems for identifying bacteria and yeasts. These systems are generally affordable and identify microorganisms in 4–24 hr. Test results of the miniaturized systems may be read and interpreted manually or in a semiautomated mode. The accuracy and reliabiliy of various systems for the identification of bacteria and yeasts have been reviewed thoroughly both by researchers and diagnosticians (1,2). Features of many miniaturized systems include the use of small amounts of media, convenient small cupules or chambers of

media, innovative inoculation and incubation techniques, and easy and quick interpretation of test results. Although the conventional method of identifying bacteria is accurate and reliable, it involves time-consuming preparation of media and inoculation procedures. In addition the conventional method requires considerable expertise in interpreting results.

Differences in the accuracy of identification of microorganisms among the various miniaturized systems are thought to be insignificant. Other criteria that should be considered in the selection of a system for a particular laboratory include the versatility of the system, the time required for preparation and inoculation of culture, the incubation time required for final test results, relative difficulty in determining positive and negative reactions, safety factors for laboratory personnel, shelf-life of the test kits, and price of the system.

Some of the more thoroughly evaluated miniaturized systems in use in the United States are listed in Table 36-1. At present, the most commonly used systems in veterinary diagnostic microbiology laboratories in the United States are the API systems.

API Systems

The API 20E system is widely used for the identification of gram-negative bacteria, especially those in the family Enterobacteriaceae. The system consists of a plastic strip with 20 cupules or chambers containing dehydrated substrates. After addition of standardized inocula (McFarland 0.5) of a test culture, the strips are incubated at 37°C for 5 hr for rapid identification or for 18–24 hr. Reagents must be added to some cupules before visually reading the color reactions of tests. The results are converted to a seven-digit profile using the manufacturer's coding system. Identification of the microorganism in question is subsequently made through the seven-digit profile index or through the computerized identification system established by the manufacturer. Reports indicate the results of the API 20E system are very reliable and show over 90% agreement with conventional methods.

The Rapid E system is similar to the API 20E except that substrates are not buffered, cupules are smaller, and the strips are incubated at 37°C for 4 hr.

The API 20A and the An-IDENT systems are for identification of gram-negative and gram-positive anaerobes. These systems also contain dehydrated substrates in cupules, as do the other API systems. The API 20A strip, after inoculation, is incubated anaerobically at 37°C for 24 hr whereas the An-IDENT strip is incubated aerobically at 37°C for 4 hr. Following incubation, test results are converted to

Table 36-1
Miniaturized Systems

System	Comments	Manufacturer
API	Most widely used in the United States and other countries; over 90% agreement with conventional procedures	Analytab Products, Inc., Plainview, New York
API 20E	Primarily used for Enterobacteriaceae	
Rapid E	Similar to the API 20E; results are read after 4 hr of incubation	
API 20A	Used for the identification of anaerobes; results are read after 24 hr of incubation	
An-IDENT	Used for the identfication of anaerobes; results are read after 4 hr of incubation	
Rapid NFT	Used primarily for the identification of nonfermenters	
API 20S	For the identification of *Streptococcus* spp.	
Rapid STREP	For the identification of *Streptococcus* spp.; results are read after 4 hr of incubation	
Staph-IDENT	For the identification of *Staphylococcus aureus* and coagulase-negative staphylococci	
API 20C	For the identification of yeasts	
Oxi/Ferm	For nonfermenters and oxidase-positive fermenters	Roche Diagnostics, Nutley, New Jersey
Enterotube II	For Enterobacteriaceae	Roche Diagnostics, Nutley, New Jersey
R/B Enteric	For Enterobacteriaceae	Roche Diagnostics, Nutley, New Jersey
Flow N/F	For nonfermenters and oxidase-positive fermenters	Flow Laboratories, Inc., McLean, Virginia
Enteric-Tek	For Enterobacteriaceae	Flow Laboratories, Inc., McLean, Virginia
Anaerobe-Tek	For anaerobes	Flow Laboratories, Inc., McLean, Virginia
Uni-yeast-Tek	For yeasts	Flow Laboratories, Inc., McLean, Virginia
Micro-ID	For Enterobacteriaceae	Organon Teknika, Durham, North Carolina
Quad Enteric Panel	For Enterobacteriaceae	Micro-Media Systems Inc., San Jose, California
Minitek	Not used as widely as the API systems; has very high percentage of agreement with conventional procedures. These systems are used for the identification of Enterobacteriaceae, nonfermenters, oxidase-positive fermenters, anaerobes, and yeasts	BBL Microbiology Systems, Cockeysville, Maryland
IDS RapID ANA	For anaerobes; results are read after 4 hr of incubation	Innovative Diagnostic Systems, Inc., Decatur, Georgia
Quantum II	For the identification of Enterobacteriaceae and oxidase-positive fermenters	Abbott Laboratories, Irving, Texas
Mini-ID	For Enterobacteriaceae	Scarborough Microbiologicals, Decatur, Georgia

a seven-digit number for identification of bacteria as in the API 20E system.

Other widely used API systems are the Rapid NFT system for nonfermenters, the API 20S and the Rapid STREP systems for the identification of streptococci, the API 20C for the identification of yeasts, and the Staph-IDENT system for the identification of *Staphylococcus aureus* and coagulase-negative staphylococci.

For information on other miniaturized systems, the reader should consult Tilton (1), the *Manual of Clinical Microbiology* (2), and journals containing articles on clinical microbiology. For information on the cost per test and the advantages and disadvantages of a particular system, the reader should contact the manufacturer's technical service department.

Immunofluorescence

The technique of immunofluorescence, commonly referred to as the fluorescent antibody or FA procedure, is now used routinely in clinical microbiology laboratories. The principles involved are now well known and thus will be dealt with only briefly below. For additional technical information on immunofluorescence, readers are referred to the supplementary references.

Fluorescent antibody reagents are prepared by coupling a fluorescent dye to specific immunoglobulin. This conjugated antibody will bind with its corresponding bacterium, virus, etc. The union is detectable by the presence of a characteristic fluorescence when viewed through a fluorescence microscope.

The great advantage of the FA technique is its rapidity. It enables an identification to be made within an hour after the smear is made. Another advantageous feature is that dead organisms can be recognized as well as live ones. The method is particularly helpful in the identification of fastidious organisms and organisms that are difficult to identify by conventional means, such as leptospires, *Clostridium chauvoei*, or *Cl. novyi*. It may obviate the need to work with cultures of organisms that are dangerous to humans. The procedure may be used effectively for the identification of organisms in clinical materials as well as from cultures. This may result in a considerable saving of time and materials, for example, in cases of tularemia, brucellosis, and psittacosis.

The major disadvantages of the FA technique are the cost of the reagents and the amount of time that is required to carry out the procedure. Isolation procedures can result in the recovery of various causal agents, but one FA reagent is specific for only one organism. Generally speaking, the FA procedures should be reserved for selected cases in

which the suspicion is strong that a particular organism is involved (e.g., blackleg, malignant edema, tularemia, leptospirosis,) or for the recognition of organisms that are difficult to propagate and identify.

Two procedures are used for the detection and identification of bacteria and fungi in smears. They are summarized briefly below:

1. Direct procedure: Labeled specific antibody plus "homologous" organism produces antigen–antibody union and fluorescence. The direct procedure is the one most commonly used for the identification of bacteria.
2. Indirect Procedure: This is carried out in two steps: (a) unlabeled specific antibody (e.g., rabbit origin) plus "homologous" organism yields antigen–antibody union; (b) adding conjugated antirabbit globulin produces fluorescence.

In the indirect test, the conjugated antiglobulin must have been prepared from the same animal species globulin as that from which the specific antiserum was obtained. For example, if a specific antibacterial serum was prepared in a goat, the antiglobulin would be prepared by inoculating an animal with goat globulin.

The indirect test has the advantage that the same conjugated antiglobulin can be used for all antisera produced in the species for which the antiglobulin was prepared. This makes it practicable, if necessary, to prepare one's own antisera (e.g., in rabbits).

Preparation and Fixing of FA Smears

The standard thickness of microscope slides is 1.2 mm, but some darkfield condensers require a slide of no more than 1 mm thickness. These "fluoroslides" have two etched circles 15 mm in diameter that facilitate the location of the specimen and aid in its retention within the circle. Number 1 coverglasses (0.13 to 0.16 mm) are suitable for most microscope objectives. Slides and coverslips should be as clean as possible.

Various smears are employed depending upon the nature of the specimen. In order to conserve reagent, small areas of the slide are usually used. If the "fluoroslides" are used, the smear is confined to the etched circles. Smears from tissue can be made by impression or by the smearing out of exudate, pus, fluid, etc., with a scalpel or an inoculating wire. If bacteria from a colony are to be examined, the smear is prepared in the same manner as for Gram staining. Experience will indicate the concentration of organisms that is desirable.

The slide is air-dried, and in the case of the smear from a colony of bacteria, it is usually lightly heat-fixed; however, other recommendations

for fixing may be called for with certain FA reagents. Smears from organs and tissue fluids are sometimes fixed in acetone for 10 min (e.g., specimens from suspected cases of blackleg and malignant edema). The use of 10% formol-buffered saline for 15–20 min is recommended by some workers.

Slides fixed in formol saline or other chemical fixatives, excepting acetone and methyl alcohol, must be washed in buffered saline and dried before the FA stain is added.

Staining and Mounting of Smears

The FA stain is added to the smear with a Pasteur pipette or with a large inoculating loop. Spreading of the stain over the whole smear may be facilitated with an inoculating loop or the end of a broken applicator stick. The slide is then placed in a moist chamber for 15–30 min. Various chambers are employed (e.g., instrument trays or a large Petri dish). Moistened filter paper is placed on the bottom, and the slide is placed on two glass rods above the moist paper.

Various washing procedures are employed. The following has been found satisfactory: (a) shake off excess stain and rinse briefly in buffered saline, pH 7.5; (b) transfer to a second container of buffered saline and leave immersed for ten minutes; (c) remove and dip in a container of distilled water; (d) drain and air dry; (e) mount by adding to the smear a drop of buffered glycerol saline (nine parts glycerol to one part (v/v) of buffered saline, pH 9.0, then coverslip. The coverslip can be fixed in position by adding a small amount of clear fingernail polish to each corner of the coverslip. If it is desired to keep FA-stained slides overnight, the mount is sealed all the way around with nail polish, then stored in the refrigerator. Some stained preparations can be stored at 4°C for several weeks or months.

In the indirect procedure, it is usually best not to dry the slide before adding the conjugated antiglobulin.

Before examining smears it is strongly recommended that operators thoroughly familiarize themselves with the instructions that accompany the fluorescence microscope. Most of the difficulties that are encountered can be avoided or resolved by reference to these instructions.

Application of the Direct and Indirect FA Techniques

The direct and indirect procedures are widely employed for the identification of microorganisms. Many reagents are available commercially. They are not all satisfactory, and they should be thoroughly

tested on known positive and negative materials before being used routinely. The monoclonal antibody technique has made possible conjugates of great specificity. Readers should consult the pertinent chapters for the use of FA for particular bacteria. The identification of fungi by FA has been summarized by Kaufman and Reiss (3).

Monoclonal Antibodies

The monoclonal antibody technique makes possible the production of antibody specific for a particular antigen, antigenic determinant, or epitope. These specific antibodies are being widely used in research and they have many practical applications including various procedures for the identification of microorganisms.

The steps enumerated below are involved in the production of monoclonal antibodies. There are a number of variations of this general procedure.

1. Mouse is injected with antigen or antigens.
2. Spleen cells (including plasma cells) are removed.
3. Spleen cells are fused to myeloma cells (antibody-producing tumor cells).
4. Resulting hybirds are grown in wells in a special medium.
5. Supernatants from wells are tested for required antibody.
6. Clones (descendants) secreting the desired antibody are selected and grown in bulk in culture or in the mouse peritoneal cavity.

Monoclonal antibodies are used in systems for the identification of *Brucella, E. coli* pilus antigens, and *Chlamydia psittaci.*

Enzyme Immunoassays

The enzyme-linked immunosorbent assay (ELISA) is now widely used. It has the advantage over radioimmune assays of not requiring a radioactive label. ELISA is used for detection of either antigen or antibody. The technique is similar to the indirect fluorescent antibody techniques except that the fluorochrome is replaced by an enzyme, usually horseradish peroxidase or alkaline phosphatase. Two techniques are used.

Sandwich Technique: The antibody is bound to plastic test tubes, beads or wells of a plastic plate. The antigen is then applied followed by an enzyme–antibody conjugate. The latter will not attach to the combined antibody–antigen. An enzyme substrate is

added to determine if enzyme is present. The latter's presence, which indicates a positive reaction, is apparent by the color change.

Double-Layer Technique: The antigen is bound to a plastic surface, such as that of a microtiter plate. The sample to be examined for antibody is then added. After incubation and washing the enzyme-linked immunoglobulin is added. The immunoglobulin used is of the same species as the antibody being tested. Addition of the enzyme substrate will detect the presence of enzyme by producing a characteristic color and thus indicate the level of specific antibody.

The colored product in both techniques can be read spectrophotometrically. As in other serologic procedures, there are many variables to control.

ELISA is widely used to detect and measure antibody to microbial pathogens and antigens. There are a number of commercial ELISA kits available for the identification of microbial pathogens and antigens. ELISA tests have been used to identify the following:

Escherichia coli (LT-labile toxin)
Brucella abortus
Yersinia enterocolitica
Staphylococcus aureus (toxin A)
Staphylococcus aureus (toxin E)
Streptococcus pyogenes
Streptococcus agalactiae
Aspergillus spp. (aflatoxin B)
Candida albicans
Cryptococcus neoformans

Counterimmunoelectrophoresis

Counterimmunoelectrophoresis (CIE) is an immunodiffusion test in which the reactants are driven by an electric current. It combines the advantages of electrophoresis and immunodiffusion. CIE is a rapid procedure most frequently used for the detection of antigen in a variety of human body fluids. The technique lacks the sensitivity of such procedures as coagglutination, latex agglutination, and immunoassay. It also lacks sensitivity in the detection and measurement of antibodies. It is rarely employed in veterinary diagnostic laboratories.

Latex Agglutination

In latex aggulination (LA), antigen or antibody is adsorbed to the surface of latex polystyrene beads. The addition of specific antibody or antigen results in agglutination. The tests are usually performed on slides.

The test is simple and rapid. Usually a drop or two of the antigen or antibody is mixed with the specific antibody or suspension of bacteria or body fluid. After it is mixed and left at room temperature for a short time, the mixture is examined for agglutination.

LA kits are available for the detection of antigens of the following:

Streptococcus groups A,B,C,D,F and G
Cryptococcus neoformans
Candida albicans
Staphylococcus aureus

In veterinary diagnostic laboratories LA tests are mainly used for grouping streptococci.

Coagglutination

Coagglutination (CA) is a phenomenon based on the ability of protein A-bearing *Staphylococcus aureus* (Cowan strain I) to bind IgG by the Fc fragment, thus leaving the antibody binding sites available to homologous antigen. If a specific antibody is attached to *S. aureus*, then the addition of the homologous antigen or bacteria will result in agglutination. In general this is a rapid, sensitive, reliable test.

A number of commercial CA kits are available for the identification of bacteria or antigens. The only one used to any extent in veterinary diagnostic laboratories has been employed for the grouping of streptococci.

Radioimmunoassay

In radioimmunoassay (RIA) techniques, antibody or antigen is detected and/or measured by labeling one of the reactants with a radioisotope, usually iodine. RIA has been largely replaced in diagnostic microbiology laboratories by the less expensive enzyme immunoassay. It has rarely been used routinely for diagnostic purposes in veterinary microbiology laboratories.

Genetic Probes

Recombinant DNA technology has developed new approaches to the identification of microbial pathogens. A number of what are called genetic probes have already been prepared for the recognition of clinically significant bacteria and viruses. The genetic probe depends upon the principle of nucleic acid hybridization for recognition.

The stages in the construction of a DNA probe are as follows:

1. Isolate the specific fragments of DNA that are characteristic of the bacterium. The specific or unique fragments are isolated by the use of restriction endonucleases.
2. When the specific DNA sequence has been isolated by restriction endonuclease analysis large quantities of the sequence are obtained by cloning.
3. Cloning involves insertion of the DNA into a multicopy vector molecule, usually a plasmid.
4. Restriction endonucleases are used to construct the recombinant plasmid containing the desired specific insert.
5. The recombinant plasmid containing the particular DNA is introduced into a host bacterium such as *E. coli* by transformation.
6. Plasmids multiply in the bacterial host.
7. Plasmids are isolated and the DNA probe fragment is isolated by restriction enzyme digestion.
8. The DNA probe is then labeled with radioactive nucleotide(s) using DNA polymerase, or with nonradioactive biotin-tagged nucleotide.

Details of the procedures used for various genetic probes will differ. In general, the procedure is as follows: (1) the clinical material or culture containing the unknown microorganism is lysed on a solid matrix such as a filter membrane; (2) the filter is then treated with alkalase to separate the double-stranded DNA into single strands of DNA (SS DNA); (3) the probe, consisting of a small segment of labeled single-stranded DNA, is added to the filter paper and allowed to reanneal with the fixed SS DNA on it; (4) the filter is washed to remove unbound DNA; and (5) the labeled DNA probe is now treated to prepare it for visualization.

To visualize a radioactive probe, a piece of film is placed over the filter. The film, when developed, will show dark spots where there is bound probe. With biotin-labeled probes, an enzyme-complexed avidin, which binds tightly to biotin, is added to the membrane. The avidin with an enzyme marker (e.g., horseradish peroxidase) can be detected when the enzyme substrate is added.

Another approach that shows considerable promise depends upon the detection of ribosomal RNA.

Although a number of genetic probes are being used in human clinical microbiology laboratories, none is as yet being widely used in veterinary clinical microbiology laboratories.

Semiautomated and Automated Systems

These systems have been developed in the last few years mainly for identification and antimicrobial testing in public health and hospital laboratories processing human specimens. Because the databases are not usually adequate for all bacteria of veterinary significance their use in veterinary diagnostic laboratories is limited. Their applicability will no doubt improve in the near future. Those instruments that do antimicrobial susceptibility testing are discussed in Chapter 35.

The various semiautomated and automated systems are referred to briefly below. They are discussed in more detail by Tilton (1). Manufacturers provide detailed information on these instruments and interested readers should obtain such information.

