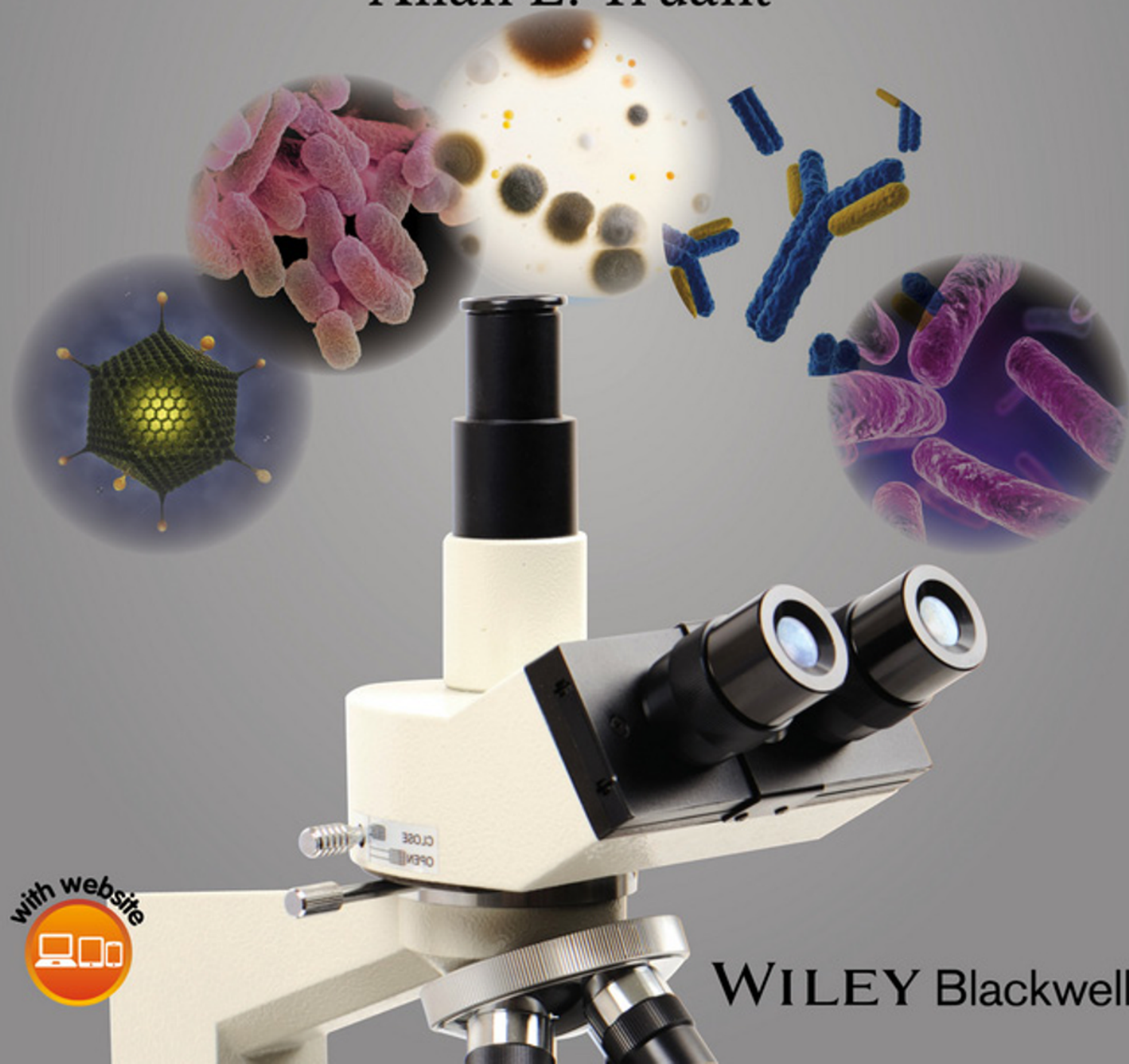


INTERNATIONAL EDITION

Manual of Commercial Methods in Clinical Microbiology

SECOND EDITION

Editor-in-Chief
Allan L. Truant



WILEY Blackwell

Manual of Commercial Methods in Clinical Microbiology

Dedication

This book is dedicated to the memory of Stephen D. Allen, MD, gentleman, scientist, innovator, pathologist, clinical microbiologist and anaerobist, author and teacher, mentor, colleague, Hoosier, and friend.

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Important Notice

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to her academic responsibilities at Hofstra North Shore-LIJ School of Medicine, Dr. Christine Ginocchio also has professional affiliations with Biofire Diagnostics, Salt Lake City, Utah, USA, and bioMérieux, Durham, North Carolina, USA. Websites and vendor contact information are current at the time of writing for each chapter/section; the reader should confirm all websites and vendor information at the time he/she is searching for current information.

Some of the tests and methods discussed in this manual have US Food and Drug Administration (FDA) clearance for selected uses; and for countries outside of the United States, some products and instruments may have approval by that specific country's equivalent to the US FDA. It is the responsibility of the laboratorian or healthcare provider to ascertain the governmental regulatory status of each product that is considered for use in his or her hospital, setting, or practice.

Preface to the Second Edition

Much has happened in the field of clinical microbiology since the first edition of this text in 2002. New organisms and new taxonomy have been described, and many new technologies have emerged from the research bench to the hospital laboratory. The principal paradigm shift has been the growth of genomic approaches led by the polymerase chain reaction (PCR) for the identification of many organisms. A second major paradigm shift currently taking place in many laboratories across the globe is the use of matrix-assisted laser desorption ionization (MALDI) combined with time of flight (ToF) mass spectrometry (MS), a proteomic approach for the identification of bacteria and fungi. The additional emergence of sequencing technologies, including real-time genome sequencing, real-time PCR, and multiplexing strategies, have further expanded our capabilities in organism detection and identification. These major approaches have, and are, changing the current design of the modern clinical microbiology laboratory and have the potential of significantly changing our long-standing basis of organism identification from a phenotypic to a genomic approach. We are still searching, however, for more rapid and accurate methods for antimicrobial susceptibility testing, since the mainstay of antibiotic testing remains a biological approach to the growth and inhibition of the organism in question (at least for bacteria). The molecular approach, however, has been helpful in the area of antiviral and selected bacterial resistance mechanisms. An explosion of point of care (POC) devices and development of total laboratory automation, are also current trends.

Clinical microbiologists interact on a daily basis with a wide variety of medical disciplines, interpret laboratory results, and work closely with specialists in infectious diseases (both the adult and pediatric sub-specialties) and infection control practitioners. For many years, those practicing our specialty have also worked very closely with pharmacists in the selection, reporting, and evaluation of antibiotics and chemotherapeutic agents; most recently a growing number of pharmacists at the doctoral level (PharmD) are specializing in infectious diseases and are working very closely with infectious disease physicians and clinical microbiologists in formulary decisions and antibiotic stewardship initiatives. Hospital administrators

have learned that hospital budgets can be more efficiently designed and implemented with careful use, reporting, and choice of antibiotics on the hospital formulary, and careful use of infection control practices.

As with the first edition, this reference is not intended to provide an exhaustive review of clinical microbiology procedures. Other references from this and other medical publishers have information related to clinical microbiology procedures, descriptions of microorganisms, and infectious diseases. We believe, however, that this is a principal reference for commercially available tests, devices, and instruments used in clinical microbiology. The most valuable up-to-date information, data, and studies still must be found in high quality peer-reviewed journals. These journal articles often review, discuss and evaluate new tests, compare new tests to current methods (and to gold standards, or enhanced gold standards) and present sensitivities, specificities, positive predictive values, negative predictive values, and other statistical information. Companies and vendors manufacturing these products also often provide early test statistics in their own package inserts from internal and occasionally funded or supported studies. Some of this internal information may also be used in the submission of the product to the US Food and Drug Administration (FDA) for product review and approval.

In this second edition of the *Manual of Commercial Methods in Clinical Microbiology* we have attempted to update all of the core chapters from the first edition, and we have added a number of new chapters such as those in Virology, Viral Hepatitis, Human Papilloma Virus, Rickettsiae and Tick-Borne Diseases, Molecular Typing Instruments and Methods, and Bioterrorism. Another major addition to this reference is an International Section, which provides information on the regulatory requirements for the adoption, evaluation, and use of clinical microbiology products in selected countries. Our group of international authors have also attempted to provide information on what products are imported, and those which are manufactured within their country and used in the field of clinical microbiology (and may not have review by the US FDA). Similar to our US FDA, many countries have agencies in their federal governments that regulate products, devices, tests, and instruments (both

domestic and imported) in the fields of clinical microbiology, pharmaceuticals, and products used in clinical trials. To our knowledge, this is the first time that a project of this magnitude has been attempted in a clinical microbiology reference and we understand the myriad of challenges that this entails. We have been limited by the number of countries that we have been able to include and we apologize in advance for any inadvertent omission of any specific regulatory requirement or product availability. Some information that we were unable to include in this final text may be available at the web site associated with the book.

The inclusion of the international section was indeed a herculean task and probably could have resulted in a separate book for each country. We have attempted to be concise and to present a snapshot of clinical microbiology products and the regulatory environment in selected countries. It is not meant as an exhaustive resource for all products commercially available. We thank all of the international authors and apologize for any omission of tests, or products. We look forward to improving this in future editions.

When feasible, the authors have attempted to include reported sensitivities, specificities, and predictive values for products, tests, devices, and instruments used in clinical microbiology. And we have attempted to include only data from peer-reviewed literature (including abstracts). Additional information that will be of value is included in chapters devoted to molecular microbiology, information management, emerging infectious diseases, and veterinary clinical microbiology.

This reference may be used as a starting point, if a test, kit, procedure, or instrument is being considered for an organism detection, identification or antibiotic susceptibility test. The user must refer to specific vendors for current pricing information since this will vary with volumes, contracts, specific products, and purchasing groups. This reference text will not universally specify the status of copyright, or trade marks for all products. The reader is referred to each specific vendor and web site for this information. Also, it is suggested that the reader review newly published peer-reviewed product information and studies, check with vendors and their web sites, and monitor the FDA clearance (if used in the United States) for any product being considered (or the FDA-equivalent outside of the United States). Discussions with colleagues and others who are using, or have experience with, a specific product may also be of significant value.

The authors and editors have attempted not to duplicate additional information regarding methods, procedures, or product reviews available in other references. Among the topics that are not exhaustively reviewed in this text include taxonomy, routine culture information (including media), swabs and transport devices, normal flora, microscopy, laboratory management, infection control, and organism

groups. This text also does not cover the plethora of antibody tests, which are reviewed in other texts. The exclusion of antibody tests does not pertain to selected chapters that rely primarily, or to some degree, on antibody testing.

The contributors to this book consist of an experienced group of practicing clinical microbiologists, infectious disease experts, pathologists, physicians, and scientists. We thank all of the authors for their diligence and completeness. We apologize for any unintentional omission of tests, data, devices, or instruments and we encourage the reader to contact the editors or the publisher with any suggestions or corrections for future editions.

The authors have done their best to describe the most commonly used products in clinical microbiology. As all clinical microbiologists readily understand, comparisons between products are quite difficult and scientifically challenging. Among these difficulties are changing reagents, pricing, software, hardware, databases, and technology used in the test, device, or instrument. Different groups of products are evaluated and described in the literature at different times, in different laboratories, or with different reference procedures. The “gold standard” is also changing rapidly, especially when evaluating molecular, genomic, or proteomic methods. In addition, many new products are entering the marketplace and others have been removed. Some products are manufactured by the same vendor, but may be marketed (or labeled) with another name under specific contractual agreements.

The contributors have attempted to sort through this maze and have tried to include only the most recent and pertinent references. Of course, each laboratory and director must thoroughly evaluate each product for its usefulness in a specific setting, and validate or verify its performance before implementation. This must be performed keeping in mind the complexity of the method, the technical expertise and quality control required, and review of the test by the FDA (or relevant governmental authority outside of the United States); additionally, the cost of the method (including reagents, personnel, and equipment) and its clinical usefulness must be evaluated. In fact, evidence based medicine (EBM) for diagnostic laboratory testing is becoming commonplace in all areas of clinical microbiology.

We hope that the information presented in this reference proves useful to clinical microbiologists, medical technologists, pathologists, infectious disease specialists, and other healthcare providers. And we especially hope that it serves as an incentive for future breakthroughs and accomplishments in clinical microbiology and infectious diseases.

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Foreword

During the past four decades, the practice of clinical microbiology has evolved from what was almost entirely a manual exercise to a discipline that is now largely dependent on various forms of instrumentation. This technologic revolution has clearly enhanced the ability of clinical microbiology laboratories to be responsive to the needs of patients with infectious diseases, however, a number of important challenges have also emerged. The work environment in clinical microbiology laboratories has clearly changed, workforce requirements are different, cost considerations have percolated into the mainstream of laboratory practice, and interactions with an ever expanding industry sector have become essential. From a practical perspective, there is the pragmatic matter of effectively navigating through the dizzying array of technological possibilities.

The first edition of the *Manual of Commercial Methods in Clinical Microbiology* (MCMCM), first published 14 years ago, quickly became an enormously important resource to laboratorians faced with having to make decisions in the midst of the technology revolution in clinical microbiology. But a lot has changed during the past 14 years. With this in mind, Allan Truant has undertaken the rather daunting task of crafting a second edition of this important text. He has been hugely successful in this endeavor. This text is entitled, *Manual of Commercial Methods in Clinical Microbiology, International Edition, Second Edition*.

This edition of MCMCM will be even more useful to practicing clinical microbiologists than the first. It begins with a review of the US Food and Drug Administration (FDA) regulatory process that attends the development and licensure of new technologies in clinical microbiology. There are 14 chapters that pertain to technologies relevant to different organism groups. The information in these chapters, most notably for blood cultures, mycobacteriology, and parasitology, is particularly instructive

and updated to current technologies. These chapters are followed by an erudite introductory chapter which addresses general concepts of molecular microbiology, a review chapter pertaining to different molecular typing technologies, a most insightful chapter on immunoassays, and even a chapter on commercial methods in veterinary microbiology. Laboratory information systems, emerging infectious diseases, and potential new technologic applications in clinical microbiology (in the “Looking Ahead” chapter) are then considered. There is also a chapter on rapid devices and instrumentation for use in antimicrobial susceptibility testing. New chapters added in this updated second edition of MCMCM include those on Bioterrorism, Rickettsiae and Tick Borne Diseases, Human Papillomaviruses, Viral Hepatitis, and Molecular Methods for the Identification of Viruses.

The reader of MCMCM is then provided with an exhaustive list of manufactures and distributors currently active in the field of clinical microbiology and, finally, in what is a welcome new addition to this edition of MCMCM, a series of most informative perspectives on the issues of clinical microbiology regulatory requirements and technology as they exist in Canada, Argentina, various countries in western Europe, Africa, Asia, and the Pacific Rim.

Simply put, Dr. Truant has hit another home run with this second edition of MCMCM. Like the first, it will undoubtedly come to occupy a prominent spot on the book shelves of practicing clinical microbiologists everywhere. It is a practical reference text that will be used repeatedly and represents an important addition to the clinical microbiology literature.

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suggestions and advice. I also owe a tremendous vote of thanks to all of the associate editors and authors (throughout the world) who have truly made this most probably the first truly international book project in clinical microbiology. We hope that it inspires others to expand on the usefulness of the information contained in this second edition of the *Manual of Commercial Methods in Clinical Microbiology, International Edition*.

Allan L. Truant
Editor-in-Chief

Rising Sun Chair



Rising Sun Chair by John Folwell, 1779. Photograph by Joseph Elliott, May 2014. (Courtesy, Independence National Historical Park.)

Since this book and its first edition were edited in Philadelphia, Pennsylvania, USA, it is appropriate to use the Rising Sun Chair as a metaphor for our own discipline of clinical microbiology. It was used by our first president, George Washington during the Constitutional Convention of 1787 in Philadelphia at Independence Hall. The chair's name was coined by Benjamin Franklin as delegates signed the newly drafted US Constitution. Franklin told those around him that the sun on the chair

was rising and not setting on this new nation. We, too, as clinical microbiologists around the world are a rising discipline and not a setting discipline in the field of diagnosing and treating infectious diseases and using clinical microbiology diagnostic techniques and methods to improve the health of people, and indeed all living things.

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CHAPTER 1

Role of the US Food and Drug Administration in the Regulation of Clinical Microbiology Devices

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1.1 Historical overview of *in vitro* diagnostics

The Medical Device Amendment (MDA) of 1976 [4] was the result of a long history of fraudulent medical devices beginning as early as the 1700s with Dr. Elisha Perkins' patent tractors, which consisted of two rods of brass and iron about three inches long purported to eliminate disease from the body. Despite the surge of fraudulent medical devices throughout the years, no substantial regulation was enacted to ensure the safety and effectiveness of devices. The enactment of the Food Drug and Cosmetics (FD&C) Act of 1938 was the first step towards eventual implementation of device regulation.

1.1.1 Device classification via the MDA of 1976

The subject matter in this section as well as the remainder of the chapter will be confined to a discussion of the regulations that affect the classification of microbiological devices, as well as the various FDA processes which enable the commercial interstate sale and distribution of clinical microbiology *in vitro* diagnostic devices (IVDs) by the Center for Devices and Radiological Health (CDRH).

The following definition of an *in vitro* diagnostic device can be found in the FDA regulation 21CFR (Code of Federal Regulations) 809.3

- (a) *In vitro diagnostic products* are those reagents, instruments, and systems intended for use in the diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae. Such products are intended for use in the collection, preparation, and examination of specimens taken from the human body. These products are devices as defined in section 201(h) of the Federal Food, Drug, and Cosmetic Act (the act), and may also be biological products subject to section 351 of the Public Health Service Act.

- (b) A *product class* is all those products intended for use for a particular determination or for a related group of determinations or products with common or related characteristics or those intended for common or related uses. A class may be further divided into subclasses when appropriate.

The MDA of 1976 required that all medical devices be divided into three product classes by virtue of the types of controls necessary to assure the safety and effectiveness of the device.

- Class I, general controls, includes devices which appear to pose relatively little risk to human health and for which a series of general controls are believed sufficient to ensure safety and effectiveness. The controls include regulations that: (i) prohibit the sale of adulterated or misbranded devices; (ii) require domestic device manufacturers and initial distributors to register their establishments with the FDA and provide the FDA with a list of all devices being sold before the passage of the law (pre-amendment devices); (iii) grant the FDA authority to ban certain devices; (iv) provide for notification to the FDA of risks and of repair, replacement, or refund (recall); (v) restrict the sale, distribution, or use of certain devices; and (vi) govern good manufacturing practices, records and reports, and inspections. These requirements also apply to Class II and Class III devices.
- Class II devices are those for which general controls alone are insufficient to assure safety and effectiveness, and existing methods are available to provide such assurances. In addition to complying with general controls, Class II devices are also subject to special controls. Special controls may include special labeling requirements, mandatory performance standards, and post-market surveillance.
- Class III devices are usually those that support or sustain human life, are of substantial importance in preventing impairment of human health, or which present a potential,

unreasonable risk of illness or injury. In many cases there is insufficient information to ensure that general controls and performance standards will provide assurance that the devices are safe and effective. New devices are assigned to Class III until they are found to be substantially equivalent to a pre-amendment device (Class I or II) or are reclassified as Class I or II devices through the *de novo* petition program.

As can be seen from the above descriptions, classification of an IVD is risk based. In addition, device classification is inherently tied to the intended use of a device. For example, when the intended use for a device is as an aid in the assessment of serological status for sexually active adults and expectant mothers the device is classified as Class II as risks may be mitigated with appropriate controls. However, when the intended use for a device is an aid in the assessment of response to therapy in immunocompromised patients, e.g., transplants recipients where there is a substantial increase in risk such as death from cytomegalovirus (CMV) infection, the device classification is Class III.

Sec. 866.3020 Adenovirus serological reagents

(a) *Identification.* Adenovirus serological reagents are devices that consist of antigens and antisera used in serological tests to identify antibodies to adenovirus in serum. Additionally, some of these reagents consist of adenovirus antisera conjugated with a fluorescent dye and are used to identify adenoviruses directly from clinical specimens. The identification aids in the diagnosis of disease caused by adenoviruses and provides epidemiological information on these diseases. Adenovirus infections may cause pharyngitis (inflammation of the throat), acute respiratory diseases, and certain external diseases of the eye (e.g., conjunctivitis).

(b) *Classification.* Class I (general controls). The device is exempt from the premarket notification procedures in subpart E of part 807 of this chapter subject to the limitations in 866.9.

Sec. 866.3390 *Neisseria* spp. direct serological test reagents

(a) *Identification.* *Neisseria* spp. direct serological test reagents are devices that consist of antigens and antisera used in serological tests to identify *Neisseria* spp. from cultured isolates. Additionally, some of these reagents consist of *Neisseria* spp. antisera conjugated with a fluorescent dye (immunofluorescent reagents) which may be used to detect the presence of *Neisseria* spp. directly from clinical specimens. The identification aids in the diagnosis of disease caused by bacteria belonging to the genus *Neisseria*, such as epidemic cerebrospinal meningitis, meningococcal disease, and gonorrhea, and also provides epidemiological information on diseases caused by these microorganisms. The device does not include products for the detection of gonorrhea in humans by indirect methods, such as detection of antibodies or of oxidase produced by gonococcal organisms.

(b) *Classification.* Class II (performance standards).

Table 1.1 Types of FDA submissions with associated device classes

Class	Premarket submission	Success metric	Action
III	PMA	Safety and effectiveness	Approval
II	510(k)	Substantial equivalence	Clearance
I	None (if exempt) 866.9 limitations to exemptions		
II (<i>de novo</i>)	<i>De novo</i> request	Safety and effectiveness	Approval

Microbiology device regulations and their classifications can be found in 21 CFR, Microbiology Regulation, Sections 866.1 through 866.3980. The following are examples of Class I and Class II microbiology devices.

Many times assay developers will look at a classification and find it exempt from section 510(k) of the act. It is important to note here that 21 CFR 866.9 Limitations of Exemptions from Section 510(k) of the Federal Food, Drug, and Cosmetic Act (the act) contains several important points that are specifically related to microbiology devices. They are:

- (6) For identifying or inferring the identity of a microorganism directly from clinical material;
- (7) For detection of antibodies to microorganisms other than immunoglobulin G (IgG) or IgG assays when the results are not qualitative, or are used to determine immunity, or the assay is intended for use in matrices other than serum or plasma;
- (8) For noninvasive testing as defined in 812.3(k) of this chapter; and
- (9) For near patient testing (point of care)

Table 1.1 illustrates the types of submissions sent to the FDA for review for each device class, the metric for the successful completion of that submission, and the final action taken to enable that device to enter interstate commerce.

1.2 Current microbiology device review regulatory pathways: practical considerations

1.2.1 Premarket notification 510(k)

After having established the regulatory class of a device and prior to introduction into the market of a device intended for human use, each assay developer must submit a 510(k) to the FDA unless the device is exempt from 510(k) requirements of the Federal Food, Drug, and Cosmetic Act (the act) and does not exceed the limitations of exemptions in .9 of the device classification regulation chapters (e.g., 21 CFR 862.9, 21 CFR 864.9 and 21 CFR 866.9).

The purpose of the 510(k) submission is to demonstrate to the FDA that the new device is at least as safe and effective, that is, substantially equivalent to, as a legally

marketed device or predicate device (21 CFR 807.92(a)(3)). While the basic content of a 510(k) has been established under 21 CFR 807.87, it is important to note that the types of information and data required may likely vary according to the analyte being detected as well as the differences between the new device and the predicate device. The FDA has 90 days for review of the submitted 510(k) in which to make a decision on whether a device is substantially equivalent.

Primary in the determination of substantial equivalence is that the new device has the same intended use as the predicate device. In addition, while the new device generally has the same technological characteristics, this is not a requirement. New devices with the same intended use yet different technological characteristics will be expected to supply appropriate clinical or scientific data if deemed necessary by the Secretary of Health and Human Services or a person accredited under Section 523, that demonstrates that the device is as safe and effective as a legally marketed device, and does not raise different questions of safety and effectiveness than the predicate device.

Following review of a 510(k) submission which the FDA finds substantially equivalent, each submitter will receive an order, in the form of a letter, from the FDA which finds the device to be substantially equivalent and states that the device can be marketed in the United States. This “clearance order” permits commercial distribution of the device.

Alternatively, following the review of a 510(k) submission, the FDA may find the device not to be substantially equivalent (NSE) to the predicate device. In general, an NSE decision is based on one of the following reasons.

- 1 No predicate device exists for the device, or the intended use is different than the predicate. In each case the device is deemed to be a Class III device and cannot be reviewed via a 510(k) submission.
- 2 The submitted information and data do not support a decision of substantial equivalence.

In the first case, the FDA will issue a letter stating that the device has automatically been classified as Class III and requires a premarket amendment (PMA), or that the device may be eligible for the *de novo* petition program (see below).

In cases where the information submitted is not sufficient to make a determination of substantial equivalence, the FDA will issue a letter identifying the deficiencies, which are usually performance related, and request additional information to resolve these deficiencies. The FDA will interactively work with developers to resolve the deficiencies within the time frame specified by regulation and guidance. If the information or justifications supplied are insufficient, the FDA will issue a NSE letter. If a sponsor does not respond at all to the request for additional information within the time frame specified by regulation and guidance, the FDA will consider the submission withdrawn. In either case above, the sponsor does have

the opportunity to make a new 510(k) submission that addresses the outstanding items from the original review. The new 510(k) submission should cite the previous 510(k) number and specify how the outstanding items have been addressed and whether the new submission contains only the original information or additional new information.

1.2.2 *De novo* petitions

In response to devices that had been found NSE due to the lack of a predicate device, the Food and Drug Administration Modernization Act of 1997 (FDAMA) added the “*de novo*” classification option as an alternate pathway to classify such devices. The formation of this pathway enables FDA to make a risk-based classification determination for the device under Section 513(a)(1) of the FD&C Act.

Devices which may be eligible for this pathway must be low to moderate risk devices and the assignment of substantial benefits to patients is not as high as that for Class III devices. Such devices should be sufficiently understood to explain all the risks and benefits of the device such that all risks can be appropriately mitigated through the application of general and/or special controls to provide reasonable assurance of safety and effectiveness.

Once a device is granted marketing authority under the *de novo* petition program it is also eligible to serve as a predicate device for new devices that can be regulated via a 510(k) submission, thus enhancing the need for an appropriate risk-benefit profile when considering the safety and effectiveness of the device.

1.2.2.1 Scientific evidence supporting a *de novo* petition

The majority of microbiology devices using the *de novo* classification option will have both analytical and clinical data in support of the petition. Nonclinical testing may include, but is not limited to, testing such as the limit of detection (LoD) of the analyte, precision and/or reproducibility of the assay, interference testing, and cross-reactivity with other microbiological or nonmicrobiological analytes. A variety of clinical testing methods are also used in support of the petition. The clinical testing may involve anything from a randomized clinical trial, comparison of the new device to a putative gold standard using clinical specimens, and comparison of the new device to a patient infected status (PIS).

1.2.2.2 Benefit-risk determinations

Since the factors that the FDA considers as part of the benefit-risk determination (as well as examples) when reviewing a *de novo* petition are detailed in the guidance document “Factors to Consider When Making Benefit-Risk Determinations in Medical Device Premarket Approval and *De Novo* Classifications” only a brief mention of them will be made here.

1.2.2.3 Benefits of a new device

For microbiological devices the types of benefits may include the impact on clinical management of a patient, for example, the use of viral load assays to predict a patient's response to therapy; the diagnosis of a specific pathogen which may have an impact on public health such as devices that identify influenza virus or *Chlamydia trachomatis*; and the ability to identify a specific pathogen to enable correct immediate treatment, such as those devices that identify *Clostridium difficile*.

Other benefit considerations may be the magnitude of the benefit, the probability of the patient experiencing a benefit and the duration of the effect(s) of the benefit.

1.2.2.4 Potential risks associated with a new device

The risks associated with microbiological devices are most often considered in terms of the risk of a false positive or a false negative result. A false positive result for a diagnostic assay may cause a patient to receive unnecessary therapy such as receiving a course of antibiotics for a viral infection, or may have severe consequences if a pregnant woman is misdiagnosed with a positive result for herpes simplex virus or rubella. A false negative result may also have either mild or severe consequences. For example, a false negative may lead to the incorrect treatment for bacterial meningitis, with severe consequences.

In addition to the individual effects, the aggregate effects of a misdiagnosis are also considered. Misdiagnosis of an individual with *C. difficile* infection may cause an infected patient to be housed with uninfected patients thus causing spread of the disease to the other residents of a hospital ward. Similarly, misdiagnosis of a sexually transmitted infection (STI) may also have significant ramifications in the spread of the pathogen in a population.

Possible mitigation of potential risks is also extremely important when determining the risk-benefit of a device. Mitigations such as restrictions in the labeling of a diagnostic device and restrictions in the use of a device to a segment of a population are two important safeguards that are often employed.

1.2.3 Premarket application

The PMA process is a scientific and regulatory review to assess the safety and effectiveness of Class III devices. Any microbiological device that involves measuring, detecting, or identifying a Class III analyte, is subject to a PMA review (see discussion above on classifying analytes). The applicant must receive FDA approval of its PMA application prior to marketing the device. Such an approval is based on a determination by the FDA that the PMA contains sufficient valid scientific evidence to assure that the device is safe and effective for its intended use(s). An approved PMA is, in effect, a private license

granting the applicant (or owner) permission to market the device. While the PMA is considered to be the most stringent type of device marketing application required by the FDA, this is primarily due to the PMA Manufacturing Quality System section, which must be included with the application. It is expected that all medical devices be manufactured under a quality system that is defined by FDA Regulation 21 CFR. 820, however, the details of the manufacturing of a specific device need only to be submitted to the FDA for Class III products. In addition a PMA may also be the subject of a review by an FDA designated microbiology panel of independent outside experts and is also subject to a quality audit by FDA's Bioresearch Monitoring Program (BIMO) of a clinical study site's compliance with a study protocol and the data generated during the study. The FDA has 180 days to review a submitted PMA and to make a decision of approval, approvable, or not approved. An approved PMA gives the applicant permission to market the device. A finding of approvable means that there are certain items outstanding, usually related to manufacturing documents that need to be corrected prior to approval, and not approved means that the application is denied and the applicant may not market the device.

1.2.3.1 When is a PMA required?

The initial step in determining whether a device requires a PMA is to search the product classification database: <http://www.fda.gov/medicaldevices/deviceregulationandguidance/overview/classifyyourdevice/ucm051637.htm>. The resulting search will yield information on device names, classifications, and any existing links to the CFR. Please note that for many devices that are novel or for which there were no existing devices prior to 1976, there will be no classification number, only the name of the device, a product code, and in some cases it will state if a PMA is required for that device.

If it is still unclear whether a PMA is necessary, the next step is to use the product code to search both the 510(k) and PMA databases, which can be found as links on the product classification database. Should a 510(k) be found in the database, and the new device has an equivalent intended use, then it is generally safe to assume that a 510(k) submission will be applicable to the device.

Finally, a device may not be found at all in the product classification database. This may occur if the analyte detected by the device is "novel," for example having only recently been discovered and isolated. For those devices not found in the database, consideration is given to whether the device is high risk, i.e., supports or sustains human life, is of substantial importance in preventing impairment of human health, or presents a potential, unreasonable risk of illness or injury. In addition, a device may have been found NSE to a Class I or Class II device. In both of these cases a PMA submission is required.

1.2.4 Major elements of an IVD submission to the FDA

For a new IVD, both “safety” and “effectiveness” of a device must be demonstrated. The broad criteria for evaluating the safety and effectiveness of a new IVD are also defined by regulation:

- Safety: are there reasonable assurances based on valid scientific evidence that probable benefits to health from use of the device outweigh any probable risks? [21 CFR 860.7 (d)(1)].
- Effectiveness: is there reasonable assurance based on valid scientific evidence that the use of the device in the target population will provide clinically significant results? [21 CFR 860.7(e)(1)].

Requirements for marketing also include the need for labeling, which includes, among other requirements, product performance characteristics “as appropriate... describing such things as accuracy, precision, specificity, and sensitivity” [21 CFR 809.10]. The following discussion provides an overview of how the FDA approaches these requirements during the development and review of a new microbiology diagnostic device.

There is significant overlap in the elements of a submission for an *in vitro* diagnostic device approval or clearance and these are presented in Table 1.2. Several of these elements will be discussed in detail below and references to guidance documents for specific elements and/or analytes are included at the end of this chapter.

1.2.4.1 Intended use

The intended use of an *in vitro* diagnostic device is possibly the most critical element of an IVD. It not only establishes the risk associated with that device but dictates what studies should be performed (both analytical and clinical) in support of the submission and, finally, drives the FDA review of that submission. In devising an intended use for a new IVD the sponsor should have a clear understanding of the conditions/disease associated with the target organism and how the results from the IVD will integrate into patient management, as well as the patient populations for whom the IVD is intended.

Table 1.2 Major elements in a submission for a new *in vitro* diagnostic device

Intended use
Device description, internal/external controls
Assay interpretation
Pre-analytical (e.g., sample preparation) and analytical performance
Clinical performance
Instrument and software, if applicable
• If multiple platforms, assay performance on each
Labeling (package insert)
PMA-specific elements
• Manufacturing
• Quality systems inspections
• BIMO inspections of clinical studies

The intended use of a device describes the organism/analyte being detected or measured in relation to the disease or condition associated with that organism/analyte. It also describes whether the assay is a qualitative assay associated with the direct detection of an organism, e.g., *Chlamydia trachomatis*, or if the assay quantitatively detects an organism, e.g., viral load molecular assays for hepatitis B. The patient specimen matrix being tested by the device is also an important part of the intended use: whether the matrix is serum or plasma (including anticoagulation types), urine or CSF, the swab type (nasal, genital), whether it is a clinician-collected or self-collected sample, and where the specimen collection may take place, such as a professional clinical high-complexity laboratory; a point of care (POC) location in a hospital (such as an emergency room or patient bedside); a facility with untrained users (e.g., a physician’s office); or an “over the counter” home test.

Limitations may also be part of the intended use. For example, “This assay has not been licensed for testing of blood donors” or in the case of many quantitative viral load assays such as for hepatitis C virus (HCV), “This assay has not been approved for diagnosis of hepatitis C.”

1.2.4.2 Device description

This section should contain all the items associated with the IVD, whether they are contained within the IVD kit or need to be obtained separately. Instrumentation and software applications may be a substantial portion of this section.

1.2.4.3 Assay interpretation

The interpretation of results from a new investigational device should be determined prior to conducting “pivotal” clinical studies. For a qualitative assay, this includes the criteria for scoring device results (e.g., as either “analyte detected,” “analyte not detected,” “equivocal,” or “invalid”). Invalid results may occur when one or more test parameters fail to meet the expected criteria. For equivocal results, rules for retesting specimens should be specified.

1.2.4.4 Analytical performance characteristics

The analytical performance characteristics are some of the initial characteristics studied on a new device. The results of these studies can provide an insight into the possibility of false negative and false positive results with the new IVD. An insight into the likelihood of false negative results can often be gleaned from the results of LoD, the effect of different matrices on the ability of the device to detect an organism, and the potential of interfering substances, either naturally occurring or introduced into the system in the case of drug interactions, to give a false result.

Analytical studies such as cross-contamination or carry-over introduced by system components may provide evidence of the potential of the IVD to generate false positive results. Similarly, cross-reactivity with organisms other than the target analyte should also be studied to ensure positive results are not obtained in their presence.

The analytical studies also need to consider the intended use of the new device. The studies for a quantitative assay, e.g., viral load assays, will need to be more extensive than those for a qualitative assay and include limit of blank (LoB), LoD, lower limit of quantitation (LLoQ), upper limit of quantitation (ULoQ), and linearity. All assays, regardless of the quantitative or qualitative output, should have their performance near the LoD or assay cutoff thoroughly validated.

In general, analytical studies should also use real clinical matrices, e.g., negative serum, or a negative vaginal swab matrix. If the new device is for pathogen detection, actual organisms should also be used, as opposed to plasmids or nucleic acid transcripts.

1.2.4.4.1 Data reporting

All raw data listings for the analytical performance section should be included in the 510(k) or PMA submission. Statistical analyses of the data are discussed in a separate section below.

There are many analyte-/device-specific FDA guidance documents which contain more details on analytical performance studies and they may be found at: <http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm070274.htm>

1.2.4.5 Clinical performance

Clinical studies in support of a 510(k) submission or a PMA application may differ somewhat but the basic concepts are the same. In each case it is essential to design a clinical study that will support the claims of the proposed intended use. Some of the more important aspects of a clinical trial are discussed below.

1.2.4.5.1 Study population

The study population in the clinical study should directly reflect the intended use population. For example, if an IVD is to be used in a population with signs and symptoms of a disease or a population which is at risk for a disease, these are the patients who should be enrolled in the clinical study. The blood donor population for example, would not be representative of the intended use population for an infectious disease diagnostic device and therefore should not be eligible for inclusion in the study.

1.2.4.5.2 Clinical samples

Any clinical study should always, where possible, use real clinical samples. In some cases for rare analytes and for biological threat (biothreat) organisms there

may be a provision for allowing the use of spiked or surrogate specimens. If multiple types of patient specimens are claimed for use in the device, all types of specimen matrices must be tested in the clinical study. The samples tested in the clinical study should be prospectively collected during the study and if not assayed immediately should be stored at the proper, validated, temperature range. For certain IVDs it may not be feasible to perform a prospective study. In that case, a set of retrospective banked samples that were at the time collected prospectively may be suitable for inclusion in the study. For example, studies found in certain PMA applications for viral load assays for hepatitis B or C may contain retrospective samples that were collected prospectively while patients were on antiviral therapy.

1.2.4.5.3 Determination of assay performance

In some 510(k) submissions the performance of the device in the clinical study may be compared to that of a predicate device that has FDA clearance for the same study population. More often, the performance of a new device is compared to a reference method such as bi-directional sequencing, viral culture, or a composite reference standard which may include both of those methods.

Another metric of assay performance is comparison to a patient infected status (PIS) which may or may not involve clinical outcome. For example, assays for *C. trachomatis* are evaluated against a PIS of infected or not infected status using an algorithm based on multiple cleared nucleic acid amplification tests (NAAT) using multiple matrices. Likewise, the performance of serological assays for hepatitis B may be judged in relation to the clinical status of a patient (e.g., acute, chronic, recovered) which has been determined by an algorithm using multiple FDA approved hepatitis B assays. In contrast, viral load assay performance characteristics are primarily based on clinical outcome, such as if a patient achieves a sustained viral load in response to drug therapy.

1.2.4.5.4 Additional studies required for assays that are run in a moderate complexity location

In order for an assay to be used in a setting such as a physician's office which is not a Clinical Laboratory Improvement Amendments of 1988 (CLIA) certified, high complexity laboratory, by operators that are untrained personnel, the assay must obtain (in addition to an FDA clearance or approval) a certificate of CLIA waiver. The basic principles that should be demonstrated are that the assay procedure is simple, has easy to understand instructions for use, and the performance when run by an untrained operator is the same as when performed by a trained laboratory technician. The additional studies that are required to attain CLIA waiver certification are outlined in the FDA guidance document entitled

Recommendations: Clinical Laboratory Improvement Amendments of 1988 (CLIA) Waiver Applications for Manufacturers of In Vitro Diagnostic Devices Human Subject Protection; FDA Regulations (21 CFR 50 and 56).

1.2.4.5.5 Institutional Review Board (IRB) approval

Both PMA and 510(k) studies require IRB approval of the clinical study protocol and also of the patient informed consent if required.

1.2.4.5.6 Data reporting

All patient data line listings should be included in the 510(k) or PMA. In addition, failed assay runs along with device failures or replacements should also be included in the submission. Statistical analyses of the data are discussed in a separate section below.

1.2.4.6 Sections specific to a PMA

The following sections are specific to a PMA, but are beyond the scope of this chapter. The quality system regulation associated with a PMA includes sections on:

- Design Controls (§820.30)
- Manufacturing Controls [§814.20(b)(4) and §820]
- Preapproval Inspections
- Postapproval Inspections

Those interested in more details can find them in the following guidance document: <http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/HowtoMarketYourDevice/PremarketSubmissions/PremarketApprovalPMA/ucm050384.htm>

1.3 Assay performance characteristics: "Statistics 101" for diagnostic device developers [1]

1.3.1 Sensitivity/specificity

The simplest approach to describing diagnostic performance characteristics is when every test result can be described qualitatively, e.g., analyte detected or not detected, and where a reference method exists. For a prospectively enrolled study where patients meet the inclusion/exclusion criteria for the intended use of the device, agreement of the test result with the reference method establishes sensitivity and specificity of the test device.

Sensitivity is the probability that the new device will have a positive result given the clinical reference method is positive; it is estimated in a clinical study as the fraction of patients that are positive by the new test among those that are positive by the clinical reference method. For example, in a study of novel H1N1 influenza, it is the percent of patients that both test positive by the new device and are positive via the reference assay divided by the number of patients positive by the reference assay. Low

Table 1.3 Sensitivity, specificity, and predictive values

Outcome	Clinical reference standard		Total
	Condition present	Condition absent	
Test positive	True positive (TP)	False positive (FP)	
Test negative	False negative (FN)	True negative (TN)	
Total	N+	N−	N

For a prospective study: prevalence is estimated as $100\% \times (TP + FN)/N$; sensitivity is estimated as $100\% \times TP/N+$; specificity as $100\% \times (TN/N-)$; PPV is estimated as $100\% \times (TP/(TP + FP))$; NPV is estimated as $100\% \times (TN/(TN + FN))$.

sensitivity to the test reflects less security with the results of a negative test, i.e., patients with the disease may be falsely labeled as negative by the new assay. One needs to be cautious in equating "analyte not detected" with the absence of disease when interpreting any assay, since that can be a reason for low sensitivity. Specificity is the converse, i.e., the probability that the new device will have a negative result given the clinical reference method is negative. Specificity is estimated in the pivotal study by the fraction of subjects that are negative by the new test among those that are negative by the clinical reference method. Higher specificity yields greater confidence in a positive result. It is important to recognize that although patients included in specificity calculations do not have the disease of interest, in most cases they are still symptomatic (depending on the specific intended use for the test); asymptomatic subjects would not be considered part of the intended use population for such a device and therefore would not be appropriate for use in a specificity calculation. The formulae for calculating sensitivity and specificity are illustrated in Table 1.3.

1.3.2 Prevalence, negative predictive values, and positive predictive values

There may be significant challenges with estimating the sensitivity of a new assay if the prevalence of the disease being tested is low. Estimates of sensitivity are dependent both on the true underlying sensitivity and sample size. In a clinical study with only 5 specimens positive for a specific pathogen (via the reference method) and an observed sensitivity of 5/5 or 100% results in a lower confidence bound of only 55.6%, meaning that diagnostic performance is very uncertain. Even for a test with perfect sensitivity, at least 35 patients with the condition of interest would be needed to yield a 95% lower bound greater than 90%. In a pivotal study for devices to detect more common conditions, it is not uncommon to see a sample size of 50 or more patients that are positive via the reference method. The FDA considers the observed sensitivity and specificity and their respective 95% lower confidence bounds as part of device performance.

Table 1.4 Positive predictive value and negative predictive value formulas

$$PPV = 100\% \times \frac{\text{prevalence} \times \text{sensitivity}}{\text{prevalence} \times \text{sensitivity} + (1 - \text{prevalence}) \times (1 - \text{specificity})}$$

$$NPV = 100\% \times \frac{(1 - \text{prevalence}) \times \text{specificity}}{\text{prevalence} \times (1 - \text{sensitivity}) + (1 - \text{prevalence}) \times \text{specificity}}$$

Table 1.5 Hypothetical example for diagnostic: low prevalence of condition clinical reference standard

Outcome	Clinical reference standard		Total
	Condition present	Condition absent	
Test positive	9	99	108
Test negative	1	891	892
Total	10	990	1000

Sensitivity = 90%; specificity = 90%; PPV = 9/108 or 8.33%; NPV = 891/892 = 99.9%.

When sensitivity, specificity, and prevalence are known, one can calculate positive and negative predictive values (PPV and NPV, respectively). The PPV and NPV ask an alternative question, i.e., given a positive or negative test result, what is the probability that the patient has (or does not have) the condition being tested for. In a prospective study, this would be estimated as the percentage of patients with the condition of interest from all patients that test positive (see Table 1.3); similarly, the negative predictive value is estimated as the percentage of patients without the condition of interest among those that test negative.

The relationship between sensitivity, specificity, prevalence, PPV, and NPV is illustrated in Table 1.3 for prospective studies and in Table 1.4 more generally. As can be seen, the PPV and NPV are expressed as percentages and, ideally, both will be close to 100%. The importance of test prevalence is illustrated with hypothetical data in Table 1.5, where, despite high sensitivity and specificity, test utility is extremely limited in the context of ruling in a diagnosis as the PPV of the test result is still only 8%, underscoring the value of tests as an adjunct to diagnosis rather than the sole means for diagnosis.

However, tests may still have substantial clinical value even when only the NPV is very high. A high NPV indicates that if the patient tests negative it is very unlikely that the disease is present and the physician may want to pursue further workup to obtain a diagnosis.

The formulas for NPV and PPV in Table 1.3 are inappropriate when prevalence is unknown (e.g., when representative cases and controls are selected for study). Instead, formulas in Table 1.4 provide a means of estimating NPV and PPV under various scenarios for prevalence. More

details on the design and analysis of diagnostic device studies can be found in FDA guidance [2] and Pepe [3].

In Table 1.4, prevalence is assumed known, i.e., measured without error. So if we had a device with a sensitivity of 90% and a specificity of 90% as in Table 1.5, but the prevalence of the target condition of interest was now 10% rather than the 1% in Table 1.5, the PPV would be 0.50 or 50% using the equation in Table 1.4 rather than 8.33% as in Table 1.5 and NPV would be 0.99 or 99%. The NPV is negligibly lower than in Table 1.5.

When a clinical study only assesses agreement to another previously cleared device rather than to a reference method, reporting NPV and PPV would be inappropriate.

1.4 Common issues with new FDA submissions

Having discussed analytical, clinical, and statistical considerations that should be present in both 510(k) and PMA submissions, it is a useful undertaking to examine some of the common deficiencies that are encountered during review of these submissions. The Safe Medical Devices Act (SMDA), the final report of the Temple Committee issued in 1993 [5], was the first such study to independently examine these issues. Many of their conclusions are still accurate today. The following examples represent the most common deficiencies that must be rectified before an application can be cleared or approved. In addition, the deficiencies are also responsible for more than one review cycle prior to clearance or approval of a device, leading to delays in ability to commercialize the device.

- Poorly designed studies that result in either intentional or unintentional biased datasets, particularly when investigational (or similar approved) test outcomes influence patient enrollment and/or disease verification procedures in a manner that is not consistent with indications for use.
- Instead of clinical accuracy (sensitivity, specificity, and predictive values), agreement of results with some other test is estimated. If these agreement measures are not high enough, it can make it difficult to conclude whether the device is safe and effective.
- Insufficient number of study samples resulting in performance estimates that are not powered statistically and clinically to justify safety and effectiveness claims made in the intended use.
- Insufficient or a complete lack of any study site monitoring during the clinical trial which often results in protocol deviations, or incorrectly recorded data.
- All workflow processes are not fully validated (e.g., IVD test-system processes multiple sample tube types, but only one type is included in the clinical trial).
- The addition of bridging studies when a clinical study for safety and effectiveness fails to include all the necessary intended-use subpopulations or types of specimens.

- The often long time lag between a preliminary contact of a device developer with the FDA for preclinical advice and the submission of the 510(k) or PMA can result in the omission of studies that may then be necessary due to a change in clinical practice.
- Device used to validate the performance during the clinical evaluation is still a prototype and not the final design.
- Organizational problems:
 - nonintuitive grouping of information;
 - lack of a single detailed table of contents (TOC) at the front of a submission, lack of sequential pagination;
 - organizational structure differs between submitted electronic and hard copies, and no TOC that aligns the two;
 - studies not described clearly or completely (especially, analytical studies);
 - important study details scattered throughout a submission;
 - electronic data are provided but not in EXCEL (it requires a lot of time to convert non- EXCEL files), and a description of variables in the data sets are not provided.

1.4.1 IVD labeling

Federal IVD regulations apply to all legally marketed commercial IVDs [21 CFR 809.10(a) and (b)], whether class I exempt or nonexempt, Class II, or Class III; devices for investigative use, IUO, [21 CFR 809.10(–)(2)(ii)] and research use, RUO, [21 CFR 809.10(c)(2)(I)].

1.4.1.1 510(k) and PMA labeling

The proposed labeling is an important part of the 510(k) notification or PMA and is reviewed by the FDA prior to issuing clearance or approval of an IVD. As noted above labeling requirements for inserts and outer packaging can be found in 21 CFR 809.10(b).

When reviewing labeling, particular attention is paid to the intended use, device description, specimen collection procedures, transport and storage recommendations, quality control recommendations, warnings and limitations, expected validation of cutoff, results and their interpretation, expected values, and specific performance characteristics. It is of particular importance that the clinical studies are clearly described and the associated performance characteristics match what was determined during review of the data submitted. The FDA is committed to “truth in labeling” in order to provide the end user with a product that performs as expected.

1.4.2 New technologies

1.4.2.1 How have FDA review policies and regulations kept up with the technological revolution in the microbiology laboratory?

Recent changes in the methodologies applied to rapid pathogen identification have been “game-changers” in microbiology laboratory practice, beginning with the move

towards nonculture methods when singleplex polymerase chain reaction and other nucleic acid amplification tests were developed and leading towards the development and use of multiplex nucleic-acid-based tests, mass spectrometry, and high-throughput sequencing technologies. New FDA regulatory science concepts on how to determine the performance of these new and more complex technologies have been open for comment and discussion by way of a series of public workshops and guidance documents.

The FDA held such a workshop in 2011 to discuss how best to establish the performance of “highly multiplexed microbiology assays” and to discuss the clinical and public health applications of such devices.

As a followup, the FDA drafted a guidance document published in 2014 describing how to validate the performance of these types of technologies as they apply to the diagnosis of infectious disease: <http://www.fda.gov/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm373750.htm>.

To date several multiplex devices with up to 30 plus microbial analytes and resistance markers have been cleared using the principles outlined in the guidance. Similarly in April 2014, the FDA held a public workshop entitled “Advancing Regulatory Science for High Throughput Sequencing Devices for Microbial Identification and Detection of Antimicrobial Resistance Markers.” The purpose of this workshop was to discuss the clinical and public health applications and performance validation of new high-throughput sequencing devices, the quality criteria for establishing the accuracy of reference databases for regulatory use, and ways to streamline clinical trials of such devices for microbial identification. This discussion was essential to establish the safety and effectiveness of high-throughput sequencing devices when used to test human specimens or clinical isolates for the diagnosis of infectious diseases and detection of antimicrobial resistance markers. A guidance document outlining the types of studies the FDA is proposing be undertaken to validate the performance of such devices is currently under development.

1.4.3 How have FDA regulations and policies fostered preparedness for new and emerging pathogens and other public health emergencies?

During public health emergencies, medical countermeasures (MCMs) including diagnostic assays may be needed to prevent or treat diseases or conditions caused by biothreat agents or emerging infectious disease threats, like pandemic influenza or MERS-CoV.

The Pandemic and All-Hazards Preparedness Reauthorization Act of 2013 (PAHPRA) provides key legal authorities’ guidelines to sustain and strengthen national preparedness for public health emergencies; and the Emergency Use Authorization (EUA) authority allows

the FDA to help strengthen the nation's public health protections against biothreats by facilitating the availability of MCMs needed during public health emergencies. Under Section 564 of the Federal Food, Drug, and Cosmetic Act, the FDA Commissioner may allow unapproved medical products or unapproved uses of approved medical products to be used in an emergency to diagnose, treat, or prevent serious or life-threatening diseases or conditions caused by biothreat agents when there are no adequate, approved, and available alternatives.

The FDA published *Guidance – Emergency Use Authorization (EUA) of Medical Products* in 2007, explaining

the FDA's policies for authorizing the emergency use of medical products under Section 564 of the FD&C Act. The guidance is intended to inform industry, government agencies, and FDA staff of FDA's general recommendation and procedures for issuance of EUAs.

During the 2009 H1N1 influenza pandemic, over 18 EUAs were granted to microbiology assays that could detect the H1N1 virus from appropriate respiratory specimens, and similarly, EUAs have been issued for assays that could detect the H7N9 influenza and MERS-CoV viruses when the *potential* for a pandemic scenario was declared in 2013 for both of these organisms.

Appendix: web sites

Statutory document	Web site
FDA history	http://www.fda.gov/AboutFDA/WhatWeDo/History/Overviews/ucm056044.htm
FD&C Act	http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCA/default.htm
CDRH Guidance Documents	http://www.fda.gov/MedicalDevices/deviceregulationandguidance/guidancedocuments/default.htm
Clinical Laboratory Improvement Amendments	http://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/index.html?redirect=/clia/
Food and Drug Modernization Act	https://www.cimit.org/news/regulatory/dlbm_fda.pdf
Device Advice: Comprehensive Regulatory Assistance	http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/

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- 3 Pepe, M.S. 2003. *The Statistical Evaluation of Medical Tests for Classification and Prediction*. Oxford University Press. New York.
- 4 Public Law. No. 94-295, 90 Stat. 539. 1976.
- 5 Temple, R., A. Witt, and N. Morgenster. 1993. *Report of the Committee for Clinical Review Based on a Review of Selected Medical Device Applications*. Food and Drug Administration, Washington, DC.