Although these systems have the advantage of speed of identification and a computer-stored database, one is locked into a specific set of biochemical data. There have been a number of reports in which the various systems have been compared and evaluated. These should be read prior to acquiring one of these expensive systems.

MS-2

This system can be used for antimicrobial susceptibility testing and bacterial identification. For the former an electrooptical system is used to determine growth patterns in the presence of antimicrobial compounds. Cartridges containing biochemical substrates are used for the identification of bacteria and yeasts.

API Aladin System

Aladin is a totally automated instrument that is designed to use a new group of miniaturized products (UniSept) from API. These consist of microcuples of dehydrated substances. The Aladin system inoculates, incubates, reads, and interprets according to the UniSept identification and susceptibility patterns.

The universal carrier, a disposable plastic frame, can contain an identification strip, susceptibility tray, and micro-MIC strip. The incubator

module can accommodate 60 universal carriers. The complete Aladin system consists of a floor model reading device and an external computer.

Autobac

In the Autobac IDX instrument the initial inoculum is standardized by a light-scattering photometer. There is an incubator–shaker module, a disk-dispenser for antimicrobial compounds, and a data terminal printer. Identification of bacteria is based on selective growth inhibition by antimicrobials, dyes, and other compounds. The instrument obtains profiles of growth inhibition by analyzing light-scattering indexes.

Automicrobic System

This automated system performs identification of gram-negative and gram-positive bacteria, yeasts, urine screening tests, and determines MIC values. It consists of a diluent dispenser, vacuum card-filling module, card-sealing apparatus, reader–incubator, computer control module, and data terminal printer. The cards used for identification of gram-negative and gram-positive bacteria contain substrates for the various biochemical tests. The substrates are automatically reconstituted with the inoculum. The card is placed in the reader–incubator module, which monitors microbial activity. The data are accumulated and interpreted and the probable correct identification is printed out.

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37

Diagnostic Approaches for Fish Diseases

Emmett B. Shotts, Jr.

When fish losses occur, the laboratory worker is often placed at a disadvantage by being presented an inadequate history along with a dead specimen. Diagnosis of maladies of fish is more involved than just the culture of a dead specimen. A knowledge of several associated disciplines is very important in determining the cause of death.

The most common problem associated with fish deaths is poor water quality, that is, insufficient O_2 or presence of toxic compounds both natural (NO_2, NH_3) and introduced. The next most frequent problem associated with fish losses is parasites, the vast majority of which are external and are usually found either on the gills or finage. Complicating these two causes of fish disease, and a third cause in its own right, are infectious diseases including those caused by bacteria, viruses, and fungi. Two other factors that often compound the problem associated with a case are nutritional deficiencies and/or stress resulting from borderline environmental quality.

This discussion will be restricted to infectious diseases and primarily to bacteria associated with deaths of fish.

Laboratory Procedures

Specimen Submission

The ideal specimen is a moribund fish collected and transported to the laboratory in environmental water. The second choice is a pithed moribund fish frozen in tap water and submitted frozen. In either case, dead specimens are not suitable for culture unless submitted as referred to above. It has been documented that environmental organisms

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tend to invade and replicate significantly within 30 min following death. In the initial submission, the water quality should also be evaluated and the fish examined for parasites. In the latter situation, only bacterial cultures of internal organs should be done.

Organs for Culture

The most common site for bacterial culture in fish is the hind kidney. This organ is usually located along the ventral side of the backbone and is protected from cavity contamination by a protective membrane. There are two methods for dissecting the fish to obtain a kidney culture. In both cases the fish should be pithed before dissection. In the first, make a scissor incision starting at the cloaca and cut in a half-moon fashion to a point just at the top of the gill. A second incision is made from the cloaca along the midline to the bottom of the gill. The tissue is pulled forward the gills and cut free from the fish. After reflection of the swim bladder(s) and visceral organs, the kidney should be apparent along the vertebrae as a dark reddish organ.

In the second method, external surface sterilization is important. The pithed fish is scaled, if necessary, along the central body area with subsequent disinfection using a paper towel soaked with either 70% alcohol or Roccal solution. The dorsal fin is clipped and using a hot searing spatula, a saddle-shaped sear is made using the center of the clipped dorsal fin for reference. A sterile incision in the seared area just through the backbone is made either using a scapel or large scissors. By downward pulling pressure, holding the head and tail portions, one can expose the fish kidney somewhat as if opening a shotgun for loading. If the fish is large and/or has been frozen this method is probably the more efficient. Very small fish often require special handling, and with some aquarium species two people are necessary to obtain suitable material for culture. Once the fish is open, a Gram stain of kidney tissue is made to determine if a bacterial cause should be considered.

Regardless of the method used, diagnosis should never be based on a single fish, but on data obtained from several moribund fish.

Diagnostic Approach

The results of the Gram stain will heavily influence how the case is handled beyond this point. The primary types of organisms associated with bacterial diseases of fish are gram-negative rods. This does not preclude the finding of gram-positive cocci or rods on rare occasions.

The gram-negative rods should be presumptively divided microscop-

ically into rods 1–3 μm × 0.5–0.8 μm and thinner, longer rods 2–12 μm × 0.4–0.8 μm. The former size is more characteristic of classic gram-negative rods, while the latter would suggest the presence of the genus *Flexibacter* (formerly myxobacteria). Gram-positive organisms that may be observed include streptococci, mycobacteria, *Nocardia* spp., and a corynebacterial-like organism belonging to the genus *Renibacterium*.

Media Selection and Bacterial Screening

Two options may be followed in media selection for the isolation of bacteria from diseased fish. The first is the use of a noninhibitory medium such as trypticase agar for transfer and identification. If this option is selected, it is strongly recommended that incubation be at 20–25°C (out of the mesophilic range) to retard rapid overgrowth of potential contaminants. While most bacterial pathogens of fish grow at 37°C, two (*Aeromonas salmonicida* and *Pseudomonas fluorescens*) are retarded at temperatures over 30°C. After 48 hr of incubation, noninhibitory isolation media may usually be examined and bacterial growth divided into two major groups on the basis of cytochrome oxidase for further identification procedures. In using noninhibitory isolation media, the laboratory worker should pick and examine a statistical cross section of all the "gram-negative like colonies" on the plate, since the colonial morphology of this group is so similar. Following this approach the oxidase-positive colonies are most likely members of *Pseudomonas*, *Aeromonas*, or *Vibrio* and may be further delineated using hanging drop motility, pigment, O-F glucose testing, and novobiocin or 0/19 susceptibility into groups pending any specific characterization by batteries of media inoculated at 48 hr. An effort should be made to establish a battery of media that does not include biochemical reactions prone to variation such as carbohydrate reactions. The oxidase-negative organisms associated with fish diseases belong primarily to the Enterobacteriaceae. Although the genera *Edwardsiella* and *Yersinia* are of primary concern, other enteric organisms may cause the death of fish and should be identified using conventional enteric procedures (see Chapter 10).

Due to antigenic similarities among the enterics and associated gram-negative rods, it is not reliable to depend too heavily on the use of conventional antisera to replace salient biochemical characteristics.

A second option available is the use of selective media for initial isolation with subsequent confirmatory tests. Media such as Pseudosel (BBL) for *Pseudomonas* isolation, Rimler–Shotts agar for *Aeromonas* (1), McConkey agar for gram-negative rods, and phenylethyl alcohol

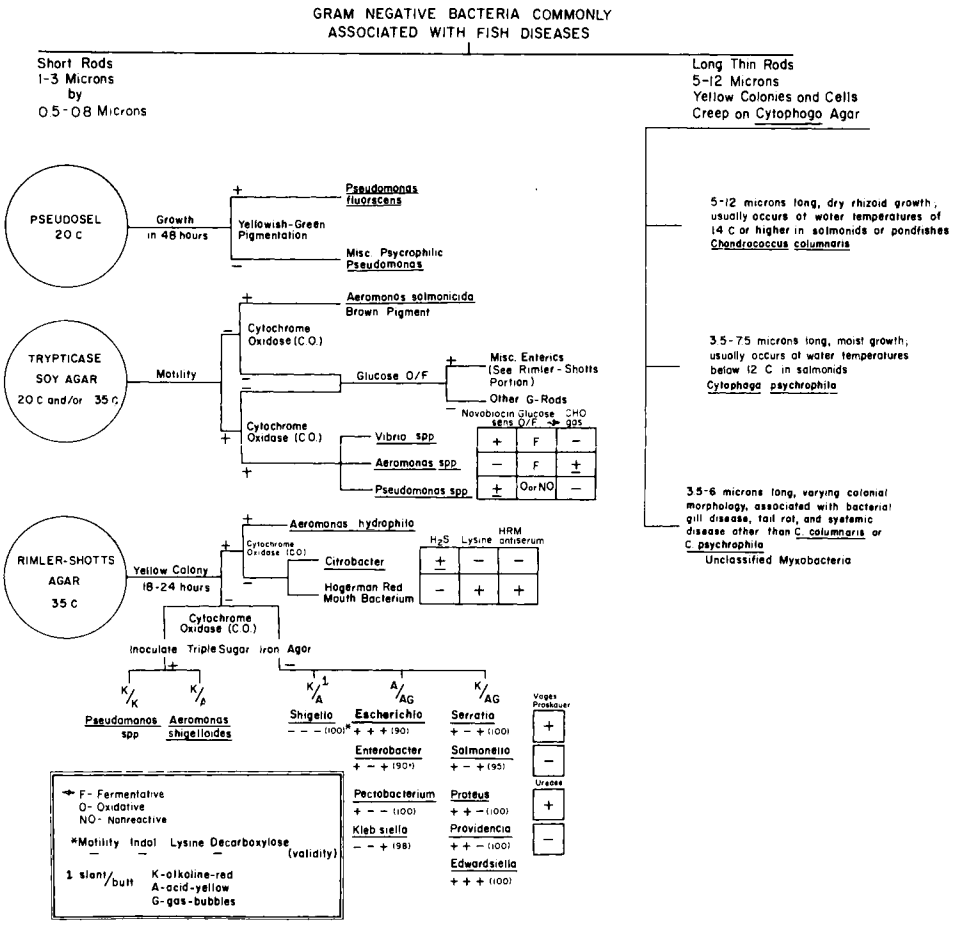

Figure 37-1. Gram-negative bacteria commonly associated with fish diseases.

agar (BBL) for gram-positive organisms are but a few that may be developed into a useful protocol. One such schematic aid is shown in Figure 37-1.

While short gram-negative rods may be identified in some detail, it is rare that organisms suggestive of flexibacteria are characterized beyond the general term of "columnaris disease." Based on recent work, there appear to be at least three genera and perhaps six species capable of producing gill rot, fin rot, or so-called "saddle back" in catfish. Diagnosis is based upon the presence of columns or "haystacks" of these organisms observed by direct microscopy of material from diseased fish.

Because of the infrequency of gram-positive organisms in bacterial diseases of fish, it is prudent that these be approached on an individual basis. The most frequent gram-positive bacterium of fish is *Renibacterium salmonis,* which causes kidney disease (KD) in salmonids. Another gram-positive rod which has been reported to cause losses in stressed spawning salmonids is *Lactobacillus piscicola,* which causes a condition sometimes referred to as a "pseudo-kidney disease."

Fish disease attributed to fungi is very uncommon in the United States and other parts of the world. Most of these fungi may be grown on either Sabouraud agar or blood agar incubated at 20°C. Microscopic observation of fruiting structures is employed for identification.

In the absence of any of the above etiologic agents, viral disease should be considered. There is one primary viral disease of warm-water cultured fish, channel catfish viral disease (CCVD). Another of some consequence in aquaria fish is lymphocystitis. In addition, a large number of viruses are associated with salmonid culture.

Some problems may arise in the identification of some of the bacterial pathogens of fish. The primary points of importance that should be given special note are discussed briefly below according to group. Consult the appropriate portions of this book for further identifying characteristics.

Significant Bacterial Pathogens

Aeromonas

Aeromonas hydrophila is perhaps the most common organism associated with the aquatic environment. It is thus important that cultures be taken with care and from moribund fish to ensure the validity of findings. This organism is easily distinguished from many isolates because it is oxidase positive and produces acid and gas in most instances from glucose. An organism with similar characteristics, *Aeromonas soberia,* has been proposed and, although DNA homologies support the division, the biochemical differentiation is unreliable. Confusion often arises as to whether or not isolates are of significance. It has been shown that strains that ferment salicin with the production of gas and cause a rapid reversion of the TSI slant to alkalinity in 24–48 hr should be considered of potential disease significance.

Aeromonas salmonicida appears to be a disease-producing obligate parasite of salmonids. Some fish carry this species in the intestine. Some atypical strains of this organism cause "ulcer" disease in goldfish and other species. While the typical strains produce a melaninlike pigment, the atypical strains as a rule do not. The atypical strains are

very fastidious and appear as small pinpoint colonies after several days incubation at 30°C or less. For this reason, it is important to streak plates carefully to assure good separation. Atypical strains produce very compact colonies that fracture when pressure is applied and remain intact when teased with an inoculating needle. Fluorescent antibody staining has been used successfully in the identification of *A. salmonicida.*

Pseudomonas

Species of this genus are found frequently in the aquatic environment and from time to time are involved in deaths. Members of the *Pseudomonas putida* group are often mistaken for *P. fluorescens* because growth temperature is not considered. *Pseudomonas fluorescens* does not grow at 37°C, while *P. putida*-like organisms do. Although Pseudosel (BBL) is used as a selective medium for *Pseudomonas* spp., some isolates will not survive on it. There have been two reports of deaths caused by an encapsulated, motile *Pseudomonas* spp.

Vibrio

This genus is primarily associated with disease in brackish or salt water, although outbreaks in fresh water have been reported. The primary organisms involved are two serotypes of *Vibrio anguillarum.* In general, they are superficially indistinguishable from *Aeromonas.* Separation is quickly made as shown in Table 37-1.

Edwardsiella

Two members of this genus, *E. tarda* and *E. ictuluri,* have been associated with fish deaths. *Edwardsiella tarda* has been reported from both septicemic outbreaks and localized lesions in warm water fish in the

Table 37-1
Characteristics Distinguishing *Aeromonas* and *Vibrio*

Reaction	*Aeromonas*	*Vibrio*
Arginine	+	−
Novobiocin susceptible	−	+
Gas from carbohydrate	±	−
0/129[a] susceptible	−	+

[a]McDaniel (2).

United States. This same organism is the primary cause of deaths in eels in the Far East. While the organism may be encountered in isolated instances, it is not of major importance in the United States. Although the majority of isolates are H_2S positive, negative strains are occasionally isolated from fish. The second species, *E. ictuluri*, a very slow-growing organism, was first described some 10 years ago in catfish fingerlings and has since resulted in a major disease problem in all ages of catfish primarily in the Southeast. Differences between these species are noted in Table 37-2.

Yersinia

Although a number of species of *Yersinia* have been associated with surface water, *Yersinia ruckeri* is the only species associated regularly with fish deaths. This organism appears to be an obligate parasite and may be isolated from the distal part of the intestine in carrier fish. Although the disease Hagerman red mouth was first associated with salmonids in the Hagerman valley of Idaho, isolates have been identified in culture collections at the National Fish Disease Laboratory, Leetown, West Virginia. The latter isolates were the first cases in the United States by some 10 years. Further studies have shown the existence of at least three serogroups, the Hagerman, the O'Leary, and Australian groups. Presently, the significance of isolations of the O'Leary group from fish is being studied in relations to certification for movement. Some diagnostic problems are involved, and inexperienced personnel may confuse this organism with *Hafnia alvei*, a common enteric bacterium of aquatic environments. These two organisms may be separated as shown in Table 37-3.

Streptococci

Streptococci, group B, have been recovered from some five extensive fish mortality problems in the past 10 years. The most recent of these occurred in Florida in 1987 (Keefe and E. B. Shotts, Jr., unpublished

Table 37-2
Characteristics Distinguishing *Edwardsiella tarda* and *Edwardsiella ictuluri*

Reaction	*E. tarda*	*E. ictuluri*
H_2S	+	−
Indole	+	−
Growth at 42°C	+	−

Table 37-3
Characteristics Distinguishing *Yersinia ruckeri* and *Hafnia alvei*

Reaction	*Y. ruckeri*	*H. alvei*
Malonate	–	+
Arabinose	–	+
Rhamnose	–	+

data). With rare exception, the streptococcal isolates from fish and reptiles are found to belong to a unique group. This is Lancefield group B, members of which are nonhemolytic and CAMP positive.

Renibacterium

For a number of years, this genus was thought to consist of a slow-growing, ill-defined species of *Corynebacterium*. Recently, it has been accepted as a new species, *Renibacterium salmoninus*. Although studied extensively, *in vitro* cultivation is a slow and exacting process. Currently, fluorescent antibody techniques are used for identification. The organism has only been found in salmonids.

Flexibacteria–Myxobacteria–Flavobacterium Complex

This very poorly defined group of bacteria have several things in common. First, they all have a yellowish orange pigment that turns red when 3% KOH is dropped on a colony. Second, they are very seldom cultured. Third, they are all interchangeably credited with causing fin rot, gill rot, and bacterial gill disease, and all are diagnosed microscopically as "columnaris disease." Actually, there are three or more genera and an unknown number of species associated with this type of disease in fish. Presently this group constitutes an ill-defined complex of organisms with G + C ratios ranging from about 30 to 70. It seems appropriate at present to refer to all these disease conditions by their common names pending further research.

Bacterial Reference Reagents

Direct fluoracien isothiocynate (FITC) conjugates for *Aeromonas salmonicida, Flexibacter columnaris, Vibrio* spp., *Edwardsiella tarda,* and *Yersinia ruckeri* have been prepared, standardized, and lyophilized

for reference purposes. They are available in limited quantities from the National Fish Health Research Laboratory, Route 3, Box 50, Kearneysville, West Virginia, 25430.

Viruses

When deaths persist in the absence of altered water quality, bacteria, or parasites, there is the possibility that losses may be due to viruses.

Very few significant viruses are currently associated with aquarium fish (lymphocystis virus) or warm water fish (channel catfish virus); however, a large number of viruses are associated with salmonids. They include both RNA and DNA viruses; however, the majority are RNA. Some of the more commonly encountered viruses of salmonids are the viruses of infectious pancreatic necrosis (IPN), of viral hemorrhagic septicemia (VHS), of infectious hematopoietic necrosis (IHN), and the herpesvirus of trout. The number of viruses currently associated with fish disease reflects the interest and research in this area of disease.