Commercial Blood Culture Systems and Methods

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Techniques for detecting pathogenic microorganisms in blood have evolved from manual methods using broth media, once prepared in individual clinical laboratories, to widely available commercial systems and media prepared and sold by manufacturers to hospitals and clinical laboratories. Currently, both manual and automated systems are available, with the market now dominated by instrument-based automated systems and methods. This chapter will emphasize those manual and automated systems and media cleared for diagnostic use in the United States, as well as developments in the use of molecular methods for detecting pathogenic microorganisms directly from blood or in conjunction with traditional blood cultures.

Over the past 15 years it has become increasingly difficult to perform controlled clinical evaluations or comparisons of blood culture products. Good studies are expensive, require a substantial investment of time and effort (studies may run for a year or more at several institutions), institutional review boards have become increasingly demanding in terms of the requirements for clinical trials of this nature, and relatively few hospitals have the numbers and types of blood culture isolates needed to generate sufficient statistical power for comparisons between products. In addition, there are few investigators with the experience and expertise required to perform these studies. Because of these factors, relatively few such studies have been published since the first edition of this book.

2.1 Manual blood cultures

The principle of blood cultures is straightforward: a defined volume of blood, obtained by sterile venipuncture, is inoculated into a broth culture medium that will support the growth of most pathogenic bacteria and fungi.

Manual methods employ this concept in its most basic form. Although there formerly were many commercial manual blood culture media, there now are relatively few. This is due, in part, to consolidations and mergers among manufacturers, elimination of some systems because of declining demand, and the availability of better evidence demonstrating which systems are acceptable for clinical use. However, broth-based manual blood culture systems, and a manual lysis-centrifugation method, still are marketed and used in the United States and other countries.

2.1.1 SEPTI-CHEK

The SEPTI-CHEK blood culture system (BD Biosciences, Sparks, MD) was developed as an alternative to older, more labor-intensive conventional blood cultures, which, in order to maximize recovery of pathogenic microorganisms, must be subcultured manually after the first overnight incubation and at the end of the incubation period. SEPTI-CHEK consists of a conventional blood culture bottle containing broth medium to which is attached an agar-coated plastic paddle, creating a biphasic system similar to that of the classic Castaneda bottle. Blood is inoculated into the culture bottle through a threaded plastic cap with a rubber diaphragm, which is then removed and replaced with an agar-coated paddle after the blood specimen is received by the laboratory. After the paddle is attached, the bottle is inverted so that the blood-broth mixture floods the paddle, thereby inoculating the agar with microorganisms that may be present. Aerobic and anaerobic bottles, the latter without the agar paddle device (which is permeable to atmospheric oxygen), are then incubated with or without agitation and inspected for evidence of microbial growth once or twice daily. The agar-coated paddle can be removed for better inspection by unscrewing it from the top of its plastic container. Following each examination of the agar paddle, the bottle is inverted,

in effect repeating the subculture of the blood–broth mixture to the agar. If growth occurs on the paddles first or, on paddles and in broth at the same time, colonies are available immediately for identification and antimicrobial susceptibility testing. Moreover, preliminary microbiologic information may be available (e.g., lactose-positive or lactose-negative colonies on the MacConkey agar).

SEPTI-CHEK bottles contain a broth medium with 0.05% sodium polyanethol sulfonate (SPS) as the anticoagulant. Bottles contain a CO₂ atmosphere. Two bottle sizes are available: larger bottles containing 70 mL of liquid medium that accommodate blood specimen volumes of up to 10 mL, and smaller bottles containing 20 mL of liquid medium that accommodate blood specimen volumes of up to 3 mL (the latter being considered pediatric bottles). Several sizes and medium formulations are currently available, including four adult (70 mL) bottles containing soybean-casein digest broth with or without resins, soybean-casein digest/Columbia broth, soybean-casein digest thioglycollate broth, and two pediatric bottles containing either soybean-casein digest broth or brain-heart infusion broth. The paddles contain both nonselective and differential selective agars (chocolate agar, MacConkey agar, and malt agar) in conjunction with traditional blood cultures [19,28].

In a number of controlled clinical evaluations, the SEPTI-CHEK system performed well [2,25,41–43]. As with any other manual blood culture system, the system is adequate for smaller laboratories processing relatively low numbers of blood cultures, as a back up for automated systems, or in circumstances where a number of broth options are desirable.

As per the manufacturer, this product has been discontinued but may be available through some distributors. Please check with your local vendors to determine availability.

2.1.2 Oxoid Signal

The Oxoid Signal blood culture system (Remel, Lenexa KS), like SEPTI-CHEK, also was developed as an alternative to older conventional blood culture systems. Unlike SEPTI-CHEK, it was developed and marketed as a one-bottle blood culture system. Similar to SEPTI-CHEK, Oxoid bottles are inoculated with blood through a conventional rubber diaphragm. Once in the laboratory, the diaphragm is removed and the plastic signal device is attached to the top of the bottle where it is anchored by a plastic outer sleeve. The device consists of a long needle that goes below the level of the blood–broth mixture and extends up into the clear plastic cylinder of the signal device. When microbial growth occurs in the bottle, gases are released into the bottle headspace, increasing the atmospheric pressure within the bottle and forcing some of the blood–broth mixture up through the needle into the signal device. Thus, in addition to the conventional macroscopic

examinations of blood culture bottles (looking for hemolysis, gas formation, colonies growing in liquid media), the Oxoid Signal system offers the potential advantage of easy detection of positive cultures. This is because fluid present in the signal cylinder can be seen without difficulty when blood culture bottles are inspected each day. Positive cultures can then be worked up in the traditional manner.

Only one medium formulation of the Oxoid Signal system has been marketed. Published controlled clinical evaluations performed in the United States comparing both manual and automated blood culture systems have been reported previously [23,36,37,39].

2.1.3 Isolator

An additional manual system currently available in the United States is the Isolator blood culture system (Wampole Laboratories, Cranbury, NJ), sometimes referred to as the “DORN Procedure.” Unlike SEPTI-CHEK and Oxoid Signal, Isolator is based on the principle of lysis-centrifugation, making it the only commercially available blood culture system that does not utilize broth culture medium. Both adult as well as pediatric tubes are available. Blood is inoculated to Isolator tubes (Figure 2.1) that contain a lysing solution consisting of saponin, an anticoagulant, and a fluorocarbon that acts as a cushion during the centrifugation step of blood processing. Once the lysis and centrifugation steps have taken place, the rubber stopper is removed from the Isolator tube and the supernatant is removed by aspiration with a disposable pipette. The pellet is then resuspended and can be inoculated directly to the culture media that will support the various potential pathogens in which detection is desired. The system can be used for detection of routine bacterial pathogens, occasionally for quantitation of bacteria and yeasts, but has been shown, in some studies, to yield decreased recovery of anaerobes, *Haemophilus* species, and pneumococci if specimens are not processed within 8 h of receipt in the laboratory [12,13,16,35]. Isolator has been shown to be an excellent system for detecting yeasts and dimorphic fungi, mycobacteria, and *Bartonella* spp. [1]. The system is labor-intensive, however, particularly in the initial processing of specimens.

2.2 Automated blood culture systems

Commercial automated blood culture systems were first introduced in the early 1970s. The first clinically and commercially successful system was the BACTEC 460 radio-metric system (Becton Dickinson, Sparks, MD). This system was succeeded in turn by the BACTEC 660, 730, and 860 nonradiometric systems during the subsequent 20 years; systems that were all based on a similar technology and instrumentation, with each generation representing an incremental improvement over the preceding

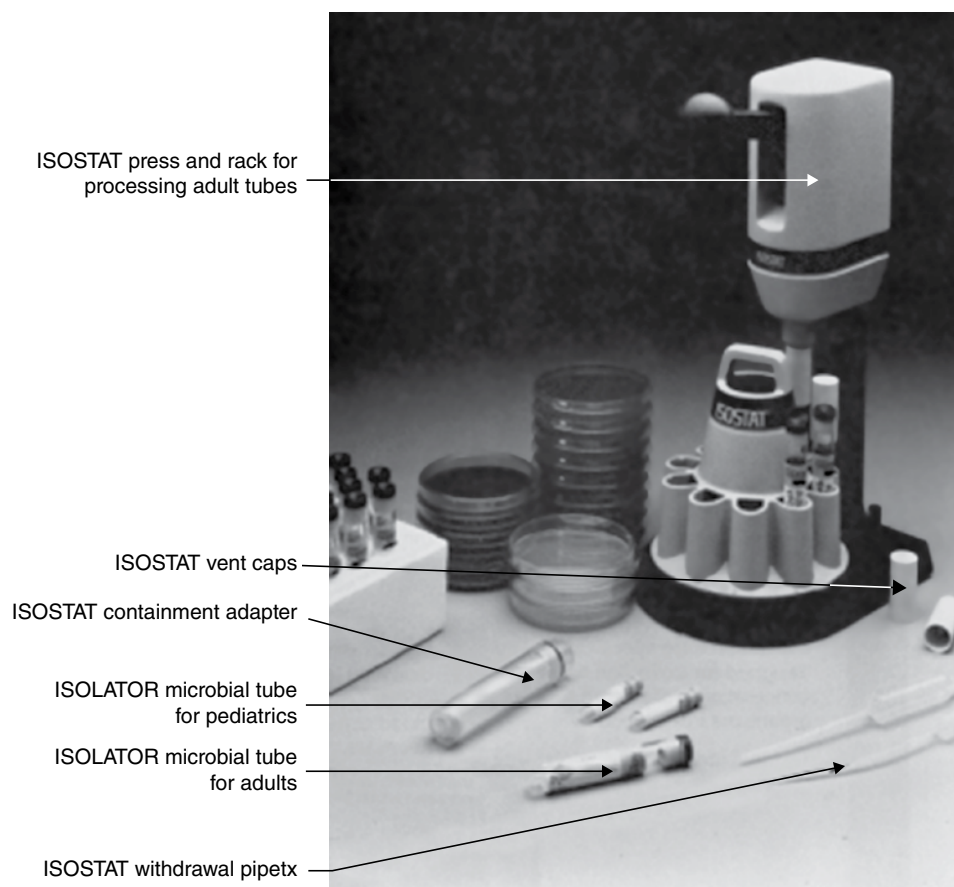


Figure 2.1 ISOSTAT and ISOLATOR microbial tubes. (Courtesy of Alere, Inc.)

one. Because they were based on the same method for detection growth of microorganisms, when the continuous-monitoring blood culture systems were introduced, the BACTEC 460, 660, 730, and 860 systems were phased out of use and are no longer available. In these systems, production of CO_2 by microorganisms was detected in the bottle headspace by detection of radioactive isotopes of CO_2 or by infrared spectrophotometry. Although they are no longer in use, the fundamental principle on which these instruments were based, detection of CO_2 production, was the basis for the development of continuous-monitoring blood culture systems.

In the early 1990s, the first continuous-monitoring blood culture system, BacT/Alert (bioMérieux, Durham, NC) was introduced [33]; it was followed shortly thereafter by the BACTEC 9000 (BD Biosciences, Sparks, MD) and ESP/VersaTREK (Thermo Scientific, Cleveland, OH) blood culture systems.

The continuous-monitoring systems have a number of characteristics in common [49]. The systems are modular, allowing a single computer to coordinate the function of one to as many as 50 incubator units. Blood culture bottles are placed in individual cells of an incubator unit, and testing is performed without further manipulation of the

bottle (until a bottle is flagged as positive and removed for Gram stain and subcultures), thereby reducing technologist hands-on time. In each system, blood culture bottles are individually monitored at intervals of 10–24 min for evidence of microbial growth. Individual bottle readings are transmitted to the system's computer for storage and analysis, and growth curves are calculated according to sophisticated instrument algorithms. Because of the frequent testing that takes place round-the-clock, it is possible to detect microbial growth approximately 1 to 1.5 days earlier than was the case for the BACTEC radiometric and nonradiometric systems [20,24,48]. The more frequent measurements and greater number of data points also seem to be associated with fewer instrument false-positive signals than was the case with the earlier automated systems [48]. Table 2.1 summarizes some of the relevant information on the current generation of commercially available continuous-monitoring systems.

2.2.1 BacT/Alert

In 1991, the BacT/Alert blood culture system was introduced as the first commercial continuous-monitoring system (Figure 2.2). In 1999, the next generation system, the BacT/Alert 3D, was introduced. Compared with the

Table 2.1 Commercially available continuous monitoring blood-culture systems

System (manufacturer)	Method(s) for detecting growth	Bottle capacity per module	Maximum number of modules (number of bottles ¹) per system	Test cycle (min)	Agitation type (speed [speed/min])	Dimensions (cm)
BacT/Alert 3D (bioMérieux)	CO ₂ detection, colorimetric	240	6 (2880)	10	Rocking (48)	90 × 49 × 61
BacT/Alert 3D 60	CO ₂ detection, colorimetric	120	6 (720)	10	Rocking (48)	87 × 87 × 55
BACTEC 9240 (BD Biosciences)	CO ₂ detection, O ₂ detection,* fluorescence	240	5 (1200) (core) 20 (4800) (Vision) 50 (12,000) (EpiCenter)	10	Rocking (48)	93 × 128 × 55
BACTEC 9120	CO ₂ detection, O ₂ detection,* fluorescence	120	5 (600) (core) 20 (2400) (Vision) 50 (6000) (EpiCenter)	10	Rocking (48)	61 × 129 × 56
BACTEC 9050	CO ₂ detection, O ₂ detection,* fluorescence	50	1 (50)	10	Continuous rotation	61 × 72 × 65
VersaTREK 240 (ThermoFisher Scientific)	Manometric, pressure change	240	5 (640)	12 (aerobic) or 24 (anaerobic)	Rotary (aerobic only) (160)	90 × 86 × 65
VersaTREK 528	Manometric, pressure change	256		12 (aerobic) or 24 (anaerobic)	Rotary (aerobic only) (160)	199 × 86 × 65

*O₂ detection is for Myco/F Lytic medium only.

¹Maximum number of bottles accommodated depends on data management system selected.



Figure 2.2 BacT/ALERT™ 3D instrument. (Courtesy of bioMérieux.)

earlier BacT/Alert systems, the 3D system is more compact and incorporates a touch screen for the instrument's computer, enabling the technologist to more easily load and unload bottles as well as to perform quality control functions on the instrument.

In the BacT/Alert system, a carbon dioxide sensor is incorporated at the base of each culture bottle, separated from the blood-broth mixture by a CO₂ semipermeable membrane that monitors the amount of CO₂ in the bottle. The sensor changes color as the concentration of CO₂

increases in the bottle [33]. At the base of the cell in which the bottle resides in the incubating unit are light-emitting and light-sensing diodes. When microbial growth occurs, the sensor changes color, and the amount of light reflected from the sensor increases; the increased reflectance is measured by the instrument as increased voltage and the data are transmitted to the central computer. The computer's detection algorithms have the ability to flag a culture as positive in several ways: (i) by recognition that the arbitrary threshold has been exceeded; (ii) by recognition of a linear increase in CO₂; or (iii) by recognition of a change in the rate of CO₂ production.

Several medium formulations are available for use in the BacT/Alert system. These include standard aerobic and anaerobic media that contain 40 mL of soybean casein digest broth and accept up to 10 mL of blood per culture vial; aerobic and anaerobic FAN media, which contain 40 mL of brain heart infusion broth, activated charcoal, and Fuller's earth (Ecosorb) designed to inactivate or bind antimicrobial agents in the blood and accept up to 10 mL of blood per culture vial; and the PF Pediatric bottle, which contains 20 mL of soybean casein digest broth supplemented with brain heart infusions solids and activated charcoal, which accepts up to 4 mL of blood. The Pediatric bottle has been promoted for use with pediatric patients and those elderly patients from whom it is difficult to obtain larger volumes of blood. The latter use is to be discouraged, as it fosters inadequate volumes of blood cultures from adult patients. The MB blood bottle for detection of mycobacteria in blood has been introduced. Refer to Chapter 14 (Mycobacteria) for additional information.

The first controlled clinical evaluation of BacT/Alert compared it to the BACTEC NR system using 5 mL of blood inoculated into standard aerobic and anaerobic bottles of each system [48]. The study found the systems to be equivalent for overall recovery of microorganisms causing sepsis, with earlier detection of positives and fewer instrument false-positive signals in the BacT/Alert. Further analysis of the data from this study revealed that BacT/Alert bottles could be processed using a 5-day rather than a 7-day incubation period without a significant decrease in the detection of microorganisms causing sepsis [46]; similar findings were reported independently by others [10] in a study that also showed that terminal subcultures of bottles shown to be negative by the instrument were not necessary. Another study showed that the BacT/Alert aerobic standard bottle inoculated with 10 mL of blood detected more microorganisms, than the same bottle inoculated with 5 mL of blood [40]. Rohner and colleagues [29] compared the BacT/Alert system (5 mL inoculated into standard aerobic and anaerobic bottles) with the Oxoid Signal (10 mL inoculated into one bottle) and demonstrated a higher yield and faster detection of microorganisms by the BacT/Alert standard aerobic bottles, but found no significant difference in overall detection of microorganisms. Hellinger et al. [11] compared the BacT/Alert standard aerobic bottle and Isolator and found no significant difference in overall detection of microorganisms. However, the Isolator recovered significantly more staphylococci and yeasts, whereas the BacT/Alert system recovered more aerobic and facultatively anaerobic Gram-negative rods.

Several studies have evaluated the BacT/Alert FAN media. Weinstein et al. [38] compared the aerobic FAN bottles and standard bottles and found superior detection of staphylococci, fungi and all microorganisms combined in the FAN system. Wilson and colleagues [47] compared the anaerobic FAN and standard bottles and demonstrated superior recovery of staphylococci, *Escherichia coli*, and all microorganisms combined but inferior recovery of nonfermentative Gram-negative rods and yeasts in FAN bottles. A subsequent report assessed the clinical importance of these two studies and concluded that use of the FAN bottles detected significantly more episodes of septicemia, especially recurrences and episodes in patients receiving theoretically effective antimicrobial therapy [18]. Potential disadvantages associated with use of FAN bottles in the studies included the greater cost of these bottles as well as enhanced detection of (and need to workup) contaminating coagulase-negative staphylococci.

The FAN bottles have been compared with culture media from both the ESP and BACTEC continuous-monitoring systems. In a study involving pediatric patients, the FAN and ESP 80A bottles were compared [45]. Overall detection rates were equivalent, but the FAN

bottle detected more *Staphylococcus aureus* organisms whereas the ESP 80A bottle detected more streptococci and enterococci. The FAN bottle detected more microorganisms from patients already receiving antimicrobial agents. Doern et al. [7] compared the aerobic FAN bottle to the aerobic 80A bottle in the ESP system in an adult population and found higher recovery rates of clinically important microorganisms in FAN bottles, in particular *S. aureus*, members of the family Enterobacteriaceae, and Gram-negative rods other than *Pseudomonas aeruginosa* from patients receiving antimicrobial therapy. The 80A bottles detected more beta-hemolytic streptococci. In this study, more contaminants were detected in the FAN bottle, and the ESP system had more instrument false-positive readings. Jorgensen and colleagues [14] conducted a multicenter comparison of the aerobic FAN and BACTEC Plus resin media and found an equivalent overall yield of clinically important microorganisms and septic episodes. *Histoplasma capsulatum* was detected more frequently in the BACTEC resin bottle. Streptococci were detected approximately 7 h earlier in the resin bottle, whereas *Candida albicans* was detected approximately 8 h earlier in the FAN bottle. A more recent study showed significant differences in recovery of organisms from patients on antimicrobial therapy [50].

2.2.2 BACTEC 9000 series

In 1992, Becton Dickinson marketed the BACTEC 9000 series continuous-monitoring blood culture system (Figure 2.3). Two instrument formats were marketed, the smaller capacity BACTEC 9120 (120 bottles per incubating unit) and the larger capacity BACTEC 9240 (240 bottles per unit). In the late 1990s, the smaller BACTEC 9050 bench top instrument (50-bottle capacity) was marketed for laboratories that processed relatively few blood cultures but wanted to use an automated rather than manual blood culture system. The most recent version of the system is called the FX, which like its predecessors is modular with the individual modules linked to a central data management system. Like the BacT/Alert system, BACTEC bottles contain a carbon dioxide sensor at the base of each culture bottle. Rather than using colorimetric detection of increased CO₂ as in the BacT/Alert, the BACTEC system utilizes fluorescence sensing to detect microbial growth. When the amount of CO₂ increases, increased fluorescence is detected by the instrument and transmitted to its computer. The principle detection criteria are a linear increase in fluorescence and an increase in the rate of fluorescence.

The BACTEC system has multiple medium formulations that can be used. These include Standard Aerobic/F and Anaerobic/F media, which contain 40 mL of soybean casein digest broth; Plus Aerobic/F and Plus Anaerobic/F media, which contain 25 mL of soybean casein digest broth

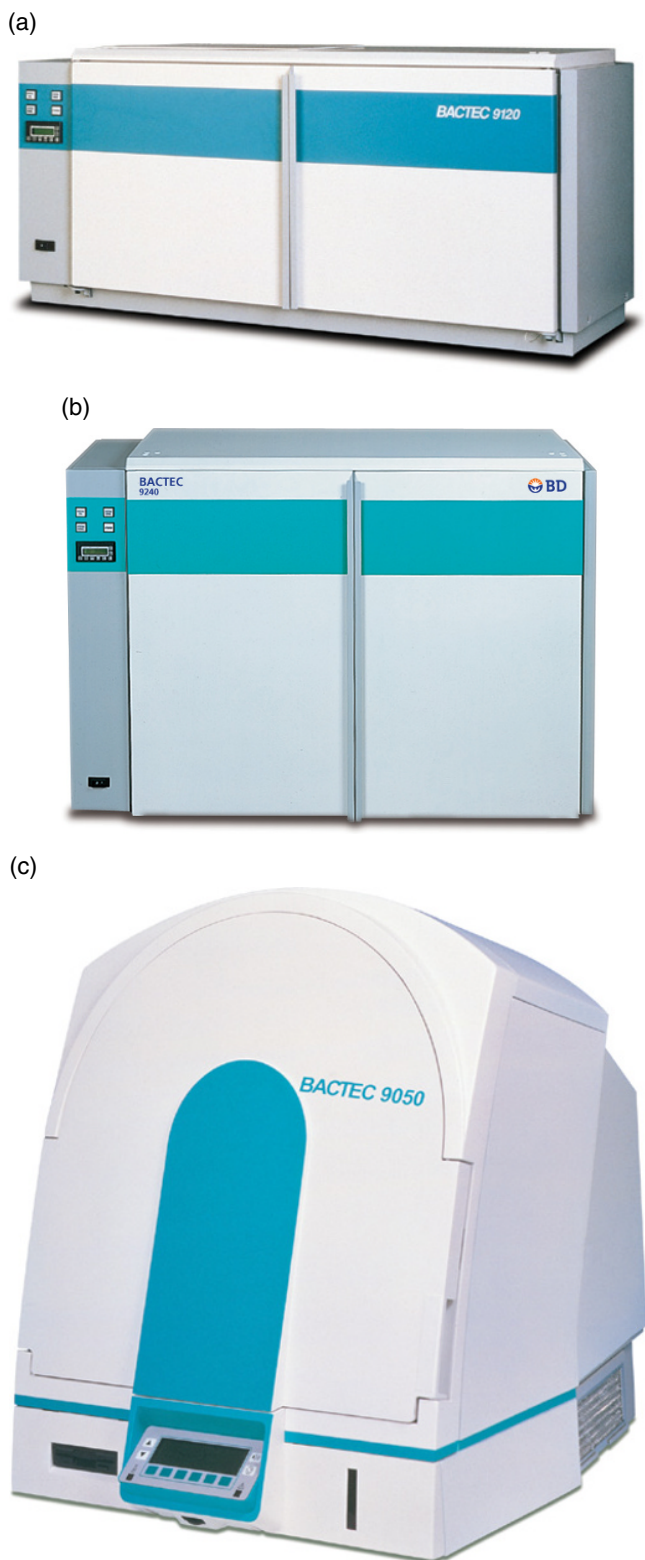


Figure 2.3 (a,b) BACTEC 9000 series continuous monitoring blood-culture system and (c) BACTEC 9050 bench top instrument. (Courtesy and © Becton, Dickinson and Company.)

plus antibiotic-binding resins on glass beads; the Lytic/10 Anaerobic/F medium, which contains 40 mL of soybean casein digest broth plus a lysing agent; the Peds Plus/F medium, which contains 40 mL of soybean casein digest

broth and resins; and the Myco/F Lytic medium, designed for improved detection of fungi and mycobacteria. All of the culture vials for these formulations accept up to 10 mL of blood with the exception of the Peds Plus/F vials, which are designed to accept 1–3 mL of blood from infants and young children.

Like BacT/Alert, a number of studies have evaluated the BACTEC 9000 series instruments and medium formulations. An initial evaluation compared Standard Aerobic/F and Anaerobic/F bottles processed in the BACTEC 9240 instrument to standard aerobic and anaerobic bottles used with the BACTEC nonradiometric 660 (660 NR) system [24]. In this study, more staphylococci and all microorganisms combined were detected in the Aerobic/F bottles, and more coagulase-negative staphylococci, members of the family *Enterobacteriaceae*, and all microorganisms combined were detected in the Anaerobic/F bottles than in the respective comparator bottles used in the 660 NR system. Moreover, positive cultures were detected earlier in the 9240 system than in the 660 NR.

Most published studies of the BACTEC 9000 system have assessed the Plus/F resin media. Schwabe and colleagues [31] compared BACTEC resin media formulated for and processed in the 9240 instrument versus that formulated for and processed in the 660 NR instrument. There were no differences in yield of microorganisms, but the 9240 instrument detected microorganisms earlier. A multicenter study [22] evaluated the aerobic and anaerobic Plus/F media processed in the 9240 versus the 9050 instruments. Phase 1 evaluated the aerobic Plus/F medium in the two instruments and found no differences in the recovery of individual microorganisms or groups thereof, with the exception of *Streptococcus pneumoniae*, which was isolated more frequently in the bottles processed in the 9050 instrument. The times to detection of positive cultures were similar in both systems. Phase 2 evaluated the anaerobic Plus/F medium and found no significant differences in the recovery of microorganisms in the two BACTEC instruments, and overall times to detection of positive cultures were similar except for those for anaerobes, which were detected more quickly in the 9240 (mean, 35 h) than in the 9050 instrument (mean, 61 h). In both phases of the study, instrument false-positive signals occurred more frequently in the 9240 than in the 9050 instrument.

Several studies have compared the Plus/F bottles processed with BACTEC 9240 and 9120 instruments to other commercially available media and systems. Smith et al. [32] compared the aerobic resin media to the BacT/Alert standard media and found superior detection of staphylococci and all microorganisms combined in the BACTEC resin bottles. The times to detection of positive cultures were similar in the two systems. In comparison of the Plus/F medium with the Septi-Chek Release medium (brain heart infusion broth supplemental with a lytic

agent, saponin), Rohner and colleagues [30] reported enhanced detection of *Haemophilus* spp. in Plus/F medium but greater detection of members of the family *Enterobacteriaceae*, Gram-negative anaerobes, and all microorganisms combined in the Septi-Check medium. Positive cultures were detected earlier by the BACTEC system. Two studies have compared the Plus/F medium and the Isolator system. In one, Pohlman and colleagues [27] reported improved detection of staphylococci, members of the family *Enterobacteriaceae*, *P. aeruginosa*, and all microorganisms combined in Plus/F medium, especially if patients were receiving antimicrobial agents at the time the blood cultures were obtained. In the second study, Cockerill et al. [3] reported better detection of staphylococci and all microorganisms combined in the resin bottle and more contaminating bacteria in the Isolator. In both studies, the BACTEC system detected positive cultures earlier than the Isolator and required less technologist time for specimen processing and examination.

Two older studies and one recent study have directly compared the BACTEC Plus/F aerobic blood culture bottle and the BacT/Alert FAN aerobic bottle. In a single-center study, Pohlman et al. [26] reported equivalent detection of clinically important microorganisms by the two systems. However, when *Pseudomonas* and the *Enterobacteriaceae* were analyzed together, the FAN bottle detected significantly more strains. In this study, more false-positive signals were detected in the Plus/F bottle than in the FAN bottle. There were no differences in times to detection or in the detection of positive cultures in patients receiving antimicrobial agents at the time of blood culture. In a multicenter study, Jorgensen and colleagues [14] reported equivalent detection of microorganisms except for enhanced detection of *H. capsulatum* in the Plus/F medium. More contaminants were detected in the FAN medium. Times to detection of positive cultures were similar overall, but streptococci were detected on average 7 h earlier by the BACTEC system, whereas yeasts were detected on average 8 h earlier by the BacT/Alert system. In a recent study by Zadroga and colleagues [50], BACTEC Plus media showed faster time to detection as well as superior recovery of bacterial pathogens compared with an aerobic BacT/Alert FAN medium. The latter was true for pathogens as a group, Gram-positive cocci, Gram-negative bacilli, and *S. aureus* [50]. Improved recovery also occurred with BACTEC Plus media for isolates from patients who were receiving antimicrobial therapy in the 48 h prior to blood culture collection [50].

BACTEC MYCO/F Lytic bottles were formulated to enhance recovery of mycobacteria and fungi while retaining the ability to detect bacteria. Unlike other BACTEC bottles developed for the 9000 series instruments, MYCO/F Lytic bottles use fluorescent sensors that detect decreasing oxygen concentrations rather than increasing carbon dioxide concentrations in the

presence of microbial growth. An early study [34] assessed the MYCO/F Lytic medium in AIDS patients with suspected mycobacteremia and fungemia. Because the number of patients with positive cultures was small, it was difficult to draw conclusions from this study. Compared with the Isolator for detection of fungemia, the MYCO/F Lytic bottle detected fewer *H. capsulatum* organisms but more *Cryptococcus neoformans* organisms. Compared with the ESP II bottle for the detection of mycobacteremia, there was superior detection of *Mycobacterium avium* complex in MYCO/F Lytic medium. Subsequent studies by Crump et al. [5,6] showed that MYCO/F Lytic medium, the Isolator 10 system, and BacT/Alert MB bottles are equivalent in yield for recovery of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex isolates, but that recovery is faster with the automated systems.

2.2.3 VersaTREK blood culture system

The third continuous-monitoring blood culture system is the VersaTREK system, first introduced by Difco Laboratories as the ESP system, and now marketed by ThermoFisher Scientific (Oakwood Village, OH) as VersaTREK (Figure 2.4). This system differs from both the BacT/Alert and BACTEC 9000 instruments in several ways. After bottles are fitted with an adapter and loaded into the instrument, growth is detected by monitoring pressure changes within the headspace of each bottle as gases (oxygen, hydrogen, nitrogen, and carbon dioxide) are either consumed or produced by metabolizing microorganisms. Aerobic bottles are agitated by use of a small stir bar (vs. the gentle rocking motion of bottles in the



Figure 2.4 Thermo Scientific™ VersaTREK™ automated microbial detection system (Model 528). (Photo courtesy of ThermoFisher Scientific, copying is prohibited.)

other systems); anaerobic bottles are not agitated. Lastly, the basal medium in the VersaTREK Redox 1 (aerobic bottle) is soy-casein peptone broth, and the medium in the Redox 2 (anaerobic bottle) is proteose-peptone broth. Similar to the other continuous-monitoring systems, bottles are monitored frequently (every 12 min for aerobic bottles and every 24 min for anaerobic bottles) and pressure changes are plotted against time to yield growth curves. Positive cultures are signaled based on the instruments proprietary algorithms. Current VersaTREK formulations include the REDOX 1 and REDOX 2 bottles, both containing 80 mL of broth medium, and the REDOX 1 EZ Draw and REDOX 2 EZ Draw bottles, which contain 40 mL of broth medium and are marketed as bottles that can be used for direct draw of blood specimens without use of a collection device, and Myco bottles that are formulated for recovery of mycobacteria. VersaTREK bottles have larger broth volumes compared with other commercial blood culture bottles; the larger broth volumes result in a greater dilution of blood, another way to overcome antimicrobial activity in blood.

In an initial multicenter clinical evaluation, the original ESP system was compared with the BACTEC 660 NR system [21]. In this study, ESP aerobic bottles recovered more pneumococci and all microorganisms combined than did the BACTEC aerobic bottles. The ESP anaerobic bottles recovered more aerobic Gram-positive bacteria, *Candida* species, and all microorganisms combined than did the BACTEC anaerobic bottles. In this study, five different BACTEC media were used in the comparison, and the volume of blood inoculated into the culture bottles was not controlled, complicating the analysis.

Zwadyk et al. [51] compared the ESP system to the BacT/Alert system and found that ESP aerobic bottles recovered significantly more *S. aureus* organisms and all microorganisms combined and that ESP anaerobic bottles recovered more anaerobes. However, significantly more bacteremia caused by streptococci were detected by the BacT/Alert system. The speed of detecting microorganisms in the two systems was variable; some microorganisms were detected earlier by the ESP system, whereas others were detected more quickly by the BacT/Alert system.

Several studies have compared the original ESP system with the Isolator system. Kirkley and colleagues [17] found that the Isolator detected more *S. aureus*, enterococci, *E. coli*, *Alcaligenes xylosoxidans*, *Stenotrophomonas maltophilia*, *C. albicans*, and *Candida glabrata* than the ESP 80A (aerobic) blood culture bottle. Kellogg et al. [15] compared the ESP aerobic and anaerobic bottles to the Isolator plus a manual Thiol broth bottle. In this system-versus-system comparison, the Isolator–Thiol-broth combination recovered significantly more staphylococci, pneumococci, members of the family *Enterobacteriaceae*,

and all microorganisms combined. Cockerill and colleagues [4] compared the ESP 80A bottle to the Isolator and found that the former recovered more coagulase-negative staphylococci causing bacteremia, whereas the latter recovered more *S. aureus*, *Candida* spp., and all microorganisms combined. In this study, the ESP 80A bottle also was compared to the manual Septi-Chek bottle and found to be equivalent in the recovery of microorganisms.

In a comparison of the ESP 80A and BacT/Alert FAN aerobic bottles, Doern and colleagues [7] reported higher rates of recovery of *S. aureus*, members of the family *Enterobacteriaceae*, and miscellaneous Gram-negative bacilli in the FAN bottles and enhanced recovery of beta-hemolytic streptococci in the ESP 80A bottles. More contaminants grew in the FAN bottles, and fewer false-positive signals were noted in the BacT/Alert instrument.

Welby et al. [44] compared the ESP system to the Septi-Chek system for the detection of bloodstream infections in children and found the systems to be equivalent in recovering pathogens. More contaminants were recovered in the ESP system, and the detection of positive cultures occurred earlier in the ESP system. A second study reported by the same group [45] compared the ESP 80A bottle to the BacT/Alert FAN aerobic bottle for use with pediatric patients. The FAN bottle detected more *S. aureus* organisms, whereas the ESP 80A bottle detected more streptococci and enterococci. The FAN bottle also detected more positives in patients receiving antibiotic therapy.

In a multicenter retrospective evaluation, Doern et al. [8] reported that a 4-day incubation period could be used in the ESP system without substantial loss of detection of positives. Subsequently, Han and Truant [9] suggested that the incubation period in the ESP system might even be shortened to 3 days.

In a more recent evaluation, Mirrett et al. [20] compared the VersaTREK system with the BacT/Alert 3D system for the detection of bacteremia and fungemia in adult patients. In that evaluation there were no overall differences in yield or in time to detection of microbial growth, although VersaTREK detected significantly more streptococci and enterococci. In addition, VersaTREK detected significantly more pathogens in blood taken from patients receiving antimicrobial therapy at the time of blood culture [20].

2.3 Molecular and other methods

At this time, no commercial molecular method is available in the United States for direct detection of pathogenic microorganisms in blood specimens. There are, however, several commercial assays available for earlier detection and/or identification of microorganisms in blood culture bottles, including the Roche Septi FAST (Roche

Diagnostics, GmbH, Penzberg, Germany), Gene Xpert (Cepheid, Sunnyvale, CA), PNA FISH probes (AdvanDx, Woburn, MA), GeneOhm Staph SR (Becton Dickinson, Sparks, MD), Verigene *Staphylococcus* Blood Culture (BC-S) Nucleic Acid Test (Nanosphere, Northbrook, IL), Biofire BCID (Biofire Diagnostics, Salt Lake City, UT), and matrix-assisted laser desorption ionization with time-of-flight mass spectrometry (MALDI-ToF/MS) (Bruker-Daltonics, Bremen, Germany, and bioMérieux, Marcy L'Étoile, France). Additional approaches and details are described in Chapters 3 and 4, and other chapters of this text.

2.4 Future directions

Although current blood culture systems have reduced the time to detecting positive cultures, clinicians and patients still would benefit from accurate assay methods that provide results in minutes to hours as opposed to days. Thus, there continues to be a need for further evolution and development of new commercial blood culture systems.

As molecular methods become more common in clinical microbiology laboratories, it is reasonable to speculate that blood culture methodology will evolve in this direction as well. The analytical sensitivity of modern blood culture systems should, in theory, be a single viable microorganism contained in a specimen of blood and inoculated in broth medium. However, because microbial growth rates vary according to genus, any antimicrobial agents present in blood can delay microbial growth, and blood itself contains antimicrobial substances, the true analytical sensitivity of blood culture systems almost certainly is higher than the theoretical analytical sensitivity. Moreover, detection systems, although greatly improved by continuous monitoring, still require on the order of 10^4 to 10^5 microorganisms per mL for signaling a positive result. A fruitful initial approach may be to couple molecular amplification with an available culture system to detect positive cultures earlier when lower numbers of replicating organisms are present. Substantial hurdles will still remain, however, including the problem of substances in the blood that inhibit molecular amplification assays, the need for determining antimicrobial susceptibility results when assays are positive, difficulties in interpreting the clinical significance of positive results if the analytical sensitivity of the new tests are greatly enhanced compared to current techniques, and added costs associated with personnel, reagents and instrumentation. In addition to possible earlier detection of common pathogens, much research needs to be performed to delineate the role of other pathogens known to be present in blood, such as many fungi, viruses, *Chlamydia*, and *Mycoplasma*, which are not detectable with current blood culture technology.

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Rapid Devices and Instruments for the Identification of Aerobic Bacteria

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3.1 Introduction

Since the first edition of this book, major advances have been made for the rapid identification of clinically significant aerobic bacteria. These include methods to definitively identify bacteria directly in clinical specimens and simpler, faster identification of isolated organisms with mass spectrometry (MS). Technologies are evolving quickly, and methods such as matrix-assisted laser-desorption ionization time-of-flight (MALDI-ToF) MS and nucleic acid detection techniques are rapidly becoming commonplace in the laboratory. Mass spectrometry and certain molecular methods for detection and identification of microorganisms in clinical specimens are discussed in this chapter, and are also reviewed in detail in other chapters in this text.

Although exciting new technologies for bacterial identification are emerging, many clinical microbiology laboratories still rely on biochemical (phenotype) testing for identification of the majority of isolates. However, the conventional biochemical tests will be relied upon less in the coming years as the new methods gain US Food and Drug Administration (FDA) approval and are implemented in laboratories. In this chapter, we update our review of the major systems available for identifying bacteria isolated in culture and directly in clinical specimens. Automation in the clinical microbiology laboratory has also improved in the past ten years. A significant improvement in the turnaround time for reporting organism identification has been seen for the phenotypic identification systems. Some of the automated systems can return results very quickly, with final identifications now being reported within hours for some groups of bacteria.

In this edition, we have updated the lists of tests, manufacturers, and references. With the rapid pace of change in the clinical microbiology marketplace, the introduction

and discontinuation of commercial kits are ongoing processes. Therefore, the omission of any tests or kits in this chapter, while likely to occur, is unintentional.

3.2 Major methods currently available

3.2.1 Conventional identification methods: phenotypic testing with biochemicals

Conventional methods for identification of bacteria rely on phenotypic characteristics and are largely based on biochemical reactions such as carbohydrate utilization and enzymatic modification of substrates. A panel of “positive” and “negative” values is then translated into a probability score based on Bayes theorem modeling [9, 84] using assumptions that (i) an unknown isolate has equal probability of belonging to any taxon, and (ii) each test is independent of another. These methods are very adaptable to different formats; conventional systems are commercially available in manual or automated formats (107). These systems perform optimally for identification of non-fastidious bacteria that grow well aerobically, although there are panels available for some groups of fastidious bacteria such as the *Haemophilus/Actinobacillus/Cardiobacterium/Eikenella/Kingella* (“HACEK”) group of organisms and anaerobic organisms.

3.2.2 Molecular identification methods

A number of different molecular methods are used for identification of bacteria, including direct probe hybridization, various iterations of nucleic acid amplification and detection, DNA microarrays, and DNA sequencing. Several methods have been FDA cleared and are available commercially. Some of the methods are discussed in this chapter, while others are discussed in the molecular diagnostics and other chapters.

3.2.3 Fatty acid analysis with gas chromatography

Fatty acid methyl ester analysis by gas chromatography (GC) has been used successfully for several groups of organisms including aerobic bacteria. Bacterial fatty acids are unique to each species. The procedure is relatively simple: fatty acids are extracted from pure isolates obtained in culture, then esterified to form fatty acid methyl esters (FAME). Since the fatty acid profile of bacteria is dependent on the media used and the temperature of incubation, standardization of the growth conditions prior to testing on the system is necessary. The FAMES are extracted, then analyzed on a gas chromatograph. The Sherlock system (MIDI, Inc., Newark, DE) automates the preparation and analysis. Its databases for aerobic bacteria include about 165 species in addition to a bioterrorism library containing 21 species in seven genera. The Sherlock system is not currently (at the time of this writing) FDA cleared for diagnostic use; laboratories interested in using the MIDI for aerobic bacterial identification must validate the method in their own laboratory.

3.2.4 Protein mass spectrometry

Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-ToF/MS) is an emerging technology for identification that is rapid, accurate, and inexpensive. The system is based on mass spectrometry. Proteins extracted from bacteria using a matrix of specialized molecules are ionized with a laser. The ionized particles are separated according to their mass-to-charge ratio, and detected using a specialized detection device (131). The characteristic patterns produced by this method can be used to classify and identify bacteria. Two major systems are currently available. The MALDI-ToF system has significant potential for use in the clinical laboratory, as it is rapid, accurate, and (after the initial outlay for the equipment) inexpensive to perform the testing in terms of reagents and labor [27, 42, 136].

3.2.5 Automated systems for the phenotypic identification of aerobic bacteria

Automated instruments for identification of aerobic bacteria use miniaturized biochemical panels that are incubated in the instrument and read at specified intervals. Software programs interpret the data collected from the results of the phenotypic reactions and generate a biochemical profile. The profiles are compared to databases to determine the identity of the organism. Results are highly dependent on appropriate inoculum preparation, incubation conditions, and the version of the software and database used. The automated phenotypic systems available with FDA clearance include Vitek 2 (bioMérieux), MicroScan (Siemens Healthcare Diagnostics), Phoenix (BD Diagnostic Systems), and Aris2X (Trek Sensititre, ThermoFisher Scientific). The Biolog OmniLog System (Biolog, Hayward, CA) uses

automated biochemical testing for identification but is not FDA cleared at the time of this writing.

A discussion by Petti, Carroll and Weinstein of manual and automated systems for detection and identification of microorganisms was recently reported [116]. The reader is referred to this report, as well as the first edition of this manual, for reviews of criteria for selecting a system, methods for evaluating an instrument or system, and other general considerations. Additional information can be found in other authoritative references that are mentioned in this chapter.

3.2.5.1 MicroScan

The MicroScan system (Beckman Coulter, Brea, CA) (Figure 3.1) includes identification panels for rapid or conventional overnight identification; panels are available for identification only, or combined with antimicrobial susceptibility testing. Panels are 96-well microdilution trays that contain biochemical substrates. Each well in the panel is essentially a small test tube that holds about 115 μ L of liquid. Conventional (overnight) panels contain chromogenic substrates, and require overnight growth of the organism in the plate, which is incubated at 35°C for 18–24 h; after adequate growth, the plates can be read visually (manually) or with an automated (WalkAway or autoSCAN-4) instrument system. Identification in conventional panels is based on the detection of pH changes, substrate utilization, and growth in the presence of antimicrobial agents. Rapid panels detect preformed enzyme using fluorogenic tests, and are incubated for 2 h at 35°C. Identification in the rapid fluorogenic panels is based on the detection of hydrolysis of fluorogenic substrates, pH changes following substrate utilization, production of specific metabolites, and the rate of production of specific metabolic byproducts after 2 h of incubation at 35°C. Reading of the Rapid Gram-negative panels must be performed on a WalkAway instrument, with the initial instrument reading at 40 min, and subsequent reads taken at regular intervals up to 7 h of incubation.

3.2.5.2 Phoenix

The Phoenix Automated Microbiology System (Becton Dickinson, Sparks, MD) was launched in US markets in 2004 (Figure 3.2). This instrument houses up to 100 identification (ID) and/or combination ID–antimicrobial susceptibility testing (AST) trays simultaneously with complete random-access flexibility. Onboard incubation is coupled with a combination of detectors for spectrophotometric, colorimetric, and fluorometric analyses, with well interrogation performed roughly every 20 min. As with other systems, preparation of organism suspension, adjustment to standardized turbidity, transfer to appropriate identification tray, and inoculation of purity check cultures are all requirements for the Phoenix system.

Somewhat distinct from other instruments, however, is an option for lower inoculum testing (0.25 vs. 0.5 McFarland) using standard Gram-negative and Gram-positive identification trays, potentially enabling a user to set up automated identifications with fewer isolated colonies [18, 37]. To expedite setup, the Phoenix AP automated processor was introduced in 2008. This instrument

automatically adjusts the organism suspension to the appropriate turbidity and, coupled with a label printer and the Epicenter data management software (BD Diagnostics Systems, Sparks, MD), significantly reduces manual “hands-on” time relative to the standard preparation process [73]. In addition to linking the Phoenix Automated Microbiology System with the Phoenix AP, the Epicenter

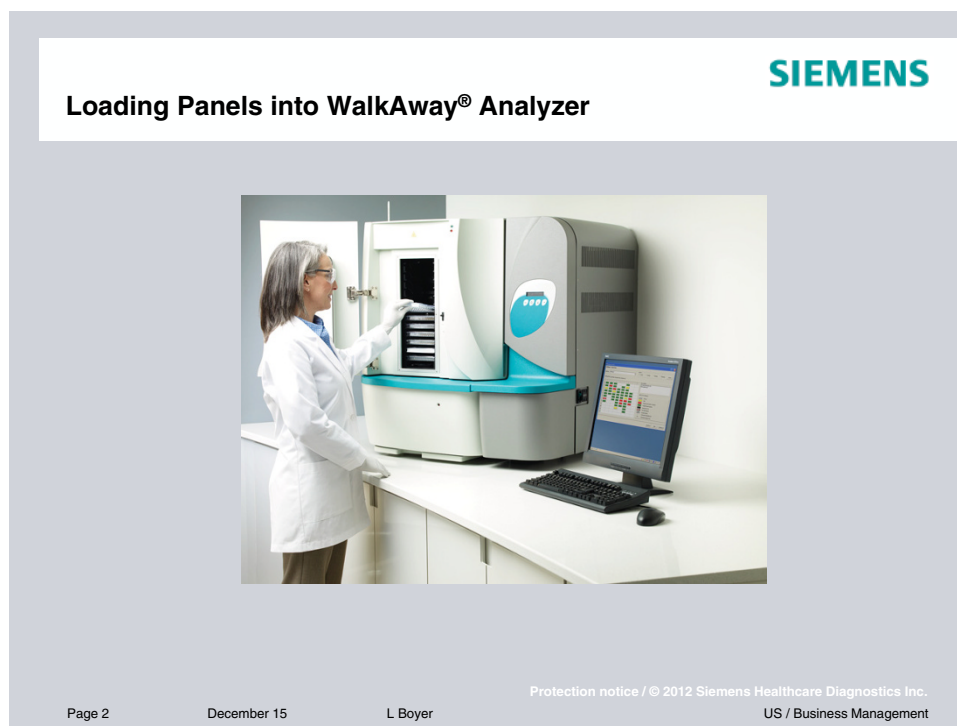


Figure 3.1 MicroScan System. (© 2015 Beckman Coulter, Inc. All rights reserved. Used with permission.)

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MicroScan® autoSCAN-4 Instrument



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Siemens Microbiology Instrument Systems



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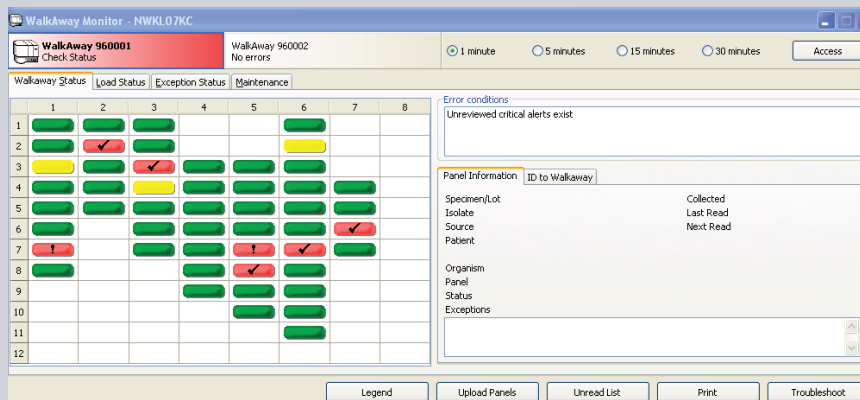
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Figure 3.1 (Continued)

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LabPro Information Manager with Alert_{EX}

Quick visual of processing panel status as seen from the WalkAway Monitor



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MicroScan® Gram-negative and Gram-positive Dried Conventional Panels

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Figure 3.1 (Continued)

MicroScan® Gram-negative Conventional Overnight Panel with Positive Reactions

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MicroScan® MICroSTREP plus® MIC panel

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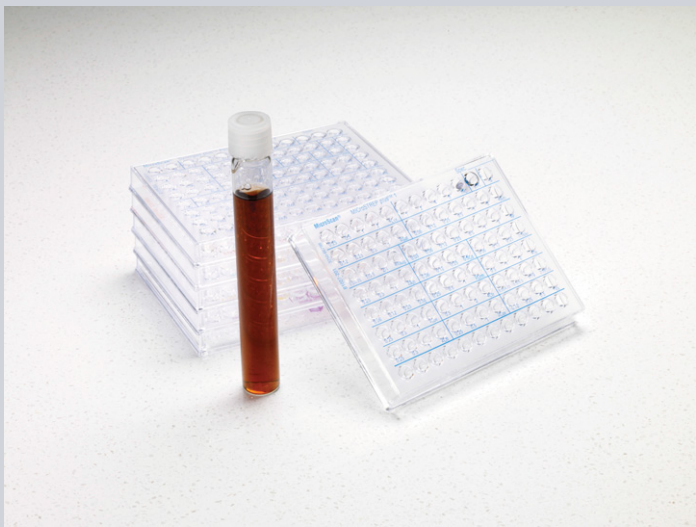


Figure 3.1 (Continued)



Figure 3.2 Phoenix System. (Courtesy and © Becton, Dickinson and Company.)



Figure 3.3 Thermo Scientific™ Sensititre™ ARIS 2X ID/AST system. (Photos courtesy of ThermoFisher Scientific, copying is prohibited).



Plus module also offers connectivity to other BD instruments and provides both user and laboratory information system interfaces as well as a variety of customizable rules and data query/reporting functions.

Reagents for phenotype-based identifications on the Phoenix system include a panel for Gram-negative aerobic and facultative organisms (NID) and another for Gram-positive organisms (PID), both of which may be purchased as ID-only kits or as combination ID/AST trays (NMIC/ID and PMIC/ID panels, respectively). An additional combination panel (SMIC/ID) has also been introduced for ID/AST of *Streptococcus* spp.

3.2.5.3 Sensititre

There are two instruments available for reading identification panels: the AutoReader and the Aris2X (Figure 3.3). The biochemical panels contain fluorometric reagents. Standard identification plates are available for Gram-negative (GNID) and Gram-positive (GPID) organisms.

Trek Diagnostics Systems (now a division of ThermoFisher Scientific) released an automated microbial identification system in the early 1980s. This included a 96-well plate format with four replicates of a 24-reaction test panel, an automated plate inoculator (now known as the Trek AIM), a mirror box for visual interpretations

and a microcomputer for data entry and taxa calculations [149]. The semi-automated system required manual preparation of organism suspension, mineral oil overlay, attachment of adhesive plate cover with puncturing of specified wells, transfer to and from incubator, and post-incubation addition of some reagents in addition to visual endpoint determination and keystroke recording of positive and negative reactions. Performance of the Sensititre system relative to other manual phenotypic kits for identification of enteric Gram-negative bacilli was good [150]. Soon thereafter, the company released a modified broth microdilution methodology for bacterial susceptibility testing that incorporated a fluorogenic substrate for endpoint determination of growth. Use of this technology allowed for automated instrument interpretation, performed by the Trek Autoreader [149], and ultimately led to development and marketing of the AP80 and AP90 trays for Gram-negative and Gram-positive identification, respectively. Cleared by the FDA in 1989, the AP80 tray performed well in a multicenter study, correctly identifying 95% of enteric and nonfermenting organisms to the genus level [151]. The AP90 tray received US FDA clearance in 1998.

The latest Sensititre identification trays marketed by Trek Diagnostics retain the automated fluorescent endpoint detection technology. These include both the GNID tray and the GPID tray, each of which may be read using the Autoreader, the updated Optiread automated system, or the fully automated ARIS instrument. Both trays employ a series of 32 enzymatic reactions such that three organisms may be tested simultaneously per 96-well tray; and manual mineral oil overlay is still required for some wells. Both systems utilize the SWIN software, with 15 genera (42 species, subspecies, or groups) present in the Gram-positive database and 51 genera (127 species, subspecies, or groups) plus one unnamed taxon in the Gram-negative database. Published studies of performance evaluations for either of these trays are not currently available at the time of this writing.

3.2.5.4 Vitek

The Vitek system (originally known as the Automicrobic System [AMS]; Figure 3.4) was developed by McDonnell Douglas in cooperation with NASA. The instrument system was released in the late 1970s and was intended for use in the direct [noncultivation dependent] detection of urinary pathogens by astronauts during space travel [141, 145]. This modular unit included individual stations for specimen dilution, inoculation and sealing of disposable plastic cards, incubation, and spectrophotometric reading of cards, as well as probability calculations and display of results; and it marked an innovative and radical departure from practice standards of the time. Improvements in the system allowed for expanded use for various microbial isolates until 1989, when the company and technology was purchased by bioMérieux (Marcy l'Etoile, France).

The bioMérieux Vitek instrument was engineered largely around the design and principles of the AMS, requiring the user to inoculate identification cards in a separate workstation prior to "onboard" incubation and reading phases. The system successively measured colorimetric or turbidometric changes within individual card wells to determine positive and negative endpoints. Several updates to the organism database (primarily reflective of shifts in prokaryotic taxonomy) as well as adjustments to the biochemical test batteries in newer card versions continued in an effort to improve system performance. As a result, the Vitek was adopted by many clinical laboratories throughout the 1990s due to its efficiency relative to traditional phenotypic testing, in addition to its accuracy compared with other manual and automated platforms [124].

In 1998, bioMérieux launched its Vitek 2 instrument, which reduced the amount of hands-on technologist time necessary by automating steps for organism suspension dilution (for antimicrobial susceptibility test cards), card-inoculation/sealing, and waste disposal. Inclusion of a card



Figure 3.4 Vitek System. (Courtesy of bioMérieux.)

carrier and “smart” docking station provided a more efficient workflow, allowing users to scan identification (and susceptibility) cards and accession labels at multiple benches, thereby linking patient demographics, lot numbers, and organism information to the Vitek software centrally and providing immediate recognition by the instrument upon loading of the card carrier. Further, the Gram-negative and Gram-positive identification cards (i.e. ID-GNB and ID-GPC, respectively) incorporated a fluorogenic indicator that reduced the time to final identification relative to the older, colorimetric cards. These were subsequently phased out of production, however, in favor of a return to colorimetric cards (i.e. GN and GP) in the mid-2000s to improve identification accuracy. A smaller version of the instrument, the Vitek 2 Compact, offers 15, 30 and 60-card capacities but is somewhat less automated than its full-size sibling. By contrast, the Vitek 2 XL doubles the capacity of the Vitek instrument (120 vs. 60 cards) while maintaining near-full automation. Each of the instruments includes similar software, data storage, and retrieval systems and interfacing capabilities. Other similarities between the original Vitek instrument and Vitek 2 models include requirements for: (i) manually preparing saline suspensions of well-isolated colonies to a specified McFarland turbidity range; (ii) inoculating purity plates; (iii) performing off-line testing (e.g., oxidase, indole, motility, pigmentation, hemolysis) for some isolates when automated discrimination values are low. Because the large majority of studies published over the past 15 years evaluating the Vitek system focus on the Vitek 2, and because the original Vitek instrument is no longer manufactured, this chapter concentrates exclusively on performance characteristics of Vitek 2.

3.2.5.5 The Sherlock system

The Sherlock system (MIDI, Inc., Newark, Delaware; Figure 3.5) is based on “fingerprints” or profiles, of bacterial fatty acids, which are first extracted from bacteria, then converted to FAMES. High-resolution gas

chromatography is used to analyze the fatty acids, generating a pattern that is unique for each species of bacteria. The patterns are analyzed with software and compared to the patterns in the database to generate the identification. As of the time of this writing the Sherlock system is not currently FDA cleared. A rapid version of the FAME, called Q-FAME, is also available.

3.3 Commercial systems for identification of Gram-positive organisms

3.3.1 Commercial identification systems for aerobic Gram-positive cocci

Table 3.1 is an overview of currently available commercial systems that can be used for the identification of Gram-positive cocci. There are significant variations in the organism databases and time to results. When considering system implementation, each laboratory should take into account what best meets its needs with respect to commonly encountered organisms, space requirements, ability to interface to a laboratory information system, amount of disposables, turn-around-time to identification, and cost.

3.3.1.1 BBL Crystal Gram-Pos ID System

The BBL Crystal Gram-Pos ID System (BD Biosciences, Sparks, MD) identifies 13 different genera of aerobic Gram-positive bacteria. The panel consists of 29 biochemical and enzymatic substrates that are reconstituted with a suspension of the organism. After overnight incubation, the panels are read using the BBL Crystal Panel Viewer (Figure 3.6), which utilizes a combination of white and UV light sources to interpret reactions and obtain a profile number.

3.3.1.2 BBL Crystal Rapid Gram-Pos ID System

The BBL Crystal Rapid Gram-Pos ID System identifies nine different genera of aerobic Gram-positive bacteria. The panel consists of 29 biochemicals and enzymatic substrates that are reconstituted with a suspension of the organism. After 4 h incubation, the panels are read using the BBL Crystal Panel Viewer, which utilizes a combination of white and UV light sources to interpret reactions and obtain a profile number.

3.3.1.3 Phoenix Gram Pos Panel

The Phoenix Gram Pos Panels (PMIC/ID and PID), developed for the BD Phoenix Automated Microbiology System, identify 30 different genera (140 species and subspecies) of aerobic Gram-positive bacteria. The identification panel incorporates wells for hydrolysis ($n = 29$) and utilization ($n = 16$) of various substrates with colorimetric and fluorometric indicators, two wells for polymyxin resistance, and one for nitrocefin degradation, with two wells serving as growth controls. The instrument reads inoculated panels



Figure 3.5 Sherlock MIDI System. (Courtesy of MIDI, Inc.)

Table 3.1 Commercial identification systems for Gram-positive cocci

Bacteria	Commercial product	Manufacturer	Time to results (h)	<i>Micrococcus</i>	<i>Streptococcus</i>	<i>Staphylococcus</i>	<i>Enterococcus</i>	<i>Aerococcus</i>	<i>Gemella</i>	<i>Lactococcus</i>	<i>Leuconostoc</i>	<i>Pediococcus</i>	<i>Rothia</i>	<i>Allioccocus</i>	<i>Globicatella</i>	<i>Helcococcus</i>	<i>Kocuria</i>	<i>Weissella</i>
<i>Staphylococcus</i> and related genera	API RAPIDEC Staph	bioMérieux	2	x														
	API Staph	bioMérieux	18–24	x	x												x	
	Microgen Staph ID	Hardy Diagnostics	18–24	x	x												x	
<i>Streptococcus</i> and related genera	RapID STAPH PLUS	Remel/ThermoFisher	4	x	x								x		x		x	
	API 20 Strep	bioMérieux	4–24			x	x	x	x	x								
	Microgen Strep-ID	Hardy Diagnostics	18–24			x	x	x	x		x							
<i>Staphylococcus</i> , <i>Streptococci</i> , and related genera	RapID STR	Remel/ThermoFisher	4			x	x	x	x	x	x	x	x		x			x
	BBL Crystal Gram-Pos ID System	BD Diagnostics	18–24	x	x	x	x	x	x	x								
	BBL Crystal Rapid Gram-Pos ID System	BD Diagnostics	4	x	x	x	x	x	x	x								
	Phoenix Gram-Pos Panel	BD Diagnostics	2–15	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
	Vitek ID-GPC and Vitek GP	bioMérieux	2–15	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
	MIDI Sherlock	MIDI	18–24	x	x	x	x			x							x	
	MicroScan Pos ID Panels	Beckman Coulter	18–24	x	x	x	x	x	x		x	x	x				x	
	MicroScan Rapid	Beckman Coulter	2	x	x	x	x	x	x								x	
	Gram-Pos ID Panels																	
	GP2 Microplate	Biolog	2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Sensititre GPID	Trek	18–24	x	x	x	x	x	x								x	



Figure 3.6 BBL Crystal and Autoreader. (Courtesy and © Becton, Dickinson and Company.)

every 20 min for up to 16 h. Time to results generally requires only 3–4 h for identification-only panels [38, 87]. The panel is sealed and self-inoculating and the Phoenix instrument can accommodate up to 100 tests. Studies published on the performance of the Phoenix system for Gram-positive identification focus almost exclusively on Gram-positive cocci despite the fact that the Gram-positive database includes 14 genera of commonly encountered (e.g., *Corynebacterium* spp., *Bacillus* spp.) and/or medically relevant (e.g., *Listeria monocytogenes*, *Rhodococcus equi*, *Erysipelothrix rhusiopathiae*) bacilli. Concordance to the species level for streptococci, staphylococci and enterococci is generally high (>90%) relative to other phenotypic methods, but appears somewhat lower relative to MALDI-ToF/MS and/or sequence ID. In a 2006 study comparing Phoenix Gram Positive panels to conventional biochemical and MIDI Sherlock, the Phoenix Gram Positive panels had good agreement with the identification to the genus level of 99.7% for staphylococci and enterococci, with 100% of *Staphylococcus aureus* and 98% of coagulase-negative staphylococci identified correctly [23]. A study in 2008 showed that correct identification of coagulase-negative *Staphylococcus* spp. by Phoenix was 90.5% [18]. Investigations designed to assess the low-inoculum (0.25 McFarland) protocol have demonstrated comparable levels of performance [19, 26, 37].

3.3.1.4 Vitek ID-GPC

The Vitek ID-GPC card, developed for the Vitek 2 System but no longer manufactured, identified 22 different genera of aerobic Gram-positive bacteria. The ID-GPC card contained a 46 fluorescent test battery, which included tests for 22 enzymes, 16 fermentation reactions, two decarboxylases and six ancillary tests, with final results available in approximately 8 h or less. Fifty-two taxa were represented in its database. In contrast to published evaluations of the

ID-GNB, studies of the ID-GPC generally focused on single or related groups of Gram-positive cocci, with correct identification to the species level reported to be approximately 80–85% for streptococci/enterococci and approximately 85–90% for staphylococci relative to phenotypic comparator assays [2, 11, 26, 36, 40, 54, 79, 90, 97, 146].

3.3.1.5 Vitek GP

Forty-six total biochemical tests are contained within the GP card, with 115 taxonomic groups defined in the Gram-positive database. Each of the cards is automatically inoculated and incubated onboard the instrument, and reaction wells are interrogated every 15 min. Mean time to organism identification is up to 8 h [49]. Studies that directly compared GP and ID-GPC cards for identification of staphylococci and pneumococci have demonstrated similar to slightly better performance for the GP card [2, 87]. Additional studies of GP card performance using various groups of Gram-positive organisms have reported correct taxonomic assignment for approximately 85–95% of all strains tested [54]. In a 2005 study comparing the Vitek 2 Gram-positive panels to the Phoenix system panels, Vitek correctly identified 100% of the 51 *Enterococcus* isolates that were tested [41]. Several other studies reported accuracies of 93% [90], and 92% [44] for *Enterococcus* spp.

3.3.1.6 MicroScan Pos ID Panels/Rapid Gram-Pos ID Panels

The MicroScan Pos ID Panels, developed for the WalkAway System, identify ten different genera of aerobic Gram-positive bacteria. A variety of 96-well microtiter plate formats are available for identification alone or in combination with susceptibility testing. Identification is based on a total of 27 different conventional and chromogenic tests. After 18–24 h of incubation, the WalkAway System interprets the reactions and provides an identification. In an evaluation of the MicroScan WalkAway with Positive Combo panels, the system identified 94.6% of staphylococci to the species level [113]. The Rapid Gram-Pos ID Panel, also developed for the WalkAway System, identifies seven different genera of aerobic Gram-positive bacteria. The 96-well microtiter plate incorporates 36 different fluorometric substrates or fluorometric indicators and relies on the detection of preformed enzymes for identification. After 2 h of incubation, the WalkAway System interprets the reactions and provides a definitive identification. A study comparing MicroScan, Vitek 2 and Phoenix showed no incorrect identification of *Staphylococcus* spp., *Enterococcus* spp., and other Gram-positive cocci [69].

Two types of panels are available from MicroScan (Beckman Coulter, Brea, CA) for identification of Gram-positive organisms, including *Staphylococcus* spp. Conventional overnight panels contain biochemical

substrates used for bacterial identification after 18–24 h of incubation. Identification in conventional panels is based on the detection of pH changes, and substrate utilization. Rapid Fluorogenic panels provide a result after 2 h of incubation. The biochemical tests on these panels are designed to detect preformed enzymes. The Rapid Fluorogenic panels are based on the detection of hydrolysis of fluorogenic substrates, pH changes following substrate utilization, production of specific metabolic substrates, and the rate of production of specific metabolic byproducts. Conventional panels can be read manually or with automated instrumentation; rapid panels must be read with the WalkAway instrument. Gram Positive ID Panels that can be used to identify *Staphylococcus* spp. include Positive ID Type 3 (conventional overnight panel) and Rapid Pos ID Type 2 (Rapid panel). Two other studies to evaluate the Microscan Gram-positive identification panel indicated that supplemental off-line testing may be necessary for accurate identification of *Enterococcus faecium* [31, 67]. Enterococci and related organisms are difficult to identify with automated systems.

3.3.1.7 Phoenix SMIC/ID Panels

Though designated specifically for classification of *Streptococcus* spp., the ID portion of the SMIC/ID trays is configured similar to the PMIC/ID and NMIC/ID trays, containing 45 individual wells for assessment of substrate utilization ($n = 14$) and enzymatic hydrolysis ($n = 31$) in addition to two growth control wells [74]. Unlike the other panels, however, the 0.25 McFarland protocol is not available for the streptococci ID panel. The most current taxonomic database includes 27 species, subspecies, and biotypes of β -hemolytic and viridans group *Streptococcus* spp.

There are no published studies reporting a direct comparison of Phoenix SMIC/ID to PID or PMIC/ID trays. However, results of four investigations, each including more than 100 streptococcal isolates, demonstrated 88–97% accuracy for species-level identifications when compared to standard phenotypic ID. Identification of *Streptococcus pneumoniae* with automated systems has also been studied recently and shown to have good accuracy. In a study comparing the Phoenix and Vitek 2 cards, both systems were comparable in their performance (96.8% for Phoenix, 95.2% for Vitek), and both compared favorably to manual identification methods. Each system returned identifications more rapidly than conventional testing [97].

The Phoenix system has been evaluated in several large studies for the identification of *Enterococcus* spp., including studies that incorporate direct inoculation of panels from positive blood culture vials [18, 23, 41]. In all studies, the Phoenix system performed well for identification of *E. faecium*, and *E. faecalis*.

3.3.1.8 Biolog MicroPlate systems

The Biolog GEN III MicroPlate and the GP2 MicroPlate panel (GEN II for Gram-positive bacteria) are available for identification of Gram-positive organisms. No recent studies describing the performance characteristics of the Biolog plates for identification of clinically significant human pathogens have been published as of the time of this writing. The Biolog system is shown in Figure 3.7.

3.3.1.9 Sensititre

A complete description of the Sensititre system (Trek Diagnostics, ThermoFisher Scientific) can be found earlier in this chapter.

3.3.1.10 Staphylococcus species

Staphylococci are Gram-positive, catalase positive organisms that appear microscopically as cocci in clusters. The genus *Staphylococcus* is comprised of approximately 39 species, which include 21 subspecies. While the isolation of *Staphylococcus* species, particularly non-*S. aureus*, may represent commensal microbiota, in the appropriate patient and/or clinical setting all species have the potential to be true pathogens. *Staphylococcus aureus* is the most common species associated with disease and the spectrum of clinical presentations is broad. *Staphylococcus aureus* is associated with skin infections (impetigo, furuncles, folliculitis), abscesses, pneumonia, osteomyelitis, endocarditis, sepsis, and many other infectious processes [94]. The increasing use of indwelling medical devices has contributed to the recognition of coagulase-negative staphylococci as pathogens as well. This group of organisms, in particular *S. epidermidis*, has been associated with bacteremia, endocarditis, surgical wound infections, and ventricular shunt infections [28, 52, 126, 127].

The ability of *S. aureus* to clot plasma is one of the key differentiators of this organism when compared to other species within the genera. One of the earliest methods used to detect coagulase production was to mix a suspension of organism with plasma, incubate for 4 h



Figure 3.7 Biolog System. (Courtesy of Biolog.) (See insert for color representation of the figure.)

at 37°C and observe for clot formation. Due to inherent issues with both false-positive and false-negative results, other methods have been developed.

Clumping factor A can also be used as a diagnostic tool. This cell-wall associated protein leads to aggregation of *S. aureus* in the presence of plasma. This reaction must be read within 10 s in order to reduce the chance of false-positive reactions. *Staphylococcus lugdunensis* and *Staphylococcus schleiferi* are also positive for clumping factor A, which can impact the specificity of this reaction.

Numerous commercial kits are available for the detection of clumping factor, protein A, and capsular polysaccharides. Table 3.2 summarizes selected products available in the United States for the rapid identification of *S. aureus* from isolated culture colonies. The predominant

methodology is latex agglutination, although hemagglutination and DNA probe technologies are also available. Performance of some assays may vary depending on whether the isolate is methicillin susceptible or resistant; the performance characteristics of these kits range from 93 to 100% sensitivity and specificity [32]. Some strains of methicillin-resistant *S. aureus* (MRSA) are thought to block clumping factor and protein A with capsular antigens. Therefore, the incorporation of capsular antibodies can improve the sensitivity for MRSA [114]. A recent publication [6] examined the performance of a “fourth generation” latex agglutination assay for the identification of *S. aureus*. It incorporated the detection of coagulase, protein A, and capsular polysaccharides, similar to third generation assays, and also a blue carboxylated microparticle. It was found to be equal to current assays and is currently not available in the United States at the time of this writing.

Tables 3.3 and 3.4 are listings of selected methods available for the rapid identification of MRSA from culture and for the identification of *S. aureus* direct from specimens. The performance characteristics of these methods are > 90% sensitivity and specificity [31, 85, 128, 153, 163].

Table 3.2 Rapid assays for the identification of *Staphylococcus aureus*

Manufacturer	Assay	Method
Arlington Scientific	Staphslide	Latex agglutination
Becton Dickinson	BBL Staphyloslide	Hemagglutination
bioMérieux	Slidex Staph-Plus	Latex agglutination
Hologic	Accuprobe	DNA probe
MKL Diagnostics	Phadebact Staph aureus	Latex agglutination
Oxoid	Dryspot Staphytest Plus	Latex agglutination
Oxoid	Staphytest Plus	Latex agglutination
Oxoid	Staphylase	Hemagglutination
Pro-Lab Diagnostics	Prolex-Xtra Staph Latex	Latex agglutination
Remel	Staphaurex	Latex agglutination
Remel	Staphaurex Plus	Latex agglutination

3.3.1.10.1 API RAPIDEC Staph

The API RAPIDEC Staph kit (bioMérieux) identifies *S. aureus*, *S. epidermidis*, and *S. saprophyticus*. A plastic strip with six cupules is inoculated with a suspension of organism and after 2 h results are available. The test includes control and test cupules for fluorogenic detection

Table 3.3 Rapid identification of MRSA from culture

Manufacturer	Assay	Method	Time to results (min)
Alere	Alere PBP2a	Immunochromatography	6
Denka Seiken and Oxoid	MRSA Latex Test for PBP2'	Latex agglutination	15

Table 3.4 Rapid identification of *Staphylococcus aureus* direct from specimens

Specimen	Manufacturer	Assay	Organism	Method	Time to results (min)
Nares Swab	BD	Geneohm MRSA ACP Assay	MRSA	PCR	120
	Cepheid	GeneXpert SA nasal Complete (MRSA SA)	MRSA MSSA	PCR	60
Skin and soft tissue	Roche	LightCycler MRSA Advanced Test	MRSA	PCR	120
	Cepheid	GeneXpert SA Wound Complete (MRSA SA)	MRSA MSSA	PCR	60
Positive blood culture bottle	AdvanDx	Staphylococcus QuickFISH Blood Culture Kit	SA	Nucleic acid hybridization	60
	AdvanDx	<i>S. aureus</i> /CNS PNA FISH	SA	Nucleic acid hybridization	20
	Alere	Binax NOW <i>Staphylococcus aureus</i> test	SA	Immunochromatography	30
	BD	Geneohm StaphSR	MRSA MSSA	PCR	75
	Nanosphere	Verigene Gram-positive Blood Culture	MRSA MSSA	Microarray	150
	Biofire	FilmArray BCID Panel	MRSA	PCR	60
			MSSA		

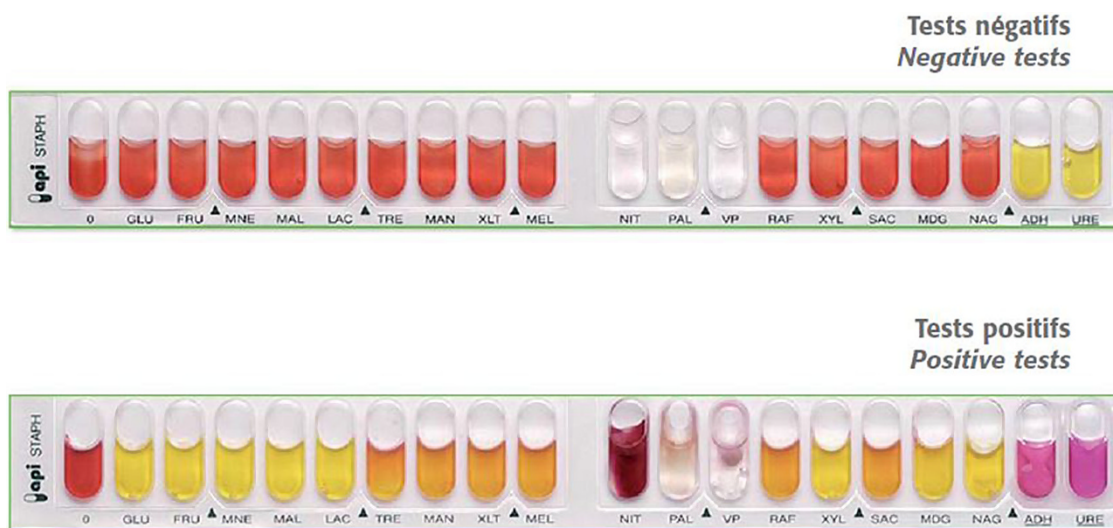


Figure 3.8 API Staph. (Courtesy of bioMérieux.) (See insert for color representation of the figure.)

of coagulase and chromogenic substrates for alkaline phosphatase and β -galactosidase. A UV lamp is required to read the fluorescent coagulase substrate. One publication demonstrated that RAPIDEC Staph correctly identified 100% of 130 *S. aureus* strains, 70.3% of 74 *S. epidermidis* strains, and 81.3% of 32 *S. saprophyticus* strains [68].

3.3.1.10.2 API STAPH

The API STAPH kit (bioMérieux) (Figure 3.8) identifies *Staphylococcus*, *Micrococcus*, and *Kocuria* spp. A plastic strip with 20 microtubes is inoculated with a suspension of organism and after overnight incubation color changes associated with each reaction are read and interpreted. The identification can be determined by locating the numerical profile in the manual database or by using APIweb identification software.

3.3.1.10.3 Microgen Staph ID

The Microgen Staph ID (distributed in the United States by Hardy Diagnostics) identifies *Staphylococcus*, *Micrococcus*, *Kocuria*, and *Kytococcus* spp. Twelve biochemical substrates in a microwell strip are reconstituted with a suspension of the organism. After overnight incubation, a numerical profile is determined based on visual inspection of biochemical reactions, and then interpreted by the Microgen Identification System software (MID-60).

3.3.1.10.4 RAPID STAPH PLUS

The RAPID STAPH PLUS system (Remel Inc., ThermoFisher Scientific, Lenexa, KS) (see Figure 3.9 for example of RAPID kit) identifies *Staphylococcus*, *Micrococcus*, *Kocuria* and *Rothia* spp. Each panel consists of 18 reaction cavities that are reconstituted with a suspension of the organism. After 4–6 h incubation the reaction cavities are visually examined for color change. Identification is obtained by comparison



Figure 3.9 RapID Kits. (Thermo Scientific™ RapID™ NH system. Photos courtesy of ThermoFisher Scientific, copying is prohibited; also see Table 3.13)

of reactions to a differential chart or by the Electronic RAPID Compendium (ERIC) software (Remel, Inc).

3.3.1.11 Streptococcus species, Enterococcus species, and related genera

Streptococci are Gram-positive, catalase-negative organisms that appear microscopically as cocci in chains and pairs. Streptococci and related organisms comprise 17 different genera. Historically, the presence or absence of β -hemolysis formed the foundation for classifying streptococcal species. Currently, the term “pyogenic” is used to describe the β -hemolytic streptococci, as well as additional species such as *S. dysgalactiae* subsp. *dysgalactiae*. Characterization of species by Lancefield grouping is also becoming less definitive. A list of the commercial kits for grouping of β -hemolytic streptococci isolated in culture is found in Table 3.5. As with the staphylococci, the clinical significance of isolating *Streptococcus* and *Streptococcus*-like organisms in culture will depend on the clinical presentation and the immune status of the host. Infectious processes vary widely

and can include pharyngitis, skin and soft tissue infections, neonatal infections, pneumonia, endocarditis, and septicemia. The “nonpyogenic” species include the α - and non-hemolytic organisms such as *S. pneumoniae* [43].

3.3.1.11.1 Direct detection of *Streptococcus pneumoniae* antigen in urine specimens

Streptococcus pneumoniae is a major cause of community-acquired pneumonia. Rapid diagnosis of invasive disease caused by *S. pneumoniae* is of importance clinically. The Binax NOW *S. pneumoniae* test (Alere, Inc., Waltham,

MA), shown in Figure 3.10, is a rapid immunochromatographic test that detects pneumococcal antigen in urine and cerebrospinal fluid (CSF) specimens, and was approved by the FDA in 2003. In a prospective clinical evaluation of the Binax NOW test, the sensitivity of the test was 82%, and the specificity was 97% [139]. A more recent study showed the sensitivity of the Binax test to be 88%, with a specificity of 96% [140]. A second method is the Trinity Biotech UNI-Gold *S. pneumoniae* immunoassay which is currently Conformité Européenne/European Conformity (CE) cleared but not yet cleared by the US FDA at the time of this writing.

3.3.1.11.2 Rapid systems for identification of Group A *Streptococcus* in clinical specimens

Rapid antigen tests for the direct detection of group A streptococcal antigen in throat swabs have been used for many years. Current practice guidelines recommend use of rapid detection methods for Group A streptococcal pharyngitis [138]. A number of rapid tests are available for the direct detection of Group A streptococcal antigen from throat swabs [62, 118, 158]. Compared to culture, the direct detection methods range in sensitivity from 75 to 85%. Negative test results should be followed with culture. An increase in self-contained direct detection tests using an immunochromatographic test format has occurred during the past 10–15 years. Many of these newer tests have been granted waived test status under the Clinical Laboratory Improvement Amendments of 1988 (CLIA 88) and they are used in physician's offices, clinics and emergency departments. These tests have important utility in point-of-care testing, with results available in less than 10 min. Early detection of Group A streptococcal pharyngitis allows the appropriate and timely use of antimicrobial agents [55, 89]. It is not uncommon for a single manufacturer of a rapid waived test to enter into marketing agreements with a few different distributors, resulting in the test being sold under different names. Accurate test results will depend on both sampling technique and the amount of organism present in the specimen [55]. A list of the currently available rapid Group A *Streptococcus* tests is found in Table 3.6.

Table 3.5 Kits for grouping β -hemolytic *Streptococcus* isolated in culture

Kit name	Manufacturer/supplier
Streptpro Grouping Kit	Hardy Diagnostics
Streptex	ThermoFisher/Remel
Prolex Streptococcal Grouping Kit	Pro-Lab Diagnostics
PathoDX Latex Agglutination Strep Grouping Kit	Diagnostic Products Corp/Thermo Scientific
Oxoid Streptococcal Grouping Kit	Oxoid
PathoDXtra Streptococcal Grouping Kit	Oxoid
Slidex StreptoPlus	bioMérieux
Streptocard	Becton Dickinson
Meritec Strep	Meridian Diagnostics
Phadebact <i>Streptococcus</i>	MKL Diagnostics



Figure 3.10 Binax NOW *Streptococcus pneumoniae* antigen test kit. (Courtesy of Alere, Inc.) (See insert for color representation of the figure.)

Table 3.6 Kits for the direct detection of Group A *Streptococcus* antigen in throat specimens

Kit name	Method	Supplier
BD Chek Group A Strep	Immunochromatographic	BD
Binax NOW Strep A	Lateral flow immunochromatographic	Alere
ICON DS StrepA Test	Dipstick immunochromatographic	Beckman-Coulter
BioStar Aceeava Strep A Test	Lateral flow immunochromatographic	Inverness Medical Professional Diagnostics
Clearview Strep A Exact	Dipstick immunochromatographic	Alere
Genzyme OSOM StrepA Test	Lateral flow immunochromatographic	Sekisui Diagnostics
QuickVue Strep A Test	Dipstick immunochromatographic	Quidel
Immunocard STAT Strep A	Dipstick immunochromatographic	Meridian
SureVue Signature Strep A Test	Immunochromatographic	Fisher Healthcare
McKesson Strep A Test Cassette	Immunochromatographic	McKesson
Polymedco Poly Stat Strep A	Immunochromatographic	Polymedco
BD Directigen EZ Group A Strep Test	Rapid chromatographic immunoassay	BD

Table 3.7 Molecular tests for the direct detection of Group A *Streptococcus* in throat specimens

Test name	Method	Manufacturer	Performance characteristics (%)	Reference
Illumigene Group A <i>Streptococcus</i>	LAMP DNA amplification	Meridian	Sensitivity 99 Specificity 99.6	5
Hologic Gen-Probe GASDirect Test	DNA probe assay	Hologic-GenProbe	Sensitivity 85.7 Specificity 97.8	115
Laboratory-Developed Light Cycler Assay	Real-time PCR	Roche	Sensitivity 93 Specificity 98	154

Table 3.8 Rapid identification of Group B *Streptococcus* direct from vaginal/rectal specimens

Manufacturer	Assay	Method	Time to results (min)	Sensitivity	Specificity	References
BD	BD GeneOhm Strep B	RT-PCR	70	94	96	129
BD	BD Max GBS	RT-PCR	60–120	95	97	128
Cepheid	Smart GBS	RT-PCR	75	78–99	90–97	68
Cepheid	Xpert GBS	RT-PCR	55	92–99	92–96	17
Meridian	Illumigene GBS	LAMP technology	<60	97	92	Data from manufacturer

3.3.1.11.3 Molecular assay systems for identification of Group A *Streptococcus* in clinical specimens

Three molecular assays for the direct detection of Group A *Streptococcus* in pharyngeal specimens are currently commercially available (Table 3.7). Two of these are FDA cleared at the time of this writing, while one is available as a laboratory-developed assay. In two studies comparing the Illumigene (Meridian Bioscience, Cincinnati, OH) Group A *Streptococcus* DNA assay to culture, the sensitivity and specificity were > 99.0% [5, 63].

3.3.1.11.4 Rapid molecular assay systems for identification of Group B *Streptococcus* in clinical specimens

Molecular methods are currently a recommended method for the rapid identification of Group B *Streptococcus* directly in vaginal/rectal specimens or in broth-enriched specimens because of their superior sensitivities and specificities [133]. Five FDA-cleared systems (Table 3.8) are commercially available: BD GeneOhm Strep B Assay, BD Max GBS Assay (Figure 3.11), Cepheid Smart GBS, Cepheid Xpert GBS, and Illumigene GBS (Figure 3.12) from Meridian. Sensitivities range from 78 to 99% and specificities range from 90 to 97%. In a multicenter study comparing the Smart Group B *Streptococcus* assay with an enrichment protocol to the BD GeneOhm StrepB direct, the Smart assay had a sensitivity of 98.6% and specificity of 90% [70]. Sensitivity of the molecular assays is increased when the test is performed on enriched samples (e.g., incubated in selected broth preparations prior to performing the assay). In an evaluation of the BD MAX GBS assay performed on broth-enriched samples, the sensitivity and specificity were 100% compared to culture [132]. Table 3.8 lists the commercial methods available for rapid identification of Group B *Streptococcus* directly from vaginal/rectal specimens.



Figure 3.11 BD Max System. (Courtesy and © Becton, Dickinson and Company.)



Figure 3.12 Meridian Bioscience Illumigene System. (Courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)

3.3.1.11.5 *Enterococcus* spp.

Enterococci are catalase-negative, Gram-positive cocci that are normally found in the intestines of humans and animals, but are pathogenic in certain circumstances. Identification of *Enterococcus* spp. is important because these organisms are intrinsically resistant to many antimicrobial agents, and they may acquire resistance to vancomycin [46]. It is also necessary

to differentiate between enterococci and the related Gram-positive cocci such as *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Vagococcus*. Commercial systems for the identification of *Enterococcus* spp. are listed in Table 3.1.

3.3.1.11.6 Manual systems

BBL Crystal Gram-positive ID system In a study comparing the BBL Crystal to API Rapid ID 32 Strep, the BBL Crystal system correctly identified 96% of enterococci [59].

API 20 Strep The API 20 Strep kit (bioMérieux) identifies *Streptococcus* spp. and seven other genera (Table 3.1). A plastic strip with 20 microtubes, which determines enzymatic activity or fermentation of sugars, is inoculated with a suspension of organism. Reactions are first read at 4 h after the addition of reagents. If a definitive identification is not obtained, the strip is incubated for up to an additional 20 h. The identification can be determined by locating the numerical profile in the manual database or by using APIweb identification software.

Microgen Strep ID The Microgen Strep ID (distributed in the United States by Hardy Diagnostics) identifies *Streptococcus*, *Enterococcus*, and *Gemella* species. Twelve biochemical substrates in a microwell strip are reconstituted with a suspension of the organism. A hippurate test and macroscopic appearance are also used for identification. After overnight incubation, a numerical profile is determined based on visual inspection of biochemical reactions, and then interpreted by the Microgen Identification System software (MID-60).

RapID STR The RapID STR system (Remel; ThermoFisher) identifies *Streptococcus* spp. and seven other genera (including *Listeria* which is not represented in Table 3.1). Each panel consists of 10 reaction cavities that are reconstituted with a suspension of the organism. After 4 h incubation, the reaction cavities are visually examined for color change. Four of the cavities are bifunctional since two substrate results are generated, one before and one after the addition of reagent. Hemolysis is also used as part of the identification schema. Identification is obtained by comparison of reactions.

3.3.2 Commercial identification systems for aerobic Gram-positive Bacilli

3.3.2.1 Commercial systems for the identification of *Corynebacterium* spp. and related genera

Corynebacteria are aerobic, Gram-positive, nonspore-forming bacteria that are normal skin flora of humans and many animals. They are occasionally found as pathogens, usually in immunocompromised patients, or in hospitalized patients. *Corynebacteria* are often found as contaminants in many types of cultures, and determining when to pursue identification can be difficult. Isolates from sterile body sites should be identified. In addition, *C. urealyticum* has potential as a urinary tract pathogen and laboratories

should have guidelines for identifying this species from urine cultures. Several other genera of bacteria that resemble *Corynebacteria* (*Arcanobacterium*, *Brevibacterium*, *Microbacterium*, *Arthrobacter*, *Rothia*, and several others) are occasionally isolated from clinical specimens and are often mistaken for *Corynebacterium* [12]. Identification of these Gram-positive rods using phenotypic and biochemical methods remains challenging. Compared with MALDI-ToF and DNA sequencing, phenotypic panels have been shown to be inaccurate for almost 30% of isolates [12]. The MALDI-ToF and DNA sequencing are more accurate and rapid for identification of *Corynebacterium* spp. [3, 12, 56].

The Vitek 2 CBC colorimetric identification cards can be used for identification of coryneform organisms. Cards contain 41 biochemical reactions; and the database includes 64 taxa (including ≥ 2 species each of *Actinomyces*, *Brevibacterium*, *Corynebacterium*, *Lactobacillus*, *Microbacterium*, and *Rhodococcus*). Additionally, eight *Corynebacterium* spp. are present in the Vitek 2 anaerobic card (ANC) database. Rennie et al. [123] reported accurate identification of 94% of *Corynebacterium* species correctly ($n = 53$) using the ANC card. Table 3.9 lists the commercial identification systems for *Corynebacterium* spp. and other related Gram-positive rods. Other products manufactured in the EU and elsewhere may be available in selected countries for this organism and for other organism groups (e.g., Merlin Diagnostika, Berlin, Germany). Please refer to other sources, the company web sites and current scientific journals and vendor information.

3.3.2.1.1 Manual systems

Most of the studies evaluating phenotypic methods for identifying *Corynebacterium* and the related genera were performed prior to the advent of MALDI-ToF and DNA sequencing. The phenotypic testing methods should be used with the understanding that the reported identification of these organisms is likely to be incorrect about 30% of the time; laboratories should probably not rely solely on phenotypic methods for definitive identification of these organisms.

3.3.2.2 Commercial systems for the identification of *Bacillus* spp.

Identification of some of the commonly isolated Gram-positive bacilli may also be accomplished using the Vitek 2 BCL. The BCL consists of a database comprising 40 taxa (including ≥ 2 species each of *Bacillus*, *Geobacillus*, *Paenibacillus*, *Brevibacillus*, and *Virgibacillus*) and cards containing 46 reactions. In a large, single-center evaluation of the BCL card, Halket et al. tested 109 strains of *Bacillus*-like organisms and documented 88% correct identification (93% including low-discrimination identities that were correctly assigned following manual off-line testing), with 5% low-discrimination, 1% unidentified, and 6% incorrect identifications [58].

Table 3.9 Commercial identification systems for *Corynebacterium* spp. and other Gram-positive Bacilli

Commercial product	Manufacturer	Time to results (h)	<i>Corynebacterium</i>	<i>Actinomyces</i>	<i>Arcanobacterium</i>	<i>Arthrobacter</i>	<i>Erysipelothrix</i>	<i>Gardnerella</i>	<i>Listeria</i>	<i>Cellulomonas</i>
Phoenix Gram-Pos Panel	BD Diagnostics Systems	2–15	x		x		x	x	x	x
BBL Crystal	BD Diagnostics Systems	18–24	x	x	x		x	x	x	
Gram-Pos ID System										
BBL Crystal Rapid	BD Diagnostics Systems	4	x	x			x	x	x	
Gram-Pos ID System										
API Coryne	bioMérieux	18–24	x	x	x	x	x	x	x	x
MIDI Sherlock	MIDI	18–24	x		x	x		x	x	x
IDS RapID CB PLUS	Remel/ThermoFisher	4	x	x	x		x		x	x
MicroScan Pos ID Panels	Beckman Coulter	18–24	x						x	
MicroScan Rapid	Beckman Coulter	2	x						x	
Gram-Pos ID Panels										
Vitek GPI Card	bioMérieux	18–24	x							
Vitek ID GPC Card	bioMérieux	18–24	x							
Biolog GP MicroPlate	Biolog		x							

3.4 Commercial systems for identification of Gram-negative organisms

3.4.1 Manual systems

A listing of selected manual systems available for identification of Gram-negative bacteria is found in Table 3.10. Most of the published evaluations of manual systems for the identification of Gram-negative bacteria were performed and published more than 15 years ago, and many systems are older than that. Therefore, a review of the many scientific comparison studies evaluating these manual tests will not be included in this chapter, and can be found in the first edition of this text and other references. Most of the manual tests have been shown to perform at a level of > 90% accuracy, and many have performed at or above 95% accuracy and are therefore considered comparable to conventional identification protocols.

3.4.2 Automated systems for the identification of Gram-negative bacteria

The accuracy of any identification systems will be influenced to some extent by several factors, some of which include: (i) the proportion of clinical versus reference isolates evaluated (since the former is more likely to demonstrate aberrant metabolic patterns or phenotypes); (ii) the constituency and overall diversity of organisms used to challenge the system (e.g., commonly recovered versus rare or unusual isolates); (iii) the reference or comparator method (e.g., phenotypic versus genotypic); (iv) whether “low-discrimination” values are considered “correct” if rapid off-line testing permits accurate classification [106]. As a result, the assimilation of data from multiple studies, each designed and conducted somewhat differently, is inherently problematic. The reader is advised to

interpret the compilation of results below with caution and in this light. A listing of the automated panels available for identification of Gram-negative bacteria is found in Table 3.11.

3.4.2.1 MicroScan

MicroScan panels for identification of non-fastidious Gram-negative organisms include the conventional overnight (Negative ID Type 2) panel and Rapid panels (Rapid Neg ID Type 3 and Type 4). Reported performance characteristics of the automated systems can vary slightly according to the panel type, software used, and the method of reading. A comparison of the BD Phoenix with the MicroScan showed identification of *Enterobacteriaceae* to be very good for Microscan (98% to the genus level; and 97% to the species level) [143]. For nonfermentative organisms, a 2011 study comparing MicroScan, Vitek, and Phoenix automated identification of 142 clinical isolates showed that the MicroScan Gram-negative panel correctly identified 95.6% of enteric Gram-negative bacilli, and 52% of the non-fermenting Gram-negative bacilli [69].

3.4.2.2 Phoenix

Gram-negative organism identification by the NID or NMIC/ID panels is achieved via colorimetric or fluorometric measurements in 45 separate reaction wells, which include substrates for enzymatic hydrolysis ($n = 19$) or utilization ($n = 24$) and polymyxin resistance ($n = 2$), in addition to two fluorometric growth control wells. The most recent taxonomic database for the 0.5 McFarland procedure contains 61 separate genera of enteric ($n = 24$), nonfermenting ($n = 28$), and miscellaneous ($n = 9$) Gram-negatives comprising 157 distinct species, subspecies, and serovars as well

Table 3.10 Manual kits for the identification of Enterobacteriaceae and other non-fastidious Gram-negative Bacilli

Name of kit	Manufacturer	Time to identification	Enterobacteriaceae	Non-Enterobacteriaceae
API 20E	bioMérieux	Overnight	x	x
API 20NE	bioMérieux	Overnight	x	x
Crystal E/NF	BD	4 h	x	x
Enterotube II	BD	Overnight	x	
RapID NF	Remel/ThermoFisher	4 h		x
UniTek N/F	Remel/ThermoFisher	Overnight		x

Table 3.11 Selected automated systems and panels for the identification of Enterobacteriaceae and other non-fastidious Gram-negative Bacilli (GNB)

System	Panel	Organism group	References
MicroScan	Gram-negative	Gram-negative	8,119,124,143
Microscan	Gram-negative	Nonfermenting GNB	103–105,154
Phoenix	Gram-negative	All Gram-negative	49,102,143,166
Phoenix	Gram-negative	Nonfermenting GNB	143
Trek	GN-ID	Gram-negative	149,150
Vitek	ID-GNB	All GNB	47,51,54,91
Vitek	ID-GNB	Enteric GNB	41,71,102,161
Vitek	GN	All GNB	50
Vitek	GN	Nonfermenting GNB	16,41,71,72,168
Vitek	GN	<i>Burkholderia</i> and <i>Ralstonia</i>	20,80,93

as four unnamed Centers for Disease Control and Prevention (CDC) nonfermentative groups. In the absence of concomitant AST, identification results are typically available within 4 h for most organisms [102]. Published reports of the accuracy of the identification panels for non-fastidious organisms show correct identifications of 80–85% [129]. A comparison study of the Phoenix with the MicroScan WalkAway reported good agreement between the two systems [143]. Chatzigeorgiou et al. also reported good agreement between the Phoenix and Vitek for identification of Gram-negative bacteria [26]. A study comparing conventional biochemical testing to the Phoenix NID card reported overall agreement of 95% to the genus level and 94% to the species level [24]. Most studies report greater than 90% accuracy to the genus level when using some phenotypic-based reference method, whereas comparisons to MALDI-ToF or sequenced-based methods, particularly when focused upon a specific taxonomic group that may be difficult to classify phenotypically, demonstrate lower accuracy rates (Table 3.11). Yan et al. report a notable exception to this, however, in a study of > 1000 clinical isolates recovered from urine sources [166]. They observed 99.5% accuracy to the genus level (98.2% to the species level) relative to MALDI-ToF and/or 16S rRNA gene sequencing for Gram-negative organisms.

3.4.2.3 Sensititre

For identification of non-fastidious Gram-negative organisms, the Sensititre GNID standard identification plate is available. These panels use a standard 96-well format. The reader is referred to the previous edition of this manual for studies and references.

3.4.2.4 Vitek

3.4.2.4.1 ID-GNB

The Vitek 2 was released in 1998, incorporating the use of fluorescent biochemical endpoints. The new format resulted in a decreased time to result reporting to approximately 3 h for most organisms [49, 90]. The ID-GNB utilized 41 distinct fluorescence-based reactions (plus two control wells), including 18 enzymatic, 18 fermentation, two decarboxylase, and three miscellaneous reactions. The corresponding database included 57 enteric and 40 nonenteric taxa in addition to three “highly pathogenic” organisms and a “various nonfermenting Gram-negative bacilli” category. Studies of the ID-GNB assessing large numbers of varied Gram-negative taxa demonstrated accurate identification for 80–85% of isolates [30, 48, 54, 65, 104, 120]. Similar work targeting subsets of Gram-negative organisms, however, revealed better accuracy rates for enteric species compared with nonenteric isolates (Table 3.11), an observation believed to be related to slower growth rates, lack of fermentative capabilities, and/or less active metabolism among the nonenteric phyla.

3.4.2.4.2 GN Card

In an effort to improve identification accuracy of the automated system, bioMérieux launched new colorimetric-based detection cards (i.e. GN and GP cards) in 2005 [117, 161], and upgraded respective taxonomic databases. The GN Card contains 47 distinct reactions plus a decarboxylase broth base well; and its database features nomenclature for 70 enteric, 72 nonenteric and six “highly pathogenic” taxonomic groups. Mean time to organism identification is reported to be 4–6 h for GN [109, 121].

Results of direct comparisons between the ID-GNB to the GN Card using the same organism set have been published [161, 168]. Both studies reported better identification accuracy for the GN Card, with better performance for enteric organisms (Table 3.11). Differing accuracy rates reported by Zbinden et al. may be attributable to the set of organisms used in the study [168]. Subsequent studies have demonstrated accuracy rates of > 90% (Table 3.11).

3.4.3 Identification methods for selected members of the family Enterobacteriaceae

3.4.3.1 Identification of toxin-producing *Escherichia coli*

Following a report of two outbreaks of hemorrhagic colitis in 1983 linked to a strain of *Escherichia coli* that had until then been encountered only rarely (i.e., O157:H7) (126), attention began to focus on pathogenic mechanisms and an etiological association with hemolytic uremic syndrome (HUS) [76, 77]. Identification of a toxin produced by some *E. coli* strains (referred to as enterohemorrhagic *E. coli*, or EHEC) that produced cytopathic effects in Vero cells (termed “vero-toxin”) led to the recognition of homology between shiga-toxin produced by *Shigella dysenteriae* (later referred to as “shiga-like toxin” or Stx), and ultimately to a more complete picture of its role in HUS [81]. Early focus in diagnostic laboratories was upon selective recovery and detection of O157:H7 among the many commensal, less virulent strains of *E. coli* that typically inhabit the gut. In the late 1980s and throughout the 1990s, the use of sorbitol MacConkey agar, which exploited the ineffective utilization of this substrate by O157:H7, became commonplace in clinical laboratories. Similarly, methods for serologically screening for and defining this strain *in vitro* were promulgated. Approaches incorporating immunofluorescence, latex-beads (e.g., assays by Pro-Lab Diagnostics, Murex, Shared Systems, Oxoid), enzyme-linked immunosorbent assay (ELISA; e.g., Lmd Laboratories), and immunochromatography (e.g., assays by Guardian Scientific, Meridian, Oxoid) all employed antibodies with specificity to O157 and could be easily transitioned into fecal culture algorithms. While their overall performance was generally good [25, 152], the observation that non-O157 serotypes were also responsible for cases of hemorrhagic colitis and HUS due to production of one or both Stx subtypes led to development of more broadly encompassing assays targeting the toxin itself. A list of the commercial assays available as of this writing for the detection of the *E. coli* toxin is shown in Table 3.12.

3.4.3.1.1 Premier EHEC ELISA

The Premier EHEC ELISA (Meridian BioScience) (Figure 3.13) integrates a 96-well plate ELISA method for simultaneous qualitative detection of shiga-like toxins 1 and 2 to a limit of 7 and 15 pg/well, respectively [78]. The assay may be read visually or by spectrophotometer, and may be



Figure 3.13 Premier EHEC ELISA. (Courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)



Figure 3.14 Thermo Scientific™ ProSpecT™ STEC (Shiga Toxin *E. coli*) Microplate Assay. (Photo courtesy of ThermoFisher Scientific, copying is prohibited.) (See insert for color representation of the figure.)

used directly on stool specimens or on organisms cultivated *in vitro* in enrichment broth or solid media. Published evaluations of this product report sensitivity ranging from 82.4 to 100% (Table 3.12) with better performance using enrichment broth compared to direct specimen testing [156]. One exception was a study by Vallieres et al. in which clinical sensitivity of 28.6% was observed, though here the comparator method was a very sensitive, laboratory-developed polymerase chain reaction (PCR) method [160].

3.4.3.1.2 ProSpecT Shiga Toxin EIA

The ProSpecT Shiga Toxin *E. coli* (STEC) Microplate Assay (Remel/ThermoFisher) (Figure 3.14) is a 48- or 96-well microplate ELISA used for qualitative detection

Table 3.12 Commercial assays for detection of toxin-producing *E. coli*

Reference	Immunoassay (IA)	Testing Performed on	Comparator method(s)	Total specimens tested	Total positive for Stx	Total O157/total STEC recovered	IA sensitivity	IA specificity	All O157 detected by IA (%)
80	Premier EHEC EIA	Enrichment broth	Vero cell cytotoxicity on stool or isolate	972	15	10/13	100	99.7	100
95	Premier EHEC EIA	Enrichment broth	Premier O157 EIA or Vero cell cytotoxicity or Stx PCR on isolates at provincial laboratory	815	58	47/57	91.4	99–100	100
101	Premier EHEC EIA	Enrichment broth	Vero cell cytotoxicity or Stx PCR on stool	488	17	8/14	82.4	100	NR
80	Premier EHEC EIA	Enrichment broth	SMAC and Stx PCR on isolates	1851	39	28/39	92.3	99.8	89.3
110	Premier EHEC EIA	Enrichment broth	SMAC and State DOH testing	NR	65	45/65	98.5	100	97.8
63	Premier EHEC EIA	Enrichment broth	SMAC and isolate Stx EIA or serotyping at State DOH	5110	66	33/50	96	99.7	93.9
29	Premier EHEC EIA	Enrichment broth	Stx PCR on broth and/or Stx PCR or IA on isolates	2328	21	8/21	90.4	NR	87.5
53	ProSpecT Shiga toxin EIA	Enrichment broth	SMAC and Stx EIA + serotyping at State DOH	2060	29	13/27	100	99.9	100
14	VTEC-Screen	Colonies	Vero cell cytotoxicity and/or Stx PCR on broth	234	59	4/43	89.8	99.4	100
111	SeikenRPLA	Colonies	Premier EHEC EIA on broth or isolate subcultures	291	43	33/43	100	100	100
159	Duopath Verotoxin ICG	Enrichment broth	Stx PCR (x2) on broth and/or isolates	632	21	5/16	28.6	100	60
159	ImmunoCard Stat!	Enrichment broth	Culture + limited serotyping	554	12	8/12	19	100	
22	Premier EHEC EIA	Enrichment broth					91.6	99.8	87.5
22	VTEC-Screen	Colonies					91.6	99.3	87.5
162	SeikenRPLA	Broth cultures	Reference isolates, previously characterized	78	78	20/78	88.5	N/A	95
162	Premier EHEC EIA						92.3		95
156	ProSpecT Shiga toxin EIA	Stool	Vero cell cytotoxicity	742 (5)	31	NR	83.9	99.8	NR
156	Premier EHEC EIA	Enrichment broth		827	81		90.1	100	

of Stx-1 and/or Stx-2. Similar to the Premier assay, it may be used on direct stool specimens or enrichment broth cultures and can be read visually or spectrophotometrically [160]. Review of two published studies demonstrates sensitivity of this assay to be 92.3–100% (Table 3.12).

3.4.3.1.3 VTEC-Screen Seiken RPLA

The VTEC-Screen Seiken (Denka Seiken Co., Tokyo, Japan) is a 96-well microplate assay that utilizes a reverse passive latex agglutination (RPLA) method to detect (but not delineate) Stx-1 and/or Stx-2 produced by colonies of bacteria tested from solid media [22]. Unlike the typical 2–4 h turnaround time for an ELISA, the VTEC-Screen must be incubated overnight in order to allow settling of latex particles [14]. The limit of detection for this assay is 25 pg/well, and agglutination is read visually. Two studies designed with different comparator methods reported similar sensitivity (89.8–91.6%) for this assay (Table 3.12).