If a viral disease is suspected, appropriate tissues should be taken from moribund fish and frozen at −70°C pending processing or forwarding to a speciality laboratory for virus isolation. One such laboratory is located at the National Fish Health Research Laboratories, Kearneysville, WV.

Fungi

A large number of fungi have been associated with disease in fish. Most of them belong to the *Oomycetes* or to the Fungi Imperfecti. The most common of these are the *Saprolegnia* spp. This group of organisms appears as a mass of rapidly growing, nonseptate, multibranched cottonwoollike mycelia. Reproduction of this organism is both sexual and asexual. The asexual form consists of diflagellated zoospores arising from a sporangium. This organism is usually associated with injured fish tissue and is most commonly noted at water temperatures of 15–20°C. If isolation is desired, material is inoculated onto either Sabouraud agar or cornmeal agar and incubated at room temperature. Diagnosis is usually based on the microscopic examination of fresh material. Identification is based upon the presence of asexual sporangia containing zoospores and nonseptate mycelia. While a wide array of other mycotic organisms have been reported in fish, this organism is by far the most common.

A number of miscellaneous fungi have been isolated from systemic mycoses of fish. They include *Exophiala salmonis*, which causes cere-

bral mycetoma of trout; general infection caused by *Heterosporium* and Scolecobasidium; swim bladder infection caused by *Phoma herbarum:* infection with *Icthyophonus hoferi;* and dermal cysts caused by several species of *Dermocystidium.*

Although rare in the United States, branchiomycosis has been reported and may produce extensive damage in fish. The branchiomyces are unique because they grow intravascularly in the gills, producing a massive infarctive necrosis. This disease is caused by *Branchiomyces sanguinus* or *B. demigrans.* It is usually found at temperatures of 20°C or higher and spreads rapidly, with mortality often in excess of 50%. Fungal infections have also been reported due to *Basidiobolus* spp. and *Fusarium* spp.

In general, fungi may be found on or in fish or fish eggs as saprophytes and/or parasites. Their occurrence is usually associated with poor environmental quality such as high organic content, algal blooms, or highly fertilized water. Usually, the rapidity of spread is enhanced by an optimum temperature for the fungus involved.

Unless workers have had considerable experience in the identification of fungi, it is advisable to submit cultures to a research of reference laboratory for final identification. The procedures manual edited by McDaniel [2] will be helpful in the identification of many of the fungi.

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Appendix A

Staining Procedures

G. R. Carter

Staining solutions should be kept in airtight plastic or glass bottles. Because dyes are light-sensitive, they should be stored in a protective container, such as an amber glass bottle, or kept out of direct sunlight. Ready-to-use solutions of a number of stains are available commercially.

Gram Stain (Hucker's Modification)

1. Stock crystal violet
   - Crystal violet 10.0 g
   - Ethyl alcohol (95%) 100.0 ml
2. Stock oxalate solution
   - Ammonium oxalate 1.0 g
   - Distilled water 100.0 ml

   Crystal violet working solution: Mix 20 ml of solution 1 with 80 ml of solution 2. Additional dilution may be made if desired.
3. Gram's Iodine Solution
   - Iodine crystals 1.0 g
   - Potassium iodide 2.0 g

   Dissolve completely in 10 ml of distilled water, then add distilled water to make 200.0 ml

   Store in amber bottle.
4. Decolorizer
   - Ethyl alcohol (95%) 50.0 ml
   - Acetone 50.0 ml

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The following combination is not as likely to overdecolorize:

Ethyl alcohol	75.0 ml
Acetone	25.0 ml

5. Counterstain

Safranin	2.5 g
Ethyl alcohol (95%)	100.0 ml

Safranin working solution: The stock safranin is usually diluted 1:4 with distilled water.

An alternative counterstain is the following:

Basic fuchsin	3.0 g
Ethyl alcohol	100.0 ml

Procedure for Gram Staining

Gram staining is best performed on young cultures because older cultures often decolorize too readily.

1. Make a thin smear or film of clinical material. Bacteria from solid media are transferred to a drop of distilled water on a slide. A loopful of broth culture is placed directly on the slide. If the slides are not clean, material may wash off. New slides can be cleaned by soaking in 95% ethyl alcohol, then wiping dry with clean gauze. Allow the film to dry in the air. Drying is accelerated in the incubator. Fix the film by quickly passing the slide through the Bunsen flame several times. Proper fixing also prevents washing off.
2. The slide is flooded with crystal violet stain. Leave on 30–60 sec.
3. Pour off the stain and wash the remaining stain off with the iodine solution, leaving the slide covered with iodine for 1–2 min.
4. Wash off the iodine and shake the excess water from the slide.
5. Decolorize with acetone–alcohol until the decolorizer flows colorless from the slide. Some organisms are easily decolorized too much with acetone alcohol.
6. Counterstain with safranin for 30–60 sec and wash with water. Weak carbolfuchsin for 60 sec can be substituted for safranin. To obtain weak carbolfuchsin, dilute Ziehl–Neelsen carbolfuchsin 1:10 or 1:20 with distilled water.

Gram Stain of Smears from Lesions, Exudates, and Tissues

1. Alkaline Gentian Violet

Solution A

Gentian or crystal violet	1.0 g
Distilled water	100.0 ml
Solution B	
Sodium hydrogen carbonate ($NaHCO_3$)	1.0 g
Distilled water	100.0 ml

2. Iodine Solution

Iodine	2.0 g
1 *N* Sodium hydroxide (NaOH, 40.01 g/liter)	10.0 ml
Distilled water	100.0 ml

3. Counterstain
   Safranin or carbolfuchsin as in Hucker's modification, referred to previously.

Staining Procedure

1. Air-dry thinly spread films.
2. Flood slide with a freshly prepared mixture of gentian violet (solution A: 1.5 ml and solution B: 0.4 ml). Leave on for 5–10 min.
3. Wash with water, and blot water from surface of smear but do not dry.
4. Decolorize with acetone–ether (1:1). Ten sec is usually sufficient.
5. Dry in air and counterstain for 5–10 sec with safranin or carbolfuchsin.
6. Wash with water, blot, dry, and examine.

Ziehl–Neelsen Acid-Fast Stain

1. Carbolfuchsin Stain

Basic fuchsin	0.3 g
Ethyl alcohol (95%)	10.0 ml
This solution is mixed with	
Phenol (melted crystals)	5.0 ml

2. Acid–Alcohol

Hydrochloric acid (concentrated)	3.0 ml
Ethyl alcohol (95%)	97.0 ml

3. Counterstain
   Loeffler's methylene blue

Methylene blue	0.3 g
Ethyl alcohol	30.0 ml
Potassium hydroxide 0.01%	100.0 ml

Staining Procedure

1. Prepare a thin smear; dry and fix as described under the procedure for Gram staining.
2. A strip of filter paper the size of the smear is placed on the slide covering the smear.
3. Flood the slide with carbolfuchsin stain and heat to steaming with a low Bunsen flame. Allow to stand for 5 min without further heating, after which the paper is removed and the slide washed in running water. The stain should not boil.
4. Decolorize to a faint pink color by several applications of acid–alcohol. The decolorizer is applied until no more color comes out. This usually takes approximately 2 min for films of average thickness. Aqueous sulfuric acid (0.05%) is recommended for decolorizing suspected *Nocardia asteroides*.
5. Wash with water and counterstain with Loeffler's methylene blue for 20–30 sec.
6. Wash with water, dry, and examine.

Modified Ziehl–Neelsen Stain

This stain is useful for staining *Brucella* and chlamydia.

Stock carbolfuchsin:

Basic fuchsin	1.0 g
Absolute methyl alcohol	10.0 ml
Phenol, 5%	90.0 ml

Staining Procedure

Smears are stained for 10 min with a 1:10 solution of the stock carbolfuchsin, then washed with tap water. Decolorize with 0.5% acetic acid for 20–30 sec., then wash and blot dry.

Brucella and chlamydia stain red against a blue background.

Kinyoun Acid-Fast Stain

Basic fuchsin	1.0 g
Phenol (melted crystals)	8.0 ml
Ethyl alcohol (95%)	20.0 ml
Distilled water	100.0 ml

Dissolve the basic fuchsin in the alcohol and slowly add the water while stirring. The melted phenol is then added. Phenol crystals can be conveniently melted in a heated water bath.

Staining Procedure

1. Stain a fixed smear for 3–5 min with the staining solution described above. No heating is required.
2. The remaining procedures are the same as for the Ziehl-Neelsen method.

Loeffler's Methylene Blue Stain

This stain, like safranin or carbolfuchsin, can be used as a general-purpose stain when morphology is the primary consideration. With this solution, the spores of spore-forming bacteria appear as unstained bodies within blue bacilli. The beading and granules of the corynebacteria may also be revealed with this stain.

After this stain ripens (12 months), it is particularly useful for staining the capsules of *Bacillus anthracis*. The capsule appears as amorphous purplish material around the bacteria (McFadyean's reaction).

Methylene blue (1% in 95% ethanol)	30.0 ml
Potassium hydroxide (0.001% aqueous solution)	1.0 ml
Distilled water	100.0 ml

Staining Procedure

The fixed smear is stained with the above solution for 1 min. Wash with water and dry.

Motility Examination

Young organisms (6–8-hr culture) from media that do not contain fermentable substrates are examined. A method more convenient than the conventional hanging drop involves suspending a drop on the underside of a coverslip over a "perimeter" of petrolatum. The petrolatum is delivered to the slide from a 10.0-ml syringe through an 18-gauge needle. The coverslip is placed on the ridges of petrolatum in the same manner that one is placed over the concavity of a hollow-ground slide.

Because of sensitivity to oxygen, the hanging drop is not always satisfactory for clostridia. They can be examined in a capillary tube preparation from cooked meat cultures, sealed at each end.

Giemsa Stain

Stock Solution

To 0.3 g of Giemsa powder (National Analine or other satisfactory source) is added 25.0 ml of glycerin and 25.0 ml of absolute, acetone-free methyl alcohol. The ready-to-use staining solution is available commercially.

If the stain does not go into complete solution, it should be filtered. One volume of the stock solution is diluted with 10 volumes of distilled water. This diluted stain is ready for use.

Staining Procedure

1. Fix smear in methyl alcohol for 3–5 min.
2. Dry in air.
3. Immerse in diluted stain for 20–30 min. The staining period may be extended to an hour or longer as indicated by results.
4. Wash with distilled water.
5. Stand on end to dry, then examine.

Wright's Stain

Staining Solution

Wright's stain powder (certified)	0.1 g
Absolute methyl alcohol (acetone-free)	60.0 ml

The stain is ground with alcohol in a mortar, then allowed to stand in a stoppered bottle for a week to ripen. The ready-to-use solution is available commercially.

Buffer Solution

Potassium phosphate (monobasic) KH_2PO_4	6.63 g
Sodium phosphate (dibasic) Na_2HPO_4	3.2 g
Distilled water	1000.0 ml

Staining Procedure

1. Thin smears are air dried.
2. Flood smear with stain, counting the drops.

3. Stain for one to five minutes. Experience will indicate the optimum time.
4. Add an equal amount of buffer solution and mix the stain by blowing an eddy in the fluid.
5. Leave the mixture on the slide for 3–7 min.
6. Wash off by running water directly to the center of the slide to prevent a residue of precipitated stain.
7. Stand slide on end, and let dry in air.

Koster's Stain for *Brucella* (Slightly Modified)

1. Films or smears are dried and fixed over a flame in the usual manner.
2. Wet smear under the tap. Add to the slide two drops of saturated safranin solution and five drops of 1 *N* potassium hydroxide solution. Mix and leave on slide for 1–2 min.
3. Wash under the tap.
4. Decolorize with 0.1% sulfuric acid solution and repeat this operation, with a total decolorization time of 10–20 sec.
5. Wash thoroughly.
6. Counterstain with ordinary carbol-methylene blue solution for 2–3 sec. (Carbol-methylene blue solution: methylene blue 1 g, absolute ethyl alcohol 10 ml, and 100 ml of 5% aqueous solution of phenol.)
7. Wash with tap water, dry, and examine.

Brucella organisms, usually seen intracellularly, stain red, while other bacteria stain blue. Colonies of organisms from the cotyledons such as streptococci are stained clearly with this stain.

Relief or Negative Staining

Staining Solution

Nigrosin	10.0 g
Distilled water	100.0 ml

Boil this solution for 30 min, add 0.5 ml of formalin. Pass through filter paper and dispense in sterile tubes in 2-ml amounts.

Staining Procedure

1. Place a loopful of the bacterial suspension or clinical material on a slide.

2. Add an equal amount of nigrosin suspension.
3. Air-dry and examine.

Bacteria appear white against a dark background. This method is especially convenient for organisms with poor staining properties such as spirochetes and vibrios.

India Ink Wet Mount

Clinical material or organisms from cultures are mixed on a slide in a drop made up of loopfuls of distilled water and India ink. Experience will indicate the amount of India ink to use. A coverslip is added, and the preparation is examined. The large capsules of *Cryptococcus neoformans* show up strikingly by this method.

An alternative procedure is to place the coverslip on the material as above then add the India ink to the edge of the coverslip. The ink will seep under the coverslip and provide areas of variable density.

Capsule Stain (Hiss Method)

Mix a loopful of a suspension (physiological saline) of growth with a drop of normal serum on a microscope slide. Allow the slide to air dry, then heat fix. Flood the smear with 1% aqueous solution of crystal violet. Steam the slide gently for 1 min, then rinse with 20% aqueous solution of copper sulfate. Capsules appear as faint blue haloes around purple to dark blue cells.

Schaeffer and Fulton Spore Stain

Staining Procedure

1. Fix smear and flood with malachite green (5% aqueous solution).
2. Steam gently over a flame for 30 sec.
3. Wash with water and stain with safranin (0.5% aqueous solution) for 30 sec.
4. Wash with water, blot, dry, and examine.

Flagella Stains

Leifson Stain (2)

Flagella stain (powder) prepared according to the formulation of Leifson along with the staining procedure is available commercially.

Reagents

1. Solution A

Fuchsin (certified for flagella staining)	0.59 g
Ethyl alcohol (95%)	50.0 ml

Shake and leave overnight to dissolve.

2. Solution B

Tannic acid	1.5 g
Sodium chloride	0.75 g
Distilled water	100.0 ml

Solutions A and B are combined and mixed thoroughly. The stain is ready to use and remains satisfactory for 1–2 months if kept at 4°C. Don't disturb the precipitate when using.

Procedure

With a needle or loop, transfer a small clump of growth from a fresh plate culture to 3–5 ml of distilled water in a test tube. After the growth is in suspension a drop is placed on a clean microscope slide and allowed to air dry. Cover the slides with stain and leave on for about 5 min. Wash the stain off with water and slant to dry.

Silver Stain (3)

Reagents

1. Solution A

Saturated aqueous aluminum phosphate	25.0 ml
5% aqueous ferric chloride	5.0 ml
10% aqueous tannic acid	10.0 ml

2. Solution B
   a. Prepare 100 ml of 5% silver nitrate solution.
   b. Two to five drops of concentrated ammonium hydroxide is added to 90 ml of the silver nitrate solution just mentioned. A brown precipitate forms, which dissolves as more base is added. Stop adding just as the solution clears.
   c. Reverse the above procedure and add the remaining 5% silver nitrate solution 1 drop at a time until the stain solution is cloudy.
   d. The solution is stored in a dark bottle at room temperature. It is stable for several months.

Procedure

Slides are prepared as for the Leifson method described above. Flood the slide with solution A and leave for 4 min. Rinse with distilled water, then flood with solution B. Heat gently over a burner until steam is emitted. After 4 min staining, rinse with distilled water and dry in a slanted position.

References

1. Christoffersen, P. A.: Personal communication.
2. Leifson, E.: *J Bacteriol, 62:*377, 1951.
3. West, M., Burdash, N. M., and Freimuth, F.: *J Clin Microbiol, 6:*414, 1977.

Supplementary Readings

Clark, G. (Ed.): *Staining Procedures.* 4th ed. Baltimore, Williams & Wilkins, 1981.
Gurr, E.: *Encyclopedia of Microscopic Stains.* Baltimore, Williams & Wilkins, 1960.

Appendix B

Culture Media and Tests

G. R. Carter

A wide variety of media are available for the cultivation of pathogenic bacteria and fungi. Only the more widely used ones are referred to in this appendix. Most are available commercially in the dehydrated form. The name of the commercial source is given if the medium is one that is not available from most of the firms listed below. Commercial sources of the well-known media are not given, as they are widely available. Because the product of one firm is specified it does not necessarily indicate a preference. An equally satisfactory product may be available from another firm. The formulations and directions for the preparation of the commercially available dehydrated media are not given. These are available in the detailed and informative manuals and literature supplied by the manufacturers.

Sources of Commercially Available Media

The sources of the commercial media described in this section are indicated by the word or letters in the right-hand column below, corresponding with the full name and address of the firm given on the left.

Albimi Laboratories, Inc. 35–22 Linden Place Flushing, New York 11354	(Albimi)
BBL Microbiology Systems 250 Schilling Circle P.O. Box 243 Cockeysville, Maryland 21030	(BBL)

Diagnostic Procedures in Veterinary Bacteriology and Mycology, Fifth Edition

Difco Laboratories, Inc. (Difco)
P.O. Box 1058A
Detroit, Michigan 48232
Gibco Laboratories (Gibco)
P.O. Box 4385
2801 Industrial Drive
Madison, Wisconsin 53713
Oxoid U.S.A. Inc. (Oxoid)
9017 Red Branch Road
Columbia, Maryland 21045
Remel (Remel)
12076 Santa Fe Drive
P.O. Box 14428
Lenexa, Kansas 66215

Media for General Purposes

Collection of Blood for Media

In the smaller diagnostic laboratories, it is usually more convenient to collect blood in an anticoagulant solution. Blood collected in this manner has been found satisfactory for general purposes. Equine, bovine, and ovine blood is used.

The site over the jugular vein is clipped and generously disinfected with iodine. Blood is drawn into a 600-ml commercially prepared blood collection bottle containing 120 ml of anticoagulant acid citrate dextrose solution by means of a sterile, also commercially prepared, 36-inch-long blood collection set with siliconed needles. The needle on the one end of the collection set is inserted into the animal's vein, and when securely in place, the needle on the other end is inserted into the blood collection bottle. Blood is drawn up to the 600-ml level. This fresh blood is distributed into sterile screw-cap tubes in 15-ml amounts and stored in the refrigerator until used.

If defibrinated blood is preferred, blood is taken sterilely into a bleeding flask containing glass beads for the collection of the fibrin. Ox, horse, and sheep defibrinated blood is available from a number of commercial sources.

Blood Agar

Various blood agar bases are available commercially. Trypticase soy agar (BBL) is widely used. Blood agar base is rehydrated, distributed in flasks in 250-ml amounts, and autoclaved. Flasks of the base medium

are melted and placed in a water bath at 50°C. When the medium has reached 50°C, 15 ml of sterile blood is added to each flask. Plates are poured after mixing, then incubated before using.
Listed below are some commonly used media:

Tryptose broth (Difco)
Tryptose phosphate broth (Difco)
Trypticase soy broth (BBL)
Brain–heart infusion broth (Gibco)

The growth of fastidious organisms is increased if 0.5–1.5 g of agar is added to each liter of broth.

Trypticase soy agar (BBL)
Tryptose agar (Difco)
Brain–heart infusion agar (Gibco)
Thioglycollate media (See under *Clostridia*)

Brain–heart infusion semisolid medium is prepared by adding 1.5 g of agar to 1 liter of brain–heart infusion broth. Swabs may be placed in this medium, which supports the growth of many fastidious aerobes, microaerophilic, and anaerobic bacteria.

Oxidation and Fermentation Tests

The base media used for the conventional fermentation tests contain peptones that are broken down during bacterial growth to substances that are alkaline in reaction. Thus, acid must be produced in excess of the alkalinity derived from the breakdown of peptones if the indicator is to be altered.

The following indicators are commonly employed in carbohydrate base media.

Indicator	Medium	Acid when pH falls below
Phenol red	Broth base	6.0
Bromthymol blue	Broth base	6.0
Bromcresol purple	Peptone water	5.0
Andrade's indicator	Peptone water	5.5

The peptone water bases yield less alkalinity than do the broth bases such as the phenol red broth base described below, but they are not as nutritious.

Although the majority of the bacteria referred to in the manual ferment carbohydrates, some oxidize these substances, for example,

Pseudomonas spp. Those organisms that oxidize carbohydrates may not produce sufficient acid in conventional liquid media to change the indicator. Oxidation may be demonstrated when these bacteria are grown on the surface of solid media. In the case of *Pseudomonas* spp., an ammonium salt as a nitrogen source may be substituted for the peptone. An ammonium salt carbohydrate broth is described by Cowan and Steel (1). The O-F test described below may be used to determine whether the breakdown of the sugar is by oxidation or fermentation.

Oxidation–Fermentation Test

This test demonstrates whether the breakdown of carbohydrates is by oxidation or fermentation. Workers are referred to Cowan and Steel (1) for a comprehensive description and discussion of the test. The bacterium in question is grown in two tubes of Hugh and Leifson's (2) broth, which is heated for 5 min in boiling water prior to inoculation. One of the tubes is covered after inoculation with Vaspar seal (one part petrolatum, one part paraffin). Those bacteria that oxidize show acid production in the open tube only, while those that ferment produce acid in both tubes.

Listed below are some important bacteria that break down sugars by oxidation rather than by fermentation.

Micrococci (some strains do not split sugars at all)
Neisseria (some strains do not split sugars at all)
Mycobacteria, *Nocardia, Pseudomonas* spp.

The results obtained with some bacteria in the oxidation-fermentation (O-F) test are given in Table B-1.

Hugh and Leifson's O-F Medium (2)

This medium is used to determine whether an organism splits sugars by fermentation or oxidation (see above). It is available commercially as Bacto-OF Basal Medium (Difco).

It is prepared as follows:

Peptone	2.0 g	
Sodium chloride	5.0 g	
Potassium phosphate (dibasic) K_2HPO_4	1.5 g	
Agar	3.0 g	
Distilled water	1000.0 ml	1.0 liter
Bromthymol blue, 0.2% aqueous solution	15.0 ml	

Dissolve ingredients by heating in water. Adjust the pH to 7.1; filter and add the bromthymol blue. Autoclave at 115°C for 20 min.

Table B-1
Results Obtained with the Oxidation–Fermentation Test[a,b]

Organism	Glucose		Lactose		Sucrose	
	Open	Covered	Open	Covered	Open	Covered
Alcaligenes faecalis	–	–	–	–	–	–
Pseudomonas aeruginosa	A	–	–	–	–	–
Bacterium anitratum	A	–	A	–	–	–
Agrobacterium tumefaciens	A	–	–	–	A	–
Malleomyces pseudomallei	A	–	A	–	A	–
Shigella dysenteriae	A	A	–	–	–	–
Shigella sonnei	A	A	A	A	–	–
Vibrio comma	A	A	–	–	A	A
Salmonella enteritidis	AG	AG	–	–	–	–
Escherichia coli	AG	AG	AG	AG	–	–
Aeromonas liquefaciens	AG	AG	–	–	AG	AG
Enterobacter aerogenes	AG	AG	AG	AG	AG	AG
Unclassified species	A	A	A	–?	Variable	
Some paracolon	AG	AG	A	–?		

[a]From Hugh and Leifson [2].
[b]A, acid reaction; AG, acid and gas formation; –, no change or alkaline reaction.

Add the desired sugars as sterile solutions to convenient amounts of the medium to give a final concentration of 1%, and dispense in 10 ml amounts in sterile tubes.

Some results obtained with this test are given in Table B-1.

Phenol Red Broth Base

This is a base medium for fermentation tests. Carbohydrates are added to give final concentrations of 0.5–1%. Some laboratories add to this base medium 0.15% agar. This aids the growth of fastidious organisms. If the carbohydrate broths are sterilized by Seitz or other filtration, they are dispensed aseptically in sterile tubes. Lactose, sucrose, xylose, arabinose, trehalose, and salicin are best sterilized by filtration. The other carbohydrate broths are tubed and, if desired, carefully sterilized in the autoclave at 116–118°C (10–12 lb) for 15 min.

Liquid media are usually dispensed in cotton-plugged tubes or in screw-cap tubes or vials. If the former are used, considerable dehydration takes place, even in the refrigerator. The small laboratory may find it convenient to dispense carbohydrate broths and other media in tubes (14 × 100 mm) closed with standard 00 rubber stoppers. The stoppers are pushed down to seal the tubes after autoclaving. The sterilized tubes of media can be stored indefinitely at room temperature if there is a shortage of refrigerator space.

Carbohydrate Differentiation Discs (Difco)

These sterile discs contain a standardized amount of carbohydrate or other substrate for use in the differentiation and presumptive identification of bacteria based on fermentative or oxidative reactions. They may be used in a variety of carbohydrate-free nutrient broths, semisolid or solid media. See the manufacturer's literature for details.

Perhaps their most important advantage, aside from affording a rapid test, is that of providing a wide range of carbohydrates for immediate use. It is difficult for the small laboratory to maintain a comprehensive stock of carbohydrate broths. The discs may also be added to the thioglycollate medium (without dextrose and indicator) for fermentation tests on the anaerobes. This use is especially convenient in view of the need for fresh media where the anaerobes are concerned.

Special-Purpose Media

Litmus Milk (Oxoid)

Overheating will cause caramelization. Some laboratories add 0.1% peptone to litmus milk for the growth of anaerobes.

The important changes seen in this medium are the following:

Acidity: Lactose is fermented, and the indicator becomes red to pink.
Reduction: Indicator turns a pale pink or white.
Coagulation: This is produced by rennetlike enzymes or by acid production.
Peptonization and digestion: This is produced by proteolytic enzymes. The medium clears as digestion proceeds.
Alkalinity: The indicator turns a darker blue.
Stormy fermentation: The acid clot is broken up by gas production.

Schaedler Broth (BBL)

This medium, which contains hemin, is useful for the cultivation of fastidious obligate and facultative anaerobes. If used instead of BHI semisolid medium, 0.15% agar is added. Both of these media are tubed in 8–10-ml amounts prior to sterilization. Swabs can be placed in either medium after plate media have been inoculated.

Stuart's Transport Medium (Oxoid)

Dispense in screw-cap tubes, leaving room for expansion during autoclaving. After cooling, tighten the caps. As a precaution against possible leakage, the caps may be taped.

Convenient vials of suitable transport media are available commercially.

Blood Culture Media

1. The diphasic medium is prepared by adding 50 ml of tryptose or trypticase broth (containing sodium citrate 5 g/100 ml) to a 5-oz or 150-ml square, clear glass screw-cap bottle containing as a slant 40 ml of tryptose or trypticase soy agar. The broth can be added from a flask or bottle prior to inoculation of the blood.
2. For the growth of anaerobes and various fastidious organisms, a thioglycollate medium is frequently used. This medium (without indicator but with dextrose) containing 0.5% sodium citrate is dispensed in 4-oz bottles in 50-ml amounts. It is a good practice to inoculate one-half of the blood specimen (heparinized, EDTA, or in sodium citrate) to the diphasic medium above and the other half to the 50-ml bottle of thioglycollate. The total blood specimen should be at least 10 ml. This amount of blood will not coagulate in 3 ml of 2% sodium citrate. Sterile Vacutainer tubes,

containing sodium polyanethol sulfonate solution are available with a drawing capacity of 8.3 ml.

Bottles of prepared blood culture media are available commercially. The Liquoid blood culture bottle containing anticoagulant sodium polyanethol sulfonate is reported to be more efficient than conventional media.

Media for Specific Types of Bacteria

Readers should also consult the pertinent chapters where additional media may be described.

Streptococcus

Edward's Medium (Modified) (Oxoid)

Beef extract	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Crystal violet (final conc.)	1:750,000
Thallous sulfate or acetate (final conc.)	1:3000
Esculin	1.0 g
Agar	15.0 g
Distilled water	1.0 liter

Final pH 7.4

After sterilization, cool to 50°C, add 5–7% sterile whole citrated blood, mix, and pour plates. A blood agar base may be substituted for the first three items and the agar listed in the formula above. The esculin and the following amounts of inhibitory compounds are then added:

Crystal violet	1:1000 solution	1.3 ml
Thallous sulfate	1:10 solution	3.3 ml

Phenylethyl Alcohol Agar (BBL)

This medium is useful for the isolation of streptococci and micrococci from materials contaminated with gram-negative organisms, particularly *Proteus*. The latter form visible colonies, but their size and number are much smaller than on ordinary media. *Proteus* does not spread on this medium.

Salt Broth

This medium is used to aid in the identification of streptococci (see Table 17-2). It is prepared by adding sufficient sodium chloride to BHI

broth to give a final concentration of 6.5%. It is tubed in 3.0-ml amounts, then sterilized.

CAMP–Esculin Agar (See CAMP test)
This medium is used for the CAMP–Esculin test (see Table 30-1 and Fig. 34-1). It consists of blood agar with 0.1% esculin and 0.01% ferric citrate added prior to sterilization. A positive CAMP test on this medium is identical to that seen on regular blood agar: a semicircular or arrowhead-shaped zone of clear lysis in the partial zone of hemolysis of the staphylococcus. A positive esculin test is indicated by a browning of the medium around the streptococcal colonies.

Streptosel Agar and Broth (BBL)
This medium is used for the selective recovery of streptococci and *Erysipelothrix rhusiopathiae.* Coliforms, *Proteus, Pseudomonas,* and *Bacillus* spp. are markedly inhibited by the sodium azide and crystal violet.

Staphylococcus

Mannitol Salt Agar (BBL)
This is a selective medium that inhibits the growth of almost all organisms but micrococci and staphylococci. Plates are inoculated and incubated for 36 hr. Colonies of nonpathogenic cocci are small and surrounded by red or purple zones, while the mannitol-fermenting pathogenic organisms have yellow zones. It should be noted that not all acid-producing colonies are *Staphylococcus aureus,* as some strains of *S. hyicus, S. intermedius,* and *S. saprophyticus* ferment mannitol under aerobic conditions.

Vogel–Johnson Medium (Difco)
This medium is used for the isolation and recognition of coagulase-positive mannitol-fermenting staphylococci. These organisms appear as black colonies surrounded by a yellow zone after 24–48 hr of incubation at 37°C. Coagulase-negative, mannitol-negative staphylococci appear as black colonies in a red zone.

Baird–Parker Medium (Difco)
This is a selective medium for the isolation and recognition of coagulase-positive staphylococci. Prior to pouring plates, EY tellurite (tellurite egg yolk emulsion) enrichment is added to the agar base medium. Coagulase-positive staphylococci form black, shiny, convex colonies surrounded by a clear zone. Coagulase-negative staphylococci occasionally grow and produce black colonies with clear zones. They are

readily distinguished by the irregular appearance of their colonies and by the wide opaque zones surrounding them.

DNase Test Agar (Difco)
This medium is used to demonstrate the deoxyribonuclease activity of microorganisms. It has been observed that DNase activity correlates closely with coagulase production in strains of *S. aureus*. For this reason, some laboratories have tested for DNase activity instead of coagulase production. It should be kept in mind that some strains of *S. hyicus* and *S. intermedius* also produce DNase.

Listeria

To suppress contaminants, sufficient potassium tellurite may be added to blood agar to give a final concentration of 1:5000.

Erysipelothrix

Blood agar is satisfactory. Sodium azide yielding a final dilution of 1:1000 may be added to supress contaminants.

Streptosel Agar and Broth (BBL)
These media (see *Streptococcus* above) are of value in the recovery of *E. rhusiopathiae* from contaminated specimens.

Clostridium

Thioglycollate Media
Some of the thioglycollate media available and their corresponding uses are tabulated below.

Fluid thioglycoliate medium (Difco) (with dextrose; without indicator)	Isolation and growth of anaerobes; growth of various fastidious bacteria
Thioglycoliate medium (BBL) (without indicator and dextrose)	Fermentation tests; anaerobes. Several drops of bromthymol blue (0.2% aq. sol.) added after incubation
Thiol medium (Difco) (with dextrose; without indicator)	*Campylobacter*; tissues containing antimicrobial agents

Cooked Meat Medium (Difco)
The most convenient way to prepare this medium is to add to each screw-cap tube 1.25 g of the dehydrated product. Ten milliliters of cold distilled water is then added to each tube. Sterilize at 121°C for 15 min.

Prior to use, the medium should be heated in a boiling water bath for 10 min to remove oxygen.

This medium can be used to maintain stock cultures of anaerobes.

Fresh Blood Agar

This is a nutrient agar base to which sterile blood (commonly horse blood) is added. Tryptose blood agar (Difco) and Columbia agar base (Oxoid) are suitable base media, the anaerobic performance of which is improved by the addition of 0.05% cysteine hydrochloride before sterilization.

Prepare and sterilize 100 ml of the agar base medium. Cool to 50°C in a water bath. Add 7 ml sterile defibrinated horse blood and mix gently to avoid bubbles. Pour into petri dishes. Sterile sheep, human or other blood may be used instead of horse blood.

Stiff blood agar is prepared as above, but the agar concentration is supplemented to 3%.

Media for Fermentation Tests

Trypticase agar base (BBL) media or fluid thioglycollate medium (without dextrose and indicator) may be used. The latter medium is preferred for the more fastidious anaerobes. The filtered carbohydrate solutions are added to the latter medium prior to inoculation. If the carbohydrates are added before sterilization, autoclave carefully for 15 min at 116–118°C. After incubation of the thioglycollate fermentative media, the presence or absence of acidity is determined by the addition of several drops of 0.2% aqueous solution of bromthymol blue.

Potassium nitrate (1%) may be added to the thioglycollate medium if nitrate broth is desired; lead acetate and oxalic acid test papers may be suspended over cultures for the detection of hydrogen sulfide and indole, respectively.

***Cl. chauvoei* blood agar (After Batty and Walker, 1966)**

Broth base	94.0 ml
Liver extract (Oxoid)	3.0 g
Glucose	1.0 g
New Zealand agar	1.6 g
Defibrinated sheep blood	5.0 ml

Mix together the broth base, liver extract, glucose and agar, and sterilize by autoclaving at 115°C for 10 min. Cool to 50°C, add the sheep blood, mix gently and pour plates.

Cl. chauvoei grows poorly on ordinary blood agar, but good growth is obtained by supplementing the medium with liver extract. Sheep erythrocytes are susceptible to *Cl. chauvoei* hemolysin.

Cl. novyi (types B-D) blood agar. (NP medium of Moore, 1968)

1. Basal medium

Neopeptone (Difco)	1.0 g
Yeast extract (Difco)	0.5 g
Proteolyzed liver (Pabryn Labs)	0.5 g
Glucose	1.0 g
New Zealand agar	2.0 g
Salts solution*	0.5 ml
Distilled water	100.0 ml

*Salts solution contains (g/liter): $MgSO_4 \cdot 7H_2O$, 40; $MnSO_4$, 2; $FeCl_3$ (anhydrous), 0.4; concentrated HCl, 0.5 ml.

Preparation of basal medium: Dissolve the neopeptone, yeastextract, liver extract, and glucose in 50 ml of the water, add the salts solution, and adjust to pH 7.6–7.8. Mix this with a hot solution of agar prepared from the agar and the remaining 50 ml water. Dispense in 18-ml volumes in 28-ml screw-capped bottles and sterilize by autoclaving at 115°C for 10 min. Store the basal medium at 4°C.

2. Preparation of the reducing solution:

Cysteine hydrochloride	120.0 mg
Dithiothreitol	120.0 mg
Glutamine	60.0 mg
Distilled water	10.0 ml

Dissolve the constituents in the water, adjust to pH 7.6–7.8, and sterilize by filtration. The solution must be freshly prepared before use.

3. Preparation of the complete medium: Melt an 18-ml volume of the basal medium and cool to 50°C. Add to it 2 ml defibrinated horse blood and 0.15 ml reducing solution, mix gently and pour plates immediately.

Egg Yolk Suspension (McClung and Toabe, 1947)

Prepare an egg yolk emulsion by mixing egg yolk with an equal volume (about 20 ml per egg yolk) of sterile normal saline solution. The egg yolk is separated from the white by the usual culinary techniques; no special aseptic precautions are necessary.

Skimmed Milk (Reed and Orr, 1941)

Skimmed milk is prepared from fresh cow's milk by centrifugation in order to separate the cream. The milk is pipetted off, distributed in 20-ml volumes in screw-capped bottles, and autoclaved at 121°C for 10 min.

Lactose Egg Yolk Milk Agar (Willis and Hobbs, 1959)
Prepare a basal medium by mixing together 100 ml broth, 1.2 g New Zealand agar, 1.2 g lactose, and 0.3 ml of a 1% solution of neutral red. Autoclave the mixture at 121°C for 15 min. Cool this base medium to 50–55°C, and add to it 3.5 ml egg yolk emulsion and 15 ml sterile skimmed milk. Mix gently and pour plates immediately.