3.4.3.1.4 Duopath Verotoxin ICG

The Duopath Verotoxin assay (Merck KGaA, Darmstadt, Germany) is a single-use, lateral flow immunochromatographic kit for the detection and delineation of Stx-1 and/or Stx-2 produced by organisms cultivated on solid media. Fewer incubation steps and faster turnaround time (10–20 min total) relative to ELISA and RPLA are advantages of this platform, although analytical sensitivity is somewhat higher (limits of detection, 25 ng/mL and 62.5 ng/mL for Stx-1 and Stx-2, respectively). The single known publication at the time of this writing evaluating this assay included a procedural modification to shorten turnaround time, but reported sensitivity of 100% relative to the Premier EHEC assay [111] (Table 3.12).

3.4.3.1.5 ImmunoCard Stat! EHEC

The ImmunoCard Stat! EHEC assay (Meridian Bioscience) (Figure 3.15) is a single-use, lateral flow immunochromatographic kit for the detection and delineation of Stx-1 and/or Stx-2 produced by organisms cultivated in enrichment broth or solid media. Similar to the Duopath assay, it offers rapid turnaround times with few incubation steps and analytical sensitivities of 25 ng/mL and 63.5 ng/mL for Stx-1 and Stx-2, respectively. Whereas published reports of the assay targeting O157 antigen from the same ImmunoCard Stat! product line are available, no such studies could be identified for the EHEC assay targeting shiga-like toxins at the time of this writing.

3.4.3.1.6 Shiga toxin Chek and Quik Chek

The Alere Shiga Toxin Chek and Quik Chek (Figure 3.16), are the latest assays cleared by the US FDA for Stx detection. The former is a 96-well microplate ELISA with limits of detection of 0.2 to 0.3 ng/mL for each toxin subtype (depending on specimen matrix) but without capability of delineating Stx type. The latter is a lateral flow immunochromatographic kit



Figure 3.15 Immunocard STAT! EHEC Kit. (Courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)

that does differentiate Stx-1 and Stx-2 subtypes. The analytical sensitivity of this test is 0.04 ng/mL for each toxin type. Both assays may be used for testing of direct fecal specimens or enrichment broth cultures, although performance characteristics of neither method have been reported in peer-reviewed medical literature to date.

A summary of the available assays and their performance characteristics is provided in Table 3.12.

3.4.4 Identification of selected Gram-negative organisms (non-Enterobacteriaceae)

3.4.4.1 *Campylobacter*

Campylobacter spp. are curved, Gram-negative, rods which are usually microaerophilic. Two species, *C. jejuni* and *C. coli*, are the most commonly isolated members of the genus *Campylobacter* associated with diarrheal disease in humans. Commercial methods for diagnosis of *Campylobacter* enteric infections include detection of the organism directly in stool specimens and isolation in culture followed by identification.

3.4.4.1.1 Tests for identification of *Campylobacter* spp. directly in stool specimens

Three test kits are currently FDA approved, as of this writing, for the direct detection of *Campylobacter* spp. in stool specimens: Premier CAMPY Enzyme Immunoassay (Meridian Bioscience, Cincinnati, OH), ProSpecT *Campylobacter* EIA (Remel/ThermoFisher, Lenexa, KS), and ImmunoCard STAT! CAMPY test (Meridian Bioscience).

In studies comparing antigen detection methods to culture on selective media, the rapid antigen test kits performed relatively well. Granato et al. [57] compared the three available EIA tests to stool culture, with PCR as a reference method. In this study, the enzyme immunoassay (EIA) tests had a higher sensitivity than culture (Premier CAMPY and ProSpecT, 99.3%; ImmunoCard STAT, 98%). Culture had



Figures 3.16 Shiga Toxin Quik Chek. (Courtesy of Alere, Inc.)

a sensitivity of 94.1%. Specificities were 98% for Premier CAMPY and ProSpecT, and 98.2% for ImmunoCard STAT. Other investigators have reported higher specificity rates [34, 64]. Molecular methods for detecting *Campylobacter* directly in stool specimens are being developed and show good sensitivity when compared to culture [13].

Automated panels have limited usefulness for identification of *Campylobacter* spp. isolated from stool specimens. In a study comparing Vitek 2 NH card to MALDI-ToF, the Vitek card identified 89.6% of *C. jejuni* and 87.7% of *C. coli*, but failed to identify the other species of *Campylobacter* [34]. New methods such as MALDI-ToF are reportedly more accurate than biochemical identification of *Campylobacter* spp. [96].

3.4.4.2 *Helicobacter pylori*

The genus *Helicobacter* contains >20 species that are known to colonize the gastrointestinal tracts of a wide variety of hosts, including mammals and birds. *Helicobacter* spp. are spiral or curved Gram-negative bacteria that are not

usually cultured from clinical specimens because of the special conditions required for recovering them in culture. The principle species of human medical importance is *Helicobacter pylori*, which is an important cause of peptic ulcer disease, and is a significant risk factor for gastric cancer. A number of different methods are available for the diagnosis of *H. pylori*, including pathology tests (for biopsies or other invasively collected samples), urea breath tests, and laboratory-based testing. The laboratory-based tests include antigen detection, PCR, and serology [135].

Two tests are available, as of this writing, for detection of *H. pylori* antigen in stool specimens: the Premier Platinum HpSA, which is an ELISA-based method, and the Immunocard STAT (Figure 3.17), which is a rapid lateral-flow membrane immunoassay. Both of these tests are marketed by Meridian Diagnostics (Cincinnati, OH). Both methods use monoclonal antibodies to capture *H. pylori* antigen. A 2009 study comparing the monoclonal HpSA assay to the older polyclonal assay showed the HpSA test to have a sensitivity of about 91% and specificity of

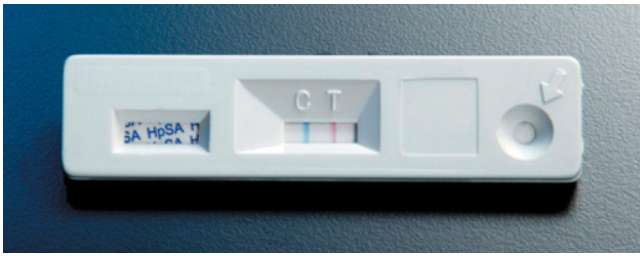


Figure 3.17 *H. pylori* Immunocard STAT! Antigen Kit. (Courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)

98% [35]. In a meta-analysis of antigen-based assays to diagnose *H. pylori* in children (published in 2011), the antigen-based tests had sensitivities of 92–97%, and specificities of 93–98% [88].

3.4.4.3 *Vibrio* and *Aeromonas*

Vibrio spp. and *Aeromonas* spp. are oxidase-positive glucose fermenting Gram-negative bacilli that can be difficult to identify in the clinical laboratory [1]. Historically thought of as being closely related, these two groups of organisms are now classified into two distinct families: *Vibrionaceae* and *Aeromonadaceae*. While most of the commercially available phenotypic identification systems (both manual and automated systems) include some species in their databases, the systems do not reliably identify members of these two genera to the species level. These systems continue to be problematic due to outdated databases, taxonomy and nomenclature changes, and the need to perform off-line manual tests in addition to the panels. Molecular methods (sequencing and restriction fragment length polymorphism (RFLP) analysis) and MALDI-ToF [39, 61] are performing much more accurately for identification of the clinically significant species of *Vibrio* and *Aeromonas*, although many clinical laboratories do not have the capability at this time for either of these two technologies. In a study evaluating six commercial systems including manual biochemical panels (API-20E and API-32GN) and automated systems (Vitek 2 ID-GN Card, Microscan NFC47, Phoenix ID69, and Omnilog GN2 plates), the percentage of organisms identified to the species level ranged from 67 to 92% [82]. Difficulties with identification of *Aeromonas* spp. are the result of recent taxonomic changes based on molecular analyses of this genus; databases based on phenotypic characteristics often differ from the genetic data, making the methods difficult to interpret [108].

3.4.5 Identification of selected fastidious Gram-negative organisms

Fastidious Gram-negative bacteria (species with special growth requirements, or those which require extra nutrients) are more challenging to identify with routine

systems available in the clinical microbiology laboratory. While phenotypic identification systems relying on biochemicals are used, they may not be optimal for all organisms. Thus a variety of different methods are available now, including antigen detection, DNA sequencing, and MALDI-ToF in addition to biochemical profiling.

3.4.5.1 *Bordetella* spp.

Bordetella spp. are catalase-positive, strictly aerobic, small Gram-negative coccobacilli, with seven species infecting mammals: *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *B. avium*, *B. hinzii*, *B. holmesii* and *B. tremat*. An eighth species, *B. petrii*, has recently been described but has not been isolated from humans or animals. *Bordetella* spp., while primarily respiratory pathogens of humans and other animals, have been isolated from other sources such as blood cultures, wounds, and ear samples. The primary human pathogen in this group of organisms is *B. pertussis*, the etiologic agent of whooping cough. Although pertussis immunization is available for both adults and humans, whooping cough remains an important disease and diagnostic methods for *B. pertussis* and the related pathogens remain important, especially in the pediatric setting.

Diagnostic strategies for *Bordetella* include detection in specimens using direct fluorescent antibody (DFA) testing, isolation in culture, and direct detection using molecular-based assays. No single method has emerged as optimal for all cases, and the choice of methods for diagnosis of *Bordetella* must be guided by the hospital setting and laboratory expertise.

3.4.5.1.1 Direct fluorescent antibody

The use of DFA testing for the diagnosis of pertussis became common in the United States in the early 1960s. Although DFA offers a rapid diagnostic approach, its sensitivity has been a limiting factor, and molecular testing has become the preferred method for diagnosis of pertussis [137]. If DFA is used, most authorities agree that it should not be used alone for *Bordetella* testing; culture and/or PCR-based assays should be performed as well. The DFA reagents are commercially available from two sources: (i) a fluorescein-labeled polyclonal antibody available for *B. pertussis* and *B. parapertussis* (Difco/BD Diagnostic Systems); (ii) dual-labeled monoclonal antibodies (Cytovax Biotechnologies, Edmonton, Alberta, Canada), combining monoclonal antibody 1H2 (*B. pertussis*) and monoclonal antibody A7D12 (*B. parapertussis*). Both monoclonal antibodies are isotype immunoglobulin G2a. This dual-fluorochrome reagent contains a fluorescein-conjugated murine monoclonal antibody specific for the lipo-oligosaccharide of *B. pertussis*, and a rhodamine-conjugated murine monoclonal antibody specific for the lipopolysaccharide of *B. parapertussis*. Using the dual reagent, *B. pertussis* will appear brilliant green and

B. parapertussis will appear brilliant orange-red. This reagent is known to cross-react with some strains of *B. bronchiseptica*, which occasionally infects humans. At this time, there are no published reports documenting the use of the Cytovax dual-fluorochrome reagent. The DFA testing is relatively insensitive when compared to molecular testing; in a 2007 study comparing DFA to PCR, the sensitivity of the DFA was 18%; specificity was 98% [137]. The fluorescent antibody reagents can also be used to identify *Bordetella* isolated in culture.

3.4.5.1.2 Molecular tests for *Bordetella*

Nucleic acid amplification assay tests have become the test of choice in most laboratories performing pertussis testing, because of their superior sensitivity [92]. At this time, there is one FDA-approved molecular-based test for the diagnosis of *B. pertussis*. This test, Illumigene pertussis, is based on loop-mediated isothermal amplification and is distributed by Meridian Bioscience. Other nucleic-acid-based methods for detection of *B. pertussis* must be developed in-house using primers and probes that are commercially available as analyte-specific reagents (ASR) reagents or by developing home-brew assays. The nucleic-acid-based assays have been shown to have excellent sensitivity but specificities may be poor [92, 125]. An evaluation of four PCR-based methods performed in France [83] reported sensitivities of 90–100% and specificities of 100%.

3.4.5.2 *Haemophilus* and *Neisseria* spp.

Haemophilus spp., a group of pleomorphic, oxidase-positive Gram-negative bacilli or coccobacilli, are the causative agents of a variety of human infections. Clinically important species include *Haemophilus influenzae*, *H. parainfluenzae*, *H. ducreyi*, *H. haemolyticus*, and *H. parahaemolyticus*. Recent taxonomic and nomenclature changes resulted in the transfer of *Haemophilus aphrophilus* to a new genus, *Aggregatibacter*. *Haemophilus paraphrophilus* was also shown to be identical to *H. aphrophilus*, and the two species were combined into a single species, *Aggregatibacter aphrophilus*. A third species, *H. segnis* was also moved to the new genus

Aggregatibacter [98–100]. Commercially available kits for the identification of *Haemophilus* and *Neisseria* spp. include rapid tests kits and overnight panels. These kits are listed in Table 3.13.

3.4.5.3 Vitek NH Card

A new version of the Vitek *Neisseria-Haemophilus* identification card (NH) was released in 2008. The card is composed of 30 biochemical reactions having colorimetric endpoints and provides results within 6–8 h [144, 159]. Its most recent database includes 26 taxonomic groups including *Neisseria*, *Haemophilus*, and *Moraxella* spp. One large single-center evaluation [159] and another multicenter study [122] of NH performance both reported accurate taxonomic assignment in approximately 85–90% of strains tested (Table 3.13), while the latter group also demonstrated acceptable cumulative interassay quality control performance, a detail seldom included in published studies of this sort. In contrast to these studies, however, Sonksen et al. subsequently reported a considerably lower accuracy rate for the NH (64%) with 21% misidentification and 15% low discrimination [144]. The disparity may be due to differences in the selection of taxa included in their respective analyses.

3.4.5.4 *Legionella*

Five assays are FDA cleared for the identification of *Legionella* antigen in urine specimens: Binax *Legionella* Urine Antigen (Alere), BinaxNOW *Legionella* urine antigen (Alere) card, TRU *Legionella* (Meridian), Xpect *Legionella* (Oxoid), and the Uni-Gold *Legionella* Urinary Antigen PLUS (Trinity Biotech). The reported sensitivity of urine antigen tests for *Legionella* ranges from 70 to 78% [167]. A recent study evaluating the Meridian TRU *Legionella* test showed the sensitivity of the assay was 73%, but was increased when incubation was extended to 1 h [21]. For identification of *Legionella* spp. isolated in culture, direct fluorescent antibody reagents are available from Remel, Trinity Biotech, BioRad, and Pro-Lab Diagnostics (Table 3.14). In addition, a latex agglutination test is available from Oxoid. The latex agglutination test is easy to perform, and results are available

Table 3.13 Commercial systems for the identification of *Haemophilus* and *Neisseria* spp.

Commercial product	Manufacturer	<i>Neisseria</i>	<i>Haemophilus</i>	<i>Moraxella</i>	<i>Eikenella/Kingella</i>
RapID NH	ThermoFisher	x	x	x	x
API NH	bioMérieux	x	x	x	x
API QuadFERM	bioMérieux	x	x	x	
Catarrhalis disk	ThermoFisher			x	
<i>Haemophilus</i> identification test kit	ThermoFisher		x		
BBL Crystal	BD Diagnostic Systems	x	x	x	x
MicroScan	Beckman Coulter	x	x	x	x
Phoenix	BD	x	x	x	x
Vitek	bioMérieux	x	x	x	x

Table 3.14 Commercial methods for rapid identification of *Legionella* spp.

Manufacturer or distributor	Assay	Matrix	Method	Species	Time to result (min)
Alere	Binax <i>Legionella</i> urine antigen	Urine	Enzyme immunoassay	LP 1*	150
Alere	BinaxNOW <i>Legionella</i> urine antigen card	Urine	Immunochromatography	LP 1	15
Meridian	TRU <i>Legionella</i>	Urine	Lateral-flow immunoassay	LP 1	30
Oxoid	Xpect <i>Legionella</i>	Urine	Immunochromatography	LP 1 and 6	45
Trinity Biotech	Uni-Gold <i>Legionella</i> urinary antigen PLUS	Urine	Immunochromatography	LP 1	15
Remel/ThermoFisher	<i>Legionella</i> Poly-ID test	Specimen and culture isolate	Direct fluorescent antibody	<i>Legionella</i> sp.	90
Trinity Biotech	<i>Legionella</i> DFA test	Specimen and culture isolate	Direct fluorescent antibody	Seven <i>Legionella</i> sp. [†]	40
Bio-Rad	MonoFluo <i>Legionella</i>	Specimen and culture isolate	Direct fluorescent antibody	LP	50
Pro-Lab Diagnostics	<i>Legionella pneumophila</i> DFA kit	Culture isolate	Direct fluorescent antibody	LP serogroups 1–14	40
Oxoid	<i>Legionella</i> latex test	Culture isolate	Latex agglutination	LP serogroups 1–14 and seven other <i>Legionella</i> sp. [‡]	2

* *Legionella pneumophila* serogroup 1.

[†] *Legionella bozemanii*, *L. dumoffii*, *L. gormanii*, *L. longbeachae*, *L. micdadei* and *L. jordanis*.

[‡] *Legionella bozemanii*, *L. dumoffii*, *L. gormanii*, *L. longbeachae*, *L. micdadei*, *L. jordanis* and *L. anis*.

rapidly. The direct fluorescent antibody reagents require more time. The culture identification reagents are highly sensitive and specific. *Legionella* may also be identified by DNA sequencing methods.

3.5 Commercial methods for identification of microorganisms directly in blood culture bottles

Given the frequency of blood culture contamination by commensal coagulase-negative *Staphylococcus* spp. coupled with a critical need to manage patients with *S. aureus* bacteremia with prompt and appropriate antimicrobial therapy, laboratory methods for quickly identifying Gram-positive cocci and delineating methicillin-susceptibility or resistance status directly from blood culture bottles were needed. Several groups reported promising results using laboratory-developed or research-use-only assays that used real-time PCR techniques. The first commercially available *in vitro* diagnostic device cleared by the US FDA for this purpose was the BD GeneOhm StaphSR in 2007. Several other molecular-based methods are now available.

3.5.1 BD GeneOhm StaphSR (Becton Dickinson)

The test couples a manual lysis/extraction step performed on positive blood bottle aliquots with a triplex real-time PCR on the SmartCycler system (Cepheid) that targets a proprietary *S. aureus*-specific sequence, a flanking sequence near the *SCCmec* insertion site and an internal control [66]. Total time

for assay completion is 60–75 min. Experimental and clinical studies showed high specificity for *S. aureus* (96.7–100%) and high sensitivity for methicillin resistance (95.6–100%), although false positive methicillin resistance results were noted among some phenotypically susceptible strains containing *SCC* elements without *mecA* (the so-called “empty cassette” phenomenon) [148]. More recent work has highlighted decreased sensitivity of *SCCmec* detection by this assay among MRSA strains or clones with variant sequences at this target site [142, 157].

3.5.2 Xpert MRSA/SA

The second system to receive US FDA clearance for this same indication was the Xpert MRSA/SA Blood Culture Assay (Cepheid) in 2008. Here, the detection strategy involved real-time PCR amplification that targeted three genes (*spa*, *mecA*, and *SCCmec* at the *attB* insertion site) and utilized 12 total primers with three individually labeled TaqMan probes in addition to an internal control [164]. Overall test time requires approximately 60 min; and the assay has been cleared for use with BACTEC Plus Aerobic/F, BacT/ALERT SA (Standard Aerobic) and VersaTREK REDOX 1(aerobic) blood culture media, as of this writing. As with other tests performed on the GeneXpert system, the MRSA/SA assay featured a cartridge format for self-contained DNA extraction, amplification, and amplicon detection as well as near-full automation and random-access capabilities (i.e., no advantage to specimen batching). A large, multicenter trial reported sensitivity and specificity (respectively) to be 98.3% and 99.4% for the detection of MRSA, and

100% and 98.6% for the detection of *S. aureus* [165]. Two additional prospective, single-center, evaluations demonstrated sensitivities of 80–100% and 100% for MRSA and methicillin-susceptible *S. aureus* (MSSA), respectively [15, 147]. The blood culture assay has been shown to have a positive impact on patient management and outcome. Using the Xpert MRSA/SA Blood Culture Assay, Parta et al. demonstrated significant reductions in unnecessary antimicrobial use and more rapid de-escalation to appropriate β -lactam therapy for MSSA bacteremia [112]. Bauer et al. also found that implementation of this assay reduced the time to appropriate antimicrobial therapy for MSSA at their institution [10]. Prompt verbal notification of PCR results by the laboratory to a physician or clinical pharmacist and timely adjustment to antimicrobial regimens by the provider were integral components in both of these studies. The updated 510(k) summary includes a disclaimer stating that any “MRSA negative/SA positive result” obtained by this assay should be interpreted as “MRSA indeterminate/SA positive” with phenotypic susceptibility confirmed by an alternate method.

3.5.3 PNA-FISH probes

Peptide-nucleic acid fluorescent *in situ* hybridization (PNA-FISH; AdvanDX, Woburn, MA) utilizes synthetic peptide nucleic acids that mimic DNA. These peptides are polyamide molecules that replace the sugar-phosphate backbone normally found in DNA; they contain the same four nucleotide bases. The polyamide molecules mimic DNA, thus they hybridize to complementary bases. The synthetic peptides are labeled with fluorescent tags and used as probes for *in situ* hybridization reactions.

The PNA-FISH kits that are FDA cleared for diagnostic use in the United States include tests for several species or groups of species (Table 3.15). The tests are designed for rapid identification of bacteria in positive blood culture bottles. The peptides are designed to be complementary to ribosomal RNA (rRNA) molecules that are normally present in high amounts in bacteria undergoing

replication, such as in blood cultures. Blood cultures that have positive Gram stains can be tested with the appropriate kit, according to the Gram-stain result.

The AdvanDX kits are sensitive and specific. In a multicenter study of the *Staphylococcus* QuickFISH test, the sensitivity for detection of *S. aureus* was 99.5%; the sensitivity of detection of coagulase-negative staphylococci was 98.8%. Combined specificities were 89.5% [33].

In a 2006 study evaluating the performance of FISH compared to conventional methods, 79% of the organisms in positive blood cultures were identified to the species level [115]. FISH identified organisms more rapidly (10 h faster than culture-based ID), allowing for appropriate antimicrobial therapy to be instituted more quickly [115]. Harris and Hata reported accuracy of 98.8% of FISH compared to culture, with a very low time to identification (11.2 h) compared to culture (83.6 h) [60]. FISH, in addition to being relatively easy for laboratories to perform and implement into a routine workflow, can potentially result in significant cost savings because correct antimicrobial therapy can be implemented much more rapidly when FISH is used [60].

3.5.4 Verigene *Staphylococcus* Blood Culture (BC-S) nucleic acid test

The Verigene *Staphylococcus* Blood Culture (BC-S) (Nanosphere, Inc., Northbrook, Illinois) nucleic acid test (Figure 3.18) was FDA cleared in December 2011. This technology relies on bacterial lysis and nucleic acid extraction followed by hybridization to probes with bacterial-specific and resistance gene-specific sequences attached to a solid phase substrate [134]. Secondary probes tagged with gold nanoparticles are then applied, creating a light scatter signal where complementary hybridization to bound DNA occurs. The position-dependent signal(s) are then translated into the presence/absence of various

Table 3.15 Bacterial taxa identified in PNA-FISH assays

Kit	Species
<i>S. aureus</i> /CNS PNA FISH	<i>Staphylococcus aureus</i> Coagulase-negative staphylococci
<i>E. faecalis</i> /OE PNA FISH	<i>Enterococcus faecalis</i> <i>Enterococcus faecium</i> Other enterococci (not speciated)
<i>E. coli</i> /P. aeruginosa PNA FISH	<i>Escherichia coli</i> <i>Pseudomonas aeruginosa</i>
GNR Traffic Light PNA FISH	<i>Escherichia coli</i> <i>Klebsiella pneumoniae</i> <i>Pseudomonas aeruginosa</i>



Figure 3.18 Verigene System. (Courtesy of Nanosphere, Inc.) (See insert for color representation of the figure.)

Table 3.16 Bacterial taxa identified in Verigene panel

Bacterial taxa identified	<i>Enterococcus</i> spp. (genus level) <i>Enterococcus faecium</i> <i>Enterococcus faecalis</i> <i>Staphylococcus</i> spp. (genus level) <i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i> <i>Staphylococcus lugdunensis</i> <i>Streptococcus</i> spp. (genus level) <i>Streptococcus agalactiae</i> <i>Streptococcus anginosus</i> <i>Streptococcus pneumoniae</i> <i>Streptococcus pyogenes</i> <i>Listeria</i> spp.
Resistance genes identified	<i>mecA</i> <i>vanA</i> <i>vanB</i>



Figure 3.19 Biofire System. (Courtesy of Biofire Diagnostics Inc.) (See insert for color representation of the figure.)

bacterial taxonomic groups and/or genes imparting antimicrobial resistance. All extraction and hybridization steps take place in the Processor SP, which handles one cartridge with one positive blood culture sample at a time. Once completed, the cartridge is manually moved to the Reader module for interrogation and interpretation. The entire process requires 5–10 min of “hands-on” time and is completed within approximately 2.5 h. Unlike the PCR assays described above, the Verigene assay targets a broad array of Gram-positive organisms in addition to three resistance genes (Table 3.16). Samuel et al. recently published the first clinical evaluation of the product [130]. For all taxa and resistance genes included in the assay, they found overall concordance rates of 93% for genus and species level identifications and 96% for resistance determinants compared to traditional, phenotypic methods. Of note, higher discordance rates were apparent among polymicrobial cultures.

Table 3.17 Bacterial taxa identified in FilmArray BCID panel

Gram-positive	<i>Enterococcus</i> spp. <i>Listeria monocytogenes</i> <i>Staphylococcus aureus</i> <i>Streptococcus agalactiae</i> <i>Streptococcus pneumoniae</i> <i>Streptococcus pyogenes</i>
Gram-negative	<i>Acinetobacter baumannii</i> <i>Haemophilus influenzae</i> <i>Neisseria meningitidis</i> <i>Pseudomonas aeruginosa</i> <i>Enterobacter cloacae</i> complex <i>Escherichia coli</i> <i>Klebsiella oxytoca</i> <i>Klebsiella pneumoniae</i> <i>Proteus</i> spp. <i>Serratia marcescens</i>
Resistance genes	<i>mecA</i> <i>vanA</i> <i>vanB</i> KPC

3.5.5 Biofire BCID Panel

The Biofire (BioFire Diagnostics, Inc, Salt Lake City, UT) (Figure 3.19) BCID Panel received FDA clearance in 2013. The BCID panel is a multiplexed PCR panel in which the assay is performed in a reagent pouch using a closed system. The sample (a small aliquot of culture media from positive blood culture vials) is placed into the pouch and the pouch is placed into the FilmArray instrument. The entire set of reactions (nucleic acid purification, nested multiplexed PCR, and detection) occurs in the pouch. The FilmArray software generates a melting curve, and generates a result for each target in the assay. Results are available in about 1 h.

The BCID panel can identify 16 species of aerobic bacteria, and four antimicrobial resistance genes (Table 3.17). There are also five species of yeast on the panel. A recent study evaluating the FilmArray BCID assay showed 91.6% of blood culture isolates were identified correctly by the BCID assay. The unidentified organisms were species that are not included in the panel. The BCID assay correctly identified more than one species of bacteria in 3.6% of the samples [4].

3.6 Commercial molecular methods for identification of bacteria isolated in culture

Nucleic-acid-based methods are increasingly being used for identification of bacteria isolated in culture. Available methods include hybridization and PCR with specific primers and probes. The 16S rRNA gene is often used as the target for molecular methods; good specificity can be obtained when using this gene for identification.

3.6.1 GenProbe hybridization probes

The GenProbe (GenProbe Inc., San Diego, CA; now Hologic, Inc.) AccuProbe Culture Identification tests are based on hybridization protection assays. They are available in kit form, and are FDA cleared. The test must be performed on pure colonies isolated in culture. The probes used in these tests are directed to the 16S rRNA gene and are highly specific. Kits are available for *Streptococcus agalactiae*, *Neisseria gonorrhoeae*, *S. aureus*, *S. pneumoniae* and *L. monocytogenes*.

3.7 Emerging technologies for the identification of organisms: mass spectrometry

3.7.1 MALDI-ToF/MS

The earliest attempts at the use of mass spectrometry for the identification of bacteria occurred in the 1970s [7], but it was the MALDI ionization method, first introduced in 1987 [75], and 1988 [155], that enabled the technology to advance to become a rapid and highly reliable analytical tool for characterization of a diverse collection of microbes encountered in the clinical laboratory [45, 86]. Within the past few years major advances in the use of MALDI-ToF/MS are changing the way microorganisms are identified. The MS approach is likely to present a significant challenge to the approach of automated phenotypic identification that is in place in many clinical microbiology laboratories worldwide.

Using MALDI-ToF/MS technology, the molecular weights of proteins and peptides are determined and used to create a unique signature that enables identification of microorganisms. Intact microbes are deposited onto the MALDI-ToF/MS sample plate, either from single colonies or liquid culture media, and are mixed with ionizing matrix [27]. Irradiation with a laser results in ionization and movement of the soluble proteins, which is measured. A spectrum of mass/charge ratio (m/z) is created and a microbe's dominant spectral peaks from highly conserved proteins are compared to a database composed of spectra from known organisms. An algorithm determines which organism is the closest match to the experimental organism, and identification is made with a corresponding confidence.

There are two instruments currently available for use in clinical laboratories: (i) the Bruker MALDI Biotyper (and also associated with BD); (ii) the bioMérieux Vitek MS instrument (formerly the Shimadzu AXIMA Microorganism Identification System); each with its own proprietary database. Both systems have received US FDA approved status. The Andromas database from Andromas SAS (Paris, France) is an additional database. While both instrument systems show fundamentally equivalent performance, the platforms differ in their respective

instrumentation scoring algorithm and databases. Antimicrobial susceptibility instruments will link with MALDI-ToF systems and culture-based testing may still be advantageous for identifying unusual strains that may not be properly detected by protein-based techniques.

The MALDI-ToF/MS approach is fast, cost efficient, broadly applicable to many microorganisms, and reduces the number of procedures and reagents required for identification of microbes. These advantages combine with the method's high analytical sensitivity, and accuracy, to make MALDI-ToF/MS a highly desirable approach to microbial identification. There is still a great amount of work required before the total benefits and impact of such technology are known; however, the future is in sight, and MS methods are likely to alter the practice of clinical microbiology as we know it [164]. For more information on emerging technologies and their potential impact on the clinical microbiology laboratory, the reader is referred to Chapter 24.

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Rapid Devices and Instruments for the Identification of Anaerobic Bacteria

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4.1 Introduction and clinical considerations

From a practical standpoint, anaerobic bacteria are defined here as those bacteria that do not multiply on the surface of nutritionally adequate solid media incubated in air or in a CO₂ incubator (10% CO₂) but grow in environments without O₂. They do not use molecular oxygen as a terminal electron acceptor. While many ferment a wide variety of carbohydrates, many others do not (i.e., they are asaccharolytic), obtaining their energy through fermentation of amino acids or various other organic compounds. Vegetative cells of anaerobic bacteria, but not the spores of clostridia, are killed by exposure to molecular oxygen in ambient air. However, as can be shown by aerotolerance testing, anaerobes vary considerably in their sensitivity or ability to tolerate oxygen [132, 140]. Some, referred to as “moderate obligate anaerobes (e.g., *Bacteroides fragilis* and *Clostridium perfringens*),” will tolerate some oxygen exposure while others, referred to as “strict obligate anaerobes (e.g., *Clostridium novyi* type B and *Clostridium hemolyticum*),” cannot survive exposure to oxygen longer than a few minutes, and lastly, the “aerotolerant anaerobes” will have scant growth in the presence of O₂ (e.g., *Clostridium tertium*, *Clostridium carnis*, and *C. histolyticum*). To successfully isolate and identify anaerobes in a specimen, the entire process is ideally done entirely under anaerobic conditions but if an anaerobic chamber is not available, the process should be done rapidly to minimize oxygen exposure.

There have been extensive changes in the taxonomic classification of anaerobes in recent years based on molecular genetic studies. The standard reference on bacterial taxonomy, *Bergey's Manual of Systematic Bacteriology*, is organized using 16S rRNA gene sequence analysis [83]. Current genera with descriptions are available on-line:

List of Prokaryotic Names with Standing in Nomenclature (<http://www.bacterio.cict.fr/classifgenrafamilies.html>). Summaries of recent taxonomic changes regarding anaerobic bacteria are available elsewhere [35, 74, 133, 137]. Keeping up with the many changes in the nomenclature and classification of anaerobes, with increased numbers of new genera and species in recent years, has been a challenge for manufacturers of commercial identification systems, as well as clinical microbiologists and clinicians alike [28, 77, 113, 130]. The databases of most of the commercial identification systems are somewhat out of date with respect to the current nomenclature for anaerobic bacteria.

The indigenous microflora of humans (e.g., upper respiratory tract, oral cavity, gastrointestinal tract, genitourinary tract and skin) is primarily composed of anaerobic bacteria [19, 21, 58, 77, 100, 131], and anaerobes take part in essentially all types of infections that involve bacteria. Although a few diseases involving anaerobes can be of exogenous origin (e.g., foodborne and wound botulism, tetanus), and pseudomembranous colitis (caused by *Clostridium difficile*), the vast majority of anaerobic infections are endogenous, arising from the body's own microbial flora. These types of infections are often polymicrobial and commonly involve anaerobes mixed with aerobes, facultative anaerobes, or other anaerobes. However, some anaerobic infections involve only a single species. Anaerobic infections are often difficult to treat, life-threatening, and are associated with high mortality rates [14, 25, 79, 109].

Anaerobic infections probably are still among the most commonly underdiagnosed bacterial infections, although clinicians and microbiologists generally know more about anaerobic infections today than they did 30 years ago [18, 79, 99]. Factors that influence the selection of

specimens submitted for anaerobic culture include the probability of whether or not anaerobes are likely to be a significant component of the infection and the perceived clinical importance of documenting the presence of anaerobes in the infectious process. Other important factors include the ability to collect an uncontaminated specimen, the cost of empirical treatment alone without anaerobic culture data, and the perceived cost benefit of having anaerobic culture data to aid in management of the patient. Several types of infections involve anaerobes relatively frequently. These include abscesses of all anatomic regions of the body (e.g., central nervous system, respiratory tract, intra-abdominal, pelvic, and other sites). In addition, anaerobes are commonly involved in aspiration pneumonia, appendicitis, bacteremia, chronic otitis media, chronic sinusitis, crepitant and noncrepitant cellulitis, dental-oral infections, endocarditis, endometritis, myonecrosis, necrotizing fasciitis, neutropenic enterocolitis (caused by *Clostridium septicum*), peritonitis, thoracic empyema, septic arthritis, subdural empyema, and many other types of infections [17, 25, 119]. Examples of infections in which anaerobes are seldom important include acute cholecystitis (although seen occasionally in diabetic patients), acute otitis media, acute osteomyelitis, acute sinusitis, bacterial meningitis, bronchitis, community-acquired pneumonia, cystitis, pharyngitis, pyelonephritis, superficial skin (surface) infections, and spontaneous (primary) peritonitis.

Some of the more frequently isolated anaerobes from properly selected and collected specimens are the *Bacteroides fragilis* group (which includes *B. fragilis*, *B. ovatus*, *B. thetaiotaomicron*, *B. uniformis*, *B. vulgatus* and *Parabacteroides distasonis* as the most commonly isolated species), *Prevotella* and *Porphyromonas* species, *Fusobacterium nucleatum* and *F. necrophorum*, the anaerobic cocci (e.g., *Peptostreptococcus anaerobius* and *Finegoldia* [formerly *Peptostreptococcus*] *magna*), *Actinomyces israelii*, and certain *Clostridium* species (e.g., *C. perfringens*, *C. ramosum*, *C. clostridioforme*, *C. septicum*, and *C. difficile*) [66, 99, 140].

There are a number of clinical clues that suggest anaerobes are probably involved in an infection [66]. For example, a putrid odor from an infected lesion or purulent discharge is strong evidence of infection with anaerobes. No other types of bacterial, mycobacterial, fungal, parasitic, or viral infections emit such foul odors. Presumably various products of anaerobic metabolism, including short-chain fatty acids (e.g., butyrate, isocaproate, and succinate), amines, hydrogen sulfide, and probably other sulfides can contribute to the unpleasant odors. However, in many if not the majority of cases, these types of odors go unnoticed. Thus, a lack of odor does not rule out infection with anaerobes. Lesions that arise in close proximity to mucosal surfaces where anaerobes reside within the flora,

or from inocula derived from these sites (e.g., dental/oral infections, necrotizing pulmonary infections following aspiration of oral contents, and infections secondary to penetrating wounds to the abdomen or pelvis) are likely to involve anaerobes. Gas in tissue, underlying disease with tissue necrosis, tissues with blackened discoloration on bloody exudate, impaired blood supply, and chronic conditions such as diabetic foot infections and malignancies, gangrenous necrosis, abscess formation, previous antibiotic treatment, septic thrombophlebitis, infections following animal or human bites, and "sulfur granules" from suspected actinomycosis also are important clues. Anaerobic infections may be suspected when microorganisms are seen on Gram stain of the original specimen but its aerobic culture is negative.

The direct microscopic examination of Gram-stained smears frequently provides presumptive evidence consistent with anaerobic infections [66, 86–88]. Since anaerobic infections are commonly polymicrobial, the presence of different morphologic forms of bacteria within aspirated pus from a deep tissue site could represent polymicrobial infection involving a combination of anaerobes and facultative anaerobes or a variety of anaerobes. Pleomorphic, long Gram-negative rods with rounded ends are typical of *Fusobacterium necrophorum*. Pale, Gram-negative, slim rods with pointed ends, particularly in material from a lower respiratory tract infection, could suggest infection with *F. nucleatum* [87]. Boxcar-shaped, nonspore-forming, relatively large, broad, Gram-positive rods could suggest the presence of *C. perfringens*. Cell size may be used as an aid for identification, as with *Parvimonas micra* (formerly *Micromonas micros*) and *F. magna* (*P. micra* is between 0.3 and 0.7 μm in diameter while *F. magna* is between 0.7 and 1.2 μm in diameter) [48, 132]. In addition, the inspection of colonies on primary isolation plates following anaerobic incubation aids in the preliminary identification of a number of different anaerobes (e.g., *Actinomyces* species, the *B. fragilis* group, *C. perfringens*, *F. nucleatum*, and others) [66].

4.2 Steps in the diagnosis of anaerobic bacterial infections

The diagnosis of anaerobic bacterial infections involves the following steps:

- 1 selection, collection, and transportation of specimens;
- 2 rejection of unacceptable specimens; rapid processing of acceptable specimens;
- 3 direct microscopic examination of clinical materials;
- 4 selection and use of selective and non-selective primary isolation media;
- 5 use of anaerobic systems for incubation;
- 6 proper incubation conditions;

- 7 inspection and subculture of colonies;
- 8 identification of anaerobe isolates;
- 9 antimicrobial susceptibility testing of significant isolates;
- 10 issuing preliminary reports at appropriate times (e.g., after above steps, 3, and 7–9) prior to the final report.

Decisions made by physicians regarding which specimens to select and collect for anaerobic culture are extremely important for both optimal patient care and the laboratory. Anaerobes commonly cause pyogenic infections with purulent exudates. Abscesses may occur within any soft tissue, including brain, lung, liver, spleen, and other tissues within the peritoneal cavity. These abscesses are usually due to mixed organisms involving both obligate anaerobes and facultative anaerobes that reflect the microbial flora adjacent to mucosal surfaces. For example, abscesses in the lung commonly involve the flora of the oral cavity while those in the organs of the peritoneal cavity reflect the organisms of the intestinal tract. Specimens must be obtained in such a manner that only those organisms involved in the disease process are collected.

More information on collection, transport, and storage of specimens is provided in the section below. Upon receipt of the specimen in the laboratory, the direct microscopic examination of clinical materials can provide important information rapidly. The processing of specimens, selection and inoculation of media for primary isolation, use of anaerobic systems for incubation, procedures for inspection and subculture of colonies, identification, and anaerobic susceptibility testing, while important topics as well, are beyond the scope of this text. These topics are covered extensively elsewhere [11, 26, 32, 54, 66, 140], including CLSI document M56-A, principles and procedures for detection of anaerobes in clinical specimens; approved guideline, July 2014.

4.2.1 Collection, transport, and storage of specimens

The optimal laboratory diagnosis of anaerobic infections includes important considerations such as (i) selecting, collecting, and transporting appropriate specimens for microbiologic examination and (ii) processing and examining the specimens in the laboratory as rapidly as possible after they are received; general information on specimen collection, transport, and storage is provided by Baron and Thomson [13]. Additional detailed information on the selection, collection, and transport of specimens related to anaerobic infections is available elsewhere [12, 27, 29, 66, 132, 140].

Careful selection of materials to be examined for anaerobic bacteria is extremely important. The specimen should be collected from the active site of infection and should not be contaminated with extraneous flora [66, 140]. Because anaerobes are abundant components of the indigenous flora of mucous membrane surfaces and

present on skin, several types of clinical materials should not be cultured for anaerobic bacteria. These include the following examples: throat or nasopharyngeal swabs, saliva, gingival swabs, expectorated sputum, bronchoscopic specimens not collected by a protective, double-lumen catheter [6, 139], gastric contents, small bowel contents (except in blind-loop and similar syndromes), feces (except in the workup of diseases due to *C. difficile* and *C. botulinum*), rectal swabs, colostomy fistulae, and colostomy stomata, surface material from decubitus ulcers, swab samples of other surfaces, sinus tracts, eschars, materials adjacent to skin or mucous membranes other than the above which have not been properly decontaminated, voided urine, and vaginal or cervical [66].

Guidelines for collection of specimens for anaerobic culture are summarized in Table 4.1. Examples of acceptable specimens for isolation of anaerobes include an adequate sample volume of pus aspirated from an abscess or a deep wound (closed), tissue (biopsy, surgical removal, autopsy), normally sterile body fluids (e.g., cerebrospinal, pleural, pericardial, paracentesis, synovial), blood, bone marrow, fine needle aspirates of lung or other body sites, and “sulfur granules”. Aspiration of liquid samples by syringe and needle or biopsies of infected tissues are the methods of choice for collecting specimens to be processed for anaerobes. Endometrial specimens taken using double-sheathed swab devices are acceptable specimens. Swabs are generally not acceptable because they retain only a very small volume of sample, dry out, and subject anaerobes to undue oxygen exposure. If swabs must be used, it is best to use swabs made of synthetic fibers such as flocked nylon, polyester fiber, rayon, or calcium alginate, and not use cotton wool swabs, which contain inhibitory fatty acids. Prior to aspiration or biopsy of clinical material, the skin or mucous membrane surface should be properly decontaminated to prevent contamination with extraneous microorganisms at the time of specimen collection [13, 66, 140].

After aspiration of pus or other liquid, the sample should be carefully injected into an oxygen-free, anaerobic transport vial or anaerobic transport tube that contains reducing agents and redox indicator [27, 66]. The syringe and needle itself should not be transported to the laboratory because of the potential danger for needlestick injury and because oxygen diffuses through the plastic in plastic syringes [30]. Tissue can be placed in a sterile container (capped loosely) and transported to the laboratory in an anaerobic bag that removes oxygen from the atmosphere inside the container [66]. Although swabs are the least desirable (of the methods described above for specimen collection), at times it is impossible to obtain an aspirate or tissue sample, and a swab may need to be used. Swabs are best transported in anaerobic transport containers that are commercially available for this purpose (Table 4.2) [66].

Table 4.1 Examples of specimens and collection procedures in anaerobic bacteriology

Site	Specimens and methods of collection
Cardiovascular system	Blood cultures
Central nervous system	Abscess material, tissue obtained surgically, cerebrospinal fluid
Dental area, ear, nose, throat, and sinuses	Carefully aspirated or biopsied material from abscesses after surface decontamination with povidone-iodine, needle aspirates, and surgical specimens from sinuses in chronic sinusitis patients
Pulmonary area	Transtacheal aspiration, fine-needle aspirate of lung, thoracotomy specimen, thoracentesis (pleural fluid), bronchoscopic specimen obtained with protective, double-lumen catheter*
Abdominal area	Paracentesis fluid, needle-and-syringe aspiration of deep abscesses under ultrasound or at surgery, surgical specimen if not contaminated with intestinal flora, bile
Female genital tract	Culdocentesis after surface decontamination of the vagina with povidone-iodine; laparoscopy specimens, surgical specimens, endometrial cavity specimen with double-lumen catheter† and microbiologic brush after cervical os is decontaminated
Urinary tract	Suprapubic aspirate of urine
Bone and joint	Aspirate of joint (in suppurative arthritis), deep aspirate of drainage material after surgery (e.g., in osteomyelitis)
Soft tissue	Open wounds (deep aspirate of margin or biopsy specimen of the depths of wound only after careful surface decontamination with povidone-iodine); sinus tracts (aspiration by syringe and small plastic catheter after careful decontamination of skin orifice); deep abscess, anaerobic cellulitis, infected vascular gangrene, clostridial myonecrosis (needle aspirate after surface decontamination); surgical specimens, including curettings and biopsy material; decubiti and other surface ulcers (thoroughly cleanse area with povidone-iodine by surgical scrub technique, and aspirate pus from deep pockets or obtain biopsy specimen from deep tissue at margin)

Source: Adapted from Jousimies-Somer et al. [66].

* Reviewed elsewhere by Allen and Siders [6].

† Technique uses a telescoping double-catheter assembly similar to that available for bronchoscopy. This procedure is inadequate to diagnose postpartum endometritis [66].

Table 4.2 Selected commercially available anaerobe transport devices: manufacturers and product numbers

Manufacturer	Description	Manufacturer's number
Anaerobe Systems San Jose, CA	Anaerobic Transport Medium (ATM) – 6 mL	AS-911
	ATM Sterile Surgery Pack – 6 mL	AS-914
	Wide Mouth ATM – 8 mL	AS-915
	Wide Mouth ATM Sterile Surgery Pack – 8 mL	AS-919
Becton Dickinson Franklin Lakes, NJ	BBL Vacutainer Anaerobic Specimen Collector	236500
	BBL Port-A-Cu Tube with Swabs – sterile pack	221607
	BBL Port-A-Cul Tube	221606
	BBL Port-A-Cul Transport Jar 3" sterile pack screw cap jar with 1" wide mouth	221602
Copan Diagnostics Murrieta, CA	Amies Agar Gel – double plastic swabs – blue cap	134C
	ESwab – sterile single use sample collection pack	480C
Starplex Scientific	Anaerobic Transport System	S120D
Etobicoke, Ontario, Canada	Modified Amies Clear Gel	SP130X

Optimally, the viability and relative proportions of anaerobic bacteria or other microorganisms should not change while the specimen is being transported to the laboratory. In order to minimize loss of viability of anaerobes or overgrowth of aerobic microorganisms within the specimen container, rapid transportation is highly recommended. Under certain circumstances, some anaerobes lose viability during refrigeration (e.g., *B. fragilis* and *C. perfringens* at 4°C) [52, 128]. However, at room temperature or warmer temperatures, some aerobic or facultative anaerobic bacteria may overgrow (e.g., *Escherichia coli* or *Pseudomonas* species), making it difficult or impossible to recover any anaerobes that could originally have been present when the sample was collected [49, 52]. For these

reasons, specimens are best maintained at 15–25°C during transportation to the laboratory.

4.2.2 Incubation, inspection and subculture of colonies

Once a properly collected and transported specimen has been received, appropriate selective and nonselective media are inoculated and incubated in an anaerobic system. Anaerobes grow best in freshly prepared, prereduced, anaerobically sterilized (PRAS) media [56, 85, 138]. The PRAS media are prepared by avoiding exposure to oxygen, which can oxidize the ingredients in the culture medium resulting in the formation of toxic oxygen intermediates. Commercial sources for PRAS media are listed in Table 4.3.

Anaerobes vary considerably in their oxygen tolerance; some are extremely oxygen sensitive and are killed within minutes while others withstand longer oxygen exposure. Cultures need to be processed under anaerobic conditions or processed rapidly to minimize the negative effects of air exposure. Then the cultures require anaerobic incubation systems that protect them from oxygen exposure. More protective systems will grow more anaerobes and, especially, more of the oxygen sensitive organisms. Anaerobic incubation systems include anaerobic chambers, jars, boxes, and pouches. These vary in the time that they allow cultures to be exposed to oxygen, convenience, space requirements, and cost [66, 140]. Several commercially available sources of systems for anaerobic incubation are provided in Tables 4.4 and 4.5. Relatively current illustrations, discussions, and recommendations on the use of a number of these products are available elsewhere [66, 140].

4.2.3 Presumptive identification of anaerobes

Colonial morphology, cellular morphology from a Gram-stain examination, aerotolerance testing, and examination of growth on primary agar media are essential for initial presumptive identification of isolates. Many common anaerobes can be identified to genus or group

by phenotypic characteristics [11, 66]. Because many anaerobes have distinctive colony characteristics, it can be useful to inspect and subculture colonies under a stereoscopic dissecting microscope (7–15× magnification). Photographs of anaerobe colonies on commercially available CDC Anaerobe Blood Agar, *Brucella* Blood Agar, or *Bacteroides* Bile Esculin Agar along with their microscopic

Table 4.5 Selected manufacturers/vendors* of anaerobe chambers and gas chromatographs

Anaerobe chambers	Gas chromatographs
Anaerobe Systems 15906 Concord Circle Morgan Hill, CA 95037 Phone: (408) 782-7557 Website: www.CoyLab.com Baker Bioscience Solutions P.O. Drawer E Sanford, ME 04073 Phone: (877) 350-6414 Website: www.bakerbio.com Coy Lab Products 14500 Coy Drive Grass Lake, MI 49240 Phone: (734) 475-2200 Website: www.CoyLab.com Sheldon Manufacturing, Inc. P.O. Box 627 300 North 26 th Avenue Cornelius, OR 97113 Phone: (503) 640-3000 Website: www.shellab.com Microbiology International Suite H 5111 Pegasus Ct. Frederick, MD 21704 Phone: (800) 396-4276 Website: www.800ezmicro.com	Agilent Technologies 5301 Stevens Creek Blvd Santa Clara, CA 95051 Phone: (800) 227-9770 Website: www.agilent.com Buck Scientific, Inc. 58 Fort Point Street East Norwalk, CT 06855 Phone: (203) 853-9444 Website: www.bucksci.com GOW-MAC Instrument Co. 277 Brodhead Road Bethlehem, PA 18017 Phone: (610) 954-9000 Website: www.gow-mac.com Shimadzu Scientific Instruments 7102 Riverwood Drive Columbia, MD 21046 Phone: (800) 477-1227 Website: www.ssi.shimadzu.com SRI Instruments 20720 Earl St. Torrance, CA 90503 Phone: (310) 214-5092 Website: www.srigc.com

* Because of the numerous sizes and configurations of these kinds of equipment, it is suggested that the interested reader contact the manufacturer/vendor for sizes/models, other descriptive information, and prices.

Table 4.3 Selected commercially available sources for PRAS media

Manufacturer	Packaging
Anaerobe Systems 15906 Concord Circle Morgan Hill, CA 95037	Plate, tube
Remel 12076 Santa Fe Drive Lenexa, KS 66215	Tube
Oxyrase, Inc. P.O. Box 1345 Mansfield, OH 44901	Plate

Table 4.4 Selected commercially available anaerobic incubation systems: manufacturers and product numbers

Manufacturer	Description	Product number
Advanced Instruments	Advanced Anoxomat Mark II System, Anaerobic Jar	AJ9025
	Advanced Anoxomat Mark II System, Anaerobic Jar (large capacity)	AJ9028
Becton Dickinson	Bio-Bag Environmental Chamber Type A: holds one 100 × 15 mm petri dish	261214
Franklin Lakes, NJ	Bio-Bag Type A Multi-Plate: holds three 100 × 15 mm petri dishes	261216
	GasPak EZ Gas Generating Pouch Systems	260683
	GasPak EZ Standard Incubation Container	260671
	GasPak EZ Large Incubation Container	260672
	GasPak 100 System	260626
	GasPak 100 System, Vented	260627
	GasPak 150 Large Anaerobic System	260626
	GasPak 150 System Large Anaerobic System, Vented	260627
Remel/Mitsubishi	AnaeroPouch Bag	R682001
Lenexa, KS	AnaeroPack Rectangular Jar 2.5 L	R684004
Remel/Oxoid	AnaeroJar 2.5 L	AG0025A
Lenexa, KS		

features of anaerobe isolates are available in several manuals and atlases [66, 140]. The following characteristics are especially useful for presumptive identification [31]. The interpretation of these phenotypic characteristics for a presumptive identification is available in several recently published manuals such as the *Manual of Clinical Microbiology* [11] and *Wadsworth-KLM Manual* [66]:

- 1 relation to oxygen (e.g., obligate anaerobe, aerotolerant anaerobe, facultative anaerobe);
- 2 colony characteristics (e.g., size, form, elevation, margin, surface, consistency, pigment, fluorescence, pitting);
- 3 microscopic features (e.g., Gram reaction, morphology, presence or absence of spores);
- 4 presence of hemolysis on blood containing media;
- 5 catalase production;
- 6 reduction of nitrate to nitrite;
- 7 indole production;
- 8 reaction to special potency antibiotic disks that include vancomycin, kanamycin and colistin;
- 9 inhibition by sodium polyanethol sulfonate;
- 10 bile resistance;
- 11 hydrolysis of esculin;
- 12 lecithin and or lipase production on egg yolk agar;
- 13 detection of short chain acid metabolic products by gas liquid chromatography (GLC).

Results of the phenotypic identification systems permit differentiation of most clinical isolates to the genus or a group level, and a few to the species level. Although such presumptive identifications may be cost effective and rapid, they may not always be sufficient for appropriate treatment or correlation with malignancy or other disease condition when a confirmatory or complete identification is needed.