In addition to serving as a half-antitoxin egg yolk plate, this medium also shows bacterial lipolysis, lactose fermentation, and proteolytic effects.

Enterobacteria

MacConkey Agar
This medium is usually put up in flasks in convenient amounts. Plates and slants are poured from the melted medium as required. Lactose-fermenting enteric bacteria produce red colonies, while nonfermenters do not. Included among those producing pale, colorless colonies are *Salmonella, Proteus* spp., and *Alcaligenes faecalis.*

Preparations without crystal violet (BBL) allow the growth of the enterococci, which appear as pink or colorless pinpoint colonies.

MacConkey Agar No. 2 (Gibco) is recommended in growth tests for *Pasteurella haemolytica* and *Actinobacillus equuli.*

Selenite Broth
This medium, which should not be autoclaved, is dispensed in screw-cap tubes or small Erlenmeyer flasks to a depth of 2 inches. They are placed in flowing steam for 30 min. This is a selective, or enrichment, medium for the isolation of *Salmonella* and *Shigella. Proteus* and *Pseudomonas aeruginosa* are not inhibited. Plate to solid media after not more than 18–24 hr incubation.

Tetrathionate Broth
The base medium can be stored indefinitely, but after the addition of iodine, it should be used the same day. This is a selective and enrichment medium for the isolation of *Salmonella* and *Shigella.* Plate to solid media after not more than 18–24 hr incubation.

SS Agar
This medium is used for direct plating and also for subculturing. It favors the growth of *Salmonella, Shigella,* and *Yersinia* while inhibiting gram-positive organisms and many coliforms. Nonfermenters produce colorless colonies, while the lactose fermenters produce red or pink colonies.

Eosin Methylene Blue Agar (without sucrose)
This medium is used for direct plating and also for subculturing from selenite and tetrathionate broths. Colonies have the following appearances:

E. coli: Colonies have a metallic sheen.
Enterobacter, Aerobacter, and *Klebsiella:* Their colonies are brownish, frequently convex and mucoid with a tendency to coalescence.
Salmonella and *Shigella:* Transparent, amber to colorless colonies are produced.

Brilliant Green Agar
This is a highly selective medium recommended for the isolation of salmonellae directly from feces, less commonly from tissues, and also from previously inoculated enrichment broths. Large inocula may be used because of the strong selective capacity of this medium for salmonelle. *Salmonella* colonies appear as slightly pinkish-white, opaque colonies surrounded by a brilliant red zone. Lactose- or sucrose-fermenting organisms produce colonies that are yellow-green and surrounded by an intense yellow-green zone.

XLD Agar (Gibco)
This xylose lysine desoxycholate agar is used for the isolation of enteric pathogens including *Salmonella* and *Shigella.*

Hektoen Enteric (Oxoid)
This medium inhibits the growth of most enterobacteria except *Salmonella* and *Shigella. Salmonella* and *Proteus* colonies are blue-green, with or without a black center. Coliform colonies are salmon-pink to orange.

Malonate Broth (Difco)
This medium is used to determine if organisms, particularly of the enteric group, can utilize sodium malonate as a source of carbon. If malonate is utilized, an alkaline reaction is produced, resulting in the broth medium turning blue. Reactions of the various enteric species are given in Table 10–5.

Lysine Iron Agar (Difco)
This medium, which is tubed with a butt and a slant, is used in the differentiation of the enteric bacteria. Lysine decarboxylase production is evidenced by an alkaline reaction. Dextrose fermentation (yellow) and hydrogen sulfide production (black) are also shown in this medium.

The usual reactions with lysine iron agar after 48 hrs are the following:

Salmonella	K/KG H_2S[a]
Arizona	K/KG H_2S
Citrobacter	K/AG or K/AG H_2S
Providencia	K/AG or K/A
Proteus	K/AG, K/AG H_2S, or K/A
Shigella	K/A
Escherichia	K/A or N, K/AG, K/K, or K/KG
Enterobacter	K/AG or K/KG
Klebsiella	K/KG

[a]K, alkaline; A, acid; N, neutral; G, gas; H_2S, hydrogen sulfide.

Kligler Iron Agar (Oxoid)
This differential medium is used for the enterobacteria. It employs double sugar fermentation and hydrogen sulfide production. Detailed information on its use and the reactions obtained with genera of enterobacteria is provided by the supplier. Many laboratories prefer triple sugar iron (see below) agar to this medium.

Triple Sugar Iron Agar
This medium is dispensed in tubes that are one-third filled. After autoclaving, they are allowed to cool in the slanted position, the slant being such that a deep butt as well as slant is obtained. The slant is streaked and the butt stabbed. Observations are made after 18–48 hrs incubation. See Chapter 10 for the appearance of the various reactions.

Simmons Citrate Agar
Those bacteria capable of utilizing citrate as a sole source of carbon will grow on this medium. Visible growth is usually accompanied by an alkaline (blue) change of the indicator.

Inhibition of *Proteus Swarming*
The separation of various bacteria from spreading *Proteus* spp, is a frequent problem in the diagnostic laboratory. Contamination by *Proteus* is often due to the careless handling of specimens at necropsy. The procedures that follow have been found effective:

General: Swarming is inhibited if the agar concentration in blood agar is increased to 4%.
Gram-positive bacteria: Plate on a medium consisting of one-half of phenyl ethyl alcohol agar and one-half trypticase soy agar. This medium inhibits the growth of *Proteus* and other gram-negative bacteria. Sodium azide (1:5000) will also prevent swarming.
Gram-negative bacteria: MacConkey agar and deoxycholate–citrate agar inhibit swarming. The inhibition by the just mentioned medium is increased if the agar concentration is raised to 4%.

Tergitol 7 Agar (BBL)
This is a selective medium for the recovery of coliforms. It has been employed in the diagnosis of colibacillosis. *Escherichia coli* produces yellow colonies while those of *Enterobacter* are greenish yellow. The spreading of *Proteus* is inhibited.

Rappaport Medium (3)
This is used as an enrichment medium for *Salmonella choleraesuis* and *S. pullorum,* both of which can be inhibited by tetrathionate broth.

It can be obtained commercially or prepared as follows:

1. Solution A

Bacto tryptone (Difco or Oxoid equi.)	5.0 g
Sodium chloride	8.0 g
Potassium dihydrogen phosphate	1.6 g
Distilled water	1.0 liter

2. Solution B
(Kept as stock)

Magnesium chloride ($MgCl_2 \cdot 6H_2O$ A.R.)	40.0 g
Distilled water – make up to	100.0 ml

3. Solution C (Stock)

Malachite green (Merck)	0.4 g
Distilled water	100.0 ml

4. For use:

Solution A	100.0 ml
Solution B	10.0 ml
Solution C	3.0 ml

Distribute the medium in 10-ml volumes and sterilize by steaming for 30 min.

Yersinia

Yersinia Selective Agar Base (Oxoid)
This is a selective medium for *Yersinia enterocolitica.*

Pseudomonas

Cetrimide Agar (Pseudosel™ Agar—BBL)
This is a selective medium for the isolation of *Pseudomonas aeruginosa.* The medium favors the production of pyocyanin and fluorescin. Most other organisms are completely inhibited by the quaternary ammonium compound cetyl trimethyl ammonium bromide (cetrimide).

Pasteurella

Selective media for the isolation of *P. multocida and P. haemolytica* have been described by Morris (4). See Chapter 11 for additional selective media.

Francisella

Blood Cystine Dextrose Agar (Difco)
This medium is used for the isolation of *Francisella tularensis.* After sterilization, the medium is cooled to 60–70°C, and 18 ml of whole rabbit blood is added for each 300 ml of medium. After mixing well, plates and slants are poured.

Bordetella

MacConkey agar with 1% dextrose added is recommended as a selective medium for the isolation of *B. bronchiseptica* from the nasal passages of pigs (5). Small, grayish-tan colonies with hard centers are produced in 48 hr. This medium was altered by the addition of 20 μg/ml of furaltadone (6) and later by adding 200 μg/ml of nitrofurantoin (7).

A more complex selective medium was used by Rutter (8).

Smith–Baskerville Selective Medium (9) This medium makes possible the identification of *B. bronchiseptica* on primary culture. It is prepared as follows:

Bacto-peptone	20.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	857.0 ml

The pH is adjusted to 7.2 and these ingredients are autoclaved at 121°C for 15 min. The mixture is cooled and the following added:

A. Gentamycin	0.5 μg/ml	final/conc.
Penicillin	20.0 μg/ml	final/conc.
Furaltadone	20.0 μg/ml	final/conc.
B. Glucose (10% stock)	100.0 ml	
Lactose (10% stock)	100.0 ml	
C. Bromthymol Blue (0.2%)	40.0 ml	

A stock solution of bromthymol blue is made as follows:

Bromthymol blue	1.0 g
0.1 *N* NaOH	25.0 ml
Distilled water	475.0 ml
Optional: Amphotericin B	10.0 μg/ml

The medium is dispensed in conventional Petri plates. See Chapter 8 for the appearance of colonies of *B. bronchiseptica* on this medium.

Brucella

Brucella spp. grow well on many media that support the growth of fastidious organisms, e.g., Brucella agar (Albimi), Tryptose agar (Difco), Trypticase soy agar (BBL), and blood agar.

A *Brucella* Differentiation Kit, which includes media containing basic fuchsin and thionin, is available commercially (BBL).

"W" Medium (10)
To a liter of Albimi or tryptose agar add:

Polymyxin B sulfate	6000.0 U
Actidione	100.0 mg
Bacitracin	25,000.0 U
Circulin	1500.0 U
Crystal violet	1.4 mg

Add the crystal violet prior to sterilization by autoclaving at 15 lb. for 20 min. The antibiotics are added after autoclaving.

This formula was modified by Weed (11) by leaving out the crystal violet (may inhibit the growth of some *Brucella* strains) and enriching with 5% blood.

Campylobacter

See Chapter 6.

Haemophilus

See Chapter 13.

Leptospira

Fletcher's Medium Base (Difco)
Used for the isolation of Leptospira.

Stuart's Medium Base (Difco)

Leptospira Medium Base EMGH (Difco)
Used for the isolation and maintenance of Leptospira. See Chapter 5 for details.

Nonsporeforming Anaerobic Bacteria

Modified Cary and Blair Transport Medium

Cary and Blair Medium (BBL)	2.5 g
$CaCl_2$, 1% solution	1.8 ml
Resazurin solution (0.1% w/v)	0.1 ml
L-Cysteine HCl	0.1 g
Distilled water	198.0 ml

Heat ingredients in a flask with glass beads until the agar has dissolved. Cool to 50°C in an atmosphere of O_2-free CO_2. Add cysteine. Adjust pH to 8.4. Continue to gas with O_2-free N_2 to maintain pH. Dispense 10 ml into tubes approximately 16 × 125 mm while gassing both tubes and flask with N_2 (or carry out this stage in an anaerobe chamber). Stopper with airtight butyl rubber stoppers. Steam in a press for 15 min. Discard any tubes showing a pink color (i.e., not anaerobic).

Vitamin K_1–Hemin Supplement

1. Stock hemin solution: Dissolve 50 mg hemin in 1 ml 1 *N* NaOH. Add 100 ml distilled water, autoclave at 121°C for 15 minutes.
2. Stock menadione (Vitamin K_1) solution: Dissolve 100 mg menadione in 20 ml 95% ethanol and filter sterilize.

Add 1 ml sterile menadione to 100 ml sterile hemin solution; add 1 ml Vitamin K–hemin solution per 100 ml sterile medium.

Cooked Meat Carbohydrate Medium (anaerobes) Chopped meat (BBL.): Prepared according to manufacturer's directions; add 0.4% (w/v) glucose, 0.1% (w/v) cellobiose, 0.1% (w/v) maltose, and 0.1% (w/v) starch.

CHO Medium

CHO base (Difco)	26.0 g
Distilled water	900.0 ml

Mix and heat to dissolve. Autoclave at 121°C for 15 min. Cool to 50°C; add 100 ml sterile distilled water for base control or 100 ml filter sterilized carbohydrate solution (6% w/v). Mix well, adjust pH to 7.0.

Dispense 7ml in 15 × 90 ml screw-cap tubes. Before use store 24–48 hr in anaerobic conditions with loosened caps, or boil for 10 min, cool, and inoculate immediately. CHO fermentation medium can be prepared by making thioglycollate medium (BBL 135-C) without dextrose or indicator and adding 2 gm/liter of yeast extract (Difco) and 1 ml of 1% aqueous bromthymol blue solution.

Esculin Broth

Dissolve ingredients by boiling, adjust pH to 7.2, dispense 7 ml into 15 × 90 mm screw-cap tubes. Autoclave for 15 min at 121°C. Store for 24–48 hr anaerobically before use, or boil for 10 min with loosened caps and inoculate immediately.

Indole–Nitrate Broth

Indole-nitrate medium (BBL)	25.0 g
Distilled water	1000.0 ml

Dissolve by boiling for 1 min. with frequent mixing, adjust pH to 7.2, dispense 7 ml into 15 × 90 mm screw-cap tubes. Autoclave for 15 min at 121°C. Store at 4°C. Before use store 24–48 hr in anaerobic environment with loosened caps, or boil for 10 min with loosened caps and inoculate immediately after cooling.

Thiogel Medium

Thiogel medium (BBL)	90.0 g
Distilled water	1000.0 ml

Heat water to 50°C, add Thiogel medium and let stand for 5 min. Boil for 1 min with mixing and dispense in 7-ml aliquots into 15 × 90 mm screw-capped tubes. Autoclave for 15 min at 118°C. Store at 4°C. Before use, store 24–48 hr in anaerobic environment with loosened caps, or boil for 10 min, cool, and inoculate immediately.

Peptone–Yeast–Glucose Medium

1. Salts solution:

$CaCl_2$ (anhydrous)	0.2 g
$MgSO_4$	0.2 g
K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
$NaHCO_3$	10.0 g
NaCl	2.0 g

Mix and dissolve $MgSO_4$ and $CaCl_2$ in 300 ml distilled water. Add 500 ml distilled water and slowly add remaining salts. Continue with swirling until salts are dissolved. Add 200 ml distilled water, mix, and store at 4°C.

2. Resazurin solution:
   Dissolve one 11 mg resazurin tablet (Allied Chemical Co.) in 44 ml distilled water, or 25 mg powder (Baker, Difco) in 100 ml distilled water.
3. Peptone–yeast–glucose medium:

Peptone	20.0 g
Yeast extract (Difco)	10.0 g
Salts solution	40.0 ml
Cysteine HCl	0.5 g
Resazurin solution	4.0 ml
Distilled water	1.0 liter

Adjust pH to 7.2, dispense 5 ml in 15 × 125 mm screw-cap tubes, autoclave at 121°C for 15 min.

Media for Susceptibility Testing

Mueller–Hinton agar (BBL) is currently the only medium recommended for antimicrobial susceptibility testing of aerobes by the disc method. It can be enriched in order to enhance the growth of fastidious organisms. Supplements recommended (12) include 5% defibrinated blood (which may be chocolatized) or 1% hemoglobin plus 1% of a synthetic stimulant mixture: Iso Vitale X (BBL) or Supplement VX (Difco).

Although a few fastidious bacteria that fail to grow well on supplemented Mueller–Hinton agar will grow adequately on trypticase soy and brain–heart infusion agars supplemented with blood and vitamins, such media should not be used for routine disc susceptibility tests (12). Uncontrolled interactions with antimicrobial agents affect inhibition zone diameters in agars other than Mueller–Hinton, so that Bauer–Kirby interpretive criteria are inapplicable to them (13).

Biochemical Tests

Nitrate Reduction Test

Reagents

Solution 1

α-Naphthylamine	5.0 g
5 *N* acetic acid (sp. gr. 1.041)	1.0 liter

Filter through clean absorbent cotton.

Solution 2

Sulfanilic acid	8.0 g
5 *N* acetic acid (sp. gr. 1.041)	1.0 liter

Procedure

Add to 5 ml of trypticase nitrate broth culture 1 ml of solution 2, followed by 1 ml of solution 1 added drop by drop. If nitrite is present, a red, pink, or maroon color develops.

In the test for nitrate reduction, if the test is negative, either the nitrate has not been broken down or nitrate has been broken down further. Whether or not nitrate is present can be determined by adding a small amount of zinc dust. The amount is critical, so the same amount of zinc dust should be added to an uninoculated control tube. If nitrate is present, a red color is obtained as a result of the zinc reducing the nitrate to nitrite. If the zinc test does not indicate the presence of nitrate and the test for nitrite is negative, the nitrite has been broken down further and the organism is considered to give a positive nitrate reduction test.

Lead Acetate Test Papers

Strips of filter paper (5 × 50 mm) are impregnated with saturated lead acetate solution, then dried in an oven at 70°C. These are suspended over a suitable medium and held in place by the cotton plug. Suitability of a medium can be determined by trial with a hydrogen sulfide-producing strain. This test is many times more sensitive than the use of lead acetate agar or triple sugar iron agar. The production of hydrogen sulfide is only meaningful if the test procedure is given.

Solubility Test for *Streptococcus pneumoniae*

Prepare a 10% aqueous solution of sodium deoxycholate containing merthiolate 1:10,000 as preservative.

Procedure

Two drops of the sodium deoxycholate solution are added to 1 ml of a 24-hr culture. If the organisms are pneumococci, clearing occurs rapidly, usually in less than 1 min. Hold for 10–15 min before discarding as negative.

Coagulase Tests for Staphylococci

Production of coagulase by staphylococci is considered an important criterion of pathogenic potentiality. Fresh or commercially available lyophilized plasma may be used. Both rabbit and human plasma are satisfactory. Rabbit plasma may be obtained from blood taken sterilely

by cardiac puncture. The blood is transferred quickly to a bottle or tube containing sodium citrate solution (10 ml blood to 1 ml of 5% citrate).

Procedure

To two drops of an overnight broth culture in a Kahn or similar tube, add 0.5 ml plasma diluted 1:5 with sterile physiological saline; mix. One loopful of organisms from a solid medium is also suitable. Bring to 37°C in the incubator, or place in a 37°C water bath. If the strain is coagulase-positive, the plasma will clot, usually within 4 hr. Observe again at 24 hr. Partial clotting is considered a positive test. Plasma should be checked before use with a known coagulase-producing staphylococcus.