4.2.4 Phenotypic identification procedures

A systematic identification system for anaerobes was described by Holdeman and Moore in their 1972 Virginia Polytechnic Institute (VPI) Anaerobe Laboratory Manual [61]. This system was based on reactions in 20–50 sugars and other substrates in PRAS media, Gram-stain morphology, and analysis of end products by gas-liquid chromatography. Isolates were tested for their ability to use each sugar in PRAS peptone–yeast broth after incubation periods of 24 h to 1 week at 35°C. The pH of each different sugar broth was measured to see if it dropped by several log units as compared to its initial pH. Gas-liquid chromatography was used to determine the short-chain fatty organic acids and alcohols that are the end products from glucose and other substrate metabolism. Bacteria were grouped on the basis that related organisms with similar metabolism produced similar types and volumes of end products [9, 36, 61]. The volumes were reported as major, minor, or trace in a semi-quantitative fashion. Although labor intensive and time consuming, the VPI system was the basic guideline for conventional identification of anaerobes for decades. It continued to be

periodically updated through the 1990s. The phenotypic testing scheme with an emphasis on clinical isolates was enhanced by addition of special potency disks and preformed enzyme tests as well as by incorporation of taxonomic changes into the identification tables as described in the *Wadsworth-KTL Anaerobic Bacteriology Manual* [66]. The PRAS biochemicals are available commercially or can be prepared according to the VPI manual [61].

Another system developed at the Centers for Disease Control (CDC) used growth characteristics on three quadrant plates (Presumpto Plates I, II, and III) to identify commonly encountered anaerobes [41]. The quadrant plates contained four differential agars per plate, with Lombard-Dowell agar as the basal medium. Following inoculation, the Presumpto plates were incubated anaerobically for 24–48 h. Characteristics used to identify anaerobic bacteria are listed in Table 4.6. Detailed instructions pertaining to the Presumpto Plates, along with identification tables, are available elsewhere [136].

Table 4.6 Media and characteristics of anaerobe isolates that can be determined using the CDC Presumpto Quadrant Plate procedure for anaerobe identification

Media	Characteristics
CDC anaerobe blood agar	Relation to oxygen,* colony characteristics, hemolysis, pigment, fluorescence with ultraviolet light (Wood's Lamp), pitting of agar, cellular morphology, Gram reaction, spores, motility (wet mount); inhibition by penicillin, rifampin, and kanamycin (disk tests)
Enriched thioglycolate medium	Appearance of growth, rapidity of growth, gas production, odor, cellular morphology
Presumpto 1 plate	
LD agar	Growth on L-lysine decarboxylation (LD) medium, production of indole, indole derivative, catalase†
LD esculin agar	Esculin hydrolysis, hydrogen sulfide, catalase†
LD egg yolk agar	Lipase, lecithinase, proteolysis on egg yolk agar
LD bile agar	Growth in presence of 20% bile (2% oxgall), insoluble precipitate under and immediately surrounding growth
Presumpto 2 plate	
LD glucose agar	Glucose fermentation; stimulation of growth by fermentable carbohydrate
LD starch agar	Starch hydrolysis
LD milk agar	Proteolysis of milk
LD DNA agar	Deoxyribonuclease activity
Presumpto 3 plate	
LD gelatin	Gelatin hydrolysis
LD mannitol agar	Mannitol fermentation
LD Lactose agar	Lactose fermentation
LD rhamnose agar	Rhamnose fermentation

Source: Modified from Winn et al. [140].

* By comparing growth on anaerobe plate with blood agar (or chocolate agar) incubated in 5–10% CO₂ incubator (or candle jar) or in room air.

† The catalase test can be performed by adding 3% hydrogen peroxide to the growth on LD agar, but the reactions of catalase-positive cultures are more vigorous on LD esculin agar.

4.2.5 Alternative procedures for characterization of anaerobes

A variety of other methods for characterizing and identifying anaerobes have been proposed as practical alternatives to the laboratory methods described above (Table 4.7) [11, 54, 66]. Compared to the more traditional approaches for identifying anaerobes, the motivation for developing

alternative procedures has often been to decrease materials costs, save time, simplify the work involved, or to find a more accurate and reproducible way to differentiate between anaerobe isolates. Among the approaches taken have been to test for certain key characteristics of isolates on differential agar media (e.g., *Bacteroides* Bile Esculin Agar; Anaerobe Systems, San Jose, CA), or to use relatively

Table 4.7 Practical and simple manual methods for characterization and presumptive identification of anaerobes

Test or procedure	Rationale, comments, commercial sources
Observations of colony characteristics on anaerobe blood agar and anaerobe selective media; Gram reactions and microscopic morphology	Morphologic observations are necessary for identification (ID) of all anaerobes. Commercially prepared formulations of anaerobe blood agar are available from several manufacturers including Anaerobe Systems, BD Biosciences Microbiology Products, PML Microbiologicals, Remel, and Springs River Biologicals
Aerotolerance tests on anaerobe blood agar	Necessary to define relationships to O ₂ . See above for sources of prepared media
Egg yolk agar (EYA) reactions	Lipase positive species include <i>C. sporogenes</i> , <i>C. botulinum</i> , <i>C. novyi</i> type A, <i>P. intermedia</i> and <i>F. necrophorum</i> . Lecithinase positive species include <i>C. perfringens</i> , <i>C. sordellii</i> , <i>C. bifermentans</i> , <i>C. limosum</i> , <i>C. subterminale</i> , <i>C. novyi</i> A and <i>C. baratii</i> . Commercially prepared EYA is available from a number of manufacturers including Anaerobe Systems, BD Biosciences Microbiology Products, PML Microbiologicals, Remel, and Springs River Biologicals
Catalase test on hemin supplemented medium (e.g. 3% H ₂ O ₂ on LD esculin agar, or slide catalase using 15% H ₂ O ₂ with added tween 80)	A positive catalase test is characteristic of some species of <i>Bacteroides</i> , <i>Bilophila wadsworthia</i> , some <i>Anaerococcus prevotii</i> , <i>Staphylococcus saccharolyticus</i> and <i>Veillonella parvula</i> , <i>Propionibacterium</i> spp. (<i>P. propionicus</i> is negative and an exception) and <i>A. viscosus</i> .
Spot indole test using <i>p</i> -dimethylaminocinnamaldehyde	Useful for all groups of anaerobes. Reagent available from several sources including Anaerobe Systems, BD Biosciences Microbiology Products, and Remel.
Enhanced growth in presence of 20% bile in medium or around disks containing bile	Key characteristic of the <i>B. fragilis</i> group, <i>Bilophila</i> spp., <i>Fusobacterium varium</i> , <i>F. ulcerans</i> , <i>Leptotrichia buccalis</i> , and <i>Mitsuokella multiacida</i> . Available commercially in quadrant plates from Remel and Smith River Biologicals
Antibiotic differentiation disks (e.g. kanamycin 1 mg; rifampin, 15 µg; penicillin, 2U; colistin, 10 µg; vancomycin, 5 µg.); used to detect growth in presence of drug or growth inhibition.	Used to aid in distinguishing between Gram-positive vs Gram-negative anaerobes and in presumptive identifications
Sodium polyanetholsulfonate (SPS) disk test	SPS inhibits <i>P. anaerobius</i> ; other anaerobic cocci are resistant to SPS. Alternatively, <i>P. anaerobius</i> is the only anaerobic coccus that produces a major isocaproic acid peak on GLC analysis. SPS disks available from Anaerobe Systems and BD Biosciences Microbiology Products
Disk test for nitrate reduction	Most if not all <i>Campylobacter ureolyticus</i> (formerly <i>Bacteroides ureolyticus</i>), <i>Veillonella</i> spp., <i>Eggerthella lenta</i> (formerly <i>Eubacterium lentum</i>), <i>Bilophila wadsworthia</i> , <i>Sutterella wadsworthensis</i> are positive; test helpful for other cocci, non-sporing rods and clostridia as well. Available from Anaerobe Systems and BD
Urease test (various procedures can be used)	A positive urease reaction is a key characteristic of <i>C. ureolyticus</i> , <i>Bilophila wadsworthia</i> , <i>C. sordellii</i> , <i>Anaerococcus tetradius</i> (formerly <i>P. tetradius</i>), <i>S. saccharolyticus</i> and certain <i>Actinomyces</i> spp. Available from Anaerobe Systems and BD Biosciences Microbiology Products
Rapid glutamic acid decarboxylase tube test	Positive reactions given with most strains of the <i>B. fragilis</i> group, many strains of <i>Fusobacterium</i> spp., <i>P. micra</i> (53% of strains), most <i>C. perfringens</i> and <i>C. sordellii</i> , some <i>C. difficile</i> and all <i>E. limosum</i> tested. Available from Remel.
Esculin hydrolysis (on LD esculin agar or using a spot test with 1% ferric ammonium citrate to PY esculin broth)	Useful for all groups of anaerobes. Available in quadrant plates from Remel and Smith River Biologicals.
Fluorescence under long-wave length (366-nm) UV light	Useful for the pigmented <i>Prevotella</i> and <i>Porphyromonas</i> species. <i>Porphyromonas gingivalis</i> does not fluoresce whereas <i>P. asaccharolytica</i> is red or yellow; <i>Prevotella intermedia</i> is bright red. <i>P. melaninogenica</i> is red-orange. Also useful for <i>Veillonella</i> spp. (red). Observations made on laked blood agar
Lack of constitutive β-glucosidase (esculinase)	<i>Fusobacterium</i> species lack the enzyme but it is produced by <i>Bacteroides</i> spp. and <i>Prevotella</i> spp.

Source: Modified from Jousimies-Somer et al. [66].

small volumes of media in small containers that can be easily and rapidly manipulated at the laboratory bench. Another convenient test format has been to use special potency disks to test for growth stimulation or growth inhibition and the use of disks or tablets for determining certain enzyme-substrate reactions. Supplies and other

Table 4.8 Selected disk tests used in presumptive identification of anaerobes

Test	Manufacturer's number	Package
SPS Disk	AS-702	50 tests
Nitrate Disk	AS-703	50 tests
Colistin Disk	AS-705	50 tests
Kanamycin Disk	AS-706	50 tests
Vancomycin Disk	AS-707	50 tests

AS, Anaerobe Systems, Morgan Hill, CA.

Table 4.9 Selected tests available in single tablet format for assessing preformed enzymatic activity of anaerobes*.[†]

Substrate and abbreviation	Enzyme, comments
Glycosidase tests (WEE-TABS) [‡]	
<i>p</i> -nitrophenyl- α -D-glucopyranoside (α -GLU)	α -Glucosidase
<i>p</i> -nitrophenyl- β -D-glucopyranoside (β -GLU)	β -Glucosidase
<i>o</i> -nitrophenyl- α -D-glucopyranoside (α -GAL)	α -Galactosidase [‡]
<i>o</i> -nitrophenyl- β -D-galactopyranoside (β -GAL or ONPG)	β -Galactosidase [‡]
<i>p</i> -nitrophenyl- β -N-acetyl-D-glucosaminide (β -NAG)	β -N-acetylglucosaminidase [‡]
<i>p</i> -nitrophenyl- α -L-fucopyranoside (α -FUC)	α -Fucosidase [¶]
<i>p</i> -nitrophenyl phosphate (PNP)	Alkaline phosphatase ^{**}
Naphthylamide-based aminopeptidase test tablets (ADD-A-TABS) [§]	
Na-benzoyl-DL arginine β -naphthylamine (BANA or TRY)	Trypsin-like activity [¶]
N-glutaryl-gly-gly-phe β -naphthylamine (CHY)	Chymotrypsin-like activity [¶]
L-Phenylalanine α -naphthylamide (PAL)	
L-Arginine α -naphthylamide (ARG)	
L-Serine α -naphthylamide (SER)	
L-Pyroglutamic acid α -naphthylamide (PYR)	
L-proline α -naphthylamide (PRO)	
Leucyl-glycine α -naphthylamide (GLY)	

* In the United States, WEE-TABS and ADD-A-TABS are available commercially from Key Scientific Products, Round Rock, TX.

[†] In Europe, tablets containing many of the substrates listed are available from Rosco Diagnostica, Taastrup, Denmark.

[‡] Glycosidases. When bound to nitrophenyl, hydrolysis of a colorless aryl-substituted glycoside substrate or phosphoester releases the nitrophenyl base. No color change at 2 h is negative; a yellow color at any time during the 2 h of aerobic incubation is a positive glycosidase test.

[§] Naphthylamide. Enzyme hydrolysis of the arylamide in the tablet releases free α -naphthylamine, which is detected and shown by the color change after adding phosphoenol pyruvate (PEP) reagent (i.e., *p*-dimethylaminocinnamaldehyde in HCl). After reading the glycosidase test, two drops of PEP are added followed by 15 min incubation; positive tests are red while negative tests are yellow.

[¶] Test aids in differentiation of *Porphyromonas* spp.

^{**} PNP: *P. micra*, *A. hydrogenalis*, and *A. prevotii* = positive; peptostreptococci = negative.

materials to perform several practical and simple tests are described in the *Wadsworth-KTL Anaerobic Bacteriology Manual* [66] and are commercially available (Table 4.8).

Many anaerobic bacteria produce constitutive enzymes that rapidly hydrolyze various chromogenic substrates. WEE-TABS (Key Scientific Products, Round Rock, TX) are stand-alone chromogenic tests for several enzyme-substrate reactions which can be used as alternative and supplemental tests for differentiation of certain anaerobes (Table 4.9; Figure 4.1). Hudspeth and colleagues found WEE-TABS to be a rapid and accurate alternative for identification of *Porphyromonas* species isolated from infected dog and cat bite wounds in humans [62]. WEE-TABS are available in single test and dual test formats with nitrophenyl and naphthylamide bound substrates. According to the manufacturer's instructions, inocula are prepared by harvesting a sufficient number of colonies into 1–2 mL of distilled water to make a suspension equal to a #5 McFarland standard. At least five or six drops of this suspension are placed into each tube containing a WEE-TAB tablet and the tube is mixed vigorously or vortexed to disintegrate the tablet and mix the suspension. The tubes are incubated aerobically at 37°C for 2 h. For the glycosidase tests, no change at 2 h is negative. The development of a yellow color at any time during the 2 h is considered a positive glycosidase test. After reading the glycosidase results (dual test format), or after incubating a single naphthylamide test, two drops of *p*-dimethylaminocinnamaldehyde reagent are added. The tube is then incubated for 15 min for color development. Positive tests will be red while negative tests will be yellow or a light peach-color. A triple test format is also available with nitrophenyl-, methylumbelliferyl-, and naphthylamide-bound substrates in each tube. Hydrolysis of methylumbelliferyl-bound substrates produces a bright, blue-green fluorescence that



Figure 4.1 WEE-TABS stand-alone chromogenic tests. Each tube contains one tablet and a small volume of bacterial suspension. (Courtesy of Key Scientific Products.) (See insert for color representation of the figure.)

is detected using a long-wavelength UV light (366 nm). When only a small battery of enzyme tests is needed for characterizing an anaerobe isolate (e.g., six or fewer tests), WEE-TABS may be more cost effective than the prepackaged commercial kits for detecting various preformed enzymes (in terms of time and materials costs). Diagnostic tablets for bacterial enzyme testing are manufactured by Rosco Diagnostica (Taastrup, Denmark) and sold by Key Scientific Products, Round Rock, TX.

4.3 Commercial kit requiring 24 h of anaerobic incubation

The API 20A (bioMérieux; Figure 4.2; Table 4.10) micro-method kit has been marketed for the identification of anaerobes isolated from clinical materials. This miniaturized system, while smaller than the conventional biochemical identification systems discussed previously, requires turbid inocula and depends upon growth of anaerobes during incubation in an anaerobic system. The system is useful for identification of saccharolytic, fast-growing anaerobes but not those that are asaccharolytic or fastidious. Interpretations of the reactions for identifications must be made by comparisons to the manufacturer’s proprietary database.

The API 20A consists of a plastic strip with 20 “micro-tubules” of dehydrated substrates to test for the following: indole, catalase, hydrolysis of urea, gelatin, and esculin, plus the fermentation of glucose and other carbohydrates. The microcupules of the strip are inoculated with a turbid suspension (i.e., ≥ #3 McFarland standard) of fresh colonies prepared in Lombard-Dowell broth and incubated anaerobically for 24–48 h. Databases exist for reading the strips at 24 h or 48 h. The indicator for the fermentation

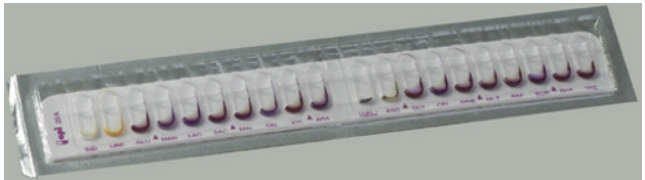


Figure 4.2 API 20A test strip. Miniaturized cupules are inoculated with a bacterial suspension and after 24–48 h of incubation, biochemical reactions are visualized. (Courtesy of bioMérieux.) (See insert for color representation of the figure.)

tests is bromocresol purple. An acid reaction is yellow (pH 5.2) or yellow-green; a negative reaction is purple. Various anaerobes (e.g., a number of *Clostridium* species) reduce the indicator to colorless, shades of green-brown or other colors, sometimes making reactions difficult to interpret. According to the manufacturer’s instructions, “other tests such as colonial and microscopic morphology, Gram stain, etc., should be performed and the results used to confirm or complete the identification.” Additional recommendations for use of the API 20A are supplied by bioMérieux along with identification tables and a numerical Analytical Profile Index.

An excellent review of the older literature by Stargel and colleagues [118] describes the development and evaluations of the packaged micromethod kit. A number of other reports have discussed the performance, advantages, and limitations of various systems [15, 55, 57, 59, 125]. It is important to note that one of the largest reports indicated that the API 20A system correctly identified only 50% of approximately 800 anaerobe isolates to the species level [125]. Although the system can accurately identify some commonly encountered species such as *Bacteroides fragilis* and *Clostridium perfringens*, many of the anaerobes that are less reactive or more fastidious (e.g., many species of *Prevotella*, *Porphyromonas*, *Fusobacterium*, other clostridia, anaerobic nonsporeforming rods, and most anaerobic cocci) require additional tests to achieve correct identification, which decreases the usefulness of the system and increases the time for identifications by another day or two. Thus, although the API 20A system can be set up relatively rapidly, it does not necessarily enable the user to identify anaerobes any faster than can be done using the conventional tests described previously. Additionally, at the time of this writing, the database for this system requires selective nomenclature updates.

4.4 Commercial enzyme kits for identification after four hours of aerobic incubation

Commercially available microsystems in kit form that have been used for characterization and identification of anaerobes following aerobic incubation for 4 h include the following:

Table 4.10 Selected miniaturized and rapid commercial packaged kit systems for identification of anaerobes: manufacturers/vendors

Kit type	System	Manufacturer’s number	Package
Miniaturized Rapid identification (≤4 h)	API 20A	20300	25 tests/box
	RapID ANA II	83-11002	20 panels
	Vitek 2 ANC ID Card	21347	20 tests/box
	MicroScan Panels; Rapid anaerobe ID	B1017-2	20 panels
	Rapid ID 32	32300	25 panels
	Crystal Anaerobe kit	245010	20 determinations

- 1 RapID-ANA II (Remel/ThermoFisher)
- 2 Vitek 2 ANC (bioMérieux)
- 3 MicroScan Rapid Anaerobe Panel (Beckman Coulter)
- 4 Rapid ID 32A (bioMérieux)
- 5 BBL Crystal Anaerobe Identification System (Becton Dickinson Microbiology Systems).

These growth-independent systems are designed to test for various miniaturized conventional reactions and preformed enzymes (e.g., aminopeptidases and glycosidases). All of them require that inocula be prepared using dense cell suspensions from the surfaces of purity plate cultures. The short incubation time of 4 h or less for each of the systems is in sharp contrast to the longer incubation times required for the growth-dependent identification systems discussed previously (Table 4.10). All of the packaged systems listed above provide numerical codes or computerized databases that aid in identification of isolates characterized using these systems.

4.4.1 RapID ANA II

The RapID ANA II tests for 18 preformed enzymes using 10 wells (Figure 4.3). The dehydrated substrates included in these wells are listed in Table 4.11.

4.4.1.1 Preparation of inocula: important considerations

A turbid inoculum is prepared from a pure culture suspension of a fresh isolate (e.g., growing on anaerobe blood agar), to be equivalent to at least a McFarland #3 turbidity standard. An important variable addressed (in part) in the package insert is the culture medium used for the preparation of inocula. Thus, Remel recommends that inocula be prepared from any of the following nonselective agar media supplemented with 5–7% sheep blood: *Brucella*, Columbia, Brain-heart infusion Lombard-Dowell, or Trypticase soy base, and CDC Anaerobic Blood Agar. Also recommended are any of the following selective or differential agar media: phenylethylalcohol, paromomycin/vancomycin, and kanamycin/vancomycin, and egg yolk agar.

The manufacturer recommends that a number of other media not be used. Schaedler blood agar and other media supplemented with disaccharides or monosaccharides should not be used because they may suppress glycolytic activity and reduce test reactivity. *Bacteroides* bile esculin and kanamycin-bile esculin agars are not recommended because a ferric-esculin complex can form that interferes with test interpretation. In addition “reducible agars” containing reducing agents or palladium chloride should not be used. Reducing agents can interfere with certain enzyme reactions.

A problem that needs to be addressed is the preparation of inocula from media that are not enriched with vitamin K, L-cystine or yeast extract (all found in CDC-anaerobe blood agar). For example, trypticase soy blood agar (TSA) may not

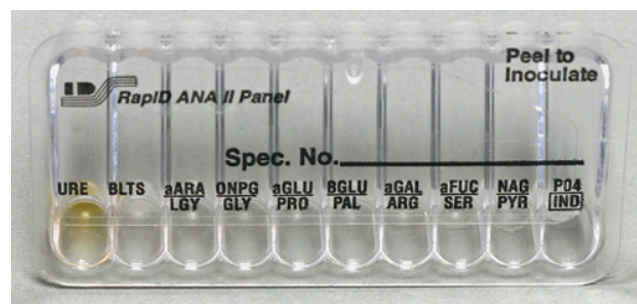


Figure 4.3 Thermo Scientific™ RapID™ ANA II System. This 4-h rapid test system for the identification of anaerobes contains 10 wells for testing the performance of 18 preformed enzymatic tests. (Photo courtesy of ThermoFisher Scientific, copying is prohibited.) (See insert for color representation of the figure.)

be adequately enriched to meet the nutritional requirements of certain pigmented *Prevotella*–*Porphyromonas* species or of certain other anaerobe groups or species. The growth of these organisms may be very poor on TSA, which could lead to incorrect test results or failure to identify certain isolates. In general, at this time, there is little published information on the effect of growth medium used to prepare the inoculum on enzymatic test results obtained using commercially packaged rapid test kits.

In a comparison of the RapID ANA II against VPI conventional methods in a large study of anaerobic clinical isolates, 68% of 566 isolates were identified correctly to species using the RapID ANA II without the use of additional tests [89]. Those correctly identified to species included 62% of 204 anaerobic Gram-negative rods, 72% of 163 anaerobic cocci, 70% of anaerobic nonspore-forming Gram-positive rods, and 74% of clostridia. Within the *B. fragilis* group, the kit correctly identified the majority of the indole negative species including 26/28 (93%) *B. fragilis*, all 19 strains of *Parabacteroides distasonis* (formerly *Bacteroides distasonis*), and 19/27 (70%) of *B. vulgatus*. The indole positive species of the *B. fragilis* group were problematic. For example, only 1/29 (3%) of *B. ovatus* and 4/14 (29%) *B. uniformis* were correctly identified to species using the RapID ANA II; they were often confused with each other or with *B. thetaiotaomicron* [37]. In addition, at the time of this writing, the database was found to be significantly out of date with respect to nomenclature of the pigmented *Prevotella* and *Porphyromonas* species. Without additional tests, the kit could identify only 1/11 (9%) strains of *F. nucleatum* and 1/4 *F. necrophorum* correctly to species, which is problematic since *F. nucleatum* is the most common *Fusobacterium* species encountered. Only about two-thirds of 24 *P. anaerobius* strains were identified correctly using the system, also problematic since *P. anaerobius* is one of the more common anaerobic cocci isolated from properly collected clinical materials. Another study showed that the RapID ANA II was only able to identify *Actinomyces* species with an accuracy of 24% [24, 91].

Table 4.11 Summary of biochemical test reactions or substrates available in the 4-h commercial packaged microsystems

Rapid ANA II	MicroScan Rapid Anaerobe Panel	Rapid ID 32 A	Crystal	Vitek 2 ANC card
Urea	<i>p</i> -Nitrophenyl- β -D-galactopyranoside	Urease	L-Arginine-7-amino-4-methylcoumarin (AMC)	D-Galactose
<i>p</i> -Nitrophenyl- β , D-lactoside	<i>p</i> -Nitrophenyl- α -D-galactopyranoside	Arginine dihydrolase	L-Histidine-AMC	Leucine arylamidase
<i>p</i> -Nitrophenyl- α , L-arabinoside	bis- <i>p</i> -Nitrophenyl phosphate	α -Galactosidase	4-Methylumbelliferon (MU)- α -D-mannoside	Ellman
Leucyl-glycyl- β -naphthylamide	<i>p</i> -Nitrophenyl-N-acetyl- β -D-glucosaminide	β -Galactosidase	L-Serine-AMC	Phenylalanine arylamidase
<i>o</i> -Nitrophenyl- β , D-galactoside	<i>p</i> -Nitrophenyl- α -D-glucopyranoside	β -Galactosidase-6- phosphatase	L-Isoleucine-AMC	L-proline arylamidase
glycyl- β -naphthylamide	<i>o</i> -Nitrophenyl- β -D-glucopyranoside	Glucosidase	4MU- β -D-mannoside	L-pyrrolidonyl-arylamidase
<i>p</i> -Nitrophenyl- α , D-glucoside	<i>p</i> -Nitrophenyl phosphate	β -Glucosidase	Glycine-AMC	D-Cellobiose
Prolyl- β -naphthylamide	<i>p</i> -Nitrophenyl- α -L-fucopyranoside	α -Arabinosidase	L-alanine-AMC	Tyrosine arylamidase
<i>p</i> -Nitrophenyl- β , D-glucoside	<i>p</i> -Nitrophenyl- α -D-mannopyranoside	β -Glucuronidase	4MU-N-acetyl- β -D-galactosaminide	Ala-phe-pro-arylamidase
Phenylalanyl- β -naphthylamide	L-Leucine- β -naphthylamide	β -N-acetyl-glucosaminidase	L-Pyroglyutamic acid	D-Glucose
<i>p</i> -Nitrophenyl- α , D-galactoside	D-L-Methionine- β -naphthylamide	Mannose fermentation	L-Lysine-AMC	D-Mannose
Arginyl- β -naphthylamide	L-Lysine- β -naphthylamide (alkaline)	Raffinose fermentation	L-Methionine-AMC	D-Maltose
<i>p</i> -Nitrophenyl- α , L-fucoside	L-lysine- β -naphthylamide (acid)	Glutamic acid decarboxylase	4MU- β -D-cellobiopyranoside	Saccharose/sucrose
Seryl- β -naphthylamide	Glycylglycine- β -naphthylamide	α -Fucosidase	4MU- β -D-xyloside	Arbutin
<i>p</i> -Nitrophenyl-N-acetyl- β , D-glucosaminide	Glycine- β -naphthylamide	Nitrate	L-Phenylalanine-AMC	N-Acetyl-D-glucosamine
Pyrrolidonyl- β -naphthylamide	L-Proline- β -naphthylamide	Indole	L-Leucine-AMC	5-Bromo-4-chloro-3-indoxyl-beta-glucoside
<i>p</i> -Nitrophenyl phosphate	L-Arginine- β -naphthylamide	Alkaline phosphatase	Ecosyl	Urease
Tryptophane	L-Pyrrolidonyl- β -naphthylamide	Arginine arylamidase	Disaccharide	5-Bromo-4-chloro-3-indoxyl- β -glucuronide
	L-Tryptophan- β -naphthylamide	Proline arylamidase	Furanose	β -Galactopyranosidase indoxyl
	3-Indoxyl-phosphate	Leucyl glycine arylamidase	Pyranose	alpha-Arabinosidase
	Trehalose	Phenylalanine arylamidase	<i>p</i> -Nitrophenyl- α -D-galactoside	5-Bromo-4-chloro-3-indoxyl- α -galactoside
	Urea	Leucine arylamidase	<i>p</i> -Nitrophenyl- β -D-galactoside	β -mannosidase
	Indole	Pyroglutamic acid arylamidase	<i>p</i> -Nitrophenyl phosphate	Arginine GP
	Nitrate	Tyrosine arylamidase	<i>p</i> -Nitrophenyl- α -D-glucoside	Pyruvate
		Alanine arylamidase	<i>p</i> -Nitrophenyl-N-acetyl-glucosaminide	Maltotriose
		Glycine arylamidase	L-Proline- <i>p</i> -nitroanilide	Esculin hydrolysis
		Histidine arylamidase	<i>p</i> -Nitrophenyl- α -L-fucoside	β -D-fucosidase
		Glutamyl glutamic acid arylamidase	<i>p</i> -Nitrophenyl- β -D-glucoside	5-Bromo-4-chloro-3-indoxyl-beta-N-acetyl glucosamide
		Serine arylamidase	L-Alanyl-L-alanine- <i>p</i> -nitroanilide	5-Bromo-4-chloro-3-indoxyl- α -mannoside
				α -L-Fucosidase
				Phosphatase
				L-arabinose
				D-ribose 2
				Phenylphosphonate
				α - L-arabinofuranoside
				D-xylose

However, Marler et al. [89] showed that it does identify most *F. magna* (formerly *P. magnus*) (26/27 or 96%), *P. micra* (formerly *Micromonas micros*) (20/20), *Propionibacterium acnes* (24/25 or 96%), *C. perfringens* (19/19), and *Clostridium ramosum* (20/20) correctly. In another large study with 480 Gram-positive and Gram-negative clinical anaerobic isolates, only 74% of the isolates were identified to species level and 17.5% to genus level, with 5% of the isolates misidentified [125]. Addition of a few simple and practical tests to the RapID ANA II can improve the accuracy of the system. For example, growth on 20% bile medium and fermentation of a few key carbohydrates can improve the accuracy of identifying *Bacteroides* species. A sodium polyanethol sulfonate disk test aids in differentiating *P. anaerobius* from the other anaerobic cocci. Reactions on egg yolk agar aid in identifying several *Clostridium* spp. [89].

4.4.2 Vitek 2 ANC

The Vitek 2 ANC identification card was developed to identify clinical anaerobes and *Corynebacterium*. The ANC card has 64 microwells with 36 colorimetric enzymatic tests (Figure 4.4; Table 4.11). There are 13 fermentative tests, 17 glycosidase and arylamidase tests, two alkaline reactions, and four other biochemical tests. The system requires several phenotypic tests not in the card that include cell morphology, Gram-stain reaction, and aerotolerance. Investigators have suggested that the Gram-stain reaction be based on the results from special potency disks (Table 4.8). A heavy inoculum consisting of a saline suspension of bacteria equivalent to a McFarland #3 turbidity standard is prepared from fresh colonies from an anaerobe blood agar plate after 24 h of incubation for fast growing anaerobes and 48–72 h of incubation for slower growing strains. Fastidious anaerobes require several blood agar plates in order to provide the heavy

inoculum necessary. The ANC cards are incorporated into the Vitek 2 automated identification system. The incubation periods are approximately 6 h and interpretations are made using the Vitek 2 algorithm. The database is proprietary. In one evaluation comparing results of the Vitek 2 ANC card with 16srRNA identifications, the ANC card was found to identify 79.4% of 301 clinical isolates to genus level, which included 100 species that were not in the proprietary database. However, the species-level identification of those 100 strains was 47% [80]. Another group of investigators evaluated the ANC card by challenging the system with only those organisms included in the database. With this more limited challenge, the cards provided 95.1% correct identifications [107]. The proprietary database does not contain all isolates encountered in clinical material but will provide identifications for relevant clinical anaerobes if they are present in the database.

4.4.3 Microscan Rapid Anaerobe Panel

The MicroScan Rapid Anaerobe Identification Panel is a clear plastic 96-well microdilution tray with 24 wells containing dehydrated substrates (Table 4.11; Figure 4.5). The system uses chromogenic tests and modified conventional tests to identify anaerobic bacteria following 4 h of incubation aerobically at 35–37°C. An inoculum suspension equal to at least a McFarland #3 turbidity standard is prepared using a suspension of fresh (24–48 h) colonies in autoclaved deionized water. As for the other 4 h rapid identification systems discussed in this section, preformed enzymes are detected and growth of the microorganism in the panel system is not required. After incubation, changes in pH, utilization of certain substrates, or other biochemical reactions are read visually, and anaerobic bacteria are identified with the aid of a “biotype codebook.” The MicroScan Rapid Anaerobe Identification Panel must be read manually as at the time of this writing the FDA have not approved it for use with the MicroScan Instrument Systems.

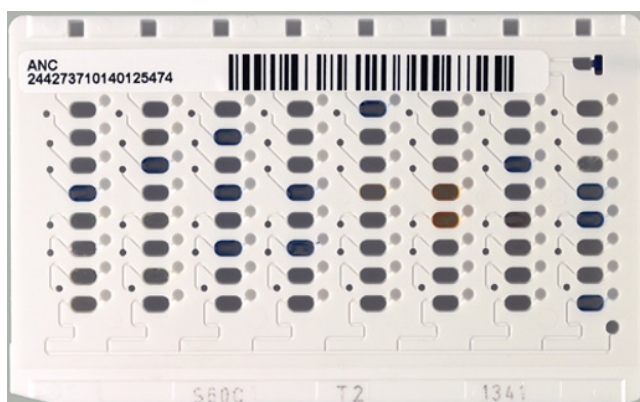


Figure 4.4 Vitek 2 ANC card. This rapid test system is incubated aerobically and comprises a single plastic card with 64 wells for performing various colorimetric and conventional biochemical tests. (Courtesy of bioMérieux.)

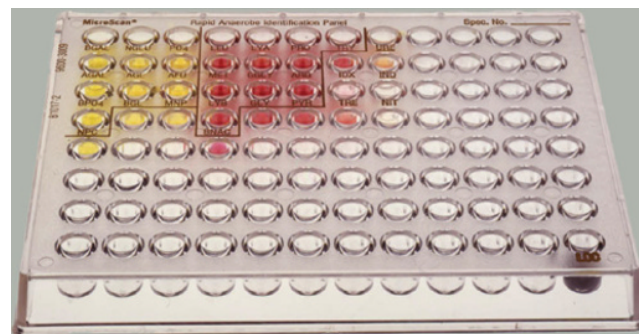


Figure 4.5 MicroScan Rapid Anaerobe Identification Panel. This 96-well microdilution tray contains dehydrated substrates and is incubated for 4 h aerobically. (© 2015 Beckman Coulter, Inc., all rights reserved, used with permission.) (See insert for color representation of the figure.)

The manufacturer has addressed certain other limitations. With regard to preparation of the inoculum, the product insert indicates that organisms to be tested should be grown only on nonselective media such as trypticase soy agar, *Brucella*, brain heart infusion, or Columbia; all supplemented with 5% sheep blood (only). Furthermore, selective media that contain antibiotics, including kanamycin/vancomycin, *Bacteroides* bile esculin agar, and phenylethyl alcohol agar, should not be used to prepare inocula for these panels. The problem that some of the media listed above (e.g., trypticase soy blood agar) may not be adequately enriched to meet the nutritional requirements of some fastidious anaerobes is a limiting factor related to this system.

Another item listed in the package insert is that, prior to testing with the Microscan panel, “each organism should be tested for aerotolerance, purity, Gram-stain reaction and colonial morphology,” and “Should more than one species identification be listed for a particular biotype number, supplementary tests may be necessary for the correct identification.”

To our knowledge, the accuracy of the Microscan Rapid Anaerobe Identification system has been addressed in the refereed literature in only a single report [123]. Compared to identifications obtained using VPI conventional methods, using this product, correct identifications were reported for 166 of 237 strains (70%) tested. The authors pointed out significant problems with the identification of *Bacteroides fragilis*, the pigmented anaerobic Gram-negative rods, and anaerobic Gram-positive bacilli (Table 4.12). The investigators emphasized that additional rapid and simple tests, including, Gram staining, gas-liquid chromatography, growth in the presence of bile, pitting of agar, production of catalase, indole, lecithinase, lipase, fluorescence, and pigment, and the hydrolysis of esculin, can aid in arriving at correct identifications [123].

4.4.4 Rapid ID 32A

The Rapid ID 32A is a miniaturized, disposable plastic strip with 32 small cupules (Figure 4.6), 29 of which are used as wells containing dehydrated substrates to test for the reactions listed in Table 4.11. Three of the small cupules are empty. The inoculum suspension, equivalent to a McFarland #4 turbidity standard, is prepared from fresh colonies growing on a 24–48 h Columbia blood agar plate (supplemented with 5% horse or sheep blood plus vitamin K3). The package insert indicates that Columbia blood agar is the only medium that can be used. Each well is inoculated with 55 µL of suspension per cupule, and then the urea cupule is overlaid with mineral oil. Panels are incubated 4 h at 37°C. Supplemental tests may be required to interpret results, which include Gram-stain

Table 4.12 Data compiled from published commercial kit evaluations on the identification of commonly encountered anaerobes to genus and species correctly without the use of additional tests*

Organisms	RapID-ANA II†	MicroScan Rapid Anaerobe Panel‡
<i>Bacteroides fragilis</i> grp.	66/221	63/54
Pigmented <i>Prevotella</i> spp.	84/32	71/17
<i>Porphyromonas</i> spp.	86/7	63/8
Other <i>Prevotella</i> spp.	85/60	84/31
<i>C. ureolyticus</i>	100/14	100/4
<i>Fusobacterium</i> spp.	26/27	73/15
<i>Finegoldia magna</i> /Parvimonas <i>micra</i>	98/66	ND
<i>Peptostreptococcus anaerobius</i>	71/31	ND
Other cocci	73/139	82/22
<i>Propionibacterium</i> spp.	94/49	ND
Other gram-positive anaerobic rods	67/101	41/17
<i>C. perfringens</i>	100/26	ND
<i>C. ramosum</i>	100/25	ND
<i>C. septicum</i>	100/5	ND
<i>C. difficile</i>	55/11	ND
Other <i>Clostridium</i> spp.	61/97	71/69

ND, not determined.
* The data are displayed as percent correctly identified to genus and species/number of isolates tested.
† RapID-ANAI composite data compiled from Celig and Schreckenberger [24] and Marler et al. [89].
‡ MicroScan data compiled from Stoakes et al. [123].

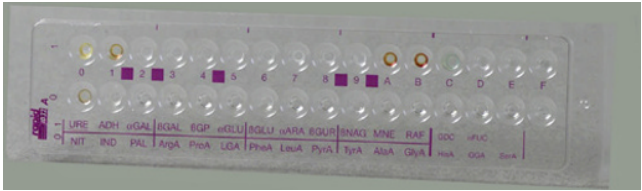


Figure 4.6 Rapid ID 32A. A plastic strip with 32 cupules, 29 of which contain dehydrated substrates for rapid biochemical characterization of anaerobes, is incubated for 4 h aerobically. (Courtesy of bioMérieux.) (See insert for color representation of the figure.)

morphology, spore production, hemolysis, motility, lecithinase production, and hydrolysis of esculin, starch and gelatin [42].

This system has been the subject of a number of evaluations with widely differing results [64, 73, 82, 102]. Without the use of supplemental tests, 73–78% of the *B. fragilis* group and 100% of the *C. perfringens* strains tested were identified correctly to species. The system had difficulty in identifying *Fusobacterium* species, the less commonly encountered anaerobic Gram-negative bacilli (many of those other than the *B. fragilis* group), *Clostridium* species other than *C. perfringens* or *C. ramosum*, and some of the anaerobic nonspore-forming, Gram-positive rods, and anaerobic cocci [93]. Data

Table 4.13 Identification of anaerobic bacteria by Rapid ID 32 A

Organism	Moll et al. 1996 [93]			Looney et al. 1990 [82]*			Kitch and Appelbaum 1989 [73]†		
	Number tested	Number identified to species in agreement with reference system	Correct ID (%)	Number tested	Number identified to species in agreement with reference system	Correct ID (%)	Number tested	Number identified to species in agreement with reference system	Correct ID (%)
<i>B. fragilis</i> grp.	52	38	73	88	69	78	73	57	78
Pig. <i>Prevotella</i>	7	4	57	11	10	91	5	2	40
<i>Porphyromonas</i>	3	2	67	3	3	100	ND		
Other <i>Prevotella</i>	2	1	50	11	9	82	18	15	83
<i>C. ureolyticus</i>	ND			4	4	100	ND		
<i>Fusobacterium</i> spp.	10	1	10	22	2	9	10	1	10
GNR totals	74	46	62	139	97	70	106	75	71
<i>P. magnus/micros</i> ‡	5	3	60	10	10	100	11	11	100
<i>P. anaerobius</i>	5	0	0	7	6	86	6	6	100
Other cocci	7	1	14	7	6	86	11	10	91
Anaerobic cocci totals	17	4	24	27	24	89	28	27	96
<i>Propionibacterium</i> spp.	15	4	27	11	10	91	11	11	100
Other GPRs	47	6	13	11	6	55	25	11	44
Nonsporing GPR totals	62	10	16	22	16	73	36	22	61
<i>C. perfringens</i>	7	7	100	16	16	100	7	7	100
<i>C. ramosum</i>	1	1	100	6	2	33	6	5	100
<i>C. septicum</i>	ND			ND			1	0	0
<i>C. difficile</i>	1	0	0	10	0	0	4	1	25
Other <i>Clostridia</i>	5	1	20	39	22	56	25	13	52
<i>Clostridium</i> totals	14	9	64	71	40	56	43	26	60
Grand Totals	167	69	41	259	177	68	214	150	70

ND, not determined; GNR, Gram-negative rods; GPR, Gram-positive rods.

* Data from Looney et al. [82] do not include one unidentified strain of *Bacteroides splanchnicus* that was listed in their paper.

† Data from Kitch and Appelbaum [73] do not include one strain of *Capnocytophaga* sp. that was identified correctly by the rapid ID 32A.

‡ *P. magnus/micros* are now called *Fingoldia magna* and *Parvimonas micra*.

compiled from three different evaluations of the system are provided in Table 4.13.

4.4.5 BBL Crystal Anaerobe identification system

The BBL Crystal Anaerobe (ANR) identification (ID) system, a miniaturized packaged kit with modified conventional, fluorogenic, and chromogenic substrates, was introduced in the mid-1990s. The kit consists of a plastic base with 30 empty reaction wells and a plastic lid with a fluorescence (negative) control and 29 dehydrated biochemical and enzymatic substrates (Table 4.11) on the tips of plastic prongs (Figure 4.7). All 30 wells are filled with a turbid bacterial suspension (prepared in inoculum fluid to be equivalent to a McFarland #4 standard). The test reactions are begun when a plastic base, with its wells filled with bacterial inoculum suspension and the corresponding lid are aligned and snapped together. Thus within each reaction well, the bacterial suspension rehydrates the dehydrated substrate on the plastic tip that is



Figure 4.7 BBL Crystal ANR ID. Thirty wells in a plastic base are aligned and snapped together with a lid containing 29 biochemical and enzymatic substrates. Some reactions are visualized with a UV light reader (upper left center), while others are read and interpreted using ambient light. (Courtesy and © Becton, Dickinson and Company.)

Table 4.14 Identification of anaerobic bacteria using the BBL Crystal Anaerobe Identification System

Organism	Cavallaro [23]			Number identified to species in agreement with reference system with retest	Total correctly identified overall includes retest (%)	Moll [93]		
	Number tested	Number identified to species in agreement with reference system initially	Correct ID initially (%)			Number tested	Number identified to species in agreement with reference system	Correct ID (%)
<i>B. fragilis</i> grp	46	34	74	42	91	52	37	71
Pig. <i>Prevotella</i>	2	1	50	2	100	7	6	86
<i>Porphyromonas</i>	2	1	50	2	100	3	2	67
Other <i>Prevotella</i>	10	10	100	10	100	2	1	50
<i>C. ureolyticus</i>	3	3	100	3	100	ND		
<i>Fusobacterium</i> spp.	26	25	96	26	100	10	9	90
Other GNRs	32	25	78	25	78	3	3	100
GNR totals	121	99	82	110	91	77	58	75
<i>P. magnus/micros</i> *	3	3	100	3	100	5	5	100
<i>P. anaerobius</i>	4	4	100	4	100	5	3	60
Other cocci	12	10	83	12	100	7	2	29
Anaerobic cocci totals	19	17	90	19	100	17	10	59
<i>Propionibacterium</i> spp.	26	26	100	26	100	15	11	73
Other GPRs	53	45	85	52	98	47	19	40
Nonsporing GPR totals	79	71	90	78	99	62	30	48
<i>C. perfringens</i>	8	8	100	8	100	7	5	71
<i>C. ramosum</i>	7	7	100	7	100	1	1	100
<i>C. septicum</i>	8	6	75	8	100	ND		
<i>C. difficile</i>	8	7	88	8	100	1	1	100
Other <i>Clostridia</i>	72	48	63	48	63	5	3	60
<i>Clostridium</i> totals	103	76	74	79	77	14	10	71
Grand totals	322	263	82	286	89	167	108	65

ND, not determined; GNR, Gram-negative rods; GPR, Gram-positive rods.

* *P. magnus/micros* are now called *Finexgolia magna* and *Parvimonas micra*.

inserted into it and this starts the reaction. According to the package insert, the following BBL media are recommended for preparation of inocula: CDC Anaerobe Blood Agar, Schaedler Agar with vitamin K₁ and 5% sheep blood, Columbia Agar with 5% sheep blood and *Brucella* Blood Agar with hemin and vitamin K₁.

Two evaluations of this system, with differences in study design and findings, have appeared in the refereed literature [23, 93]. The reported performance of the system is summarized in Table 4.14. In both of the published reports, the authors indicated that the BBL Crystal ANR ID system was easy to use. In the most recent evaluation by Cavallaro and colleagues, the results obtained using the ANR system for clinically significant anaerobic bacteria were compared to those obtained using conventional methods of the anaerobe reference laboratory at the CDC [23]. Inocula were prepared using CDC anaerobe blood agar. Upon initial testing, the ANR correctly identified 263 of 322 (81.7%) of the strains tested and 49 were identified correctly only to the genus level. Upon repeat testing, 23 of the latter 49 strains were identified correctly to genus and species. A total of 26 strains (8.5% of 322) were misidentified at the species level, and an additional 10 strains (3.2%) were not identified.

In the study of the ANR system by Moll and associates, results were obtained for anaerobes grown on BBL Schaedler and BBL Columbia agar plates [93] and compared against those obtained using conventional procedures. In general the ANR performed better with anaerobes grown on Schaedler than it did with anaerobes grown on Columbia agar. The performance of the ANR, obtained by Moll et al. using Schaedler agar, is summarized in Table 4.14. Accordingly, for the ANR, a higher percent of correct identifications to the species level was obtained by Cavallaro and colleagues than by Moll and colleagues. Explanations for differences in results in these studies probably relate to differences in the media used to prepare the inocula, differences in bacteria included in each of the studies, differences in the conventional procedures and databases used as gold standards for comparisons, and perhaps other variables.

4.4.6 Interpretation of identification results obtained using commercial kits

As a note of caution, a species name generated from testing with a commercial packaged kit should be interpreted carefully. For example, if a resulting appellation of an isolate is that of an infrequently encountered species,

the microbiologist should not accept this identification without repeat testing, ideally with alternative procedures, or perhaps confirmation by a reference laboratory if warranted clinically. Proprietary databases that accompany commercial kits need to be updated frequently and carefully because a genus and species not included in the database, will not be identified by the system. Moreover, it is very important to compare the results obtained by the commercial test database with the Gram-stain reaction and morphology as well as with cultural reactions of the isolate. Additional supplemental tests described previously (e.g., the Presumpto system or the Wadsworth approach) may be required to identify the less common species with accuracy. Thus, the determination of colonial characteristics and microscopic features of isolates, production of catalase, lipase and lecithinase reactions on egg yolk agar, growth in the presence of bile, the reduction of nitrate, inhibition by sodium polyanetholsulfonate (SPS), inhibition by special potency disks (e.g. vancomycin, kanamycin, and colistin), and metabolic product analysis (GLC) improve the identification accuracies of the kits.

4.5 Identification by gene sequencing

The gold standard test for bacterial identification today is gene sequencing. It is faster, more specific, and more accurate and reproducible than conventional phenotypic testing because phenotypes are essentially mutable and susceptible to differing interpretations [68]. The section of the bacterial gene recommended for taxonomic purposes by ASM (American Society for Microbiology) is the conserved and universal 16S rRNA gene sequence [28]. This sequence has been determined for thousands of strains, and *Bergey's Manual of Systematic Bacteriology*, the standard for bacterial taxonomy, is organized using 16S rRNA gene sequence analysis [83].

The 16S rRNA gene is approximately 1550 bp long with conserved and variable regions. Some or all of the gene can be used for taxonomic purposes. Usually the entire gene is sequenced for new species descriptions, and the initial 500 bp or less sequence is used for identification of most clinical isolates. The sequence identification process begins by extraction of DNA from cells in isolated colonies, amplification of the 16S rRNA sequence, removal of excess primers and nucleotides, cycle sequencing, and generating a DNA sequence. The final sequence is compared to a database library using analysis software. Reliable, up-to-date proprietary databases are freely available via the World Wide Web, and commercial kits are available for each step of the process [28]. Gene sequencing is used for identification of aerobes and anaerobes alike [141] and is further discussed in other chapters of this text.

Gene sequencing technology is seen as a way of avoiding erroneous and delayed identifications of anaerobic isolates that may adversely affect patient outcomes as well as

increase the economic burden for hospitals [20, 115, 143]. In one study [68], 127 anaerobic bacterial isolates from blood cultures were identified by gene sequencing and by conventional phenotypic tests. The conventional tests included Gram stain, hemolysis, fluorescence, susceptibility to special potency disks, bile resistance, and carbohydrate fermentation and enzyme tests, and their results were interpreted using the identification tables in the *Manual of Clinical Microbiology*. Even with careful phenotypic testing, conventional identification could only identify 52% of the isolates to species level as opposed to sequencing which identified 89% to species level. The time difference that the two methods required for a final report was significant, with sequencing requiring 1.5 days versus conventional testing requiring 2.8 days.

Clinical microbiologists are especially interested in sequencing bacterial genes directly in specimens such as blood, cerebrospinal fluid, pleural fluid, or joint fluid where infections usually involve a single strain. A study of bacteremia and blood cultures [135] and a study of bone/joint infections [45] demonstrated the increased power of detection by polymerase chain reaction (PCR) and also the occasional confusion in the clinical significance of results when organisms are subsequently not detected by culture or are organisms that are regarded as contaminants, are present at low numbers, or are nonviable.

Although not perfect, 16S rRNA gene sequencing is the current most accurate and reliable method of anaerobic bacterial identifications [105]. Gene sequencing is being performed in large clinical laboratories as well as in reference laboratories. Because of its cost and complexity, bacteriologists in smaller laboratories may continue relying on phenotypic testing for the identification of common clinical isolates and sending isolates to reference laboratories for definitive identifications in special cases. Gene sequencing should be considered in those situations in which an isolate identification is from a patient with a serious infection such as endocarditis and the identification is not usually associated with that disease. It would also be appropriate to sequence an isolate that cannot be identified by phenotypic testing and for organisms that are slow growing. The technique has been successfully used to identify the causative pathogens in patients with suspected infections who have negative anaerobic and aerobic cultures [71].

4.6 Identification by chemical methods

4.6.1 Mass spectrometry systems

Mass spectrometry (MS) analyzes large molecules such as proteins, sugars, lipids, or DNA. It provides rapid, reproducible, and accurate identifications. The two MS technologies are matrix-assisted laser desorption ionization time of flight (MALDI-ToF) and electrospray ionization combined with PCR (PCR/ESI). Both are soft ionization

techniques that charge and separate ionized particles according to their mass-to-charge ratio. A mass analyzer and detector determine the relative abundance of molecular fragments by their ratios to generate a mass spectrum [47, 129].

4.6.2 Matrix-assisted laser desorption/ionization time of flight

MALDI-ToF MS technique is used to generate protein fingerprint signatures from whole bacterial cells. The technique has been found to be less costly and complex than sequencing as well as more accurate, rapid, and reproducible than conventional phenotypic testing. Currently there are several MALDI-ToF systems available including Microflex LT (Bruker Daltonics, Bremen, Germany) (Figure 4.8), Vitek MS (bioMérieux, Marcy l'Etoile, France), (also see Chapter 13) and the Shimadzu/SARAMIS (Shimadzu Corporation, Kyoto, Japan and AnagnosTec GmbH Potsdam-Golm, Germany). Each system has its own proprietary database. Manufacturers' instructions must be followed.

MALDI-ToF/MS uses isolated colonies and requires minimal sample preparation. Basically, intact bacterial cells from a culture plate are smeared on a stainless steel target plate (or grid) and allowed to co-crystallize with a UV-absorbing matrix. The preparation is dried and then placed into the spectrometer. Bacteria in the matrix receive a pulse from a nitrogen laser device, the matrix absorbs energy, and surface macromolecules of the bacteria are desorbed and ionized. Mass analysis of the ionized surface

macromolecules follows, and the results are reported as a mass spectrum with mass plotted on the x axis versus abundance on the y axis. A composite spectrum is produced from bacterial mass spectral fingerprints and is matched with reference strains in the software database. Similarity scores for the top matches are used to identify the test isolate (Figure 4.9).