A slide procedure is used as a presumptive test. Sufficient bacteria are emulsified in a drop of water on a microscope slide to yield a thick suspension. The suspension is stirred with a straight wire that has been dipped in suitable plasma. A positive reaction is indicated by clumping within 5 sec. The factor tested for is sometimes referred to as the "clumping factor" rather than coagulase.

Methyl Red Test

Reagent

The methyl red solution is prepared by dissolving 0.1 g of methyl red in 300 ml of 95% ethyl alcohol and diluting to 500 ml with distilled water.

Procedure

To 5 ml of culture (incubated 5 days at 37°C in MR-VP broth) add 5 drops of methyl red solution. A positive reaction is indicated by a distinct red color, indicating acidity (pH 4.4–6.0). A yellow color constitutes a negative reaction.

Voges–Proskauer Test

This test is for acetylmethylcarbinol.

Reagents

Solution 1

5% alpha-naphthol in absolute ethyl alcohol

Solution 2

40% potassium hydroxide containing 0.3% creatine

Procedure

Transfer 1.0 ml of a 48 hr culture (37°C) grown in MR-VP broth to a Wasserman tube and add 0.6 ml of solution 1, then 0.2 ml of solution 2. Shake well and leave 5–10 min. A bright orange-red color develops and gradually extends throughout the broth if acetylmethylcarbinol has been produced.

An alternative procedure is to add 5 ml of 10% potassium hydroxide to 5 ml of culture. Mix well and allow to stand exposed to air. Observe at intervals of 2, 12, and 24 hr. A positive test is indicated by the development of an eosin pink color.

Indole Test

Reagent

p-Dimethylaminobenzaldehyde	2.0 g
Ethyl alcohol, 95%	190.0 ml
Hydrochloric acid (conc.)	40.0 ml

Store in dark bottle in the refrigerator.

Procedure

One milliliter of ether is added to a 5-ml portion of a 48 hr culture of organisms (media: trypticase nitrate broth, peptone water, or beef-heart infusion broth). Shake well, then allow to stand until the ether rises to the top. Gently run 0.5 ml of the reagent down the side of the tube so that it forms a ring between the medium and the ether. If indole has been accumulated by the ether, a brilliant red ring will develop just below the ether layer. If there is no indole, no color will develop. The use of ether makes for a more sensitive test but it can be omitted.

Spot Indole Test

1. For each day saturate a strip of filter paper (e.g., Whatman No. 1) with the indole reagent described above. This reagent is available from Sigma Chemical Co.
2. With a wooden stick or an inoculating loop rub colonial growth on the filter paper. A positive test is indicated by a blue color, which usually appears within 30 sec.

Oxalic Acid Test Paper for Indole

Soak a piece of filter paper in saturated oxalic acid solution. Dry and cut into strips approximately 10 mm × 50 mm. These are suspended over the medium (e.g., SIM medium, trypticase, nitrate broth) and held in place by the cotton plug. Indole is indicated by the presence of a pale pink color at the lower end of the test paper.

SIM Medium (Difco)

This medium is useful in testing for the production of indole and hydrogen sulfide and to detect motility. The medium is inoculated with a straight stab to a depth of about 2 inches.

To test for indole, an oxalic acid test paper is suspended over the medium at the time of inoculation and held in place by the cotton plug (see above for preparation of the test paper). The formation of a pink color on the paper indicates indole production.

Motility is evidenced by diffuse growth producing a turbidity throughout the medium. Hydrogen sulfide production is indicated by darkening or blackening of the medium.

MIO Medium (Difco)

This medium is useful in the identification of enterobacteria on the basis of tests for motility and for ornithine decarboxylase and indole production. The medium is tubed in 5-ml amounts and sterilized. The butt is inoculated with a straight wire to the bottom of the tube. After incubation for 18–24 hr at 35–37°C, the motility and ornithine decarboxylase reactions are read. Motility is evidenced by a clouding of the medium extending from the stab line. A positive ornithine decarboxylase test is indicated by a purple color throughout the medium. Three or four drops of Kovac's reagent are added to the top of the butt, and the tube is shaken gently. The appearance of a pink to red color in the reagent indicates the presence of indole.

Indole Nitrate Medium (BBL)

This medium is employed to demonstrate indole production and the reduction of nitrate by aerobic, microaerophilic and anaerobic bacteria.

Urease

Urea Agar Base (Difco)
This is Christensen's medium, which is used widely in the form of slants for the detection of urease production.

The entire slant is inoculated heavily. Incubate for 4 days before reporting as negative.

If negative there is no color change. A positive reaction is indicated by a pink or red color, which with some organisms may appear in several hours.

Spot Urease Test

1. Prepare a solution of the urea reagent (10% urea agar base concentrate available from Difco or BBL) and store in the refrigerator.
2. A circular filter paper (Catalogue No. F2915-50, American Scientific Products) is placed in a petri dish then moistened with several drops of the reagent.
3. A portion of a colony is rubbed onto the moist filter paper with a wooden stick. A color change to pink or red, usually within 2 minutes, indicates urease activity.

Patho Tec Urease Strip
A Patho Tec strip is available commercially for a rapid urease test (Organon Teknika, Durham, North Carolina).

Urease Test Broth (BBL)
This medium, which is employed similarly to the urea agar base above, is suitable for demonstration of urease production by a wide variety of bacteria including *Brucella* and *Mycobacterium.*

Gelatinase

Nutrient Gelatin (BBL)
The medium when prepared should gel at room temperature. It is inoculated heavily and incubated up to 5 days before being considered negative for gelatinase. The gelatin has been liquefied (i.e., gelatinase produced) if the medium remains liquid after being kept in cold water or in the refrigerator until cold.

X-Ray Film Method
Exposed but undeveloped X-ray film is cut into small strips. A small strip is dropped into a tube containing a heavy suspension of the bacte-

rium in Tryptose or Trypticase Soy Broth. After incubation for up to 48 hr the film is observed for removal of the gelatin layer, leaving pale blue clear plastic film. This removal of the gelatin layer constitutes a positive test for gelatinase.

Oxidase Test

This is a test for production of cytochrome oxidase.

Reagent

Prepare a 1% solution (0.1 g in 10 ml distilled water) of *p*-aminodimethylaniline monohydrochloride or 0.5% of *N, N, N, N'*, *N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Eastman Organic Chemicals, Rochester, New York).

The dye is added to the distilled water and allowed to stand for 15 min prior to using. The solution should be kept in a brown bottle and refrigerated. Solutions of the first compound are satisfactory for approximately a week, while solutions of the latter compound may be used for up to a month.

Procedure

The dye solution is added to portions of plate cultures containing suspicious colonies. Colonies producing cytochrome oxidase become pink, changing to red, then finally to black when solutions of the first compound are used. The reagent will turn blood and other agar media pink, then black. The colonies themselves must change color. Solutions of the other compound stain cytochrome oxidase-positive colonies a dark purple. Colonies treated with the dye can be used for Gram staining.

Patho Tec Strips

Test papers or discs are available commercially for the cytochrome oxidase test (Organon Teknika, Durham, North Carolina). These are not considered as sensitive as the plate test . They are employed by adding a suspension of the test organism to the paper or disc or by placing the disc on the medium adjacent to isolated colonies. In the latter procedure, a drop of sterile distilled water is added to the disc. Diffusion of the reagent will turn oxidase-producing colonies a pink color that becomes black on standing. Oxidase negative organisms are unchanged.

Spot Oxidase Test

A rapid test can be carried out by applying growth to filter paper strips moistened with 1% tetramethyl-*p*-phenylenediamine dihydrochloride

(Kodak, Sigma and other suppliers). In a positive test there is a color change to blue or purple within 10 sec.

Catalase Test

A slant culture is used. One milliliter of a 3% solution of hydrogen peroxide is poured over the growth. The slant is tilted so that the solution covers the growth. A rapid ebullition of gas indicates a positive reaction.

The test can also be performed transferring a small amount of growth, preferably a single colony, from solid medium to a microscope slide. A drop of fresh hydrogen peroxide (3%) is added, then a coverslip is applied. The production of gas bubbles constitutes a positive reaction.

The following procedure has been used for the demonstration of catalase, production by *Campylobacter*. Organisms are grown for 3 days in semisolid medium, after which 5.0 ml of 3% hydrogen peroxide is added to the culture. After mixing by inversion, the tube is observed for the presence of gas bubbles. A culture is considered positive if 2–3 mm of gas bubbles are produced.

CAMP Test

This test is used for the presumptive identification of *Streptococcus agalactiae*. This species is able to complete the partial lysis of red cells produced by the beta-hemolysin of a *Staphylococcus* strain. A β-hemolytic staphylococcus can be streaked heavily across a blood plate, or β-hemolysin can be supplied by a filtrate of a *Staphylococcus* broth culture. If the latter procedure is used, the hemolysin is swabbed onto the surface of the agar and allowed to dry for at least 30 min. Strains of streptococci to be examined are streaked at right angles up to the hemolysin or to the *Staphylococcus* streak line.

Occasional strains of *S. dysgalactiae* and *S. uberis* will also give positive reactions. The latter splits esculin, while the former and *S. agalactiae* do not. *Streptococcus dysgalactiae* does not coagulate litmus milk, while the other two species do. Colonial appearances on Edward's medium are also differential.

The procedure outlined below has also been found satisfactory.

1. Draw a line across the center of a blood agar plate.
2. Streak the *Streptococcus* culture at right angles and across the line.
3. Streak a β-hemolytic *S. aureus* culture across the plate, directly over the line.

4. Incubate for 18–24 hr and observe. If the *Streptococcus* is a Group B, it produces a characteristically shaped clear zone of hemolysis in the β zone of hemolysis produced by the *Staphylococcus* (see Fig. 34-1).

Phenylalanine Deaminase

Patho Tec Strip
Apply a heavy loopful of the culture to one of the two reagent zones of the test paper (Organon Teknika, Durham, NC) by rubbing with the loop for 15 sec to bring the culture into intimate contact with the reagents. The second reagent zone is used to run a negative control. Positive reaction: A darkened stain develops in the area within 5–10 min. Color may vary from brownish to gray black.

Phenylalanine Agar (Difco)
Phenylalanine deaminase can be demonstrated in this medium. The test organism is grown on a slant of phenylalanine agar at 35–37°C for 18–24 hr. Production of phenylpyruvic acid from phenylalanine is determined by adding 5 drops of the test reagent to the growth on the slants in such a way as to cover the slant and loosen the growth. The production of a green color in 1–5 min indicates a positive test. The test reagent is prepared by dissolving 2.0 g of ammonium sulfate and 1.0 ml of 10% sulfuric acid in 5 ml of a half-saturated solution of ferric ammonium sulfate.

Decarboxylation of Arginine, Ornithine, and Lysine

Decarboxylase Test Medium, Moeller (Difco)
This is a base to which lysine, arginine, and ornithine or other amino acids may be added to demonstrate decarboxylase activity. Ten grams of the amino acid to be tested is added to 1 liter of the basal medium. Where D,L-amino acids are employed, a 2% concentration is used rather than 1%. No further adjustment of reaction will be required when lysine or arginine are used. Ornithine, being highly acidic, requires the readjustment of pH with sodium hydroxide before sterilization. Usually 1 liter of the medium in which 10 g L-ornithine has been dissolved requires the addition of 4.6 ml 10 *N* sodium hydroxide. Dispense the medium in 5-ml amounts into screw-cap tubes and sterilize in the autoclave for 10 min at 15 lb pressure (121°C).

Procedure
Inoculate the prepared media lightly from a 24-hr agar slant culture. Aseptically overlay the inoculated broth and controls with 4–5 ml

sterile mineral oil. Incubate at 35–37°C for 4 days. Positive decarboxylase reactions will be indicated by a change in the indicator to a reddish violet color. A negative reaction is indicated by a yellow color in the tube.

The reaction of the medium containing the amino acids should be pH 6.0 after sterilization.

A test paper is available for the detection of lysine decarboxylase (Organon Teknika, Durham, North Carolina).

Sodium Hippurate Hydrolysis

Reagent

Twelve g of ferric chloride ($FeCl_3 \cdot 6H_2O$) are dissolved in 100 ml of a 2% aqueous solution of hydrochloric acid.

Medium

Sodium hippurate	10 g
Heart infusion broth	1000 ml

Dissolve the sodium hippurate in broth, dispense into tubes in 1 ml amounts, and sterilize at 115°C for 20 min.

Procedure

Transfer 0.8 ml of the culture to a small tube, then add 0.2 ml of the reagent and mix immediately. After 10–15 min, observe for the presence of a permanent precipitate. The latter indicates the presence of benzoic acid resulting from hydrolysis of hippurate. If the culture is particularly turbid, the organisms can be separated by centrifugation and the test carried out using supernatant.

It is important that the prescribed amounts of culture and reagent be used because the benzoic acid can be dissolved by an excess of reagent. A control in which the sterile broth is used should be included.

Optochin or Ethylhydrocuprein Inhibition Test

Ethylhydrocuprein (EHC) specifically inhibits the growth of *Streptococcus pneumoniae*. It is available impregnated in paper discs (Difco) that are applied to inoculated plates in the same manner as a sensitivity disc. The zone of inhibition should be at least 5 mm. Small zones, as may be observed with some viridans streptococci, are ignored.

O-nitrophenyl-β-D-galactopyranoside Test

The o-nitrophenyl-β-D-galactopyranoside (ONPG) test is used to detect potential lactose fermenters that in ordinary media either take

several days to produce acid or do not produce it at all. This test detects the induced intracellular enzyme β-galactosidase, one of two enzymes involved in lactose fermentation. The preparation of ONPG broth and the performance of the test is described by Cowan and Steel (1).

Turbidity Standards

McFarland Nephelometer Standards

These tubes of graded turbidity are useful for the rough quantitation of bacterial suspensions. They are available commercially (Difco).

Preservation of Bacteria

Lyophilization

Although somewhat laborious, lyophilization or freeze-drying is a very useful process for the preservation of almost all bacteria and mycoplasmas. Several apparatuses are available commercially, including the Bellco all-glass ampoule freeze-drying apparatus (Bellco Glass, Inc., Vineland, New Jersey). Ampoules with firing-off constrictions are convenient when using a "crossfire" ampoule torch for sealing off. Although freeze-drying can be carried out using a good vacuum pump without a vacuum gauge, it is advantageous to have the latter. The lower the vacuum, at least 10 μm or less of mercury, the less residual moisture and consequently the greater survival.

Prior to being dispensed in ampoules, organisms are suspended in a protective medium such as the well-known Mist Dessicans®: one volume nutrient broth with 30% glucose, plus three volumes sterile inactivated serum. After lyophilization, the ampoules are sealed, then tested for vacuum using a high-frequency coil (Tesla type). Those that fail to "light up" are discarded.

Maintenance in Media

Many gram-negative organisms and some gram-positive ones can be maintained in a viable state in Stock Culture Agar® (Difco). The medium is dispensed in screw-cap or rubber-stoppered tubes. The unslanted medium is stabbed several times, incubated, then stored in the dark at room temperature. Some species, including *Pasteurella multocida*, will die if stored at refrigerator temperature for several weeks. Although many organisms can be stored for months in this medium, occasional cultures of a species will die.

Many aerobes and anaerobes, including clostridia, remain viable for many months in tubes of sealed cooked meat medium at room temperature.

Deep Freeze

The procedure outlined below will maintain many fastidious organisms for long periods.

1. Grow on a blood plate or other suitable medium.
2. Place 0.5 ml of defibrinated blood in a small sterile tube.
3. Suspend loopfuls of bacteria in the blood.
4. Store in deep freeze, −70°C preferred.
5. To recover, remove the tube and allow the edge of the blood to thaw: Remove loopfuls of the melted blood from between the frozen plug of blood and the wall of the glass tube.
6. Plate on a suitable medium, then return the incompletely thawed tube to the freezer.

An alternative procedure is to employ glass beads as follows:

1. Prepare "lawn" of pure culture of bacteria on blood agar plate. Incubate 48 hr.
2. Add 1 ml of a sterile 2% glycerol–water solution to the surface of the plate.
3. Pour 20–30 sterile glass beads (4 mm in diameter) onto the plate and swirl beads to coat with the bacteria.
4. Sterilize forceps by dipping into 95% alcohol and flaming, and then pick up beads. Place them in a 1.8-ml capacity Nunc tube or other suitable sterile tube for freezing.
5. Store in deep freeze, −70°C preferred.
6. A frozen bead can be removed and placed on a blood agar plate when a fresh culture is desired.

Liquid Nitrogen

Freezing in liquid nitrogen (−196°C) is the preferred method of long-term storage. The procedures involved in the storage of bacteria in liquid nitrogen and also in other ways have been described in detail (14).

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Appendix C

Mycological Techniques

G. R. Carter

Only a small number of useful media are described below. All are available commercially. Readers are referred to Chapters 28, 29, 30, 31, and 32 and their supplementary references for additional media, reagents, and procedures.

Media

Sabouraud Dextrose Agar

This medium without inhibitors is used routinely for the isolation of dermatophytes. It is available from several commercial sources.

A modified Sabouraud dextrose agar containing less carbohydrate is available commercially. It is preferred for the isolation of pathogenic fungi other than dermatophytes.

Sabouraud Dextrose Agar (with Inhibitors)

This medium is similar to the regular Sabouraud agar except that it contains chloramphenicol for the reduction of bacterial growth and cycloheximide (actidione) for the suppression of saprophytic fungi.

It is available under various trade names [e.g., Mycosel® (BBL) and Mycobiotic® (Difco)]. Sabouraud media are usually dispensed in Petri dishes, large tubes as slants, or small prescription bottles. After inoculation, they are incubated at room temperature only. It should be remembered that the growth of *Allescheria boydii, Cryptococcus neoformans, Aspergillus* spp., *Mucor, Absidia, Rhizopus,* and some saprophytic fungi is inhibited on this medium.

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Brain–Heart Infusion Agar

This is a good medium for the growth of the yeast phase of dimorphic fungi. The addition of blood may aid in the recovery of *Histoplasma capsulatum* (see Chapter 32).

Chlamydospore Agar (Difco)

If a selective medium is desired onto which material can be inoculated directly, add per milliliter 40 U of penicillin, 40 μg stroptomycin, and 20 μg of aureomycin. (See Chapter 30 for appearance of chlamydospores in this medium.)

Birdseed Agar (Staib Agar)

Readers are referred to the *Manual of Clinical Microbiology* (1) for the preparation of this medium. *Cryptococcus neoformans*, unlike other *Cryptococcus* spp., produces brown colonies on this medium. Several formulations of this medium are available commercially.