There have been several studies to evaluate MALDI-ToF/MS systems on their ability to identify anaerobes. Dubois et al. [43] tested 767 routine clinical isolates comparing the identifications obtained by the Vitek MS system with identifications obtained by phenotypic tests and, if discordant, by molecular tests. Only 30 isolates of the total isolates tested were anaerobic. The Vitek MS system identified 25 of the 30 (83%) anaerobes to species or genus level. The authors suggest that identifications could ultimately have been reached by supplementing with conventional tests such as Gram stain, cell morphology, and cultural characteristics. They also discussed the need for more species included in the databases and greater diversity of the species by adding information of at least 10 strains of each species. A larger study with 1129 clinical isolates including 73 anaerobes using two MALDI-ToF/MS systems demonstrated that the Bruker system database identified 61.6% of anaerobes to species level while the Vitek MS database identified 75.3% to species [90].

In another study, two different MALDI-ToF systems, the Bruker and the Shimadzu/SARAMIS systems, were



Figure 4.8 Microflex LT. (Courtesy of Bruker Daltonics.) (See insert for color representation of the figure.)

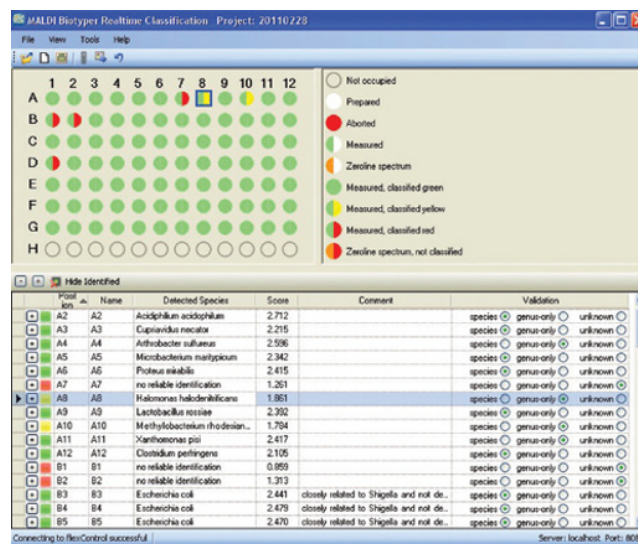


Figure 4.9 MALDI Biotyper software (Courtesy of Bruker Daltonics, Bremen, Germany) is used to generate an identification. Mass spectra of the unknown isolate are correlated with a database of reference strain spectra. An initial score, from 0 (no match) to 1000 (perfect identity), is generated and then converted into a log score from 0 to 3. A score of ≥ 1.7 is considered an identification result of high confidence.

challenged with 290 clinical anaerobic isolates [67]. In this challenge, the Bruker system identified 67.2% of isolates to species level versus the Shimadzu/SARAMIS system, which identified 43.8–49% of isolates. A study of the Bruker system's ability to identify *Prevotella* species showed that the system identified these anaerobes at 62.7% [142]. In every study evaluating the use of MALDI-ToF/MS systems in routine microbiology laboratories for the identification of clinical isolates, investigators have discussed their potential to facilitate the process while emphasizing the need to improve the databases with more genera, species, and strains. Aspects of MALDI-ToF/MS systems that are being determined include whether specimen extraction should be included into the process and whether the similarity score needed to assign an organism to a species or genus should be re-evaluated [72, 110].

4.6.3 PCR combined with ESI/MS

PCR-ESI/MS instruments generate amplicons by multiplex PCR and then measure the mass/charge ratio of amplicons by MS. PCR targets both conserved and species-specific gene sequences, which are compared to known sequences in a database. Commercial instruments include the Abbott PLEX-ID and the Ibis T5000 [141]. The technology has tremendous potential since it can be used for isolated organisms or mixed cultures of dissimilar organisms. However, problems can arise if the mixed cultures involve similar organisms such as staphylococci and streptococci and if there are large differences in the numbers of the different organisms present [69].

4.6.4 Gas chromatography

4.6.4.1 MIDI Sherlock Microbial Identification System

The MIDI Sherlock Microbial Identification System (MIDI, Inc., Newark, DE), or the "MIDI," is a fully automated gas chromatographic (GC) analytical system dedicated to the analysis and identification of bacteria (and certain yeasts) by means of cellular fatty acid (CFA) analysis. Unlike the short chain fatty acid metabolic products (e.g., volatile fatty acids) of two to eight carbons in length that accumulate in broth cultures during fermentation processes, the CFAs are from nine to 20 carbons in length and are major constituents of bacterial cell membranes [134]. The CFAs differ from the long-chain mycolic acids (24 to 90 carbons in length), or the isoprenoid quinones and menaquinones that also occur in cell membranes. CFA analysis requires a different chromatographic setup than that which can be used for short-chain fatty acids, and it differs from the high-pressure liquid chromatographic procedure that is used for long-chain mycolic acid analyses.



Figure 4.10 MIDI Sherlock Microbial Identification System, an automated GC analytical system. Included in the photograph are the following: an Agilent 6890 GC with an automatic sampler, a computer, and a printer. The GC contains a fused-silica capillary column and a flame ionization detector. (Courtesy of MIDI.)

The MIDI system hardware (Figure 4.10) consists of an Agilent 6890 gas chromatograph with an automatic sampler and a fused silica capillary column, a computer, and a printer. For CFA analyses, anaerobic bacteria are grown in a specially formulated PRAS peptone–yeast extract–glucose (PYG) with additional supplements added according to Gram reaction. The PYG broth culture is incubated until it reaches maximum turbidity (e.g., usually overnight for *Clostridium* spp.). The cells are then harvested, saponified with the use of NaOH and heated, methylated (using methanol-HCl and H₂SO₄-methanol plus heat) and extracted into organic solvent (i.e., hexane and methyl tert-butyl ether). The organic layer remaining in the tube is washed with diluted NaOH and transferred to a GC vial. The sealed vial is loaded onto the automatic sampler of the GC, and the analysis of CFAs is performed. The MIDI computer compares the CFA results for an unknown isolate using a database of known reference strains. The identification results are expressed in terms of a similarity index indicating how closely the CFA composition of an unknown isolate matches that of the most closely matched strains in the library.

In an analysis of 216 strains representing 18 species of *Clostridium*, the MIDI identified 86% of the strains correctly to the species level without the need for supplemental tests, and identified an additional 6% of the strains with the aid of a few supplemental tests. Only 3% of the strains were identified to the genus level incorrectly [8, 9]. Cellular fatty acid profiles of *Eubacterium lentum* and species of the genera *Bacteroides*, *Prevotella*, *Porphyromonas* and *Fusobacterium*, and *Propionibacterium*, as well as several other genera of clinically encountered anaerobes, have been studied extensively [16, 36, 95, 96].

4.6.5 The Biolog AN MicroPlate

The Biolog Identification System (Biolog, Inc., Hayward, CA) is based on the principle of carbon-source utilization; the system utilizes a small-well microtray format for the characterization of microorganisms. The Biolog AN MicroPlate is a test panel (with 95 tests/MicroPlate) designed specifically for the identification of a wide array of anaerobic bacteria. The anaerobic bacteria database (Release 4.01B) contains identification patterns for 363 anaerobic bacteria species/taxa. The list of clinically important anaerobes included in this database is extensive. In addition, anaerobes important in industrial and environmental bacteriology are also included in the database. The Biolog AN MicroPlate was found to be useful in differentiating between *Porphyromonas uneonis*, *P. asaccharolytica* and *P. endodontalis* [46].

4.7 Immunodiagnostic and molecular methods for diagnosis of *Clostridium difficile* infection

Since the publication of the first edition of this book in 2002, much progress has been made towards the development of an optimal laboratory test for the diagnosis of *C. difficile* infection (CDI). New FDA-approved molecular assays are now commercially available and provide enhanced test sensitivity and specificity for detection of toxigenic *C. difficile* compared to virtually all previously used test methods. The new molecular assays detect the *C. difficile* toxin B gene (*tcdB*), and/or other genes (e.g., *tcdC*, *tcdA*), in patient stool specimens. If performed on liquid or unformed stool specimens from patients meeting the diagnostic criteria for CDI, molecular assays can now be used as stand-alone tests since they have very good sensitivity and specificity for detecting toxigenic *C. difficile* [44, 53, 75, 101, 111, 112, 114].

New rapid immunodiagnostic tests, i.e., enzyme immunoassays (EIAs), have been developed for detection of glutamate dehydrogenase (GDH), a cell-wall enzyme (common antigen) found in both toxigenic and nontoxigenic *C. difficile* [112]. GDH is secreted in stool containing *C. difficile*. Enzyme immunoassay testing for GDH is used to “screen” stool specimens to determine if additional tests are needed to detect or rule out the presence of toxigenic *C. difficile*, e.g., by detecting toxin A (TcdA) and/or toxin B (TcdB) [78, 111]. For GDH-positive specimens, *C. difficile* toxins can be detected using additional EIAs, a molecular test, or the cytotoxin assay (described below) [111]. Specimens that test GDH-positive and then negative for toxins using EIAs or the cytotoxin assay should be further tested by molecular methods or toxigenic culture. One new lateral flow rapid membrane EIA can detect both GDH and toxins simultaneously (Figure 4.11) [106, 112, 124, 126]. Such new EIAs may



Figure 4.11 *C. Diff* Quik Chek Complete. This lateral flow rapid membrane enzyme immunoassay can detect *C. difficile* GDH antigen and toxins A and B simultaneously. (Photograph courtesy of Alere, Inc.)

be used in lieu of molecular assays to decrease the cost per laboratory test (since the EIA tests cost less), and for small laboratories that are simply unable to implement molecular test methods. Compared to previously used tests for GDH, the new EIAs for GDH appear to have enhanced sensitivity [78, 106, 112, 124], and although a recent study raised concern about poor sensitivity for certain test kits for detecting non-027 ribotype strains [126], a subsequent study of 77 different ribotypes found no effect of ribotype on the detection of GDH-producing *C. difficile* using the same test kits [22]. Using a first line stand-alone EIA for detecting *C. difficile* toxins (TcdA and/or TcdB) is no longer commonly performed because EIA tests lack adequate sensitivity for the diagnosis of CDI [76, 78, 111, 126].

The cytotoxin assay uses cytotoxicity testing of cell cultures inoculated with stool filtrates for the detection and specific neutralization of TcdB. The cytotoxin assay has often been considered the gold standard when new test-detection methods are evaluated, and has been considered the most specific test since its specificity depends on neutralization of the cytotoxic effect of TcdB [132] (Figure 4.12). If sufficient TcdB is present in the patient's specimen filtrate, cell cytopathic effect (CPE) is observed when the specimen is added to cell cultures. Positive results are confirmed by adding TcdB-specific antitoxin to the specimen filtrate. After incubation, a lack of CPE (compared to a reactive duplicate of the patient's specimen without TcdB-specific antitoxin), confirms the presence of TcdB. There are multiple limitations for the cytotoxin test, and few laboratories in the United States currently perform it [34, 106]. The cytotoxin assay is relatively slow,



Figure 4.12 *C. Difficile* Tox-B cytotoxin assay. (Photograph courtesy of Alere, Inc.) (See insert for color representation of the figure.)

and requires 48 h of incubation before a test result can be reported as negative. Most positive cytotoxin assay results are generated within 18 to 24 h. Due to the requirement for neutralization and slow turn-around time for test results, and its cost, not every laboratory should perform the cytotoxin assay for detection of CDI [120]. Expertise of the bench microbiologist/medical technologist is also an issue for cytotoxin and cell culture testing. Finally, although historically considered a gold standard for new test method evaluations, the cytotoxicity test itself has poor sensitivity compared to toxigenic culture and new molecular assays that detect the toxin B gene (*tcdB*) [78, 124].

Stool culture for *C. difficile*, followed by testing of isolates for toxin production (toxigenic culture), is the most sensitive test for detection of toxigenic *C. difficile* [38, 103, 104, 114]. Toxigenic culture is therefore a superior gold standard compared to the cytotoxin assay, when new test-detection methods are evaluated. Use of enriched toxigenic culture (that often involves inoculation of broth media containing taurocholate) further enhances sensitivity. Toxigenic culture has nearly equivalent specificity compared to the cytotoxin assay, but it also has the problem of a relatively long turnaround time (two or more days) and is labor intensive [5, 65, 94, 108]. Similar to the cytotoxin assay, toxigenic culture is appropriate for routine use only in specific laboratories. Routine culture for isolation of *C. difficile* using selective agar such as Cycloserine Cefoxitin Fructose Agar (CCFA) may be useful for recovery of isolates for subsequent strain subtyping and epidemiological studies [120].

The most accurate diagnosis of CDI requires a combination of both clinical diagnostic criteria for CDI and supporting laboratory results. Only stool specimens

that are liquid or loose enough to conform to the specimen collection container (unformed stool) should be tested, unless the patient is suspected of having ileus, in which case formed stool is acceptable for testing.

Recognizing the need for a rapid, sensitive, specific, and simple test for CDI, manufacturers have brought numerous molecular assays and immunodiagnostic tests to the marketplace (Tables 4.15 and 4.16). The following is a detailed description of each test type.

4.7.1 Tests for glutamate dehydrogenase

4.7.1.1 Latex agglutination

A rapid latex agglutination test for the detection of *C. difficile* in stool was developed in the 1980s. While the test detected virtually all cytotoxic and noncytotoxic *C. difficile* strains, it also produced immunologic cross-reactions with other anaerobes (*C. sporogenes*, proteolytic *C. botulinum*, and *P. anaerobius*), and it was determined that the protein that was reacting in commercial latex tests for *C. difficile* was a GDH and not a toxin [84, 92]. Subsequent studies demonstrated that latex tests for GDH have poor sensitivity for diagnosing *C. difficile*-associated diarrhea (CDAD), e.g., 47–67% [63, 108]. Latex tests for GDH and *C. difficile*, i.e., the Culturette CDT (Beckton Dickinson Microbiology Systems, Cockeysville, MD) and the Meritec *C. difficile* (Meridian Bioscience, Cincinnati, Ohio) have been discontinued and are no longer commercially available in the United States (Table 4.15). Latex tests for the rapid presumptive identification of *C. difficile* from selective or enrichment broths, or colonies from solid media, are still available, i.e., the *C. difficile* Test Kit (Oxoid Ltd., Basingstoke, UK) and the Microgen *C. difficile* test (Microgen Bioproducts Ltd., Camberley, UK).

4.7.1.2 Antigen detection

GDH is present in the cell wall of *C. difficile* and is secreted into the stool. In clinical trials the specificities of tests for GDH antigen detection have been very good, i.e., in the 98–99% range [8, 78]. As a result, many laboratories are increasingly screening stool for the presence of *C. difficile* using EIA test methods for GDH antigen [120]. All GDH-positive specimens should subsequently be tested for TcdA and TcdB as soon as possible by using EIAs, molecular tests, or the cytotoxin assay [111].

Specimens that test GDH-positive and then negative for toxins using EIAs or the cytotoxin assay should be further tested by molecular methods or toxigenic culture (i.e., for the *tcdB* gene or toxin-producing *C. difficile*, respectively). Molecular assays are optimal due to their short test result turn-around times (results available within a few hours) and superior sensitivity compared to the cytotoxin assay [78, 112, 122, 124].

GDH antigen detection kits may take the form of microplate EIAs, e.g., Premier *C. difficile* GDH (Meridian

Table 4.15 Selected tests for the detection of *C. difficile* antigens in stool specimens

Entity detected	Method	Principal advantage	Principal limitation(s)	Available (and discontinued) test(s)	Source(s)
<i>C. difficile</i> isolates	Culture	Sensitive, specific	Efficiency varies from laboratory to laboratory; must perform additional method for toxin detection	CCFA is available from various suppliers; pre-reduced media is preferred	Anaerobe Systems, Morgan Hill, CA (www.anaerobesystems.com) and others
GDH	LA	Rapid, simple	Low sensitivity for direct specimen testing; does not distinguish between toxigenic and nontoxigenic <i>C. difficile</i> strains	<i>C. difficile</i> test kit (used for presumptive identification of <i>C. difficile</i> from broth) Culturette CDT (discontinued)	ThermoFisher Scientific-Remel Products, Lenexa, KS (www.thermofisher.com; www.remel.com) Becton Dickinson Microbiology Systems, Cockeysville, MD
	EIA	Rapid, simple	Does not distinguish between toxigenic and nontoxigenic <i>C. difficile</i> strains Microplate assays allow batch testing of multiple samples, but are less rapid versus rapid membrane EIAs	Meritec <i>C. difficile</i> (discontinued) Immunocard <i>C. difficile</i> GDH Premier <i>C. difficile</i> GDH microplate <i>C. DIFF QUIK CHEK</i> <i>C. DIFF CHEK-60</i> microplate	Meridian Bioscience, Cincinnati, Ohio Meridian Bioscience, Cincinnati, Ohio (www.meridianbioscience.com) Alere, Inc. – TechLab Products, Blacksburg, VA (www.alere.com; www.techlab.com) Biosite Diagnostics, San Diego, CA Becton Dickinson Microbiology Systems, Cockeysville, MD
GDH + toxin A	EIA	Rapid, simple	Does not distinguish between toxin B-positive and B-negative strains	Triage <i>C. difficile</i> panel (discontinued)	VA (www.alere.com; www.techlab.com) Biosite Diagnostics, San Diego, CA
Toxin A	LA	Rapid, simple	Low sensitivity	Culturette CDT (discontinued)	Becton Dickinson Microbiology Systems, Cockeysville, MD
	EIA	Rapid, simple	Does not detect A-B+ isolates	<i>C. difficile</i> toxin A (discontinued)	ThermoFisher Scientific-Remel Products Lenexa, KS
	ICA	Rapid, simple	Less sensitive than EIA	ColorPAC toxin A test (discontinued) Clearview <i>C. difficile</i> toxin A (discontinued) <i>C. DIFFICILE TOX-B</i>	bioMérieux, Marcy-l'Etoile, France Alere, Inc. - Inverness Medical Professional Diagnostics, Princeton, NJ Alere, Inc. – TechLab Products
Toxin B	CTA	Specific	Suboptimal sensitivity; Requires 24–48 h to complete; inactivation of toxin B may yield false-negative results	Premier toxins A and B ProSpect <i>C. difficile</i> toxin A/B microplate	Meridian Bioscience ThermoFisher Scientific-Remel Products
Toxins A and B	EIA	Rapid; detects toxins A and B	Suboptimal sensitivity	ImmuoCard toxins A and B Xpect <i>C. difficile</i> toxin A/B <i>C. DIFFICILE TOX-A/B II</i> <i>TOX-A/B QUIK CHEK</i> VIDAS <i>C. difficile</i> A & B <i>C. DIFFICILE QUIK CHEK COMPLETE</i>	Meridian Bioscience ThermoFisher Scientific-Remel Products Alere, Inc. – TechLab Products bioMérieux, Marcy-l'Etoile, France Alere, Inc. – TechLab Products,
GDH + toxins A and B	EIA	Rapid, simple	Automated May have lower sensitivity for detection of GDH for ribotypes other than 027.		

CTA, cell culture-based cytotoxin test; LA, latex agglutination; EIA, enzyme immunoassay; ICA, immunochromatographic assay.

Table 4.16 Selected commercially available products related to *C. difficile* testing

Test for	Product	Manufacturer's number	Package contents	Manufacturer
Glutamate dehydrogenase only: EIA	Immunocard <i>C. difficile</i> GDH	716050	50 tests	Meridian Bioscience, Cincinnati, Ohio
	Premier <i>C. difficile</i> GDH	611096	96 wells/kit	Meridian Bioscience, Cincinnati, Ohio
	<i>C. Diff</i> Quik Chek	T5029	25 tests	Alere, Inc. – TechLab Products Blacksburg, VA
	<i>C. Diff</i> Quik Chek-60	T5025	96 wells/kit	Alere, Inc. – TechLab Products Blacksburg, VA
<i>C. difficile</i> toxins toxin B tissue culture toxins A and B	<i>C. Difficile Tox-B</i>	T5003	96 wells/kit	Alere, Inc. – TechLab Products Blacksburg, VA
	Premier toxins A and B	616096	96 wells/kit	Meridian Bioscience, Cincinnati, Ohio
	ProSpecT <i>C. difficile</i> toxin A/B	R244596	96 wells/kit	ThermoFisher Scientific – Remel Products Lenexa, KS
	ImmunoCard toxins A and B	712050	50 tests	Meridian Bioscience, Cincinnati, Ohio
	Xpect <i>C. difficile</i> toxin A/B	R24640; R24650	40 tests; 20 tests	ThermoFisher Scientific – Remel Products Lenexa, KS
	<i>C. Diff</i> Tox-A/B II	T5015	96 wells/kit	Alere, Inc. – TechLab Products Blacksburg, VA
	Tox-A/B Quik Chek	T5033	25 tests	Alere, Inc. – TechLab Products Blacksburg, VA
	VIDAS <i>C. difficile</i> A & B	30118-01	60 tests	bioMérieux, Marcy-l'Etoile, France
	<i>C. Diff</i> Quik Chek Complete	30525C; 30550C	25 tests; 50 tests	Alere, Inc. – TechLab Products Blacksburg, VA
Glutamate dehydrogenase and toxins A and B				

Table 4.17 Performance characteristics of commonly used enzyme immunoassays (EIAs)

Test	Source	Performance for detection of <i>C. difficile</i> toxins or GDH compared to toxigenic culture			
		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Premier Toxin A&B test*	Meridian Diagnostics, Cincinnati, OH	48–69.6	94.7–100	68.9–88	87–91.9
ImmunoCard Toxins A&B test†	Meridian Diagnostics, Cincinnati, OH	66.7–80	95.1–99	66.7–91	88.1–98
Xpect Toxin A/B test‡	ThermoFisher Scientific–Remel Inc., Lenexa, KA	48–49	84–95.8	46–82	83–85
<i>C. Diff</i> Quik Chek Complete test§	Alere, Inc.–TechLab/Wampole	GDH 97.6–100 Toxins 61.1–78.3	GDH 88.1–100 Toxins 99.2–100	GDH 81.8–100 Toxins 100	GDH 100 Toxins 95

* Data compiled from references 101, 114, 124. Gold standard used was a combination of PCR and toxigenic culture for reference 123.

† Data compiled from references 2, 4, 60, and 114. Gold standard used was a combination of cytotoxin test and toxigenic culture for references 2 and 4.

‡ Data compiled from references 4 and 114. Gold standard used was a combination of cytotoxin test and toxigenic culture for reference 4.

§ Data compiled from references 106, 112, 121, and 124. Gold standard used was a combination of PCR and toxigenic culture for references 106, 112, and 124.

Bioscience, Cincinnati, OH) and *C. Diff* Chek-60 (TechLab Products, Blacksburg, VA), or rapid membrane EIAs, e.g., Immunocard *C. difficile* GDH (Meridian Bioscience, Cincinnati, OH) and *C. Diff* Quik Chek (TechLab Products, Blacksburg, VA). When deciding on which GDH antigen test kit to use (Tables 4.15 and 4.17), it is important to consider that the sensitivity and negative predictive value of the test method may decrease over time due to antigenic drift and/or reagent variability. It has also been proposed that the reason why some studies found the GDH antigen test to be very sensitive for the

detection of *C. difficile* (and therefore useful for screening purposes), while other studies have not, may be in part due to geographical differences in the reactivity of strain ribotypes [112, 126], and also freezing and thawing the stool specimen prior to testing [112]. Nonetheless, the GDH component of a new rapid lateral flow EIA, i.e., the *C. Diff* Quik Chek Complete test (TechLab Products, Blacksburg, VA) (Figure 4.11) was reported to have very good sensitivity and specificity compared to molecular tests and toxigenic culture (97.6–100% and 88.1–100% respectively) [106, 112, 121], and also to the cytotoxin

test $\pm$ toxigenic culture (98.2–100% and 93.6–100% respectively) [3, 124]. It should be noted that a similar test, i.e., the *C. Diff* Quik Chek test for GDH only (GDH-Q, TechLab Products, Blacksburg, VA), exhibited lower sensitivity (86.3%) and specificity (92.7%) compared to a molecular and cytotoxin gold standard, after a false negative rate was extrapolated and applied to all initially GDH-Q-negative specimens [78]. In addition, a study by Tenover et al. found that one laboratory site that used the GDH-Q to test 114 specimens reported a low sensitivity (61.5%) compared to enriched toxigenic culture [126]. The study also found that the GeneXpert *C. difficile* molecular assay was significantly more sensitive compared to the combined averaged sensitivity of the GDH-Q and *C. Diff* Chek-60 test for GDH (TechLab) for detection of ribotypes other than 027. The lower sensitivity of the GDH tests could not be attributed to any specific ribotype, and the individual sensitivities of the two GDH tests were not reported. In contrast, in a subsequent study 104 *C. difficile* isolates comprising 77 different ribotypes and 25 nontoxigenic ribotypes were tested for GDH using the GDH-Q, *C. Diff* Chek-60 test, and *C. Diff* Quik Chek Complete, but no effect of ribotype on the detection of GDH-producing *C. difficile* was found [22]. Antigenic variation in GDH also did not affect the detection of GDH-producing *C. difficile*. Additional studies are needed to confirm the effect of ribotype (or lack thereof) and/or other factors mentioned above on GDH test sensitivity. The above study results indicate, however, that before implementing GDH screening tests for first line detection of *C. difficile*, it would be optimal for laboratories to establish the sensitivity of the GDH test compared to a molecular assay or toxigenic culture (i.e., in order to validate adequate detection of toxigenic *C. difficile* using the proposed GDH screening test). In summary, the performance characteristics of each type of test kit for GDH varies, and thus the laboratory director should evaluate the merits of each kit on an individual basis.

The ImmunoCard *C. difficile* (Meridian Diagnostics, Cincinnati, Ohio) was a rapid enzyme immunoassay that detected GDH and was used as a screening test for detecting *C. difficile* in stool specimens from patients suspected of having CDI. The sensitivity of the ImmunoCard (84–92%) was reported to be much better than that of latex agglutination, and the specificity of the ImmunoCard ranged from 95 to 100% [5, 108]. The card was discontinued by the manufacturer. A new card, the ImmunoCard *C. difficile* GDH (Meridian Bioscience, Cincinnati, OH) (Figure 4.13) was recently approved by the FDA for use in the United States. According to the manufacturer, compared to culture the ImmunoCard's sensitivity and specificity ranged from 95.1 to 100% and 80.5 to 95.2%, respectively, when the test was evaluated at five test sites.



Figure 4.13 ImmunoCard *C. difficile* GDH. (Photograph courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)

4.7.2 Enzyme immunoassays for toxin A and toxin B

As mentioned above, using a stand-alone enzyme immunoassay (EIA) for detection of *C. difficile* toxins A and B testing is no longer commonly used because EIA tests lack adequate sensitivity for the diagnosis of CDI [78, 111]. Multiple EIAs are marketed by various manufacturers for detection of TcdA and TcdB in stool (Tables 4.15 and 4.16). Most of these products are in the form of manual test kits, and at least one is designed for use with an automated laboratory instrument that can perform other tests, i.e., the VIDAS system (bioMérieux, Marcy l'Etoile, France; Figure 4.15) [7].

In general, these EIAs vary in sensitivity (45–95%) and specificity (75–100%) compared to the cytotoxin assays [78, 112, 132], but when compared to enriched toxigenic culture they are considered even more insensitive and should not be relied upon as the sole laboratory test for diagnosing CDI [10, 50, 111, 114, 126]. All EIAs detect toxin A efficiently, but not all EIAs detect toxin B well due to various TcdB isoforms [119]. Therefore, the laboratory director should evaluate the merits and performance of each EIA test kit on an individual basis.

Testing for both toxins A and B in the clinical laboratory is clinically relevant. Testing only for toxin A is not usually performed, due to the increased incidence of toxin A-negative-toxin B-positive strains of *C. difficile* involved in CDI [84]. New cases and outbreaks with these strains are being reported with increasing frequency worldwide [1, 33, 70, 81, 94, 98], and in these cases, EIAs for toxin A are repeatedly negative.



Figure 4.14 ImmunoCard Toxins A&B. (Photograph courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)

The EIA tests for both toxins A and B can be used to aid in detecting CDI caused by toxin A-negative-toxin B-positive strains [39]. Toxin A-positive-toxin B negative stains are only rarely encountered, but they still may cause CDI [120].

According to a recent College of American Pathologists (CAP) proficiency testing survey for stool pathogens, the most common EIA test kits used by the majority of participants for the detection of *C. difficile* toxins, and/or GDH, were the ImmunoCard Toxins A&B test (Meridian Diagnostics, Cincinnati, Ohio), the Premier Toxin A&B EIA (Meridian Diagnostics, Cincinnati, Ohio), the Xpect Toxin A/B test (Remel Inc., Lenexa, Kansas), and a new lateral flow assay, i.e., the *C. Diff* Quik Chek Complete test (TechLab/Wampole) [34], and these test systems will be discussed below.

The ImmunoCard Toxins A&B (Meridian Bioscience, Cincinnati, Ohio) is a rapid membrane enzyme immunoassay that detects toxins A and B and takes only 15–20 min to perform (Figure 4.14). Compared to toxigenic culture (with or without the cytotoxin assay as part of the gold standard), studies have shown that the sensitivity and specificity of the ImmunoCard have ranged from 48 to 80% and 95.1 to 99% respectively [2, 4, 60, 114]. Some authors suggest the true sensitivity of the ImmunoCard Toxins A&B is about 67.7% when larger numbers of specimens are tested [2]. Compared to toxigenic culture, the ImmunoCard Toxins A&B and the automated VIDAS Toxin CDA/B assay (bioMérieux, Marcy l'Etoile, France) (Figure 4.15) yielded similar results [2, 60]. In one study when compared to the Xpect Toxin A/B (sensitivity of



Figure 4.15 VIDAS immunoassay analyzer. (Photograph courtesy of bioMérieux.)



Figure 4.16 Premier Toxins A&B test. (Photograph courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)

49%) (Remel Inc., Lenexa, KA) and the Tox A/B Quik Chek (sensitivity of 54.9%) tests using toxigenic culture as the gold standard, the ImmunoCard Toxins A&B was the quickest and the most sensitive test (66.7%) [4].

The Premier Toxins A&B test (Meridian Bioscience, Cincinnati, OH) (Figure 4.16) is a microwell EIA that detects toxin A at levels of ≥ 1 ng/mL and toxin B at ≥ 2.4 ng/mL in stool from adult patients. Past reports indicate that when the cytotoxin test was used as the gold standard, the sensitivity and specificity of the Premier Toxins A&B test was high (96.8–98.7%, 94.3–97.3%) [97, 130]. More recent studies, however, that used GDH screening tests (performed on specimens in parallel with the Premier Toxins A&B test and followed by cytotoxin testing of discordant samples) yielded significantly lower sensitivity and comparable specificity, 59.5–73% and 98–99.4%, respectively [40, 51]. When toxigenic culture was used



Figure 4.17 Thermo Scientific™ Xpect™ Toxin A/B test. (Photo courtesy of ThermoFisher Scientific, copying is prohibited.) (See insert for color representation of the figure.)



Figure 4.18 *C. Difficile* Tox A/B II. (Photograph courtesy of Alere, Inc.) (See insert for color representation of the figure.)

as the gold standard, the sensitivity and specificity of the Premier Toxins A&B test ranged from 48 to 58.3% and 94.7 to 98% respectively [101, 114]. These studies further demonstrate that the specific gold standard used to assess EIA test performance will greatly affect the calculated sensitivity and specificity of the EIA test being evaluated, and that when toxigenic culture is used as the gold standard, EIA tests used as stand-alone tests lack adequate sensitivity for the diagnosis of CDI [76, 78, 111].

The Xpect Toxin A/B test (Remel Inc., Lenexa, Kansas) (Figure 4.17) is a rapid immunochromatographic test for the direct, qualitative detection of toxin A and/or B. The kit was evaluated by Alcalá et al. along with the ImmunoCard Toxins A&B, and the *C. difficile* Tox A/B II test (TechLab Products, Blacksburg, VA) (Figure 4.18), for the diagnosis of CDI using a combination of the cytotoxin assay and cytotoxic (toxigenic) culture as the gold standard [4]. The sensitivity and specificity for the Xpect

Toxin A/B test was 49% and 95.8%, respectively, and the median time to test five stool specimens was 28 min.

The *C. Diff* Quik Chek Complete test (TechLab Products, Blacksburg, VA) (Figure 4.11) is a third generation rapid lateral flow assay (rapid membrane enzyme immunoassay) for the simultaneous detection of *C. difficile* GDH antigen and toxins A and B in a single reaction well. The kit was reported to have very good sensitivity and specificity for the detection of CDI compared to molecular tests and toxigenic culture (97.6–100% and 88.1–100% respectively) [106, 112, 121], and also to the cytotoxin test ± toxigenic culture (98.2–100% and 93.6–100% respectively) [3, 124]. Sharp et al. found 88% of 284 stool specimens were accurately screened as either positive or negative for the presence of toxigenic *C. difficile* in less than 30 min, and use of a molecular test (GeneXpert *C. difficile* PCR) to analyze discrepant results allowed easy and highly sensitive and specific diagnosis of CDI [112]. Use of this two-step algorithm has been proposed by others [124]. It should be noted, however, that in the study by Sharp et al. that the sensitivity and specificity for toxin A and/or toxin B detection component of the kit was 61.9% compared to enriched toxigenic culture. Potential issues concerning the sensitivity of the GDH component of the kit have been discussed above.

The *C. difficile* Tox A/B II test (TechLab Products, Blacksburg, VA) (Figure 4.18) is a second-generation microwell EIA for the detection of *C. difficile* toxins A and B in fecal specimens. The test is completed within 1 h, alternatively, a rapid format may be used for results within 30 min. In a recent study, the *C. difficile* Tox A/B II test exhibited identical sensitivity compared to the Premier Toxins A&B test (96.3%) and similar specificity (87% versus 93.5%, respectively), using the cytotoxin assay as the gold standard [97]. To meet the criterion for a positive result, the study required that a single patient specimen test positive by EIA to be considered a positive result (in contrast to older studies that required three specimens to be tested, and if any of the three specimens tested positive, the result was classified as positive). In other words, testing in the study by Musher et al. simulated the initial EIA test result that would be reported to physicians. The study's conclusion was that the *C. difficile* Tox A/B II test, Premier Toxins A&B test, and the ProSpecT *Clostridium difficile* microwell EIA (Remel Inc., Lenexa, KS), exhibited relatively good sensitivities and specificities compared to the cytotoxin assay. Using a single specimen, the three test kits were found to generate a highly reliable result compared to the cytotoxin assay, and the authors concluded that multiple specimens need not be submitted for analysis. Of course, the laboratory director must also consider the fact that the cytotoxicity test has poor sensitivity compared to toxigenic culture and new molecular tests that detect the toxin B gene (*tcdB*) [78, 124], and using a stand-alone enzyme immunoassay (EIA) for *C. difficile* toxin testing is no longer commonly used

because EIA tests lack adequate sensitivity for the diagnosis of CDI [76, 78, 111].

The ranges of sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) for commonly used EIAs are as listed in Table 4.17.

4.7.3 Molecular tests

Molecular tests are commercially available that detect the toxin B gene (*tcdB*) within the *C. difficile* chromosomal pathogenicity locus (PaLoc), and overcome the poor sensitivity of EIA tests for detection of *C. difficile* toxins. These real-time PCR assays include the BD GeneOhm Cdiff using the SmartCycler (Cepheid, Sunnyvale, CA) (Figure 4.19) and BD MAX Cdiff assay (Becton Dickinson, Franklin Lakes, NJ), Xpert *C. difficile* (Cepheid, Sunnyvale, CA) (Figures 4.20 and 4.21), the Prodesse ProGastro Cd (GenProbe, San Diego, CA), the Simplexa *C. difficile* Universal Direct Kit (Focus Laboratories, Cypress, CA), and the Quidel Molecular Direct *C. difficile* assay (Quidel Corporation, San Diego, CA). The Quidel Molecular Direct *C. difficile* assay is a qualitative multiplexed PCR test for the direct detection of the toxin B gene or toxin A gene (*tcdA*) sequences. In addition to the toxin B gene, the Xpert *C. difficile* assay also targets the binary toxin gene and deletion of the *tcdC* gene to presumptively identify the 027/NAP1/BI strain. The Verigene *C. difficile* test (Nanosphere, Inc., Northbrook, IL) detects both *tcdB* and *tcdA* within the PaLoc. The Illumigene *C. difficile* (Meridian Bioscience, Cincinnati, OH) is a loop-mediated isothermal amplification assay that targets *tcdA* within the PaLoc (Figure 4.22). The AmpliVue *C. difficile* Assay is an isothermal helicase-dependent amplification



Figure 4.19 BD GeneOhm Cdiff using the SmartCycler. (Photograph courtesy of Cepheid and courtesy and © Becton, Dickinson and Company.)



Figure 4.20 Xpert *C. difficile* Cartridge. (Photograph courtesy of Cepheid.)



Figure 4.21 Xpert *C. difficile* GeneXpert instruments. (Photograph courtesy of Cepheid.)



Figure 4.22 Illumigene. (Photograph courtesy of Meridian Bioscience.)

(HDA) assay (Quidel Corporation, San Diego, CA) that utilizes a self-contained disposable amplicon detection device for the detection of the toxin A gene, i.e. a conserved DNA fragment which is intact in all known A+B+ and A-B+ toxinotypes of *C. difficile*.

Multiple studies have shown that results from the above molecular assays correlate reasonably well with results of enriched toxigenic culture, with sensitivities ranging from 77.3 to 100% [76, 112, 116, 117, 126, 127]. Importantly, a recent study by Tenover et al. found that the GeneXpert *C. difficile* assay had equivalent sensitivity for the detection of ribotype 027 strains compared to two GDH test methods (i.e., the *C. Diff* Chek-60 and *C. Diff* Quick Chek, TechLab, Blacksburg, VA) but the molecular assay was significantly more sensitive compared to the combined averaged sensitivity of the GDH tests for detection of ribotypes other than 027. The lower sensitivity of the GDH tests could not be attributed to any specific ribotype [126]. Individual sensitivities of the two GDH tests were not reported. Each test was performed exclusively at a single study center on 268 stool specimens (*C. Diff* Chek-60) and 114 stool specimens (*C. Diff* Quick Chek). In contrast, in a subsequent study 104 *C. difficile* isolates comprising 77 different ribotypes and 25 nontoxigenic ribotypes were tested for GDH using the GDH-Q, *C. Diff* Chek-60 test, and *C. Diff* Quick Chek Complete, but no effect of ribotype on the detection of GDH-producing *C. difficile* was found [22]. While further studies are needed, the results of the study indicate that individual laboratories implementing GDH screening tests for first line detection of *C. difficile* first establish the sensitivity of the GDH test compared to a molecular assay.

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Rapid Antigen Devices and Instruments for the Detection and Identification of Viruses

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Diagnostic virology has experienced a great evolution over the past decade. When the first edition of this book was published, there was a limited menu of molecular assays for the detection and identification of viruses. Cell culture remained the “gold standard” for most viral assays. Due to the time required to grow most viruses and the technical expertise this method requires, rapid antigen detection tests (RADT) were being developed at a fast pace and becoming very popular as adjunct detection assays to cell culture. These included numerous monoclonal antibody reagents for immunofluorescence analyses, direct and indirect fluorescent antibody assays (DFA and IFA) for direct specimen testing as well as cell culture confirmation. In addition, many enzyme immunoassay (EIA) kits were being developed in both manual and automated formats. The diagnostic virology literature contained many studies evaluating these new assays, most often using cell culture as the gold standard. Over the past decade there has been a surge in the development of molecular-based diagnostic methods for all viral pathogens. These assays were relatively expensive in the beginning and required technical skills and expertise that were not available in many diagnostic laboratories. In the past several years as more economical, simpler, and automated molecular assays have become available, their use in diagnostic laboratories has become much more common, with many laboratories adopting molecular assays as their primary or exclusive testing method. As the improved analytical sensitivity of the molecular assays became apparent, they gradually replaced cell culture as the gold standard. Many rapid antigen detection assays were found to be significantly less sensitive when compared to molecular tests rather than cell culture. Currently, rapid antigen assays are primarily used as rapid screening tests where negative results are further evaluated by the more sensitive

molecular assays. A rapid assay that can be performed in outpatient clinics, or in laboratories lacking adequate staff or resources to provide molecular testing 24 h a day, with immediate results often available before the patient leaves the clinic can impact treatment decisions. RADTs are simple to perform and give results in 15–30 min, but are limited to detecting influenza A and B, respiratory syncytial virus (RSV), and a few others [24, 30, 36]. DFA can be completed in 30–60 min and can detect influenza A and B, RSV, parainfluenza types 1, 2, and 3, adenovirus, and human metapneumovirus [36]. DFA techniques require a higher level of expertise to interpret and equipment and may not be available outside larger hospitals and reference laboratories. Because of this, RADTs are still used as the “front-line” diagnostic tests in many smaller laboratories, clinics and physician offices. However, the lack of sensitivity (and specificity in some instances) greatly limits the actual clinical utility of many of these tests [5, 7, 13, 14, 19, 24, 29]. Reported sensitivities for influenza viruses and RSV range from 10–85% and 50–98% respectively [24]. The broad range in sensitivity is due to numerous factors, including sample type, age of the patient, the time of specimen collection after the onset of symptoms, transport and storage of the specimen prior to testing, strain variations in the virus being tested for, and the particular assay used for testing and for comparison [31, 33]. In general, RADTs require 10^5 to 10^6 virus particles to give a positive result. Cell culture and molecular assays may detect as few as 10 virus particles [32].

Nasopharyngeal (NP) flocked swabs have been shown to provide improved specimens for the detection of respiratory viruses due to their ability to collect and release respiratory epithelial cells [1, 8, 35]. (Figure 5.1) It is important that specimens be collected within 3–5 days from the start of symptoms then be transported and stored

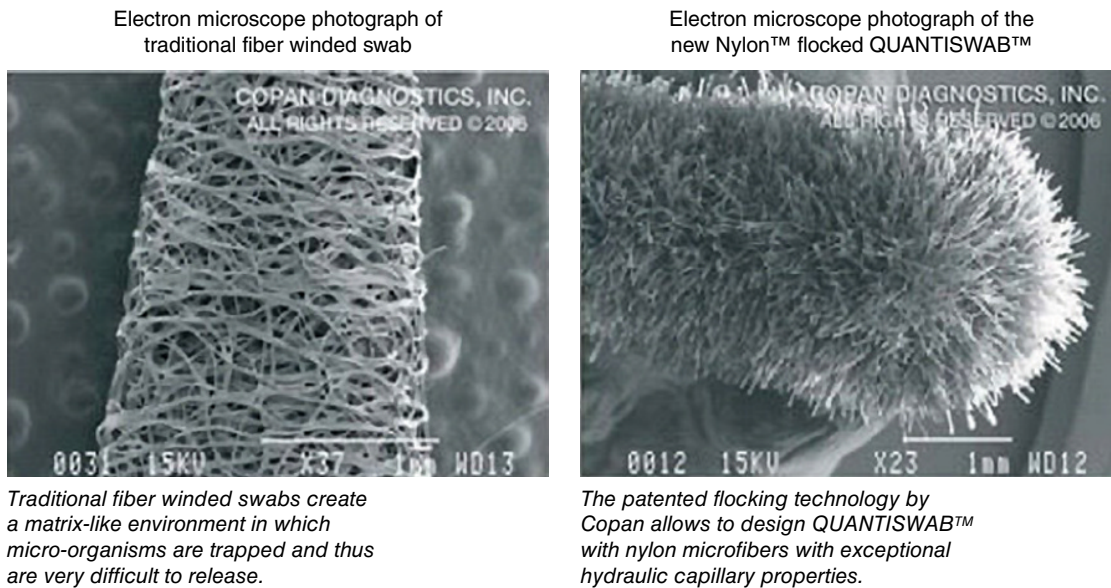


Figure 5.1 Copan flocked swab versus traditional swab. (Courtesy of Copan Diagnostics.)

refrigerated to improve test performance [31, 33]. In the past couple of years, new antigen detection assays have become available that utilize inexpensive instruments to evaluate test results. These assays have been demonstrated to have increased sensitivity when compared to the earlier assays [23, 28].

This chapter will review current literature evaluating the more common commercially available reagents and kits that have Food and Drug Administration (FDA) clearance as *in vitro* diagnostic (IVD) assays. Studies that compare the performance of these assays to molecular methods will be included when available. Numerous testing strategies are used in these assays, some are Clinical Laboratory Improvement Amendments (CLIA) waived and can be used as point-of-care (POC) tests. New pooled antibody reagents labeled with different fluorescent dyes, and improved specimen collection devices using flocked swabs have aided in improving the performance of the rapid methods. Molecular methods for detecting virus are reviewed in this manual in a separate chapter.

Due to the continual development on new assays, reviewing current literature and references such as those provided by the American Society for Microbiology, the Centers for Disease Control and Prevention (CDC) and the FDA website (<http://www.accessdata.fda.gov/scripts/cdrh/devicesatfda/index.cfm>) for the most up-to-date listing of IVD assays is strongly recommended. The Participant Summaries from the College of American Pathologists (CAP) Proficiency Testing Surveys provide excellent comparisons of the most frequently used assays in a broad multilaboratory “real-world” setting. Simply reviewing an assay’s performance in literature published years ago or reading package inserts may not give an accurate indication of how the assay will perform in your

laboratory. Although most new diagnostic assays are directed at molecular testing, new and improved assays for direct antigen detection are continually becoming available and reviewing the resources listed above is useful in selecting the “best” assay for your laboratory’s needs.

5.1 Influenza viruses

Influenza viruses are enveloped, single-stranded RNA viruses. They belong to the family *Orthomyxoviridae* and they are divided into three different groups (A, B, and C) based on the antigenic reactivity of the group-specific antigens found on the nucleocapsid and matrix proteins. The influenza A group is further subtyped based on the antigenic reactivity of the hemagglutinin and neuraminidase proteins found on the viral envelope (e.g., H1N1). The influenza genome is segmented, with A and B having eight segments and C seven segments. These segments may reassort *in vivo*, which may result in the appearance of new strains of influenza. This may result in the development of an influenza pandemic, as occurred recently in 2009. In addition to reassorting the RNA segments, the genome is subject to frequent gene mutations, which may circumvent a patient’s immunity plus reduce the sensitivity of antibody-based assays [2].

Influenza virus outbreaks occur every year, usually during the winter months. The 2009 pandemic was unusual due to the outbreak occurring during the summer and fall. Initially, the columnar epithelial cells of the upper respiratory tract are infected, commonly producing illness characterized by fever, pharyngitis, and myalgias. More severe disease results from the virus spreading to the lower respiratory tract resulting in tracheobronchitis

and pneumonia. Frequently, pneumonia is the result of a secondary bacterial infection. Other complications of influenza virus infection include encephalopathy, myocarditis, pericarditis, and myositis.

In the distant past, serology and cell culture were the only laboratory methods for detecting an infection with influenza virus. Virus isolation may take 1–2 days up to 2 weeks [2]. To address this long delay in virus detection, numerous commercially developed kits utilizing monoclonal antibodies became available in the mid-1980s. These kits were based on either the immunofluorescence of directly stained cells observed microscopically, or numerous enzyme-linked immunosorbent assay (ELISA) methods to detect viral antigens. Cell culture, immunofluorescence and ELISA assays have remained the primary methods for detecting respiratory viruses, however, these assays are gradually being replaced by molecular assays due to the greater sensitivity and the shorter turnaround times (TAT) when compared to cell culture [20, 37]. The commercially available FDA-approved assays are listed in Table 5.1. The sensitivity and specificity of these assays vary greatly depending on the type of assay they are compared to. Those compared to cell culture had much better performance characteristics than when they were compared to molecular assays. When investigating an assay, review the most recent literature to determine how it performs against a molecular assay in detecting the current strains.

Test performance is often affected by the appearance of new strains of influenza. Simple point mutations and gene rearrangements can greatly affect the performance of rapid detection assays that rely on specific antibodies to detect the virus. RADTs, DFA, and cell culture assays were all demonstrated to have suboptimal performance in detecting the new Flu A H1N1 strain [4, 15, 19, 29]. At the beginning of the 2009 pandemic, the CDC evaluated three widely used RADTs. Sensitivities ranged from 40 to 69% for the new H1N1 strain compared to 60 to 83% for the seasonal H1N1 strain. Following the pandemic, the CDC released a guidance report for using rapid diagnostic testing for influenza to highlight many of these issues (http://www.cdc.gov/flu/professionals/diagnosis/clinician_guidance_ridt.htm). In the report, 11 commercially available Rapid Influenza Diagnostic Tests, United States 2011–2012 were evaluated and summarized (Morbidity and Mortality Weekly Report (MMWR) November 2, 2012; Vol. 61, No. 43). The sensitivities for detecting 23 different strains of influenza A and B were very diverse. Assays with relatively good sensitivities for some strains had much lower sensitivities for detecting other strains.

Regardless of the sensitivity and specificity issues with the RADTs, they remain the most commonly used assays due to their ease of use, rapid TAT and relative low cost

Table 5.1 Rapid diagnostic tests for influenza virus

Procedure (manufacturer/distributor)	Influenza virus types detected	Approved specimens	Test time (min)
3M Rapid Detection (3M)	A and B	NP swab/aspirate Nasal wash/ aspirate	15
BD Veritor System for Rapid Detection of Flu A&B* (Becton Dickinson)	A and B	NP swab/nasal swab	10
BinaxNOW Influenza A&B (Alere) (Figure 5.2)	A and B	NP swab Nasal aspirate/ swab/wash	15
BioSign Flu A+B* (Princeton BioMedTech)	A and B	NP aspirate/ swab/wash, nasal swab	15
Clearview Exact Influenza A&B (Alere)	A and B	Nasal swab	15
Directigen EZ Flu A+B (Becton Dickinson) (Figure 5.3)	A and B	NP aspirate/ swab/wash Throat swab	15
OSOM Influenza A&B (Sekisui Diagnostics)	A and B	Nasal swab	10
QuickVue Influenza Test* (Quidel)	A or B	Nasal aspirate/swab/ wash	10
QuickVue Influenza A+B* (Quidel) (Figure 5.4a and b)	A and B	NP swab Nasal aspirate/ swab/wash	10
SAS FluAlert A&B* (SA Scientific)	A and B	NP swab Nasal aspirate/ swab/wash	15
SAS FluAlert A* (SA Scientific)	A only	Nasal wash/ aspirate	15
SAS FluAlert B (SA Scientific)	B only	Nasal wash/ aspirate	15
Sofia Influenza A+B (Quidel)	A and B	NP aspirate/swab/ wash Nasal wash	15
TRU FLU (Meridian Bioscience)	A and B	NP aspirate/swab Nasal wash	15
XPECT Flu A&B (Remel/ThermoFisher)	A and B	Nasal wash/swab Throat swab	15

* CLIA-waived test that can be used in any office setting.

compared to molecular methods. For many laboratories and clinics, lack of experienced staff and funds dictate these assays are the only tests available. Educating clinicians of the diagnostic performance issues of these tests is an important yet challenging responsibility of the laboratory director and staff. More recently, the FDA has approved three new RADT systems for detecting influenza A and B. These lateral flow assays are unique in that they use readers to evaluate the results, increasing sensitivity and reducing the subjectivity being interpreted by technologists. In addition, both systems provide printouts of the results that may be kept as a record of the patients test.

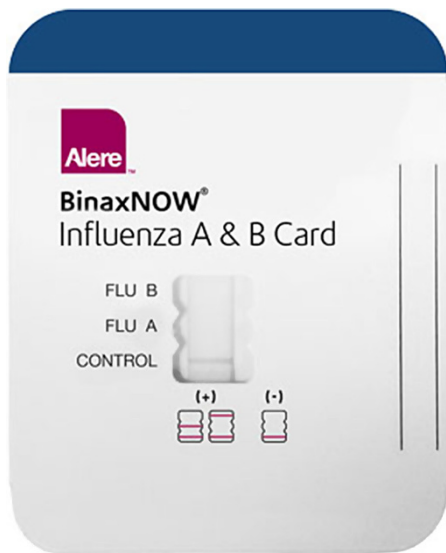


Figure 5.2 BinaxNOW Influenza A+B card. (Courtesy of Alere, Inc.)



Figure 5.3 BD Directigen EZ Flu A+B kit. (Courtesy and © Becton, Dickinson and Company.)

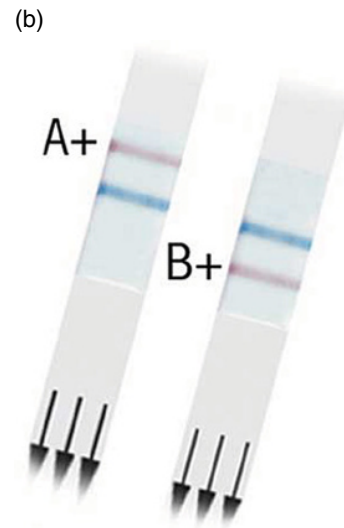


Figure 5.4 (a) QuickVue Influenza A+B Kit. (b) Quidel QuickVue Influenza A+B test strips. (Courtesy of Quidel Corporation.)