Levine Eosin–Methylene Blue (BBL)

Candida albicans produces characteristic germ tubes on this medium when incubated at 37°C for 2–3 hr.

Dermatophyte Test Medium (Pitman-Moore, Pfizer, Difco)

This is a selective and differential medium for dermatophytes. Bacterial and fungal contamination is greatly reduced. Dermatophytes change the color of the medium from yellow to red. Those bacteria and saprophytic fungi that grow on the medium do not change the yellow color of the medium, or they change it to red very slowly. Dermatophytes can be readily recovered on this medium and identified as such with a reliability as high as 97%. The medium should enable veterinarians to recover dermatophytes in practice, thus making possible a more rapid, positive diagnosis of ringworm. Cultures should be examined microscopically to confirm the presumptive identification of a dermatophyte.

Stains

Lactophenol Cotton Blue Stain

Staining Solution

Phenol crystals	20.0 g
Glycerin	40.0 ml
Lactic acid	20.0 ml
Distilled water	20.0 ml

The ingredients are dissolved by heating gently over a steam bath. When in solution, add 0.05 g of cotton blue dye.

Staining Procedure

A small portion of the mycelium is transferred to a drop of the staining solution on a slide. The mycelium is teased apart with needles, then covered with a coverslip. Permanent mounts are made by applying nail polish along the edges of the coverslip.

Clear Lactophenol

This preparation is prepared in the same manner as the lactophenol cotton blue, except that the cotton blue stain is not included. It is useful for mounts of clinical materials.

Ultraviolet Lamp or Wood's Lamp

Small, portable so-called black-light lamps[1] are available to provide the required long wave ultraviolet (wavelength: 3650 Angstrom units). These lamps are most effective at a close range (within twelve inches). Materials are examined in the dark.

Materials infected with *Microsporum canis, M. audouinii,* and *Trichophyton equinum* fluoresce under a Wood's lamp. It should be kept in mind that a variety of substances fluoresce, including certain drugs, keratin, petrolatum, porphyrins, etc.

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[1]Stroblite Co., Inc., 75 West 45th Street, New York, New York. Burton Medic-Quipment Co., El Segundo, California.

Preparation of Slide Cultures

The following materials are required:

1. Deep Petri dish
2. A short piece of glass rod or tubing (approximately ⅜ inch in diameter) with a V bend. It must be short enough to sit in the bottom of a Petri dish. It serves as a support for a microscope slide.
3. One plate of cornmeal agar or other suitable medium for the promotion of sporulation
4. Microscope slides and small coverslips (22 × 22 mm)

A microscope slide is placed in each Petri dish along with the V-shaped rod. The dish is wrapped in paper, then autoclaved.

The various steps are shown in Figure C-1. A square of cornmeal agar (approximately 15 × 15 mm) is aseptically cut and transferred to a sterile microscope slide within a sterile Petri dish. The slide should be sitting on the V-shaped rod.

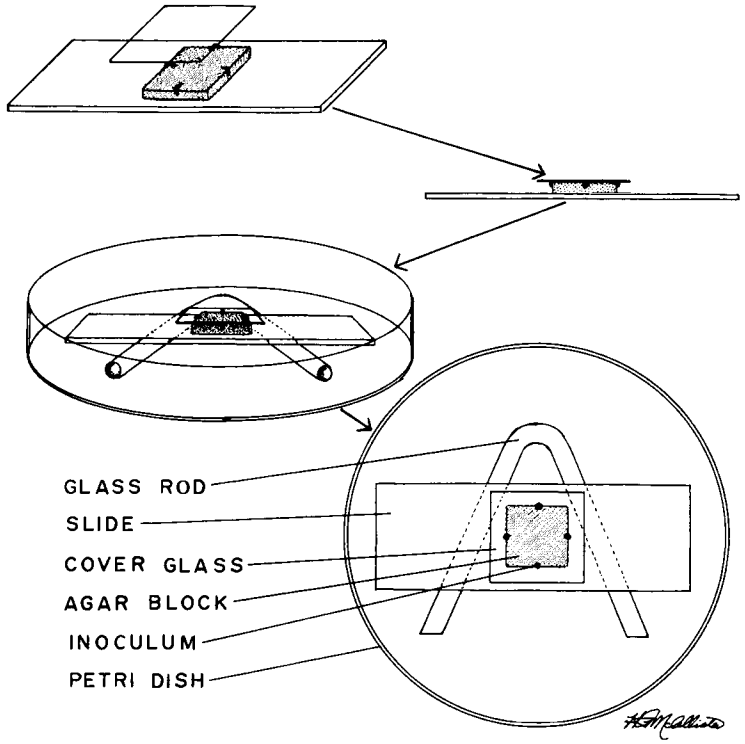

Figure C-1. Steps in preparing a slide culture of a fungus (H. A. McAllister).

By means of an inoculating loop with a short right-angle bend near its end, the agar block is seeded with the fungus to be cultured. This is done by making a short cut with the bent end of the needle into the dorsal central point of each side of the block. The amount of fungus colony applied to these cuts needs to be just perceptible to the naked eye.

A coverslip is grasped with forceps and passed through the flame several times, then placed on the block of agar. The fungus will usually grow outward under the coverslip and inward between the coverslip and the medium. The bottom of the Petri dish is covered with water to keep the agar block from drying out.

Incubate at room temperature.

With the more rapidly growing fungi, growth is apparent in several days, and the reproductive elements can be seen *in situ* with the low-power objective of the microscope.

When development is considered optimal, the coverslip is removed with forceps and placed on a drop of lactophenol cotton blue stain previously added to the center of a microscope slide. The coverslip is then ringed with clear nail polish, thus furnishing a permanent mount.

Preservation of Fungi

Many fungi can be maintained at room temperature for several months provided the cultures are not allowed to dry out. They should be transfered to fresh media every 2–3 months.

Some fungi, but not all dermatophytes, may be maintained at refrigerator temperature with an interval between transfers of about 4 months.

Lyophilization is suitable for some fungi but not those that produce large spores. This rules out most of the pathogenic fungi.

Cultures on sealed Sabouraud agar slants can be maintained successfully in deep freeze at −20°C. Another procedure that is widely used is to cover slant cultures with a good grade of heavy, sterile mineral oil.

Preparation and Use of Gastric Mucin

Hog gastric mucin is used in animal inoculations to enhance the pathogenicity and to promote conversion to the yeast phase of some fungi.

1. Put 5 g of gastric mucin in 95 ml of distilled water; emulsify with a blender for 5 min.
2. Autoclave for 15 min at 120°C; cool.

3. Adjust pH to 7.3 with NaOH (sterile).
4. Check for sterility and store in refrigerator.
5. Use equal parts of the gastric mucin and the fungus suspension and inject the mixture (1 ml) intraperitoneally into the laboratory animal.

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Appendix D

Laboratory Methods for Rickettsiae and Chlamydiae

Johannes Storz

Staining Methods

Preparation of Smears

The following clinical specimens are examined:

1. Fluids or discharges: use 1 drop and smear over a small area of slide.
2. Tissues: section and make several impression smears from cut surfaces.
3. Yolk sac: use the yolk sac membrane, previously washed with saline to remove excess yolk material and blotted on bibulous paper. Small pieces of yolk sac membrane are impressed onto the slide with forceps.

Several stains are satisfactory for demonstrating organisms of the *Rickettsiales* in impression smears from infected material. The stains considered most reliable are those of Gimenez, Macchiavello, Castaneda, and Giemsa. Characteristics of stained rickettsiae and chlamydiae are summarized in Table D-1.

Gimenez Stain

Staining Solutions

1. Carbol basic fuchsin: stock solution		
Basic fuchsin in 95% ETOH	10% w/v	100.0 ml
Aqueous phenol	4% v/v	250.0 ml
Distilled water		650.0 ml

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Table D-1
Appearance of Rickettsial and Chlamydial Developmental Forms in Stained Cytological Preparations

Stain	Rickettsiae	Chlamydiae[a]	Host cells or background debris
Giemsa	Purple to dark blue	EB purple; inclusion purple	Cytoplasm blue; nuclei dark purple
Gimenez	Bright red or greenish red	EB bright red; inclusion greenish-red	Cytoplasm faint green-blue; nuclei intense green-blue
Macchiavello	Red	EB red; inclusion reddish-blue	Cytoplasm blue; nuclei intense blue
Castaneda	Blue	EB blue	Cytoplasm red; nuclei intense red

[a]EB, elementary body.

Keep at 37°C for 48 hr before use. Basic fuchsin should be about 94%, not 84%. Harleco #677 99% and Allied Chemical #NA043 92% work equally well.

2. Sodium phosphate buffer 0.1 *M*, pH 7.45

NaH_2PO_4	0.2 *M*	3.5 ml
Na_2HPO_4	0.2 *M*	15.5 ml
Distilled water		19.0 ml

3. Working solution basic fuchsin

Stock solution	4.0 ml
Na phosphate buffer	10.0 ml

Mix fresh just before use; filter through paper.

4. Malachite green oxalate, cert. 0.8%w/v

Staining Procedure

1. Make thin impression smear of tissue on a slide.
2. Dry and lightly heat fix to inactivate agent.
3. Cover 5 min with working solution basic fuchsin.
4. Rinse with warm distilled water.
5. Counterstain 20 sec with 0.8% malachite green oxalate.
6. Wash and counterstain again for 20 sec.
7. Wash and blot the slide.
8. Examine under oil. Intracytoplasmic and cell-free chlamydial elementary bodies will stain red, and background material will stain green.

Modified Macchiavello's Stain

Staining Solutions

Basic fuchsin	0.5 g in 200 ml distilled water
Citric acid	0.5 g in 200 ml distilled water
Methylene blue	2.0 g in 200 ml distilled water

Staining Procedure

1. Smears are air dried, then lightly fixed with heat.
2. Stain for 5 min with basic fuchsin filtered through paper.
3. Wash with water and decolorize for 15 sec with citric acid.
4. Wash with water and stain with methylene blue for 20 sec.
5. Wash with water and dry by gently blotting.
6. Examine with the oil immersion objective. If the slide has been properly stained, the elementary bodies, which appear singly or in clusters, will stain red. If overdecolorized, the elementary bodies will appear blue.

Castaneda's Stain (Bedson Modification)

Staining Solutions

1. Weiss mordant solution

Formalin	100.0 ml
Glacial acetic acid	7.5 ml

2. Formol blue

M/15 phosphate buffer, pH 7.0	180.0 ml
Azure II or Unna's blue (1% in methyl alcohol)	20.0 ml
Formalin	10.0 ml

3. Safranin 0.25% aqueous solution

Staining Procedure

1. Smear is air-dried and fixed in Weiss' mordant solution for 2 min.
2. Wash thoroughly with water.
3. Stain with formol blue for 10–70 min.
4. Wash thoroughly with water.
5. Counterstain with safranin for 5–8 sec.
6. Wash thoroughly with water.

7. Examine with the oil immersion objective. Elementary bodies stain blue, while the cellular material appears red.

Giemsa Stain

The preparation and staining procedure is given in Appendix A. Elementary bodies appear purple, but they may be difficult to differentiate from the similar staining of tissue cells and background.

Preparation of Inocula from Clinical Samples

Clinical specimens received for the isolation of *Rickettsia* and *Chlamydia* are varied. Specimens include the following:

1. Blood: grind clot in Bovarnick's buffer or tissue culture fluid using a Ten Broeck tissue grinder or a mortar and pestle.
2. Other fluids, discharges: use undiluted unless the fluid is viscous. Otherwise, dilute and process as for blood.
3. Feces: suspend feces in Bovarnick's or tissue culture fluid to give a 10% concentration. Grind and centrifuge (2000 *g*) to remove debris and fecal particles; collect the supernatant and proceed as described below.
4. Tissues: mince well with scissors, then grind thoroughly using a Ten Broeck tissue grinder or a mortar and pestle. Sufficient buffer is employed to give approximately a 10% suspension. Centrifuge at 2000 *g* for 20 min to remove debris and fragments, then collect the supernatant.

If specimens are contaminated with bacteria, they have to be specially processed prior to being inoculated into cell cultures or chicken embryos. Several procedures are described below:

1. If the rickettsial organisms being sought will propagate in laboratory animals, the specimen is centrifuged to eliminate major contaminants. The animals can eliminate minor bacterial contamination.
2. Antibiotics are employed as follows: 500 μg/ml streptomycin, 75 μg/ml vancomycin, 50 μg/ml gentamycin, and 500 U/ml mycostatin.
3. Selective filtration
4. Differential centrifugation (see Fig. 25-9)
   a. Centrifuge for 30 min at 2000 *g*.

b. Withdraw 5 ml of the supernatant, being careful not to disturb the layers, and dilute 1 : 1 in buffer solution.
c. The above procedure is repeated twice.
d. Following the third cycle of centrifugation, withdraw several milliliters of supernatant and make two 10-fold dilutions if chickens embryos are infected.

Isolation and Cultivation

For practical purposes, all organisms in these orders must be cultivated in living host systems. Procedures for the inoculation of mice, the yolk sacs of developing chicken embryos, or cell cultures are given below. See Table 25-1 for the preferred host systems and route of inoculation for specific organisms.

Isolation of *Chlamydia psittaci* in Cell Culture under Enhancing Conditions through Centrifugation

1. Use mouse L cells (929) that have been grown on minimum essential medium (MEM) with 500 μg streptomycin and 75 μg vancomycin/ml for several passages; use NO PENICILLIN!
2. Soak 3-dram shell vials in appropriate detergent, then wash thoroughly. Add alcohol-cleaned circular coverslip and foil cap. Dry sterilize in the oven.
3. Plant L cells to monolayer the coverslip, about 3×10^5 cells per vial. Use 1.2 ml of cell suspension in MEM plus 10% fetal calf serum (FCS). Prepare at least two vials per sample for isolation. Inoculate within 24 hr of planting.
4. To inoculate, remove cell culture medium and add 0.5–1.0 ml of inoculum. Place a UV-sterilized plastic cap on the vial and centrifuge 20–30 min at 2000*g* Attempt to maintain cells at 37°C as much as possible. (Centrifuge shields may be prewarmed to 37°C to aid in maintaining the temperature, or centrifuge may be moved into a walk-in incubator.)
5. Remove the inoculum. If the inoculum contained debris, wash the cells with Dulbecco's solution plus streptomycin.
6. Add MEM plus additives (as follows). For primary isolation or when bacterial contamination is possible, use gentamicin (50 μg/ml) and vancomycin (75 μg/ml).

Additives to MEM for maintenance medium:

0.5% glucose
10% heat-inactivated FCS
1–2 μg cycloheximide/ml (use in one set of the vials if isolating unknown strain and run parallel set without cycloheximide)
0.25 *M* HEPES
10 m*M* $NaH_2PO_4 \cdot H_2O$

7. Reading the results:
   Stain one set of coverslips with Giemsa 40–60 hr after inoculation. When isolation and characterization of a strain is intended, save a second vial for subpassage. If inclusions are small, allow further incubation of 12–24 hr before subpassage. Break cells to release infectious agent with ultrasound treatment or freeze and thaw.

Enhancement of Chlamydial Uptake by Cultured Cells through DEAE–Dextran Treatment

1. Prepare a 1% stock solution of diethylamine-ethyl-dextran (Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178) in Dulbecco's phosphate-buffered saline.
2. Add 0.2 ml of the 1% stock solution of DEAE-D to 100 ml of Dulbecco's solution for a final concentration of 20 mg/ml.
3. Wash the cell monolayer two to three times with DEAE-D Dulbecco's solution. Allow the last rinse to remain on the monolayer for about 30 min.
4. Decant the first rinse and add the inoculum for adsorption as usual.
5. Wash inoculated cell cultures with Dulbecco's solution, add suitable cell culture medium, and incubate.

Inoculation of Mice

Intraperitoneal Method

1. Young mice, 3–5 weeks of age, are preferred. A firm hold is obtained by gripping the tail between the little finger and the ring finger and by holding the loose skin at the back of the neck between the index finger and the thumb.

2. The abdomen is swabbed with a suitable disinfectant such as 70% ethanol or tincture of iodine. Using a tuberculin syringe with a 25-gauge needle, 0.2 ml of the inoculum is administered.
3. The mice are observed daily for evidence of infection.

Harvesting Tissues

Spleen, liver, and kidneys of mice are harvested as follows:

1. The dead mouse is swabbed with a disinfectant, then secured to a dissecting board by pinning all four legs. With sterile scissors and forceps, the walls of the abdomen and thorax are reflected to expose the viscera.
2. Using another pair of sterile scissors and forceps, the spleen, liver, and kidneys are collected (taking care not to cut the intestine) and placed in a sterile Petri dish.
3. Impression smears are made from sections of the organs for staining. Tissues are prepared for further passaging or frozen and stored for future reference (− 70°C).

Intranasal Inoculation

1. Young mice, 3–5 weeks of age, are lightly anesthetized with ether. This facilitates the inhalation of the inoculum, as mice are not as likely to sneeze. It is easily accomplished by placing the mouse in a glass jar with a loose cap. The mouse is ready for inoculation when it shows signs of loss of equilibrium.
2. The mouse is held in the hand, abdomen up. Using a tuberculin syringe with a 22-gauge needle, several drops of the inoculum are placed at the openings of the nostrils. Each drop should be inhaled before the next is administered.
3. The inoculated mice are observed daily for such evidence of infection as hunched posture, labored respiration, and general depression.

Harvesting Lungs

The lungs of mice that have died and the lungs of living mice with and without signs of infection are harvested. The procedure followed is essentially the same as that described previously for liver, spleen, and kidney.

The lungs are placed in a sterile Petri dish. Impression smears for staining are made from sections of the lungs. Tissue is prepared for further passaging if indicated or frozen and stored for future reference.

Yolk Sac Inoculation

Fertile eggs should be from a flock receiving antibiotic-free feed. The temperature of incubation should be 36–37°C, and the humidity should be near the point of saturation.

1. Eggs, with 5–7-day-old chicken embryos, are candled and the position of embryo and air space are marked.
2. The surface of the egg over the air sac is swabbed with tincture of iodine.
3. A hole is either punched (a needle pushed through a cork stopper works well) or drilled through the shell over the air space posterior to the embryo.
4. An inoculum of 0.3–0.5 ml is administered with a 22-gauge, 1-inch needle. The needle is inserted vertically to the hilt (Fig. D-1).
5. The needle is removed and the hole sealed with paraffin, collodion, or nail polish.
6. The inoculated chicken embryos are placed in the incubator and candled daily for evidence of loss of motility or death. Deaths occurring within 24 hr are normally due to contamination or trauma, and the eggs should be discarded.