5.1.1 BD Veritor System for rapid detection of influenza A and B

This is a CLIA waived assay that detects and differentiates influenza A and B in approximately 10 min (Figure 5.5a and b). Specimen preparation is minimal and the results may be read on the instrument and visually. When compared to reverse transcription polymerase chain reaction (RT-PCR), the assay had a sensitivity of 72% for influenza A and 69.3% for influenza B [10]. A study that compared the Veritor, Sofia, and the Binax Now with RT-PCR on specimens collected from patients < 18 years of age found sensitivities of 93.8% for influenza A and 94.2% for influenza B with the Veritor assay. The Sofia assay gave similar results, with sensitivities of 95.8% for influenza A and 98.1% for influenza B [26]. A similar study looking at

frozen specimens from pediatric patients reported sensitivities of 89.6% for influenza A and 98.8% for influenza B for the Veritor assay [17]. When evaluating the sensitivities of these studies it is important to note that all the specimens were collected from pediatric patients who are known to shed higher titers of virus than adults.

5.1.2 Sofia Influenza A & B

The Quidel Sofia Influenza A & B fluorescent immunoassay (FIA) is similar to the BD Veritor system in that specimen preparation and test setup are simple and fast and both are performed using lateral flow devices and interpreted using a reader (Figure 5.6a and 5.6b). The Sofia assay uses immunofluorescent technology to detect positive results and therefore can only be evaluated using

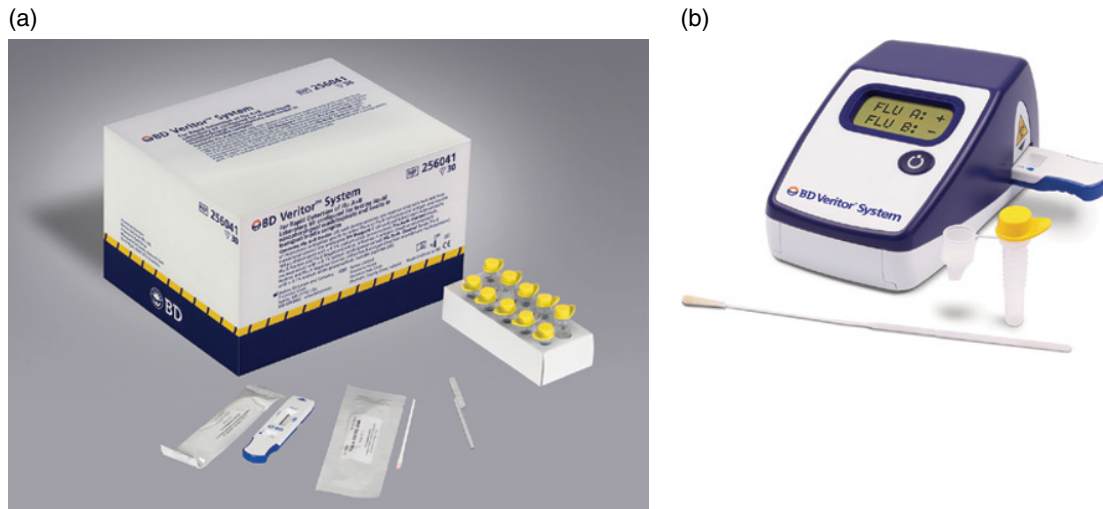


Figure 5.5 (a) BD Veritor kit. (b) BD Veritor Instrument and cartridge. (Courtesy and © Becton, Dickinson and Company.)

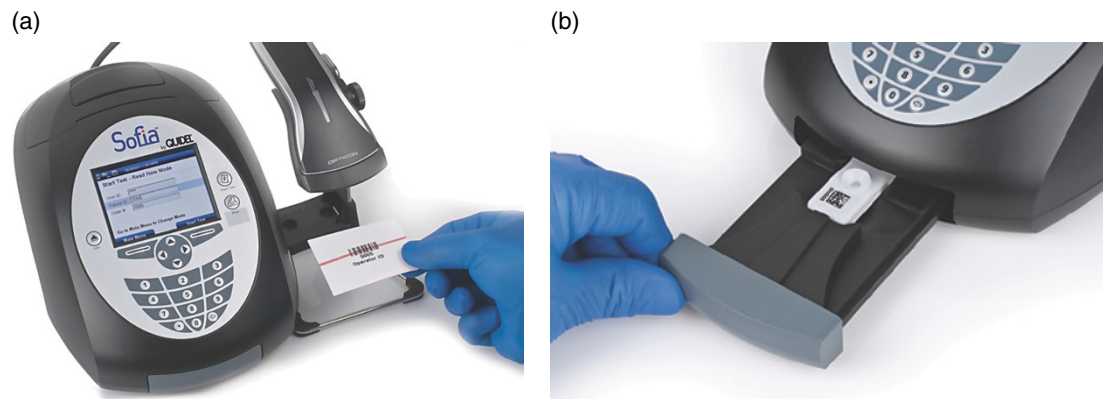


Figure 5.6 (a) Sofia FIA instrument. (b) Inserting cartridge into Sofia Reader. (Courtesy of Quidel Corporation.) (See insert for color representation of the figure.)

the reader and not visually. When evaluated using specimens from all age groups, a study comparing it to RT-PCR found sensitivities of 82.2% for influenza A and 77.8% for influenza B [23]. A similar study performed with frozen pediatric specimens from two respiratory seasons found similar results, with sensitivities ranging from 79.8% to 80.2% for influenza A and 62.5% to 67.5% for influenza B. When only specimens with cycle threshold (CT) values of < 34 were considered, sensitivities increased to 86.7–97.7% for influenza A and 81.7–86.7% for influenza B [28].

5.1.3 3M Rapid Detection Flu A+B Test

The 3M Rapid Detection Flu A+B Test assay is similar to the Sofia in that it also uses immunofluorescence and a reader to detect positive results. It was evaluated using 500 fresh respiratory samples, collected from patients aged 5 days to 99 years and compared to R-Mix cell culture. Sensitivities of 70.1% for influenza A and 86.5% for influenza B were reported [14].

5.2 Respiratory syncytial virus

Respiratory syncytial virus (RSV) is a large, pleomorphic enveloped virus with a single-stranded, negative sense RNA genome, which is approximately 300–350 nm in diameter and composed of approximately 15,000 nucleotides [37]. It is a member of the family *Paramyxoviridae* and subfamily *Pneumovirinae* (which contains metapneumovirus (MPV), human MPV, and pneumovirus, which contains RSV). The proteins of RSV are quite similar to the proteins of other viruses in the paramyxovirus group. These proteins include a phosphoprotein, a nucleocapsid protein, a large protein that exhibits polymerase activity, two surface proteins (F, the viral fusion protein, and G, the attachment glycoprotein), and a matrix protein. Two major antigenic groups comprise RSV, A and B, and further minor antigenic variability exists within these groups. Subgroup A has been reported to be more virulent than subgroup B, leading to more severe illness in hospitalized children [34].

The viruses in the RSV group are a significant cause of lower respiratory infections in both infants and young

Table 5.2 Rapid diagnostic tests for RSV virus

Procedure (manufacturer/distributor)	Approved specimens	Test time (min)
BD Veritor System for Rapid Detection of RSV* (Becton Dickinson)	NP swab/nasal swab	10
BinaxNOW Influenza RSV (Alere)	NP swab Nasal aspirate/swab/ wash	15
Clearview Exact RSV (Alere)	Nasal Swab	15
Directigen EZ RSV (Becton Dickinson)	NP aspirate/swab/wash Throat swab	15
QuickVue RSV Test* (Quidel)	Nasal aspirate/swab/wash	10
SAS RSVAAlert* (SA Scientific)	NP swab Nasal aspirate/swab/ wash	15
Sofia RSV (Quidel)	NP aspirate/swab/wash	15
TRU RSV (Meridian Bioscience)	NP aspirate/swab Nasal wash	15
XPECT RSV (Remel/Thermofisher)	Nasal wash/swab Throat swab	15

* CLIA-waived test that can be used in any office setting.

children, and increasingly recognized and identified in the adult population throughout the world. Since the virus is somewhat labile, it is occasionally difficult to recover in cell culture and may be killed during transport from the patient to the laboratory. Additionally, RSV, when grown in cell culture may take up to a week to recover and detect either by cytopathic effect (CPE) and/or by immunofluorescence. During the peak of RSV season, RADTs can be useful in detecting the virus in ill children. However, these assays have been shown to be insensitive when used for adults due to lower titers and shorter periods of shedding [11, 16, 18, 27].

RSV was one of the first viruses targeted for immunoassay development and many assays were developed and evaluated in the early 1980s. These early studies emphasized the importance of proper specimen collection, primarily the number of respiratory cells obtained, as an important factor in test efficacy. The reader is referred to the first edition of this manual for additional information on immunoassay development and published test performance. Presently there are numerous RADTs, many CLIA waived for POC testing (see Table 5.2). Similar to selecting an assay for detecting influenza, consulting the FDA and CAP sites for the most current information is strongly recommended. The sensitivities and specificities of these assays vary widely [6]. The BD Veritor and Quidel Sofia systems also have RSV assays with similar performance characteristics as the influenza assays [3, 25]. Other assays may be available; the user should refer to current sources for additional tests, kits, performance characteristics, and availability.

5.3 Immunofluorescence staining of respiratory viruses

Over the past 20, to almost 40, years a number of methods have been used to detect respiratory and other viruses from clinical specimens. These include cell culture, using a number and variety of different cell cultures and methods (e.g., traditional cell culture and shell vial methods), rapid antigen detection by EIA or ELISA, immunofluorescence (both direct and indirect immunofluorescence), and a variety of molecular amplification techniques. The gold standard for virus detection, previously accepted as cell culture, is rapidly changing to a molecular gold standard (or at least considered an enhanced gold standard). It is readily understood that these methods, reagents, and protocols can vary considerably in many contributing factors such as: (i) variety of viruses detected and identified; (ii) performance characteristics of the test, such as specimen collection, time of test performance, type of result obtained; (iii) whether an instrument is needed; (iv) the cost of many components of the test such as reagents, instruments, and technologist time. An additional factor is whether the method/test kit is readily available as a US FDA-approved kit or whether the laboratory must develop its own primers, and optimize testing methods. At the time of this writing several proposals are being entertained in the United States to bring laboratory-developed tests more under the umbrella of the US FDA and its regulatory control.

In the early 1980s Gardner and McQuillan studied aspects of immunofluorescence and its impact on early diagnosis of viral respiratory infections [12]. At the time, they used polyclonal antibodies, which were not readily available to most investigators. Later in the 1980s, monoclonal antibodies became more readily commercially available and this led to the development and widespread use of immunofluorescence as a common tool in the rapid diagnosis of viral respiratory infections. Monoclonal antibodies are now widely available for the detection of most respiratory viruses (influenza A and B, RSV, parainfluenza viruses 1,2, and 3, adenovirus, and human metapneumovirus) [21]. These reagents may be used for direct identification of infected cells in NP swabs, aspirates, and washes, or for identification and typing of viruses growing in cell culture. The use of a cytocentrifuge in preparing the cell spots has been demonstrated to greatly enhance the sensitivity of immunofluorescence staining assays [9]. The DFA assays have been reported to be more sensitive than other types of rapid detection assays, plus they offer the advantage of allowing the adequacy of the specimen to be evaluated [22].

Numerous companies provide a range of fluorescent antibody FDA-approved reagents for detecting respiratory viruses. Quidel DHI has single mouse monoclonal antibodies for all the respiratory viruses listed above as well

as kits that combine multiple monoclonals into pools that allow for the detection of two viruses (D³ Duet) in one well by labeling the different antibodies with different fluorescent tags. The D³ Ultra detects seven viruses using a pooled antibody reagent (Figure 5.7). When a specimen is positive, specific antibodies are then used to identify which virus is present (Figure 5.8a and b). The D³ FastPoint detects eight viruses directly from patient specimens. Flocked swabs are recommended for use with all these reagents.

Bartels also produces a respiratory virus screening and detection kit which contains an antibody pool for influenza A and B, parainfluenza 1,2, and 3, and RSV. Individual monoclonal reagents are used to identify which virus is present. Monoclonal reagents are also available for the individual viruses, which are useful during outbreaks or epidemics. These reagents use the indirect immunofluorescent method. Light Diagnostics has a similar kit that detects the same six viruses listed above and is available in both DFA and IFA configurations. Millipore has immunofluorescence assays for human metapneumovirus (Light Diagnostics Human Metapneumovirus Direct Immunofluorescence Assay) and a combination assay (SimulFluor) for detecting both hMPV and RSV in a

single staining reaction using two fluorescent dyes to label the specific antibodies.

5.4 Herpes simplex virus, varicella zoster virus, cytomegalovirus

The *Herpesviridae* family is classified into three subfamilies, the alphaherpesviruses (including herpes simplex virus (HSV) types 1 and 2, varicella zoster virus (VZV), and herpes B virus), the betaherpesviruses (including cytomegalovirus (CMV), human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7)), and the gamma-herpesviruses (including Epstein-Barr virus (EBV), and human herpesvirus 8, HHV-8 also known as Kaposi's sarcoma-associated herpesvirus, KSHV).

Herpes simplex and varicella zoster viruses are among those that can cause exanthems in humans. Other disease manifestations caused by these viruses include ocular disease (e.g., keratitis and acute retinal necrosis), infections in the newborn and in the immunocompromised host (including transplant recipients), and encephalitis.

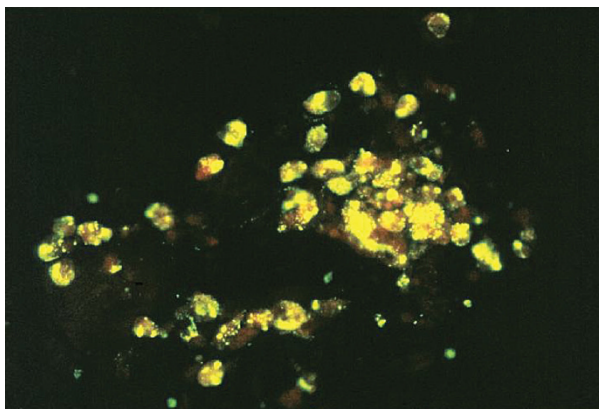
HSV is a double-stranded DNA virus with two antigenic serotypes (HSV-1 and HSV-2). Monoclonal antibodies or molecular analysis are commonly used to differentiate between the two serotypes. HSV is commonly and readily detected in cell cultures from dermal or tissue specimens within 1–3 days post-inoculation. At the present time, using cell culture, these viruses can be easily identified by immunofluorescence (commercially available products include those from Trinity BioTech, Meridian, Diagnostic Hybrids, and Remel).

Although cell culture is still commonly used throughout the world for HSV, those thought to have HSV central nervous system (CNS) disease, are best diagnosed using molecular amplification techniques, which are now the gold standard for HSV meningitis and encephalitis. In fact, molecular amplification techniques are used commonly for non-CNS manifestations of HSV as well.



Figure 5.7 DHI D³ Ultra staining kit. (Courtesy of Quidel Corporation.) (See insert for color representation of the figure.)

(a)



(b)

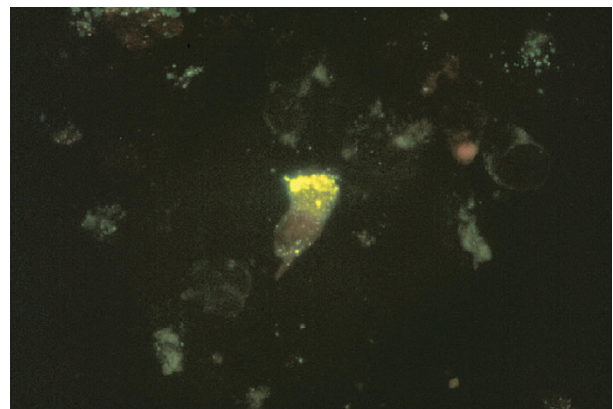


Figure 5.8 (a) Microscopic detection of influenza A in a NP specimen by DFA. (b) Microscopic detection of RSV in a NP specimen by DFA. (See insert for color representation of the figure.)

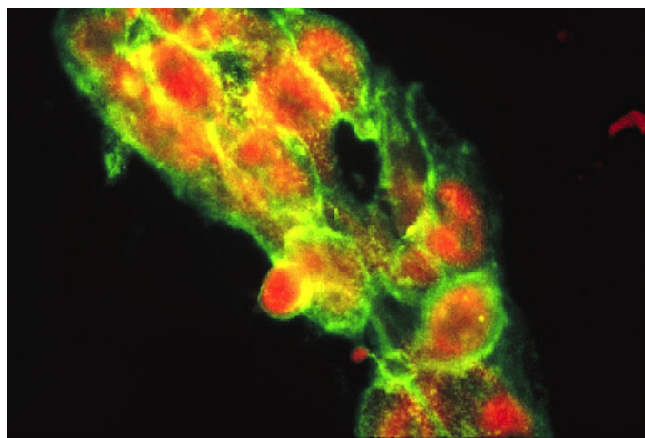


Figure 5.9 Microscopic detection of VZV in a skin scraping by DFA. (See insert for color representation of the figure.)

Varicella zoster virus is also a double-stranded DNA virus, but in contrast to HSV has only one serotype. VZV, also in contradistinction to HSV, is somewhat difficult to culture and identify in cell culture, often taking 5–10 days post-inoculation. DFA methods can detect VZV in skin scrapings in less than an hour (Figure 5.9). The more rapid shell vial approach can be used for VZV (commonly using immunofluorescence to confirm), however, the time to identification can still be delayed. Millipore supplies a kit (Light Diagnostics SimulFluor HSV/VZV) that stains for HSV and VZV simultaneously in a single reaction. When a fluorescein isothiocyanate (FITC) filter set is used, the HSV antigen–antibody complex will exhibit an apple-green fluorescence and the VZV antigen–antibody complex will fluoresce yellow-gold.

Similar to HSV, the introduction of molecular amplification techniques has greatly improved the rapid identification of VZV from clinical specimens. Several studies (see the previous edition of this manual and other reviews) have documented the increased sensitivity of PCR over cell culture. Improved diagnosis using PCR has been demonstrated using blood, serum, plasma, eye specimens, cerebrospinal fluid (CSF), and specimens from various lesions.

Cytomegalovirus is a double-stranded DNA virus which contains an envelope and is antigenically complex, with only one known serotype. CMV can cause a very broad array of clinical disease in humans, ranging from asymptomatic virus shedding to severe disease including congenital newborn infection, a mononucleosis-like syndrome, and a variety of infections in both solid organ and hemopoietic transplant recipients (e.g., pneumonia, retinitis, hepatitis, and CNS and gastrointestinal (GI) manifestations). CMV has been detected and identified using cell culture (both routine and shell vial), antigenemia assay (using pp65), and more recently molecular amplification methods. For some time the antigenemia

assay was commonly used since it was thought to be two to three times more sensitive than cell culture and therefore detected CMV infections earlier and more reliably than cell culture. Numerous kits are available for performing CMV antigenemia assays, including Bartels CINAKit CMV Antigenemia (Trinity Biotech, Jamestown, NY), D3 DFA Cytomegalovirus Immediate Early Antigen Identification Kit (Quidel Corp, San Diego, CA), Light Diagnostics CMV FA Kit (EDM Millipore, Billerica, MA), PathoDX CMV Test Kit (Remel, Inc., Lenexa, KS), and BioTest CMV Bright Kit (Biotest Diagnostics, Denville, NJ). However, the past decade has experienced a shift from the antigenemia assay to the even more sensitive PCR assay, which is now commonly used in many transplant centers. This allows the earlier detection of CMV infections and the use of antivirals much earlier and more effectively. Currently, quantitative molecular assays are commonly used to diagnose CMV disease, to monitor the effectiveness of antiviral therapy, and to initiate pre-emptive therapy.

5.5 Gastrointestinal viruses

A number of viruses, including *Caliciviridae* (Calicivirus and Norovirus, formerly Norwalk and Norwalk-like viruses), *Astroviridae* (including Mamastrovirus, which contains the human astroviruses, and Avastrovirus, which includes avian viruses), *Adenoviridae* (more than 100 adenoviruses from vertebrates, and 51 human adenovirus serotypes), *Coronaviridae* (containing two genera, Coronavirus and Torovirus) and *Reoviridae* (including rotavirus) may be capable of causing gastrointestinal disease in humans (and animals as well; refer to Chapter 19). The severe acute respiratory syndrome Coronavirus (SARS CoV) will not be discussed in this chapter. Many of these viruses have proven to be quite difficult to propagate and detect/identify in cell culture. In earlier studies many, if not most, of these viruses were detected in the feces of symptomatic individuals by electron microscopy (EM), by scanning EM, or transmission EM, or immune EM (i.e. using specific antibodies to cross-link a specific virus). A few laboratories may still offer different types of EM, however, immunoassays and molecular amplification techniques have taken over as the primary methods of detection for many viruses in this group.

Rotavirus is a major cause of gastroenteritis in infants, young children and occasionally adults, and the elderly, and is a member of the *Reoviridae* family of viruses. The *Reoviridae* consists of *Coltivirus*, *Seadornavirus*, *Orthoreovirus*, and *Orbivirus*, in addition to *Rotavirus*. Only *Rotavirus* (of the *Reoviridae*) will be considered in this chapter. The virus does not contain an envelope, and has a double-shelled protein capsid that contains a

segmented, double-stranded RNA genome. Five known rotavirus groups have been described (A to E), with most of the human pathogens residing in groups A, B, and C, with A being the most common. Highly effective vaccines for rotavirus have dramatically reduced the prevalence of this virus and the volume of testing from what it was only a few years ago. As with most viral diagnostics, recent developments in the detection of rotavirus have involved the development of molecular diagnostic tests, in particular multiplex assays for enteric pathogens including viruses, bacteria, and parasites. For laboratories that still wish to offer rotavirus antigen testing, several assays are still being manufactured, including the Premier Rotaclone and Immunocard Stat Rotavirus (Meridian Diagnostics, Cincinnati, OH), and the SAS Rota Test (SA Scientific, Inc. San Antonio, TX). Additional tests may be available, including those from Coris Bioconcepts (Rotavirus and Adenovirus, Belgium), Xpect Rotavirus kit (ThermoFisher Scientific), QuickStripe Rotavirus (Savyon Diagnostics, Israel), Rotavirus Antigen Rapid Test (Diagnostic Automation/Cortez Diagnostics, Inc., Calabasas, CA), and Rapid Response Rotavirus Antigen Test kit (from various medical supply companies). The user should review the FDA approval status of any test kit under consideration, the performance characteristics and availability of all products in their locale, and they should refer to recent peer-reviewed manuscripts containing evaluations of diagnostic products. An extensive review of some of the older immunodiagnostic tests and formats are included in the first edition of this manual.

Adenoviruses are double-stranded DNA viruses that do not contain an envelope, and contain a viral capsid that is composed of 252 capsomeres arranged as an icosahedron. More than 45 distinct serotypes of adenovirus have been described. The group of adenoviruses can cause a very wide variety of clinical disease manifestations, including pneumonia, pharyngitis, eye infections, cystitis, and gastrointestinal disease. Adenoviruses have been described as being associated with approximately 10% of pediatric diarrhea, with serotypes 40 and 41 commonly detected. Several antigen detection assays are available for detecting the enteric adenoviruses including the Adenoclone Type 40-41 EIA Diagnostic Test (Cambridge Bioscience, Worcester, MA), and the Adenovirus Antigen Detection Elisa Test (Immuno Probe, Inc., Fredrick, MD). Also, an antigen detection assay for detecting adenovirus in ocular specimens is available (RPS Adeno Detector Plus, Rapid Pathogen Screen, Inc., Sarasota, FL).

There are numerous viruses (including many respiratory and enteric viruses) for which rapid antigen testing is not available: these are addressed in Chapter 6. New developments in molecular testing have been made that greatly reduce the complexity and cost of these assays,

making them more available to a greater number of laboratories and offering attractive options to many of the antigen detection assays due to their increased sensitivity.

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Molecular Tests for the Identification of Viruses

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6.1 Introduction

The transition of nucleic acid based technologies from basic research tools to everyday clinical diagnostic tests has greatly enhanced many aspects of laboratory medicine. However, few fields have changed more than infectious disease diagnostics, especially in the area of clinical virology. In particular, nucleic acid amplification tests (NAAT) can achieve levels of sensitivity for critically important diagnoses that often cannot be obtained using traditional antigen- or culture-based methods.

Careful assay design allows NAATs to be highly specific for a targeted virus, or conversely enables the detection of different strains within a family of viruses or not yet identified viral pathogens. Using NAATs, the laboratory can detect viruses that cannot be grown in traditional cell culture (e.g., hepatitis B virus [HBV]) or viruses simply too hazardous to routinely grow in the laboratory (e.g., variola). Importantly for patient care, NAATs can detect a virus within hours compared to days or weeks typically needed for traditional culture methods [130].

NAATs are used for a variety of applications in clinical virology. Qualitative assays are primarily used for diagnosis of common viral pathogens (e.g. herpes simplex virus [HSV]) as well as emerging viral pathogens such as the severe acute respiratory syndrome (SARS) coronavirus (CoV) in 2003, and for screening blood products (e.g., human immunodeficiency virus [HIV]) [60,92,199]. Quantitative viral assays, on the other hand, have become indispensable laboratory tests for predicting patient prognosis, monitoring response to antiviral therapy, and for the determination of antiviral resistance in patients with HIV, viral hepatitis, influenza, and organ transplantation [16,18,28,161]. NAATs are available as single analyte assays (monoplex) or as syndromic panels (multiplex) for viral pathogens causing respiratory tract,

gastrointestinal tract, genital tract, and central nervous system infections. The relatively limited number of known clinically relevant viruses, and the capability to accurately quantify viruses in patient samples has contributed to the broad acceptance of NAATs as the new “gold standard” in clinical virology [60,70,213,216]. The increasing acceptance and utilization of NAATs not only in large university hospital and commercial laboratories, but also in small community hospital laboratories has been due in large part to the growing number and types of US Food and Drug Administration (US FDA) cleared/approved *in vitro* diagnostic (IVD) NAATs, increased automation, and decreased test complexity.

This chapter is divided into several sections: 6.2–6.6 will discuss commercially available molecular testing for viruses that cause respiratory, gastrointestinal, central nervous system, and genital tract disease, as well as post-transplant monitoring. The final section will consider requirements for assay implementation, verification/validation, and continued quality assurance.

6.2 Respiratory viral infections

6.2.1 Background

Viruses are the most common cause of respiratory tract infections (RTI) in children and adults [65,153,218]. RTIs are one of the major reasons for absenteeism at school and work as well as visits to clinics and emergency departments. RTIs are traditionally divided into upper respiratory infections (URI) and lower respiratory infections (LRI). URIs include nasopharyngitis, also known as the common cold, as well as sinusitis, pharyngitis, and tracheitis. LRIs involve the lungs and the respiratory tract below the trachea. Examples of LRI include bronchitis, bronchiolitis, and pneumonia. In infants and children,

most cases of community-acquired pneumonia (CAP) are viral [111,196]. Viral infections decline during adulthood to about one-third of pneumonia cases, but still constitute the second most common cause of CAP in adults (after the bacterial pathogen *Streptococcus pneumoniae*) [64,196]. In older patients viral pneumonia again increases in frequency [134,215]. Children, the elderly, and immunocompromised patients may also experience more severe RTIs and shed virus for longer periods of time [35,68,95].

Respiratory viruses are diverse with different seasonal activity, targets, clinical presentations, severity, response to treatment, and predilection for mutation. The characteristics of RTI viruses are summarized in Table 6.1. The main causes of RTIs (see Table 6.1) are adenovirus (ADV), bocavirus (BoV), coronaviruses (CoV-229E, NL63, OC43, HKU-1), human metapneumovirus (HMPV), human rhinoviruses (HRV), influenza A (FluA), influenza B (FluB), parainfluenza viruses 1–4 (PIV-1, PIV-2, PIV-3, PIV-4), and respiratory syncytial virus (RSV). Mixed viral infections are present in about 5–20% of RTIs [19,59,60,85,171,207]. Furthermore, immunocompromised patients are vulnerable to more atypical respiratory infections with viruses such as HSV or cytomegalovirus (CMV) in addition to common respiratory virus [113].

Determination of the cause of RTIs is based on a number of factors including patient's signs and symptoms, age, medical history, immune status, sick contacts, time of the year and the related seasonality of viral infections, as well as awareness of the prevalence of circulating viruses in the community at the time of presentation. Though these factors can narrow the differential to certain pathogens, clinical symptoms can overlap significantly, making an accurate etiologic diagnosis challenging and often inaccurate [144,172]. Differentiation between a viral, bacterial, or fungal RTI is necessary to appropriately treat the patient. For example, an outpatient with a viral URI would not benefit from antibiotic therapy. Unfortunately, it is estimated that about half of ambulatory visits to clinicians for URIs result in an antibiotic prescription being given despite the lack of efficacy against viruses, the most common cause of URIs [21,86,104,170]. Furthermore, excessive and unnecessary antibiotic treatment increases selective pressure for antimicrobial drug resistance in healthcare institutions and the community [141,234]. Although currently FDA approved antiviral treatments are available only for influenza and RSV, antiviral compounds that target other common respiratory viruses are under development. Prompt identification (within 48 h of symptom onset) is crucial for guiding treatment [66,112]. Finally, viral strain typing can be important in therapy selection. For example, prior to the emergence of the 2009 influenza A H1N1 pandemic strain, the circulating seasonal influenza A H1N1 strain was resistant to the neuraminidase inhibitor oseltamivir, but was susceptible to zanamivir. In contrast,

the subsequent 2009 influenza A H1N1 pandemic strain was sensitive to oseltamivir, so identification of the strain could be used as a proxy for susceptibility [66,110]. In addition to treatment, viral respiratory pathogens are often highly infectious and can cause outbreaks that are a threat to public health, best exemplified by the emergence and spread of SARS in 2003 [92]. Nosocomial spread of viral RTIs among patients in healthcare settings increases morbidity, mortality, and duration of hospitalization [206]. Therefore, early and accurate identification of the pathogen(s) can aid in appropriate isolation or cohorting of patients with similar infections thus avoiding further spread to both patients and staff.

Immunocompromised patients who develop respiratory viral infections often have more severe disease and shed virus for a longer period of time [113]. These patients can develop significant RTIs from viral pathogens not usually associated with RTIs in immunocompetent persons, including CMV, HSV, and varicella zoster virus (VZV) [225].

6.2.2 Detection of respiratory viruses

Conventional tests for the detection of respiratory viruses include rapid antigen direct tests (RADT), direct fluorescent antibody (DFA), and cell culture. A large variety of RADTs are available to detect FluA, FluB, and RSV.

In general, NAATs have better sensitivity than RADT's, DFA, and viral cell culture and a faster time to results when compared to cell culture [104,145,159]. In addition, NAATs can detect viruses such as BoV, CoV, HMPV, and PIV-4 that do not grow in cell culture [6,83,103]. Studies have demonstrated that the sensitivities of detecting a virus in respiratory samples vary significantly based on test method. For example, during the 2009 influenza A H1N1 pandemic, NAAT identified viruses in 64% of the samples, compared to 17% for RADTs, 22% for DFA and 36% for cell culture [82].

The performances of all test methods are affected to varying degrees by multiple factors, including appropriate sample collection, storage, and transport. Influenza and other respiratory viruses have high levels of replication in the columnar cells of the nasopharynx (NP) [139]. Therefore, samples collected from the nasopharynx are superior to samples from the nares or the oropharynx alone [133]. Therefore, NP swabs, NP aspirates, and NP washes are recommended specimens. However, variations can occur depending on the particular virus. For example, a combination of both NP and oropharyngeal (OP) swabs placed in a single viral transport media are the recommended sample for avian influenza [49]. Lower respiratory tract samples were found to be positive in intensive care unit (ICU) patients with acute 2009 influenza A H1N1, yet NP swabs were negative [128]. Appropriate specimens for LRIs include sputum, bronchial alveolar lavage (BAL), and bronchial washings. Synthetic flocked swabs

Table 6.1 Respiratory virus summary

Virus	Family	Genome	Subtypes	Season*	Clinical Significance	Treatment	Reference(s)
Adenovirus (ADV)	Adenoviridae	DNA	More than 50 serotypes. Genetically diverse	Year-round	URI, conjunctivitis, pharyngitis in children and adults. Pertussis-like syndrome. Pneumonia especially in children. Acute respiratory disease (ARD) in military recruits. Severe or disseminated disease in immune compromised patients	Supportive; cidofovir used for immune compromised patients	5, 166, 181, 237
Bocavirus (BoV)	Parvoviridae	DNA	Uncertain, may be two genetic clusters	Uncertain	More commonly associated with respiratory infections in children, less commonly in adults. Isolated frequently in coinfecting patients; significance still uncertain	Supportive	6, 111
Coronavirus (CoV)	Coronaviridae	RNA	229E NL63 OC43 HKU1 SARS	Winter and spring	Second most common cause of common cold (10–20%). Can also cause LRI especially in infants, elderly, and immune compromised patients. 229E and NL63 may predominate in alternate years. SARS outbreak in 2003 was a coronavirus	Supportive	62, 91, 92, 98, 182, 214
Enterovirus (EV)	Picornaviridae	RNA	More than 60 serotypes. Current types: www.picornaviridae.com. Shares 5' UTR with some rhinoviruses	Summer and fall	Common cold-like syndrome, hand-foot-mouth disease, pharyngitis, less commonly bronchiolitis, and pneumonia especially in children	Supportive; pleconaril, a drug with antipicornaviridae activity, is not approved in the United States	99, 157, 195, 198, 212
Human metapneumovirus (HMPV)	Paramyxoviridae	RNA	HMPV A HMPV B	Winter and spring	Lower respiratory infections especially in infants and children. URI and LRI in children and adults	Supportive; ribavirin <i>has in vitro</i> activity against HMPV, but efficacy in patients is still unclear	63, 105, 111, 170
Human rhinovirus (HRV)	Picornaviridae	RNA	More than 100 serotypes. Shares 5' UTR with some enteroviruses	Fall and spring	Most common cause of common cold (30–50%). LRI in infants, elderly, and immune compromised patients. Associated with exacerbation of asthma and COPD	Supportive	62, 91, 102, 168, 170
Influenza A virus (FluA)	Orthomyxoviridae	RNA, 8 segments	H1N1 H3N2 Novel 2009 Pandemic H5N1	Winter	Annual epidemics of upper and lower respiratory infections in children and adults. FluA periodically undergoes antigenic shifts causing pandemics, sometimes with high morbidity and mortality. 1918 Pandemic strain and the novel 2009 Pandemic strains were influenza A viruses	Neuraminidase inhibitors Amantadine derivatives	13, 21, 22, 37, 66, 85, 153, 172, 178, 206, 218, 235, 241
Influenza B virus (FluB)	Orthomyxoviridae	RNA, 8 segments	NA	Winter	Can cause annual epidemics like FluA, but less likely to undergo antigenic shift	Neuraminidase Inhibitors	
Influenza C virus (FluC)	Orthomyxoviridae	RNA, 7 segments	NA	Winter	More mild and is not associated with epidemics	Supportive	
Parainfluenza virus (PIV)	Paramyxoviridae	RNA	PIV-1 PIV-2 PIV-3 PIV-4	PIV-1 in summer and fall on odd years PIV-2 in fall PIV-3 in spring PIV-4 in summer and fall	Group in children, bronchiolitis in infants. Viral pneumonia in adults especially elderly and patients with underlying disease. URI in children and adults	Supportive; ribavirin has been used in immunocompromised patients	71, 97, 103, 149
Respiratory syncytial virus (RSV)	Paramyxoviridae	RNA	RSV A RSV B	Fall and winter	Most frequent cause of bronchiolitis and pneumonia in children. LRI in elderly and immunocompromised patients. URI in children and adults	Ribavirin Palivizumab prophylaxis	21, 61, 97, 170, 241

* Seasonal activity for the Northern Hemisphere. Viral activity patterns will differ in other regions of the world.

have been shown to increase recovery of epithelial cells and virus compared to traditional fiber swabs [106,228]. Swab samples should be placed into an appropriate transport media that will maintain nucleic acid stability during storage and transport to the laboratory. The laboratory should consult the manufacturer's package insert for acceptable sample types and the variety of transport media that are approved for the molecular assays. Sample types and transport media other than those recommended by the manufacturer can be used as long as the laboratory performs an in-house validation to demonstrate that the alternative transport media have comparable performance and results.

In general, respiratory specimens for NAAT should be transported on wet ice and stored 2–8°C if testing is to be performed within 48 h. If testing is delayed beyond 48 h, specimens should be stored at ≤ −70°C to preserve specimen integrity [83]. Finally, the timing of sample collection also has a significant effect on the detection of respiratory pathogens [83,130]. Respiratory virus shedding is highest a few days after the onset of symptoms, so generally specimens should be collected within 3 days after onset of symptoms in adult patients and 5 days in pediatric patients. The larger time window for respiratory specimen collection from pediatric reflects the higher viral levels and longer shedding periods in children [97,106,128]. Since NAATs are not dependent on viable virus, only the presence of nucleic acids, the recovery of virus nucleic acids is not as susceptible to timing or transport delay as other traditional test methods.

6.2.3 Commercially available *in vitro* diagnostic tests

Currently there are over a dozen FDA-cleared IVD NAATs for the detection of a variety of respiratory viruses. The characteristics of each NAAT are listed in Table 6.2. Currently these tests can detect as few as a single viral target (e.g., RSV) to up to 18 viral targets in one panel. Assay formats vary from all-inclusive unit-based tests to assays that require multiple instruments and multiple steps. Acceptable sample types for each test are also listed in the table. Technical hands-on-time varies from minutes to hours and time to results range from 1 to 10 h. All assays contain an internal control (IC).

Many assays use standard formats consisting of nucleic acid extraction and amplification/detection on generic real-time platforms. Excluding NA extraction (if required), many of these assays can have results available within 75 min. Assay formats are conducive for low- to high-volume batch testing. Tests by Hologic/Gen-Probe/Prodesse (San Diego, CA) include ProFlu+ (FluA, FluB, RSV), ProFluFAST (FluA H1, FluAH3, FluA 2009-H1N1 typing), ProAdeno+, ProPara+ (PIV-1,-2,-3) and ProhMPV+ [129,138,203]. Nucleic acid (NA) extraction is

performed using the NucliSENS easyMAG (bioMérieux, France), Magna Pure or Magna Pure LC (Roche Molecular Diagnostics, Pleasanton, CA) platforms and real-time amplification/detection performed on the SmartCycler II (Cepheid). Each assay uses fluorogenic probes to detect the specific viral targets [129,138,203]. Tests by Focus Diagnostics (Cypress, CA) include the Simplexa influenza A H1N1 and the Simplexa influenza A/B and RSV tests, both of which require initial NA extraction using either the NucliSENS easyMag or Magna Pure LC [4]. Real-time amplification/detection is performed on the rapid 3M Integrated Cycler (3M, St. Paul, MN), using bifunctional fluorescent probe-primers together with corresponding reverse primers and internal control RNA. The Simplexa influenza A/B & RSV Direct Test is a version of the assay that is also performed on the 3M Integrated cycler, however, an upfront NA extraction step is not required as samples are loaded directly into direct amplification discs. The Qiagen *artus* Infl A/B RG RT-PCR Kit (Qiagen, Valencia, CA) requires a separate NA extraction followed by amplification and fluorescent detection of the FluA and FluB targets and an internal control, using the Rotor-gene Q MDx instrument (Qiagen) [77]. Quidel (San Diego, CA) currently has two respiratory virus assays, one that detects and differentiates FluA and FluB and one that detects HPMV. Both Quidel assays require NA extraction using the NucliSENS easyMAG and real-time detection on SmartCycler II or ABI 7500 Fast DX (Applied Biosystems, Foster City, CA). Additional selected platforms with unique characteristics, varying formats and levels of complexity will be described below.

The Luminex xTAG Respiratory Viral Panel Version 1 (xTAG RVP v1) (Luminex, Austin, TX) is approved for the detection of ADV, FluA (pre-2009 H1, H3 and 2009 H1), subtyping of pre-2009 FluA H1 and FluA H3, FluB, HMPV, HRV, PIV-1, PIV-2, PIV-3, and RSV [15,38,69,78,151,163,164]. The Conformité Européenne (CE) marked version of the assay also includes the CoVs (OC43, NL63, 229E, HKU-1), PIV-4, and enterovirus (EV, grouped with HRV). Purified viral nucleic acids are amplified by multiplex reverse transcription polymerase chain reaction (RT-PCR), followed by target specific primer extension. The 5' ends of the primers contain a unique Tag sequence that allows the amplicon to bind to a complementary anti-Tag sequence on a target-specific color coded microbead. During the primer extension process, biotinylated deoxycytidine triphosphates (dCTP) are incorporated into the amplicons, which will in turn bind via streptavidin to a phycoerythrin reporter dye moiety. The Luminex 100/200 detection platforms (Figure 6.1) use one laser to identify the target-specific bead and a second laser to detect if the bead fluoresces, hence indicating the presence of target amplicons on the beads. The xTAG RVP Fast version of the assay reduces the number of steps to four and the amount

Table 6.2 Respiratory virus FDA-cleared/approved NAAT assays. Information in this table was current as of the time of writing

Manufacturer	Assay	Extraction instrument	Amplification and detection	Target(s)*	Specimen(s) [†]	Method	Reference(s)
Abbott Chicago, IL	Abbott Ibis PLEX-ID Flu Assay	Thermo-KingFisher System	Abbott Ibis T5000	Pan-flu, FluA, FluB, FluA oseltamivir resistant	NPA	Real-time RT-PCR, fluorescent detection	217
BioFire Diagnostics Salt Lake City, UT (formerly Idaho Technology)	FilmArray Respiratory Virus Panel	Integrated	Idaho Technology Film Array	ADV, CoV (OC43, NL63, 229E, HKU-1), hMPV, FluA (H1, H3, 2009-H1N1), FluB, HRV/EV, PIV (1,2,3,4),	NPS	Real-time RT-PCR, fluorescent detection	14, 100, 138, 175, 180, 184
CDC Influenza Division Atlanta, GA	CDC Human Influenza Virus Real Time RT PCR Diagnostic Panel	Qiagen QIAamp Qiagen QIAcube Roche Magna Pure bioMérieux NucliSENS easyMAG	ABI 7500 FastDx	FluA H1, H3, 2009 H1N1, H5N1	NPS, NS, NA, NW, NPS/TS, BAL, TA, BW, VC	Real-time RT-PCR, fluorescent detection	203
Cepheid Sunnyvale, CA	XpertFlu Assay	Integrated	Cepheid GeneXpert	FluA, H1/2009-H1N1, FluB	NPS, NA, NW	Real-time RT-PCR, fluorescent detection	117, 160, 179, 197
Focus Diagnostics Cypress, CA	Simplexa Influenza A H1N1	Qiagen QIAmp	3M Integrated Cycler	FluA 2009-H1N1	NPS, NA, NPA	Real-time RT-PCR, fluorescent detection	NA
	Simplexa Flu A/B & RSV	No extraction for Direct		FluA, FluB, RSV			
	Simplexa Flu A/B & RSV Direct			FluA, FluB, RSV			
Gen-Probe/Prodesse San Diego, CA	ProFlu+, ProFAST+	bioMérieux NucliSENS easyMAG	Cepheid Smartcycler II	FluA, FluB, RSV, H1, H3, 2009-H1N1	NPS	Real-time RT-PCR, fluorescent detection	129, 138, 203
	ProAdeno+	Magna Pure LC		ADV			
	ProParaFlu+	Magna Pure Total		PIV -1,-2,-3			
	ProhMPV+			hMPV			
Iqum Marlborough, MA	Liat Influenza A/B	Integrated	Iqum Liat Analyzer	FluA, FluB	NPS	Real-time RT-PCR, fluorescent detection	NA
Luminex Austin, TX	xTag Respiratory Virus Panel	NucliSENS easyMAG	Thermocyclers	ADV, hMPV, HRV, FluA (H1, H3), FluB, PIV (1,2,3), RSV (A, B)	NPS	RT-PCR primer extension	14, 15, 83,
	xTag RVP Fast	NucliSENS miniMAG	Luminex	ADV, hMPV, HRV, FluA (H1, H3), FluB, RSV (A, B)		xTag bead array	143, 151,
		MagnaPure	Lx100/200			fluorescent detection	163, 165, 186, 229
Nanosphere Northbrook, IL	Verigene Respiratory Virus Nucleic Acid Test Plus	Integrated	Nanosphere Verigene System	FluA (H1, H3, 2009-H1N1), FluB, RSV (A, B)	NPS	Real-time RT-PCR, gold nanoparticle detection	4, 115
Qiagen Artus Valencia, CA	Influenza A/B Rotor-gene RT-PCR Kit	Qiagen QIASymphony RGQ	Qiagen Roto-Gene Q MDx	FluA and FluB	NPS	Real-time RT-PCR, fluorescent detection	77
Quidel San Diego, CA	Molecular Influenza A+B Assay	NucliSENS easyMAG	SmartCycler II	FluA and FluB	NPS, NS	Real-time RT-PCR, fluorescent detection	NA
	Molecular hMPV Assay		ABI 7500 Fast Dx	hMPV			
US Army JBAIDS Fort Detrick, MD	Influenza A/H56	Integrated	Idaho JBAIDS	H5N1 (Asian lineage)	NPS, TS	Real-time RT-PCR, fluorescent detection	NA
	Influenza A&B Detection Kit6			FluA and FluB	NPS, NPW		
	Influenza A Subtyping Kit6			H1, H3, 2009-H1N1	NPS, NPW		

* NA: none available; ADV: adenovirus; CoV: coronavirus; FluA: all influenza A types; H1: seasonal H1N1; H3: seasonal H3N2; 2009-H1N1: 2009 pandemic influenza A (H1N1); H5N1: avian influenza A H5N1; Flu B: influenza B; hMPV: human metapneumovirus; EV: enterovirus; HRV: human rhinovirus; PIV: parainfluenza virus; RSV: respiratory syncytial virus.

[†] NPS: nasopharyngeal swab; NPW: nasopharyngeal wash; NPA: nasopharyngeal aspirate; NS: nasal swab; NA: nasal aspirate; NW: nasal wash; NPS/TS: dual specimen consisting of nasopharyngeal swab and throat swab. These specimen types are specified in product package inserts cleared by the US Food and Drug Administration.



Figure 6.1 The Luminex 100/200 detection platforms. (Images provided by Luminex Corporation) (See insert for color representation of the figure.)



Figure 6.2 GenMark e-sensor platform. (Image provided courtesy of GenMark Diagnostics) (See insert for color representation of the figure.)

of “hands-on” time; the turnaround time is improved from 10 to 6 h as compared to xTAG RVP v1 [204]. However, there are fewer targets available in the Fast version of the assay (ADV, FluA generic, pre-2009 FluA H1, FluA H3, HMPV, HRV, RSV) and some targets have reduced sensitivity as compared to xTAG RVP v1 [14,165]. Assays that require the opening of samples with amplified products such as the xTAG, necessitate separation of testing areas and careful attention to procedures to prevent contamination. This assay is best suited for medium- to high-volume testing since the assay is performed using microwell plates and must be batched [143].

The GenMark Respiratory Virus Panel (GenMark Dx, Carlsbad, CA) assay consists of four separate steps, nucleic acid extraction, conventional end-point RT-PCR, amplicon denaturation and hybridization, and then detection using the eSensor platform (Figure 6.2). Single stranded DNA products are hybridized to ferrocene-labeled signal probes specific for the different viral targets and added to the detection cartridge. Cartridges are then loaded onto the eSensor XT-8 instrument for data acquisition and automated analysis based on

electrochemical detection. The eSensor platform consists of a base module and up to three processing towers that contain eight cartridge slots each, allowing up to 24 samples to be analyzed simultaneously. Total hands-on-time is less than 60 min and over 3.5 h of walk-away time. The assay detects ADV (B, C, E) CoV (229E, HKU-1, OC43, NL63); FluA with subtype determination, FluB, HMPV, HRV, PIV-1, PIV-2, PIV-3, PIV-4, and RSV (A, B) [175].

The Verigene Respiratory Plus Test (Nanosphere, Northbrook, IL) is a closed modular system and consists of a nucleic acid extraction and amplification Processor SP module and a Verigene Reader unit. Extraction tray, amplification tray, tip-holder assembly, and test cartridges are loaded into the Processor SP module and the sample is added into the test cartridge. Within the processor nucleic acids are extracted, amplified, and amplicons are captured by sequence-specific oligonucleotides immobilized onto glass slides. Sequence-specific mediator oligonucleotides attached to gold particles hybridize to the immobilized amplicons. The glass slide array is then removed from the cartridge and loaded into the Verigene Reader, which detects the target gold particles through scattered light. Time from sample loading to results is approximately 3 h. The Respiratory Plus assay can identify generic FluA, pre-2009 FluA H1, FluA 2009 H1N1, FluB, RSV (A, B), and the FluA H275Y mutation that confers oseltamivir resistance [4].

The FilmArray Respiratory Panel (Biofire Diagnostics, Salt Lake City, UT) is a pouch based, highly multiplex, real-time nested PCR assay which runs on the FilmArray System. The assay can detect and identify ADV, CoVs (OC43, NL63, 229E, HKU-1), HMPV, FluA (H1, H3, 2009-H1N1), FluB, HRV/EV, PIV-1, PIV-2, PIV-3, PIV-4, and RSV (A, B) as well as the pathogens *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Bordetella pertussis*. Sample and hydration solution are added to the reagent pouch and the pouch is loaded into the Film Array instrument (Figures 6.3 and 6.4). Inside the pouch, nucleic acids are extracted and undergo a two-step nested PCR; first a multiplex PCR then the amplified products are diluted and separated into multiple reaction chambers where secondary monoplex PCRs are performed. Target amplicons are fluorescently labeled by SYBR green and the identity is confirmed by melt-curve analysis. Currently, one pouch with one patient sample can be processed on a FilmArray instrument with time to results approximately 1 h [138,174,180,184].

The Xpert Flu (Cepheid, Sunnyvale, CA) is a cartridge based, random access, totally integrated multiplex, real time RT-PCR assay which runs on the GeneXpert system (Figure 6.5). The Xpert Flu assay detects generic FluA, 2009 FluA, H1N1, and FluB. The patient sample and an elution reagent are added to the disposable cartridge,

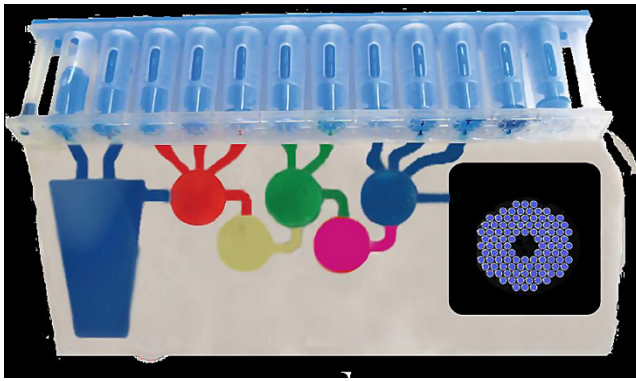


Figure 6.3 Biofire FilmArray pouch. (Courtesy of Biofire Diagnostics, Inc.) (See insert for color representation of the figure.)



Figure 6.4 Biofire FilmArray instrument. (Courtesy of Biofire Diagnostics, Inc.) (See insert for color representation of the figure.)



Figure 6.5 Xpert Flu. (Courtesy of Cepheid.) (See insert for color representation of the figure.)

which is then manually loaded into a single bay of the GeneXpert system. The GeneXpert Systems have different capacity models that include slots for 1, 2, 4, and 16 cartridges. Alternatively, the expandable walk-away Infinity system can load and test up to 80 cartridges simultaneously. The Xpert Flu test takes about 75 min to complete [117,160,197].

The Liat is a cartridge based, multiplex, real time PCR system manufactured by Iquum (Marlborough, MA). The cartridge consists of a tube containing all assay reagents needed for extraction, amplification, and fluorescent detection prepacked in tube segments, separated by removable seals. The analyzer is small, lightweight, and portable (battery or AC powered). The Liat Influenza A/B assay is FDA cleared and can detect FluA and FluB in just 20 min.

The PLEX-ID Flu (Abbott, Chicago, IL) assay utilizes RT-PCR to amplify target nucleic acids for FluA (H1, H3, 2009-H1N1) and FluB [45]. Following amplification the samples undergo post-PCR desalting, purification, and then analysis by using electrospray ionization mass spectrometry (ESI/MS). Spectral analysis determines the nucleotide base composition of the single-stranded oligonucleotides complementary to the initial target and compares the spectra to a database of viral targets. The PLEX-ID Flu is FDA-cleared.

6.2.4 Laboratory developed, investigational, and research-use only tests

Many new assays and platforms are currently in development for the detection of respiratory viruses. A few selected assays are described below and in Table 6.3. The Icube 2.0 (Icube, Huntsville, AL) is a modular cartridge-based system. The processor unit prepares the sample, performs ARM (amplicon rescued multiplex)-PCR, hybridization, and washing procedures. The processor can run one to four cassettes in a random access fashion and up to 12 units can be linked. The cassette contains all the reagents necessary to perform multiplex amplification and detection. Cassettes are transferred to a reader containing a high-speed rotating plate, laser, and photomultiplier tube, which allow the rapid acquisition of data from each cassette. The proposed targets in the assay will include ADV-3, ADV-4, ADV-7, BoV, CoV (NL63, 229E, OC43 HKU-1), coxsackievirus (A, B), echovirus, FluA, 2009 FluA H1N1, H274Y mutation, FluA H3, FluB, HMPV, HRV, PIV-1, PIV-2, PIV-3, PIV-4, and RSV (A, B). An influenza typing assay will differentiate FluA, FluB, pré-2009 FluA H1, 2009 FluA H1N1, FluA H3, FluA H5, FluA N1, and FluA N2.