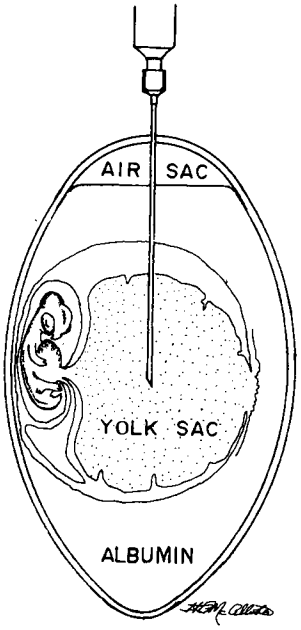

Figure D-1. Technique for inoculating 6 day-old chicken embryos (H. A. McAllister).

Yolk Sac Harvest

1. The top of the eggs are swabbed with a tincture of iodine.
2. The shell is cut, using sterile scissors, along the margin of the air space, thereby exposing the shell membrane.
3. The shell and chorioallantoic membranes are ruptured, and the chicken embryo with yolk sac is gently teased with sterile forceps into a sterile Petri dish.
4. The yolk sac is cut from the embryo, ruptured, and allowed to drain. The yolk sac membrane is stripped with dental forceps to remove remaining excess yolk.
5. Impression smears are made from small pieces of the yolk sac membrane, which are gently washed in saline and blotted on bibulous paper.
6. The rest of the yolk sac membrane is used for subpassage or frozen at $-70°C$ for future reference after homogenization in Bovarnick's buffer.

Bovarnick's Buffer

Sucrose	0.218 *M*	74.62 g
KH_2PO_4	0.0038 *M*	0.52 g
K_2HPO_4	0.0072 *M*	1.64 g
Na glutamate	0.0049 *M*	0.82 g

Dissolve in 1000 ml distilled water and autoclave. Add 1% bovine serum albumin, adjust to pH 7.0, and add appropriate antibiotics.

Sulfadiazine Sensitivity

1. Grind one yolk sac with 2.5 ml Bovarnick's sucrose–phosphate buffer containing 500 μg streptomycin per ml (yields 1 : 10 dilution).
2. Transfer 0.2 ml of the 1 : 10 dilution to 1.8 ml Bovarnick's sucrose–phosphate buffer containing 500 μg streptomycin per ml, which gives a 10^{-2} dilution. Continue in like manner to obtain dilutions of 10^{-3} through 10^{-8}. Use these dilutions as controls.
3. Transfer 0.2 ml of the 1 : 10 dilution to 1.8 ml Bovarnick's sucrose–phosphate buffer containing 500 μg streptomycin and 2 mg sulfadiazine per ml to prepare a 10^{-2} dilution. Continue in like manner to obtain dilutions of 10^{-3} through 10^{-8}.

4. Inoculate each of three or more 7-day-old developing chicken embryos with 0.5 ml of each dilution. Incubate until death occurs or for 12 days.
5. Compare the median chicken embryo lethal dose ($CELD_{50}$) for each of the two chlamydial preparations. A reduction of a hundredfold or more in the number of $CELD_{50}$ in suspensions of 10^6 or more chlamydiae per ml indicates sulfadiazine susceptibility of the strain. The test can be adjusted for infectivity assay in L cells or other cell cultures.

Enzyme-Linked Immunoassay for Detecting Chlamydial Antibodies

1. Use nonirradiated Immulon I microtiter plates.
2. Coat wells with particulate antigen consisting of partially purified chlamydial elementary bodies propagated in L cells; lysates of noninfected L cell are used as cell control antigen.
3. Plates coated with chlamydial and L-cell antigens are incubated in a humidified chamber for 18 hr at 37°C, treated with formaldehyde for 5 min and stored in a desiccator at 4°C until used.
4. Employ affinity-purified antibovine-specific IgG(H + L) labeled with horseradish peroxidase at appropriate dilution as determined by titration.
5. React with substrate 2,2-azino-di-3-ethylbenzthiazoline sulfonic acid.
6. Test reaction: Antigen plus 1:100 test serum plus conjugate in duplicate wells.
7. Controls: (a) L cell antigen plus 1:100 test serum plus conjugate in duplicate wells; (b) L cell antigen plus conjugate in duplicate wells; (c) Antigen plus 1:100 positive serum plus conjugate in duplicate wells.
8. Reaction time: Read optical density (OD) at 410 nm when 1:100 dilution of positive serum reaches a value of 0.800. Calculate net OD value of each test serum by subtracting the average OD (n = 2) of L-cell antigen from the average OD (n = 2) obtained with chlamydial antigen. Base line of significance is calculated and should be at an OD of 0.100 representing the average L-cell antigen reaction of 50 random determinations of normal serums.

Indirect Inclusion Fluorescence Test for Detecting Chlamydial Antibodies

1. Use as antigen L cells infected with cell-culture adapted strain of *Chlamydia psittaci* for 30 hr, place on 12-well Teflon-coated slides.

2. Fix cells with cold paraformaldehyde for 5 min and store at −70°C. Type- and strain-specific protein surface antigens are reactive in this test.
3. Test serum is applied as 1:8 and two-fold serial dilutions and allowed to interact with the antigen for 1 hr in a humid chamber at room temperature.
4. Bovine IgG (H + L)-specific antiserum produced in rabbits and FITC-labeled is used to probe binding of primary antibody.
5. Controls include (a) Infected L cells plus normal serum plus conjugate; (b) L cells plus positive serum plus conjugate; (c) L cells plus normal serum plus conjugate; (d) L cells plus conjugate.

Indirect Immunofluorescence for Detecting Antibodies against Ehrlichiae

1. Propagate ehrlichiae in the murine macrophage cell line P 388 D1 in medium 199 supplemented with 10% fetal bovine serum and 1% glutamine and adjusted to a pH of 7.4 with sodium bicarbonate.
2. Harvest cells with a scraper when 60–80% of the cells have developed ehrlichial inclusions. Wash cells three times at 400 *g* for 10 min in 50 volumes of Hank's balanced salt solution.
3. Suspend cell sediment in 10 volumes of 2% fraction V of bovine serum albumin in 0.15 *M* phosphate buffered saline, pH 7.2. Place 10-μl drops of antigen suspension on acetone-cleaned microscope slides, dry at 37°C for 1 hr, wrap in lint-free tissue, and store at −20°C.
4. Take slide from −20°C and defrost. Make ring around cell spots with Trichem pen. Let dry.
5. Add 25–30 μl of antiserum dilution per ring. Use cell culture fluid or PBS for dilutions.
6. Incubate in humid incubator at 37°C for 20 min, prevent drying out. Wash twice with PBS for 10 min each on shaker, and dry slides.
7. Add about 30 μl/ring of diluted conjugate (appropriate fluorescent anti-IgG in PBS). Dilution of conjugate varies with lot.
8. Incubate in humid incubator at 37°C for 20 min.
9. Wash twice with PBS for 10 min each on shaker, quick rinse with distilled water, dry slides.
10. Mount slides with 90% glycerol in Tris buffer (pH 8.5) and cover-slip. Examine preparation with microscope equipped for fluorescence.

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Appendix E

Laboratory Methods for Mycoplasmas: Avian

Harry W. Yoder, Jr.

Mediums for Growth

Some typical formulations of mycoplasma mediums are as follows.

Mycoplasma Medium (Frey) (1)

Mycoplasma broth base*	22.5 g
Dextrose	10.0 g
Swine or horse serum	100–150 ml
Phenol red	25.0 mg
Penicillin G potassium	1000 U/ml
Thallous acetate (1:4000–1:2000)	2.5–5.0 ml 10% sol.
Distilled water	1.0 liter

Adjust pH to 7.8 and filter sterilize.

Mycoplasma gallisepticum and *M. synoviae* ferment dextrose so readily that only 3 g/liter need be used for most routine purposes. Thallous acetate is used at the 1:2000 level for potentially highly contaminated specimens like those collected on tracheal swabs. Reduced NAD must be added for the cultivation of *M. synoviae* (1.0 ml of a 1% solution of NAD plus 1.0 ml of a 1% solution of cysteine hydrochloride per 100 ml of medium).

In general, horse serum should be used in media for the cultivation of *M. meleagridis*, swine serum for *M. synoviae*, and either swine or horse serum may be used in media for *M. gallisepticum*. Serum used

*Sources of Mycoplasma Broth Base (Frey): Microbiology Systems, Cockeysville, Maryland 21030

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in mycoplasma medium is usually heat-inactivated (56°C for 30 min). Laboratory passaged cultures can be adapted to more variable ingredients.

Difco brain–heart infusion broth, beef-heart infusion broth, or PPLO broth may be used as the primary base medium supplemented with yeast autolysate, proteose peptone No. 3, dextrose, NAD, and various serums.

Avian meat infusion broth with 20% avian, swine, or horse serum plus yeast autolysate and 2,3,5-triphenyltetrazolium chloride is an excellent medium, especially for *M. gallisepticum* but is more difficult to prepare.

Agar Plate Medium

To 500 ml of any of the above described broth base media should be added 7.5 g (1.5%) agar, boiled to dissolve, adjusted to pH 7.8, and autoclave-sterilized. The following filter-sterilized enrichments should be added after the agar base cools to 45° C:

Per 500 ml base

Swine or horse serum	50–75.0 ml
Penicillin G potassium	1000 U/ml
Thallous acetate (1:4000)	1.25 ml 10% sol.
1% NAD solution	5.0 ml
1% Cysteine HCl solution	5.0 ml

Agar plates or slants should be allowed to solidify.

References

1. Frey, M. L., Hanson, R. P., and Anderson, D. P.: *Am J Vet Res 29*:2163, 1968

Appendix F

Laboratory Methods for Mycoplasmas: Animal

Ole H. V. Stalheim

Modified Hayflick's Medium

Bacto PPLO broth (Difco) w/o CV	2.1 g in 70 ml H_2O
Horse serum	20.0 ml
Fresh yeast extract	8.0 ml
Penicillin	1000 IU/ml (final conc.)
Deoxyribonucleic acid (Sigma)	0.002% (final conc.)
Thallium acetate	1:2000 (final conc.)

pH 7.6–7.8
Total volume 100 ml

Filter sterilize by passing through a 0.22-μm filter. Dispense in 1.8-ml quantities and store at 5°C. Optional additions are glucose (0.5–1.0%) and phenol red (1:5000).

Modified Hayflick's Medium with Agar

Bacto PPLO agar (Difco)	3.5 g in 70 ml H_2O
Horse serum	20.0 ml
Fresh yeast extract	8.0 ml
Penicillin G	1000 IU/ml (final conc.)
Thallium acetate	1:2000 (final conc.)

pH 7.2–7.6

Sterilize agar in an autoclave. Combine and filter sterilize balance of components. Heat fluid portion to 56°C in a water bath, combine with agar, and dispense in plates of individual choice. Store in a sealed bag at 5°C.

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Modified Hayflick's Medium for Ureaplasmas

Mycoplasma broth base (BBL)	2.1 g
Distilled water	66.0 ml

Adjust pH to 5.4 with 2 *N* HCl. The final pH after sterilization and addition of all components should be 6.0 ± 0.2. The exact pH required before sterilization depends upon the effect of the horse serum.

For semisolid medium, add 0.05 g Ionagar #2 or #2S; for solid medium, add 1.0 g.

Autoclave at 15 lb pressure for 15 min. Cool (to 50°C for agar containing media), and add aseptically:

Horse serum	20.0 ml
Yeast extract, 25% (w/v), pH 6.0	10.0 ml
Urea, 25% (w/v)	1.0 ml
Phenol red, 0.5%	0.2 ml

Omit urea and phenol red for solid medium. Penicillin (500 U/ml) may be added.

Gourlay and Leach Medium (1)

Hank's buffered salt solution	40.0 ml
Lactalbumin hydrolysate, 5% (w/v)	10.0 ml
Broth (Difco)	20.0 ml
Fetal calf serum (inactivated 30 min., 56°C)	20.0 ml
Glucose, 50% (w/v)	2.0 ml
Calf thymus DNA highly polymerized, 0.2% w/v	1.0 ml
Ampicillin (for injection)	0.15 mg/ml
Thallium acetate, 5% (w/v)	0.5 ml
Phenol red, 1% (w/v)	0.2 ml

Adjust pH value to 7.8; sterilize by filtration.

Friis Medium for Swine Mycoplasmas (2)

Hank's balanced salt solution	500.0 ml
Bacto brain–heart infusion (Difco)	8.2 g
Bacto PPLO broth without c/v (Difco)	8.7 g
Water	750.0 ml

Autoclave 2–5 min at 121°C.

Add the following sterile preparations:

Yeast extract	60.0 ml
Phenol red, 0.5%	4.5 ml

Bacitracin	250.0 ml
Meticillin	250.0 mg
Thallium acetate	1:10,000
Porcine serum	250.0 ml

pH 7.4

Preparation of Yeast Extract

To 1800 ml of distilled deionized water, heated to 70°C, add 425 g yeast (bakers' cake form). Adjust pH to 4.6. Gently heat with continual agitation until boiling point is attained. Cool to refrigerator temperature, then freeze to −20°C. After a period in excess of 3 days, thaw at room temperature and adjust pH to 7.8. Centrifuge at 950 G for 60 min. Remove supernatant and filter first through No. 1 Whatman filter after addition of Celite followed by sterilization using Seitz sterilizing or Millipore (0.22-μm pores) filter. Dispense sterile yeast extract in 100 ml volumes and store in freezer at −20°C.

M-96 Broth (Frey's Medium) (3)

Peptone CS (Albimi)	4.0 g
Peptone B (Albimi)	2.0 g
Peptone G (Albimi)	2.0 g
Yeast autolysate (Albimi)	2.0 g
Yeast extract	2.0 g
NaCl	5.0 g
KCl	0.4 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Catalase	0.001 g
HEPES buffer	3.5 g
L-Arginine HCl	0.06 g
L-Glutamine	0.09 g
DNA	0.02 g
Distilled water	1 liter

Dissolve dry ingredients.

100× Eagle's MEM vitamin solution	10.0 ml
Glycerol	0.15 ml
Cholesterol (0.1% emulsion)	2.0 ml
Thallium acetate, 1%	25.0 ml
Penicillin	500,000 U

Adjust pH to 7.4–7.5 with NaOH.

Porcine serum (clarified) and inactivated (30 min, 56°C)	170 ml

Filter sterilize and dispense in desired aliquots. Add 2 ml of DPN-cysteine solution to each 100 ml of broth on the day of use. Final concentration in broth, 0.01%.

M-96 Agar

Peptone CS (Alibimi)	4.0 g
Peptone B (Alibimi)	2.0 g
Peptone G (Alibimi)	2.0 g
Yeast autolysate (Alibimi)	2.0 g
NaCl	5.0 g
KCl	0.4 g
$MgSo_4 \cdot 7H_2O$	0.2 g
Catalase	0.001 g
HEPES buffer	3.5 g
L-Arginine HCl	0.06 g
L-Glutamine	0.09 g
Distilled water	500 ml

Dissolve dry ingredients.

100× Eagle's MEM vitamin solution	10.0 ml
Glycerol	0.15 ml
Cholesterol (0.1% emulsion)	2.0 ml
Thallium acetate, 1%	25.0 ml
Penicillin	500,000 U

Adjust pH to 7.4–7.5 with NaOH.

Porcine serum (clarified) and inactivated (30 min, 56°C)	170 ml

Filter sterilize.

Ionagar #2	17 g
Distilled water	500 ml

Sterilize at 15 lbs pressure for 15 min.

Bring both broth and agar to 50°C and mix together. Add DPN-cysteine solution for a final concentration of 0.01%. Dispense into Petri dishes.

Preparation of Cholesterol Emulsion

1. Cholesterol (200 mg) is put in a sterile Petri dish and dissolved in diethyl ether. The ether is allowed to evaporate, and the process is repeated once more.

2. The sterile, recrystallized cholesterol is dissolved in 6–8 ml warm 95% ethyl alcohol.
3. The alcohol solution is drawn into a prewarmed glass Luer-Lok® syringe fitted with an 18- or 20-gauge needle.
4. Hold needle tip under the surface of 200 ml of distilled, deionized water and eject the cholesterol into it.

Clarification of Porcine Serum

1. Adjust pH of porcine serum to 4.4 with 1 *N* HCl (do not drop pH below 4.2).
2. Allow serum to stand at 4°C for 18–20 hr.
3. Centrifuge at 9000 *g* for 30 min; discard sediment.
4. Filter through No. 1 Whatman filter paper.
5. Adjust pH to 7.0 with 1 *N* NaOH.

Preparation of DPN-Cysteine Solution

Prepare a 1% solution (1 g/100 ml distilled water) of each of the following: Cozymase (coenzyme I) oxidized (Nutritional Biochemical Corp., Cleveland, Ohio) and Cysteine HCl hydrate, A grade (Calbiochem, La Jolla California) Mix the solution together; the cysteine reduces the DPN. Filter-sterilize, dispense in appropriate amounts, and store frozen at −20°C.

Dienes's Stain

Staining Solution

Methylene blue	2.40 g
Maltose	10.0 g
Azure II	1.25 g
Sodium chloride	0.25 g
Distilled water	100.0 ml

This solution is available commercially (Hyland Laboratories, Los Angeles, Calfornia).

Staining of Coverslips
Apply a thin film of the stain to clean coverslips by means of a cotton swab. The film should be uniform and light. When dry, they are ready for use and may be stored indefinitely.

Staining Procedure

A 1-cm-square block is cut from an area containing suspected colonies and transferred to a microscope slide, colony side up. Place a treated coverslip, stain side down, on the agar block. The staining reaction is complete within a few minutes. All colonies stain, but in about 15 min the bacterial colonies decolorize while the mycoplasma colonies retain their color. The preparation is examined under low or higher powers employing transmitted light.

It should be remembered that this stain does not distinguish L-type colonies from mycoplasma colonies.

References

1. Gourlay, R. N., and Leach, R. H.: *J Med Microbiol, 3:*111, 1970.
2. Friis, N. F.: *Nord Veterinaermed, 27:*337, 1975.
3. Frey, L. M., Thomas, G. B., and Hale, P. A.: *Ann NY Acad Sci, 225:*334, 1973.

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