The Qiagen Resplex II (Qiagen, Valencia, CA) assay detects ADV (B, E), BoV, CoV (229E, NL63, 229E, HKU-1), coxsackievirus/echovirus, FluA, FluB, HMPV, HRV, PIV-1, PIV-2, PIV-3, PIV-4, and RSV (A, B) [15,78,100,132,146,229]. After a separate NA extraction step, amplification is performed using TEM (target-enriched multiplex)-PCR. For each viral target, two sets of nested gene-specific primers at very low concentration are used to enrich for the target in the first few cycles. The inner nested primer has a Tag sequence recognized by universal super primers (biotin labeled), at a concentration

Table 6.3 Respiratory virus non-FDA-cleared/approved NAAT assays. Information in this table was current as of the time of writing

Manufacturer	Assay	Extraction instrument	Amplification and detection	Target(s)*	Specimen(s) [†]	Method	Reference(s)
Abbott Chicago, IL	Abbott Ibis PLEX-ID Respiratory Viruses	Thermo-KingFisher System Other extractors not specified	Abbott Ibis T5000	FluA, FluB, ADV, CoV, hMPV, PIV (1, 2, 3), RSV	NPA	Real-time RT-PCR, fluorescent detection	39, 45, 53, 68
Argene/bioMérieux Marcy l'Etoile, France	R-Gene Adenovirus Assay	bioMérieux NucliSENS easyMAG	Cepheid SmartCycler II	ADV	NPS, NPA, NPW	Real-time RT-PCR, fluorescent detection	NA
Autogenomics Vista, CA	Infiniti RVP Plus	Not Specified	Thermal cyclers: Autogenomics Infiniti Analyzer	ADV (A, B, C, E) Flu A, 2009-H1N1, Flu B, RSV, PIV, (1, 2, 3, 4), HRV (A, B), EV (A, B, C, D), CoV (HKU1, OC43, NL63, 229E), hMPV	Respiratory samples (not specified)	RT-PCR, BioFilmChip microarray	187
bioMérieux Marcy l'Etoile, France	NucliSens Influenza A+B Nuclisense RSV A+B Nuclisens hMPV	bioMérieux NucliSENS easyMAG	bioMérieux NucliSENS easyQ	Flu A and Flu B RSV (A, B) hMPV	NPA, NPS, NS, NA, BAL	NASBA, fluorescent detection	52, 148, 154, 220
GenMark Carlsbad, CA	Genmark Respiratory Virus Panel	NucliSENS easyMAG	Thermal cycler GenMark Dx eSensor XT-8 System	Flu A (H1, H3, 2009 H5N1), FluB, PIV (1, 2, 3, 4), RSV (A, B), hMPV, HRV, Pan-Adeno (B, C, E), CoV (OC43, NL63, 229E, HKU-1)	Respiratory samples (not specified)	Real-time RT-PCR, fluorescent detection	175
Gentura/Luminex Austin, Texas	Gentura Respiratory Virus Panel	Integrated	Gentura Dx ID Box	Flu A (H1, H3, 2009-H1N1), Flu B, RSV	NPS	Real-time RT-PCR, fluorescent detection	NA
Icubate/Qiagen Valencia, CA	Icubate Respiratory Panel V Icubate Flu Typing	Integrated	Icubate Processor Incubator	Flu A (H1, H3, 2009 H1N1), FluB, PIV (1, 2, 3, 4), RSV (A, B), hMPV, HRV, ADV (3/7, 4), EV (Cox A, B, Echovirus), HRV, CoV (OC43, NL63, 229E, HKU-1), BoV Flu A, Flu B, H1, H3, 2009 H1, H5 (avian) N1, N2	Respiratory samples (not specified)	arm-PCR End Point Detection	NA
Pathofinder Maastricht, Netherlands	Respifinder 15 Respifinder 19 Respifinder Smart 22	Not specified	Thermocyclers: ABI310, ABI3100, ABI3130, ABI3730, ABI3500 Beckman CEQ Roche Lightcycler 480 Corbett RotorGene 3000/6000 Qiagen Rotor-Gene Q	Flu A (H5N1), FluB, PIV (1, 2, 3, 4), RSV (A, B), hMPV, HRV, ADV, CoV (OC43, NL63, 229E, HKU-1), HBoV, <i>B. pertussis</i> , <i>C. pneumonia</i> , <i>L. pneumophila</i> , <i>M. pneumonia</i> Flu A (H1N1-2009), FluB, PIV (1, 2, 3, 4), RSV (A, B), hMPV, EV/HRV, ADV, CoV (OC43, NL63, 229E, HKU-1), HBoV, <i>B. pertussis</i> , <i>C. pneumonia</i> , <i>L. pneumophila</i> , <i>M. pneumonia</i>	NPW, NPA, NPW, BAL, sputum	RT-PCR/PCR, capillary electrophoresis, multiplex PCR, melt-curve analysis	46, 186, 188
Qiagen Valencia, CA	Qiagen Resplex II	QIAmp QIASymphony QIAxtractor	GeneAmp PCR system 9700 LiquiChip 200 Workstation	Flu A, FluB, PIV (1, 2, 3, 4), RSV (A, B), hMPV, HRV, Cox/Echo, Pan-Adeno (B, E), CoV (OC43, NL63, 229E, HKU-1), HboV	Respiratory samples (not specified)	RT-PCR/PCR, bead array hybridization detection	15, 78, 100, 146
Seegene Seoul, South Korea	Seeplex Respiratory Assay Group of 12 Seeplex Respiratory Assay Group of 15	NucliSENS easyMAG Qiagen BioRobot MDx iNTRON	Qiagen Rotor-gene Applied Biosystems GeneAmp PCR system 9700	Set A: ADV, CoV (229E/NL63), PIV (1, 2, 3) Set B: Flu A/B, RSV (A, B), HRV A, CoV OC43 Set C: ADV (A/B/C/D/E), PIV (1, 2, 3), CoV (229E/NL63) Set B: OC43, HRV (A/B/C), Flu A, RSV (A, B) Set C: BoV (1/2/3/4), Flu B, hMPV, EV, PIV 4 Flu A (generic), H1, H3, 2009-H1N1	Respiratory samples (not specified)	Real-time RT-PCR, fluorescent detection	55, 78, 128, 192
Seeplex Typing Influenza A Virus							

* NA: none available; ADV: adenovirus; CoV: coronavirus; FluA: all influenza A types; H1: seasonal H1N1; H3: seasonal H3N2; 2009-H1N1: 2009 pandemic influenza A (H1N1); H5N1: avian influenza A H5N1; Flu B: influenza B; hMPV: human metapneumovirus; EV: enterovirus; HRV: human rhinovirus; PIV: parainfluenza virus; RSV: respiratory syncytial virus.

[†] NPS: nasopharyngeal swab; NPA: nasopharyngeal aspirate; NS: nasal swab; TS: throat swab; NW: nasal aspirate; NPW: nasal aspirate; NPW: dual specimen consisting of nasopharyngeal swab and throat swab.

for exponential amplification. Allele-specific detection probes are attached to the Luminex bead and detection is performed using the Luminex 100/200 instruments, as previously described [132,151].

The Infiniti RVP System (Autogenomics, Vista, CA) (Figure 6.6) requires a separate NA extraction and then uses RT-PCR to amplify ADV (A, B, C, E), CoV (229E, NL63, 229E, HKU-1), EV (A, B, C, D), FluA, FluA 2009-H1N1, FluB, HMPV (A, B), HRV (A, B), PIV-1, PIV-2, PIV-3, PIV-4, and RSV (A, B). Detection of the target is performed using the INFINITI analyzer, which measures fluorescence signals of the labeled DNA target hybridized to BioFilmChip microarrays via molecular zip codes (attached to array) and anti-zip codes (attached to amplified target) [7,187].

The next generation Multicode-PLx by Luminex (formerly Eragen) uses standard and asymmetric PCR, and high-resolution melt. Each module consists of two independent bays that can run up to six individual tests per bay. The technology is based upon the unique MultiCode synthetic base pairs, isoC and isoG, with properties that enable site-specific incorporation of the

isobases during amplification. MultiCode-RTx primers include a fluorescent reporter in close proximity to an isoC base on the 5' end. During the amplification reaction, the MultiCode isoG base that is covalently attached to a quencher present in the reaction mix is incorporated opposite to the isoC. This site-specific interaction of the reporter-labeled isoC and the quencher-labeled isoG results in a decrease in fluorescence. Since the reporter quenching is reversible, melting-curve analysis can be used to confirm the presence of target. The panel detects FluA, FluB, ADV, PIV-1, PIV-2, PIV-3, PIV-4, RSV (A,B), HMPV, HRV, and CoV (229E, NL63, 229E, HKU-1) [15].

The PLEX-ID Respiratory Virus Assay (Abbott, Chicago, IL) [45] was formerly the Ibis T5000 Respiratory Virus Surveillance II kit by Ibis Biosciences, now a part of Abbott. The assay utilizes RT-PCR to amplify target nucleic acids for ADV (A-H), BoV, CoV, FluA, FluB, HMPV, PIV-1, PIV-2, PIV-3, PIV-4, and RSV in a microwell plate [217]. Following amplification the samples undergo post-PCR desalting, purification, and then analysis by using ESI/MS on the Ibis T5000 universal biosensor platform. Spectral analysis determines the nucleotide base composition of the single-stranded oligonucleotides complementary to the initial target and compares the spectra to a database of pathogens including viruses. In addition, the relative concentration of the target or targets present can be obtained by comparing the peak heights with the internal PCR calibration internal mass standard present in every well [39,53,68].

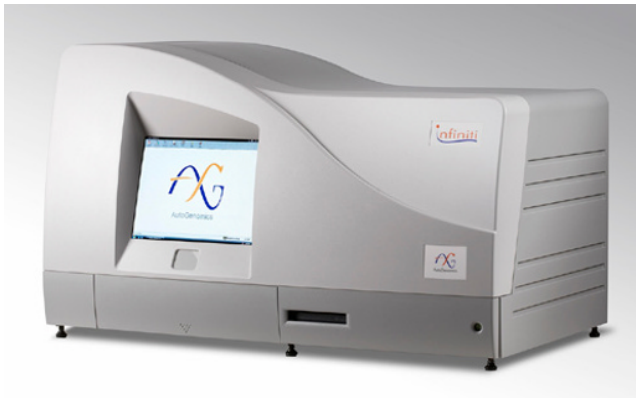


Figure 6.6 Infiniti RVP System. (Reproduced by permission of Autogenomics, Inc.) (See insert for color representation of the figure.)

6.3 Enteric viruses

6.3.1 Background

The most common causes of viral gastroenteritis worldwide include rotaviruses, noroviruses, sapoviruses, astroviruses, and the enteric ADVs 40 and 41 and are summarized in Table 6.4 [24,127,232]. Rotaviruses are

Table 6.4 Enteric virus summary

Virus	Family	Genome	Age	Clinical significance
Adenovirus 40 and 41	Adenoviridae	DNA, double stranded	Children	Usually mild diarrhea compared to other enteric viruses, but have been associated with large outbreaks and can cause severe disease in immune-suppressed patients
Astrovirus	Astroviridae	RNA, single stranded	Children and elderly	Generally more mild than rotavirus or norovirus. Associated with outbreaks in nursing homes
Cytomegalovirus	Herpesviridae	DNA, double stranded	Immunocompromised	Gastroenteritis in immunocompromised patients such as HIV and transplant patients
Norovirus	Caliciviridae	RNA, single stranded	All ages	Nausea and diarrhea. Associated with food borne outbreaks and clusters in schools and long-term care facilities
Rotavirus	Reoviridae	RNA, segmented double stranded	Infants	Associated with outbreaks of severe diarrhea in infants and children worldwide. Estimated to cause > 500,000 deaths among children < 5 years old annual worldwide
Sapovirus	Caliciviridae	RNA, single stranded	Children and elderly	Gastroenteritis primarily in children, but recently found to cause outbreaks in nursing homes

References: 24, 87, 109, 127, 147, 155, 167, 169, 232, 236.

the most common global causes of pediatric gastroenteritis and responsible for the deaths of over 500,000 children < 5 years old annually [232,236]. Although rotavirus disease tends to be longer in duration and more severe than the other enteric viruses, all are responsible for significant morbidity and mortality. Noroviruses are genetically diverse and are associated with foodborne infections, large outbreaks (e.g., cruise ships) causing moderate to severe diarrhea [155]. Sapoviruses, first identified in 1977 in a cluster of gastroenteritis in Sapporo, Japan, primarily cause outbreaks of diarrhea in children, but have also been associated with institutional outbreaks [155,169]. Astroviruses primarily affect children < 2 years old and the elderly and generally cause milder and more limited disease than rotaviruses or noroviruses [232]. Astroviruses can be detected in asymptomatic children [147]. Astroviruses are prevalent in the winter months in temperate regions and during the rainy season in tropical regions [147]. Adenoviruses can also cause gastroenteritis especially in children. Serotypes 40 and 41 account for a majority of ADV associated gastroenteritis in children and may be responsible for anywhere from 3 to 20% of pediatric gastroenteritis in the United States [127]. Other viruses associated with sporadic gastroenteritis are BoV, CoV, toroviruses, and CMV in immunocompromised patients [6,24,109,127].

Transmission is usually through a fecal–oral route including food and water that have been contaminated during production, transport, or preparation [24]. Outbreaks of viral gastroenteritis can occur at restaurants, schools, day care centers, nursing homes, and other long-term care facilities [24,167]. Viral gastroenteritis is also a major concern in regions with inadequate potable water and sanitation, such as sites of natural disasters, refugee camps, and other areas of extreme need. Clinical presentation can include fever, nausea, vomiting, abdominal pain, and diarrhea that can range from mild self-limited diarrhea to life threatening dehydration, particularly in infants and young children. Immunocompromised patients may experience more severe disease and can shed viruses in their stool for an extended period of time. Although viral gastroenteritis is generally managed with oral or intravenous hydration fluid to replace electrolytes and water, laboratory testing can still have significant clinical utility. Confirmation of an enteric virus can prevent unnecessary antibiotic treatment and reduce testing for other causes of diarrhea. Pathogen identification in an outbreak is important for determining the source, halting the spread of disease, and preventing future outbreaks [24].

6.3.2 Molecular assays for enteric viruses

In general, the enteric viruses are difficult to grow in cell culture, therefore diagnostic methods have been limited to either rapid antigen tests or laboratory developed NAATs

[155,232]. Consequently, the cause of most cases of viral enteritis is never established. Recently, highly multiplexed commercial NAATs have been developed to detect the enteric viruses, in addition to the common bacterial and parasitic pathogens. One assay, the Luminex xTAG GGP panel, is FDA cleared (see details below) but there are also a number of assays in clinical trials or reagents available as analyte-specific reagents (ASRs). Ideally a comprehensive enteric panel consisting of viral, bacterial, and parasitic targets would be able to replace the need for multiple tests such as antigen detection, stool culture, and microscopic examination. In addition, an acceptable test would have to amply address challenging matrix issues in stool, in particular the presence of amplification inhibitors and the instability of nucleic acid targets, which can lead to false negative results. Robust extraction procedures and the use of an internal control that goes through the entire testing process are necessary to guarantee assay performance.

The xTAG gastrointestinal pathogen panel (xTAG GPP, Luminex) is a highly multiplexed PCR assay that is FDA cleared for the detection of rotavirus A and norovirus GI/GII, in addition to *Campylobacter*, *Clostridium difficile* toxin A/B, enterotoxigenic *Escherichia coli* (ETEC) LT/ST, *E. coli* O157, Shiga-like toxin producing *E. coli* STEC (*stx* 1/*stx* 2), *Salmonella*, *Shigella*, *Giardia*, and *Cryptosporidium* from fresh stool [135,136,224]. Positive results should be considered presumptive and additional confirmation testing is required. The CE marked version of GPP also detects ADV 40/41, *Vibrio cholerae*, *Yersinia enterocolitica*, and *Entamoeba histolytica*. Assay format is similar to the xTAG RVP Fast (see section 6.2), with results available in 5 h including extraction. Detection platforms include the Luminex 100/200 instruments and in Europe and Canada the small compact MAGPIX instrument (Luminex) (Figure 6.7).

The Verigene Enteric Pathogens assay (Nanosphere) uses the same test format and instruments as the Verigene RVP assay. This assay is not currently FDA cleared at the



Figure 6.7 Luminex Magpix. (Image provided by Luminex Corporation) (See insert for color representation of the figure.)

time of writing. The assay detects ADV 40/41, noroviruses, and rotaviruses plus the nonviral targets: *Campylobacter*, *Salmonella*, *Shigella*, *Y. enterocolitica*, *Vibrio*, STEC Shiga Toxin Gene 1 (stx1), and Shiga Toxin Gene 2 (stx2).

The FilmArray GI Panel (Biofire) is a pouch-based, highly multiplex, real-time nested PCR assay which runs on the FilmArray System [135,136]. The test system and assay format is the same as their Respiratory Virus Panel, previously described. The assay can detect adenovirus types 40/41, astroviruses, noroviruses GI/GII, rotavirus A, and sapoviruses. Nonviral targets included in the FilmArray GI Panel are *Aeromonas*, *Campylobacter*, *Clostridium difficile*, ETEC, enteropathogenic *E. coli* (EPEC), STEC, enteroinvasive *E. coli* (EIEC), enteroaggregative *Escherichia coli* (EAEC), *E. coli* O157, *Plesiomonas shigelloides*, *Salmonella*, *Shigella* spp, *Shigella dysenteriae*, *Vibrio* spp, *Yersinia enterocolitica*, *Cryptosporidium*, *Cyclospora cayentanensis*, *Entamoeba histolytica*, and *Giardia lamblia*. Fresh stool and stool in Cary-Blair transport media are both acceptable specimen types. After the sample and hydration solution are added to the reagent pouch, results are available in approximately 1 h [135,136].

There are two Argene/bioMérieux PCR assays (bioMérieux, Marcy l'Etoile, France) for enteric viruses, the Adenovirus Consensus PCR kit and the Calici/Astrovirus Consensus RT-PCR kit, which detect noroviruses, sapoviruses, and astroviruses [119]. These assays require NA extraction using the NucliSENS easyMAG, standard RT-PCR, and end-point detection using biotinylated probes. Both assays are not FDA cleared but are CE marked at the time of writing.

6.4 Enterovirus and parechovirus

6.4.1 Background

Enteroviruses (EV) and parechoviruses (PeV) are non-enveloped, RNA members of the *Picornaviridae* virus family (for the most current categorization: <http://www.picornaviridae.com/>). Parechoviruses were formerly classified as enteroviruses until nucleic acid sequencing determined that they were divergent enough from other enteroviruses to be placed into a separate genus within the *Picornaviridae* virus family [99]. Poliovirus, the causative agent of poliomyelitis, is limited to a few unvaccinated regions in the world and is targeted for global elimination by the Global Polio Eradication Initiative [12]. Enterovirus and parechoviruses infections are ubiquitous worldwide and commonly present as nonspecific fevers or as asymptomatic infections in children < 5 years of age [99,195,198]. EVs/PeVs are the most frequent (85–95%) cause of aseptic meningitis in the United States and may also cause encephalitis, especially in children [194,195]. Additionally, EV/PeV can cause paralysis, pericarditis, myocarditis, conjunctivitis, herpangina, respiratory

infections, and exanthems. Newborns, born to mothers with active EV/PeV infections, can develop an EV/PeV sepsis-like syndrome, with or without meningitis [233]. Enterovirus 71 has been associated with outbreaks of severe rhombencephalitis in children in Southeast Asia in recent years [212]. Both EVs and PeVs are usually transmitted through a fecal–oral route as the virus is shed through the intestinal tract [198]. In temperate regions, EV activity tends to be seasonal with a higher number of cases and outbreaks during the summer and fall [198].

While some EV/PeV infections can be diagnosed clinically or just treated symptomatically, diagnosis of EV/PeV-associated meningitis and encephalitis requires laboratory testing for proper patient management. Although EV and PeV are the most common cause of meningitis, initial clinical presentation can be difficult to differentiate from bacterial meningitis or HSV meningitis/encephalitis. Early EV/PeV meningitis can present with neutrophil predominance in the cerebrospinal fluid (CSF) resembling a bacterial meningitis [156]. A diagnosis can be particularly difficult in infants or immunocompromised patients who may lack CSF pleocytosis during infection [48,201]. Therefore, differentiation of usually benign EV/PeV meningitis from serious bacterial meningitis or HSV encephalitis is critical to ensure prompt appropriate treatment with antibiotics or acyclovir. Conversely, EV/PeV meningitis can be managed with supportive care and often does not require hospitalization [191,198].

6.4.2 Assays for enterovirus and parechovirus

Specimens for the detection of EV and PeV infections can include CSF, throat swabs, rectal swabs, pericardial fluid, and blood. Traditionally EV was isolated and identified using cell culture, requiring multiple cell lines for optimal recovery due to strain diversity [41,130]. Growth usually requires 4–8 days [133] and commercial panenterovirus DFA reagents are used to confirm cytopathogenic effect (CPE). However, some enteroviruses, Coxsackie A viruses and PeV-3 in particular, grow poorly in cell culture and therefore are rarely recovered. In general, due to low concentration of virus in the CSF, the sensitivity of culture for EV is less than 50% for CSF in cases of acute meningitis or encephalitis [51,88,159].

Therefore in suspected cases of viral meningitis or neonatal EV/PeV sepsis NAATs are the standard of care. NAATs have both sufficient sensitivity and rapid enough turnaround time (1–6 h) to clinically manage patients [80,124]. The 5' UTR region of EV and PeV are the standard target for NAATs [157,194]. Rhinoviruses share sequence homology with EVs in this region and there is a risk of cross-reactivity with HRVs in EV assays [31]. However, since HRVs are not typical CSF pathogens, this is not a significant problem in testing CSF. On the other

hand, inference of an EV meningitis/encephalitis based on an EV NAAT positive throat or rectal swab must be interpreted cautiously as the NAAT may be detecting HRV and not EV. There are only two FDA-cleared NAATs for the qualitative detection of EV in CSF and only LDTs for the detection of PeV's. The NucliSENS EasyQ Enterovirus assay (bioMérieux, France) uses NASBA and real-time molecular beacon fluorescent detection [126]. The NA extraction is performed using either the NucliSENS miniMAG or easyMAG platforms and amplification and detection steps are performed simultaneously on the NucliSENS EasyQ instrument (bioMérieux, France). The assay shows minimal cross-reactivity with HRV (only at very high titers) and takes approximately 4 h to complete [31,80,126]. The format of the assay is amenable to batch testing. The sensitivity and specificity of the molecular beacon FDA version of the assay are 95.5 and 100%, respectively, compared to the original NucliSENS EasyQ detection by electrochemiluminescence (ECL) version, which in turn was 30% more sensitive than viral cell culture [31,80,124–126]. The Xpert EV (Cepheid) is a cartridge-based, multiplex, real-time RT-PCR assay which runs on the GeneXpert system (described in section 6.2) [48,205]. The assay takes approximately 2.5 h to complete and is more amenable to random-access rapid testing. However, no residual extracted nucleic acid sample remains after the testing, whereas a separate extraction step in the NucliSENS EV assay allows for additional CSF testing for other viruses such as HSV. This can be especially beneficial when limited CSF volume is available and multiple NAATs are requested. The Xpert EV assay's sensitivity was 94–97% and specificity was 100% for EV isolates [120,158].

There are no FDA-cleared assays for the detection of EV in non-CSF specimens such as blood, respiratory, or stool. Enterovirus R-gene and parechovirus R-gene ASRs are available from bioMérieux [176]. Both assays use real-time RT-PCR with detection by Taqman probes and require a separate NA extraction step; the assays are performed on a variety of instruments. The Seeplex Meningitis ACE Detection Kit (Seegene, Gaithersburg, MD) is a RT-PCR kit that can detect five bacterial targets (*Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Listeria monocytogenes*, *Streptococcus agalactiae*) and seven viral targets (human HSV-1, HSV-2, VZV, CMV, Epstein-Barr virus (EBV), HHV-6, enteroviruses) as causes of meningitis and encephalitis [210]. However, this assay is currently not yet available in the United States.

6.5 Herpesviruses

6.5.1 Background

Herpesviruses (HV) are enveloped nonsegmented double stranded DNA viruses and include HSV-1 and HSV-2, VZV, CMV, EBV, HHV-6, HHV-7, and HHV-8 [238].

Herpesviruses are involved in variety of diseases in a wide range of tissues and can cause severe or fatal disease, especially in vulnerable populations such as neonates, HIV positive, and transplant patients [25,209]. HVs are also noteworthy for being able to enter a latent state after primary infection that can persist for decades and later reactivate to cause disease [209,238].

HSV-1 is the main cause of the common oral herpetic lesion, a common self-limiting vesicular cutaneous infection [231]. After initial infection, HSV-1 can maintain latency in the trigeminal nerve ganglion and can reactivate causing recurrent lesions. More significantly HSV-1 can present as viral encephalitis and accounts for 10–20% of encephalitis cases in the United States [216]. Mortality from HSV encephalitis can be as high as 70%, with many survivors having long-term neurological sequelae [222]. Effective antiviral treatment such as acyclovir is available and timely diagnosis can significantly improve clinical outcome [51,216,222]. In contrast to HSV-1, HSV-2 primarily causes genital lesions and is an important sexually transmitted disease; approximately 16% of persons under the age of 50 are infected in the United States [36,94]. HSV-2 via maternal transmission is also responsible for neonatal herpes, which is a medical emergency [51,67,231]. Neonatal herpes can present as a CNS and/or disseminated disease with a high morbidity and mortality; timely diagnosis and prompt antiviral treatment improves clinical outcome. HSV-2 can also cause CNS infections, although it presents as aseptic meningitis or recurrent meningitis, known as Mollaret's meningitis [222] rather than encephalitis. Recently the incidences of HSV-1 in genital lesions and HSV-2 in oral lesions have increased so that the site tropism of HSV is no longer as clear [94]. Frequency and severity of both HSV-1 and HSV-2 infections are increased in immunocompromised patients [209].

VZV is the causative agent of chickenpox and herpes zoster (shingles) [10]. After primary infection, VZV remains in a latent state in the dorsal root ganglion. Herpes zoster is reactivation of latent VZV that presents with frequently painful skin lesions in a dermatome pattern [84]. VZV can present as either meningitis or encephalitis in primary or reactivation disease. Infection of a seronegative woman during pregnancy can cause serious congenital disease in the fetus [123]. Infections in adults are more likely to be more severe with potential CNS involvement, pneumonitis, and disseminated disease [10].

Cytomegalovirus and EBV commonly present as a febrile childhood infection or mononucleosis-like illness in older patients [72,140,162]. Seroprevalence studies have shown that the majority of people are infected by adulthood [30,140,150]. Human herpesvirus-6 is the cause of the common pediatric infection roseola infantum, otherwise known as exanthema subitum or sixth disease. Less commonly HHV-6 can cause febrile

seizures and meningoencephalitis [96,239]. HHV-7 is thought to cause similar diseases as HHV-6, but less is known about its pathogenesis and epidemiology [34,221]. Human herpesvirus 8-(HHV-8) is responsible for Kaposi sarcoma and Castleman's disease, which most frequently occurs in HIV-positive patients [2,57]. CMV, EBV, and HHV-6 also can be found in CNS infections, particularly in immunocompromised patients [51]. These herpesviruses are also important in reactivation disease of post-transplant patients and are discussed in the next section that describes transplantation testing [209].

6.5.2 Molecular assays for qualitative detection of herpesviruses

NAATs are the "gold standard" for diagnosing herpesvirus CNS disease due to high sensitivity (generally in the range of 5–50 viral copies/reaction) and the rapid time to results (generally within several hours) [51,216]. CSF samples that are PCR negative upon initial clinical presentation should be repeated after several days, as virus quantities can increase to detectable levels several days post-symptom onset [51]. In cases of suspected maternal–neonatal transmitted disseminated disease, samples from multiple sites such as CSF, blood, eyes, mouth, pharynx, rectum, and skin lesions, should be tested. Currently, there are no FDA-cleared tests for the detection of herpesviruses in CSF. Most NAATs in use are LDTs with varying sensitivity and scope of analytes detected.

The Herpes Consensus Generic kit (bioMérieux) uses step primers and end-point PCR to screen for six herpesviruses (HSV-1, HSV-2, VZV, CMV, EBV, HHV-6) [29,227]. The amplicons are hybridized to biotinylated probes, which bind to streptavidin–peroxidase enzyme conjugates which produce a color in the presence of kit substrate. The PCR products are detected by using a standard colorimetric microplate reader. If the generic assay is positive, the Hybridowell Herpes Identification (bioMérieux) kit is used to specifically identify which herpesvirus(es) is present. The assay can be validated for use with CSF, vitreous fluid, tissue, lesions and blood [29,227].

Several other commercial vendors manufacture ASRs for the various herpesviruses, examples of which include the Cepheid HSV 1/2 tracer kit [177,193], artus Qiagen HSV1/2 TM PCR kit [189,193], and Roche Lightcycler HSV 1/2 and VZV Primer/Hybridization Probes [199,202].

There are three FDA-cleared NAAT assays for the detection of HSV-1 and HSV-2 in genital lesions. The BD ProbeTec Herpes Simplex Viruses Qx Amplified DNA Assay (Becton Dickinson) detects HSV-1 and/or HSV-2 in anogenital specimens [223]. The BD ProbeTec Herpes Simplex is a batch mode microwell assay performed on the BD Viper system, which automates specimen preparation, extraction, amplification, and detection. After NA extraction, target DNA is amplified by SDA and HSV-1 and HSV-2 are detected in separate microwells using

fluorescent probes. When compared to viral culture, the sensitivity and specificity when collected with the Qx Swab Diluent were 96.7 and 95.1%, respectively, for HSV-1 and 98.4 and 80.6%, respectively, for HSV-2 [223].

The IsoAmp HSV Assay (BioHelix, Beverly, MA) is a FDA-cleared manual assay for the detection of HSV from genital and oral specimens and uses helicase-dependent amplification [226]. There is no extraction; the sample is diluted in an elution buffer, added to an amplification tube with reagent, and then incubated isothermally (64°C) in a heat block for HDA amplification. The amplification tube is attached to a disposable cassette where the target amplicons are visualized using a lateral flow chromatographic strip; the assay does not differentiate between HSV-1 or HSV-2 [131,152]. The turn-around time is approximately 1.5 h and had 98.8% agreement when compared to enzyme-linked virus inducible system (ELVIS) cell culture (Diagnostic Hybrid, Inc.) [152,183]. This assay is notable for not requiring an extraction instrument, thermal cycler, or detection instrument.

The Multicode RTx HSV 1&2 Kit (Luminex) is a PCR-based FDA cleared test for vaginal specimens [202]. The technology is based upon the unique patented MultiCode synthetic base pairs isoC and isoG with properties that enable site-specific incorporation of the isobases during amplification. MultiCode-RTx primers include a fluorescent reporter in close proximity to an isoC base on the 5' end. During the amplification reaction, the MultiCode base isoG that is covalently attached to a quencher present in the reaction mix is incorporated opposite to the isoC. This site-specific interaction of the reporter-labeled isoC and the quencher-labeled isoG results in a decrease in fluorescence. Since the reporter quenching is reversible, melting-curve analysis can be used to confirm the presence of target [202].

6.6 Viral infections associated with transplantation

6.6.1 Background

Hematopoietic stem cell transplant (HSCT) or solid organ transplant (SOT) recipients require long-term immunosuppressive therapy to prevent rejection of the graft. Therefore, transplantation patients are vulnerable to opportunistic infections from a variety of organisms, including HSV-1, HSV-2, VZV, CMV, BK virus (BKV), JC virus and HHV-6 [25,56,107,108,209,230] (Table 6.5). Primary viral infection can occur in seronegative recipients either through exogenous exposure or reactivation of virus present in the donor tissue. Transplant patients are also at increased risk of disease from reactivation of latent infections, leading to cutaneous, systemic, and CNS disease with significant morbidity and mortality.

CMV is one of the most common and significant sources of disease in transplant patients [72,122,137]. CMV is highly

Table 6.5 Post-transplant virus summary

Virus	Family	Genome	Subtypes	Latency	Clinical significance post-transplant	Specimen(s)	International standard	Treatment	Reference(s)
Adenovirus (ADV)	Adenoviridae	DNA	Genetic diverse, more than 50 serotypes	Lymphoid tissue Other tissue probable	Encephalitis, pneumonitis, colitis, hepatitis, nephritis, hemorrhagic cystitis, and disseminated disease. Persistent viral load rise in BMT patients correlate with adverse outcome	Plasma, whole blood	NA	Cidofovir IVIg Immune suppression reduction	58, 73, 74, 108, 114
BK virus (BKV)	Polyomaviridae	DNA	At least 4 subtypes	Renal tissue Lymphoid tissue Other tissue probable	BK virus associated nephropathy (BKVN) in renal transplant patients; possible renal dysfunction or renal graft failure. Hemorrhagic cystitis other transplant patients	Urine, plasma, whole blood	NA	No FDA approved antivirals, but cidofovir has been used. Immune suppression reduction	20, 107, 185, 190, 211
Cytomegalovirus (CMV)	Herpesviridae	DNA	NA	Neutrophils, macrophages, other tissues	Pneumonitis, retinitis, colitis, hepatitis, leukopenia, thrombocytopenia, graft versus host disease, disseminated disease, and failure of organ graft	Plasma, serum, whole blood	WHO International Standard Available	Ganciclovir Valganciclovir Valacyclovir Cidofovir Foscarnet IVIg Immune suppression reduction	27, 72, 122, 137, 211
Epstein-Barr virus (EBV)	Herpesviridae	DNA	Types A and B	B-cell lymphocytes	Post-transplant lymphoproliferative disorder (PTLD)	Plasma, serum, whole blood	WHO International Standard Available	Anti-CD20 monoclonal antibodies Chemotherapy Immune suppression reduction	9, 44, 75, 90, 93, 209
Human herpes virus-6 (HHV-6)	Herpesviridae	DNA	HHV-6a and HHV-6b	Macrophages other tissues probable	Encephalitis, pneumonitis, hepatitis, bone marrow suppression, graft versus host disease, associated with CMV disease	Plasma, whole blood	NA	No FDA approved antivirals, but ganciclovir, foscarnet, and cidofovir have been used. Immune suppression reduction on	47, 50, 56, 137, 240

prevalent in the general population with about 60–70% of adults in the United States serologically positive for CMV [30]. CMV seronegative transplant recipients are at a very high risk to develop primary CMV disease through either donor tissue or blood transfusions from CMV seropositive persons. When the cellular immune system, which normally controls CMV, is suppressed, CMV can either reactivate and replicate at low levels without causing disease or manifest clinically in a variety of ways including a mononucleosis-like syndrome, gastrointestinal disease, multiorgan failure, pneumonitis in lung transplantation, or hepatitis in liver transplantation, with a risk of allograft dysfunction or failure [72,121]. Antiviral treatment such as ganciclovir or foscarnet can be used either preemptively (e.g., seronegative recipient and a seropositive organ donor) or prophylactically, once a patient is found to be at risk for progression to CMV disease [3,121]. Evaluating the actual amount (viral load [VL]) of CMV present in a patient's blood rather than just the absence or presence of virus is an important tool in differentiating insignificant viral shedding from active disease, for assessing need for prophylactic therapy, and for monitoring the effectiveness of treatment [32,122,137].

EBV is ubiquitous, with most individuals showing serological evidence of infection increasing by age [44,173]. Reactivation of EBV in transplant patients can cause fever, adenopathy, renal dysfunction, hepatic dysfunction, and weight loss. EBV maintains latency in host B-lymphocytes and immunosuppressed transplant patients are at increased risk of lymphoma or post-transplant lymphoproliferative disorder (PTLD) [8,75,219]. In transplant and HIV patients, EBV replication in the CSF is also associated with risk of CNS B-cell lymphoma. EBV NAAT VL testing in transplant patients has shown that elevated or sustained levels of EBV in plasma or whole blood correlates with an increased risk for PTLD [1,75,93]. Whole blood usually yields a higher viral load and may be more sensitive and detect EBV earlier [75,76]. In contrast, using plasma to measure EBV viral load may be more specific in detecting EBV disease instead of asymptomatic shedding [75]. Abnormally high levels of EBV can be followed up with imaging for masses, tissue biopsy of suspicious masses, and adjustments of the level of immune suppression. EBV associated lymphoma can be treated with rituximab (an anti-CD20 monoclonal antibody), chemotherapy, and radiation [90].

HHV-6 most likely maintains latency in macrophages and possibly other cells after primary infection in childhood [56,240]. Reactivation in HSCT patients can result in encephalitis, pneumonitis, hepatitis, bone-marrow suppression, graft versus host disease, and increased risk of concurrent CMV disease [56,240]. Blood and CSF HHV-6 viral loads have been used to monitor for active HHV-6 disease or risk of disease. However, HHV-6 viral loads need to be interpreted with caution as

patients with integrated HHV-6 normally have high levels of HHV-6 in the blood without associated disease. While there are no FDA-approved drugs for HHV-6, ganciclovir, foscarnet, and cidofovir have been used to treat HHV-6 disease in immunosuppressed patients [47].

Adenovirus, as described earlier in sections 6.2 and 6.3, infects a wide variety of tissues, and has been shown to persist latently in lymphoid tissue and most likely other tissues as well. If reactivated in an immunosuppressed transplant patient, ADV can present as encephalitis, pneumonia, hepatitis, colitis, nephritis, and hemorrhagic cystitis [58,108,114]. Patients during the first 100 days after HSCT are at greatest risk for disseminated ADV disease, with a mortality rate that can approach 80% [108]. Adenovirus detection in plasma or whole blood, especially in patients with hematological disease, can predict progression to disease. Patients with a persistently elevated, or with a sustained rise in ADV viral load levels, are at increased risk of poor outcome and increased mortality [73,137]. However VL levels are not always proportional with severity of symptoms. The utility of monitoring ADV VL in SOT patients though is less clear [108,114]. ADV VL can be used to monitor response to treatment with cidofovir, intravenous immunoglobulin (IVIG), or reduction in immunosuppression [108,114].

BK virus is a DNA virus and member of the polyomavirus family. BKV is ubiquitous and seroprevalence studies have demonstrated infection in the majority of people worldwide [20,190]. After primary infection BKV appears to persist in urinary epithelium and lymphoid tissue. Reactivation in renal transplant patients can cause pyuria, ureter stenosis, ureter ulcerations, and BKV-associated nephropathy (BKVN), which can result in renal dysfunction or in some cases even renal graft failure [20,107]. Detection of high levels of BKV in urine, plasma, or whole blood can be used to screen for patients at risk for BKVN. NAATs can detect BK virus earlier than the presence of decoy cells in urine cytology, which was the traditional method [185]. These results can be used in conjunction with other laboratory tests such as proteinuria and creatinine to monitor for renal dysfunction. Hirsch et al. recommended that renal transplant patients be monitored for BKV periodically for the first 2 years post-transplant. They also suggest the threshold for disease was 10^7 copies/mL urine or 10^4 copies/mL plasma [107]. Definitive diagnosis of BKVN is confirmed by renal biopsy and is usually managed with adjustment of immunosuppression.

6.6.2 Molecular assays for transplantation monitoring

The vast majority of CMV, EBV, ADV, BKV, and HHV-6 VL tests are currently laboratory developed tests. The College of American Pathologists (CAP) proficiency

testing results have demonstrated a wide interlaboratory variation in quantification when identical samples are tested using different LDTs. The CAP Microbiology Resource Committee evaluated a multivariate analysis of parameters associated with VL testing in 198 laboratories [101]. This study demonstrated that the marked variability seen in VL results was linked to multiple aspects of the test design and performance, including the selection of different genetic targets, primers, probes, and calibrators. Not assessed in the CAP study but also critically important is the variation in quantification due to the use of different specimen types (plasma, whole blood, peripheral blood mononuclear cells, urine, etc.). Most importantly, until recently, no international standards, such as those available for HIV, hepatitis C virus (HCV) and HBV quantification, were available by which both commercial manufacturers and laboratories could calibrate their assays so that VL results between laboratories would be comparable [142,211]. Therefore, due to the high variation in VL results from one laboratory to another, the type of transplant, and the intent of the assay (diagnosis, pre-emptive monitoring, assessment of treatment response, etc.), specific guidelines for interpretation of VL results need to be established at each institution based on clinical experience. Recently, World Health Organization (WHO, Geneva, Switzerland) and National Institute of Standards and Technology (NIST, Gaithersburg, MD) international standards became available for CMV quantification and WHO standards for EBV quantification [28]. As demonstrated by the high levels of interlaboratory correlation with HIV, HCV, and HBV VLs, the international standards for CMV and EBV should help to standardize the interpretation of VL values and create consensus guidelines, independent of the assay used [28,122]. In the near future international quantitative standards for BKV and ADV will also likely be available.

In the past CMV monitoring was performed using an antigenemia assay in which polymorphonuclear cells (PMNLs) are separated from whole blood, fixed onto microscope slides, stained with anti-pp65 CMV fluorescent antibodies, and the number of infected PMNLs counted. CMV antigenemia testing has been shown to have good clinical utility, but the assay is labor intensive, requires significant expertise in performing, is limited in leukopenic patients, and requires a fresh blood sample (<24 h) as PMNLs deteriorate quickly [33,79]. Quantitative CMV NAATs are now the standard of care for monitoring CMV VL due to target stability (DNA instead of PMNLs), which allows for testing at a reference laboratory and the ability to test leukopenic patients such as HSCT patients. Comparisons between CMV VL and antigenemia have shown good agreement at predicting active CMV disease and monitoring treatment, though viral levels between the two methods often did not correlate closely [122].

Currently, the only quantitative FDA-approved NAAT assay is the COBAS TaqMan CMV Test (Roche, Basel, Switzerland). The COBAS TaqMan CMV Test was approved for quantifying CMV in plasma, and is used for the management of patients at risk for CMV infection and/or undergoing antiviral therapy after SOT or HSCT [17,32,107]. The COBAS TaqMan CMV Test is a batch assay that uses the COBAS Ampliprep instrument for automated specimen processing and the COBAS TaqMan analyzer or the COBAS TaqMan 48 analyzer for automated amplification and detection. The assay targets a conserved region of the polymerase gene (UL54) and can quantify CMV from 137 to 9,100,000 IUs/mL of plasma. The Abbott RealTime CMV kit (Abbott) and Artus PCR kit (Qiagen) are ASRs that have been used to develop LDTs using the Abbott m2000sp automated processor in conjunction with the Abbott m2000rt real-time amplification and detection instrument [23,40,42,79]. Other assays not FDA approved include the LightCycler CMV Quantification kit (Roche) and the CMV R-gene assay kit (Argene/bioMérieux), which also can be used to validate an in-house laboratory assay [27,89].

There are several commercial reagents available for developing quantitative EBV, ADV, BKV, and HHV-6 VL assays, but currently there are no FDA-approved tests [137]. Many ASR reagents or research-use only (RUO) kits are not available in the United States, thus laboratories should consult the company websites for the latest availability. Reagents include: EBV:EBV R-gene real-time PCR kit (bioMérieux, France); Light Cycler EBV Quantification kit (Roche); the Abbott EBV PCR Kit (Abbott); BKV:BKV R-gene real-time PCR kit (bioMérieux, France); Eragen BKV ASRs (Eragen Biosciences, Madison, WI, now part of Luminex); Elitech BKV ASRs (formerly Nanogen now part of Elitech, Princeton, NJ); HHV-6:HHV-6 R-gene real-time PCR kit (bioMérieux, France); ADV:Adenovirus R-gene quantification kit (bioMérieux, France) [118]. The ADV assays generally target conserved regions of the hexon or fibril genes. However, due to ADV genetic diversity, multiple primer probes are needed to detect most of the strains [74]. As with all LDTs the laboratory should establish the performance of the assays with divergent serotypes of ADV, as the assay may quantify certain serotypes differently. Unfortunately there are few quantitative reference standards for the different ADV species and serotypes.

6.7 Implementation and additional considerations

The Laboratory must have appropriate procedures in place to assure that the performance characteristics of NAATs are either verified (FDA cleared/approved assays) or validated (LDTs, RUO kits, investigational use only (IUO) kits, or

laboratory modified FDA-cleared/approved kits) prior to clinical use. Guidelines for what is required for verification/validations and ongoing quality control are defined by the Clinical Laboratory Improvement Act (CLIA), the CAP, and local regulatory authorities [26,43,60,116,200]. Verification means that the laboratory is able to reproduce the expected performance characteristics of the test as put forth by the manufacturer. The laboratory should verify that they can achieve accuracy, precision, and analytical measurement range, including linearity if quantitative, comparable with the standards established by the manufacturer of the assay [26,65,124]. Validation requires the laboratory to establish additional performance characteristics of the assays such as analytical sensitivity (lower limit of detection (LOD) for qualitative assays, LOD and lower limit of quantification (LOQ) for quantitative assays), analytic specificity (interference), and normal references ranges in addition to the requirements listed above for verification of FDA-cleared tests [26,60,116]. Finally, both verification and validation require the testing of positive and negative clinical samples. Non-FDA-cleared/approved tests require a statement with test results that confirms the laboratory developed and determined the performance of the assay and that it has not been cleared/approved by the FDA [26,60,116].

Ongoing performance of the reagents and instruments must be additionally verified by running appropriate positive and negative controls according to local, state and national regulatory requirements. Laboratories also need to be conscious of the fact that genetic variation does occur, especially with RNA viruses [54]. Target sequences can change over time through mutations (e.g., HIV-1), or new viral variants can emerge (e.g., 2009 FluA H1N1) thereby decreasing the performance of both FDA cleared/approved and LDTs by suboptimal primer/probe binding. Primer/probes sequences of LDTs should be verified yearly against an international database such as GenBank [81]. If possible, clinical results from FDA-cleared/approved tests should be occasionally correlated to another assay, or clinically, in order to verify continued assay performance. Participation in a proficiency-testing program, either commercial or in-house, is required. Instruments must be up to date with servicing and calibrations according to the manufacturer's requirements [26,60]. Laboratories should have a process in place to prevent cross-contamination, either from sample to sample or by amplicons. Appropriate laboratory design, workflow, barriers, good laboratory practices, cleaning of work areas, random placement of negative controls, and swipe tests, can serve to prevent, or detect potential contamination [11]. Finally, technical competency needs to be assessed prior to clinical testing and thereafter yearly [60,116,208].

Finally, when incorporating a new NAAT assay, the laboratory must also consider the appropriate usage of the

assay in various clinical settings and, if indicated, how it should be used in combination with other NAATs or conventional testing. A more rapid, but less sensitive, method may need to be supplemented with a slower, but more sensitive method. In addition, if an assay has a narrow range of targets, the laboratory may need to supplement with additional methods such as cell culture to test for other clinically relevant viruses or resistance markers. Laboratories must ensure that clinicians are aware of the performance characteristics of the assays, the limitations of the assays, and, just as importantly, the interpretation of the assay results.

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Viral Hepatitis

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7.1 Introduction/background

Hepatitis means inflammation of the liver; it can be caused by infections, toxins, or other systemic conditions. While many viruses (including cytomegalovirus (CMV), Epstein–Barr virus ([EBV), adenovirus, yellow fever virus, and others) can cause hepatitis, this chapter will focus on the viruses that cause hepatitis as the primary clinical manifestation; namely, hepatitis A, B, C, D and E. Patients with acute viral hepatitis usually develop nausea, anorexia, malaise, and abdominal pain. Patients often develop fever, jaundice, dark urine, and clay-colored stools. Laboratory studies demonstrate elevated aminotransferase levels. Occasionally, extrahepatic manifestations including rash, arthralgias, arthritis, cryoglobulinemia, and glomerulonephritis occur. Diagnostic studies include serology, antigen detection, nucleic acid quantitation, genotyping, and sequencing, and they vary for each hepatitis virus.

Epidemics of jaundice have been described as far back as the 1600s. It was noted during treatment for smallpox in 1885 that clinics with poor infection control practices, including syringe reuse, had more outbreaks of hepatitis. Work done by Krugman et al. in the 1950s established two distinct clinical entities of hepatitis, one with fecal–oral transmission and the other with parenteral transmission; his work also established the incubation period of both viruses [44]. In 1965, the hepatitis B surface antigen was isolated [14]. In 1973, routine screening of blood donors for hepatitis B was started. In 1977, hepatitis D virus was isolated. In 1979, hepatitis A virus was grown in tissue culture. In 1980, hepatitis E was discovered. Hepatitis C was isolated and blood supply screening initiated in 1991 [3].

In 2012, an estimated 4.4 million Americans were living with chronic hepatitis and many do not know they are infected. Additionally, viral hepatitis is the leading cause of liver cancer and the most common reason for liver

transplantation [15,39]. The epidemiological, diagnostic considerations, and commercial laboratory methods used in the management of viral hepatitis will be discussed in this chapter; however, histological techniques used to diagnose and stage viral hepatitis are beyond the scope of this chapter.

7.2 Clinical manifestations, epidemiology, and diagnostic considerations by virus

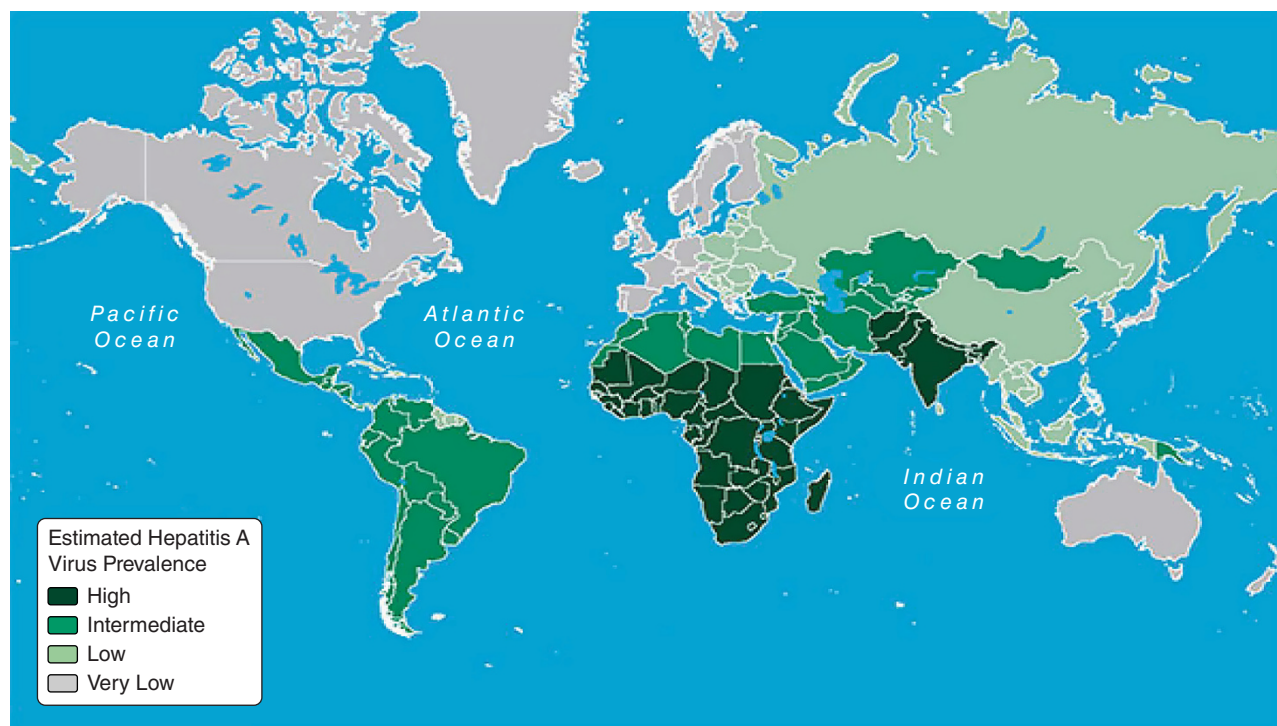
7.2.1 Hepatitis A virus

Hepatitis A virus (HAV) is a nonenveloped, single-stranded RNA virus in the *Picornaviridae* family (Table 7.1). Infection with HAV generally causes an acute, self-limited, inflammatory disease of the liver. The spectrum of clinical disease ranges from asymptomatic infection to, rarely, fulminant disease. Patients with HAV infection usually describe a mild prodrome of fevers, chills, headache, malaise and fatigue followed (usually within 1–7 days) by jaundice, dark urine, and pale or clay-colored feces [41]. One study found that 66% of patients had recovered from acute infection with hepatitis A within 2 months, 85% within 3 months, and nearly all recovered by 6 months [68]. Infection with HAV does not cause chronic liver disease, but disease relapse has been reported in some cases [63,68]. Clinical manifestations vary by age, with older persons having more symptoms (e.g., jaundice) while children less than 6 years of age are usually asymptomatic [32,76]. According to the Centers for Disease Control and Prevention (CDC), the mortality rate from HAV infection in the United States was 0.12 deaths per 100,000 population in 2008 [17].

The number of reported cases of HAV infections has been decreasing each year in the United States since the vaccine was first introduced in 1996. In 2006, the Advisory

Table 7.1 Summary of hepatitis A, B, C, D, E viruses

	Hepatitis A	Hepatitis B	Hepatitis C	Hepatitis D	Hepatitis E
Virus family	<i>Picornaviridae</i>	<i>Hepadnaviridae</i>	<i>Flaviviridae</i>	<i>Deltaviridae</i>	<i>Hepeviridae</i>
Nucleic acid	Single-strand RNA	Partially double-strand DNA	Single-strand RNA	Single-strand RNA, lacks RNA polymerase	Single-strand RNA
Envelope	No	Yes	Yes	Yes	No
Incubation period, median	32 days [44]	65 days [44]	14–160 days	2–8 weeks	15–45 days
Modes of transmission	Fecal–oral, person-to-person	Blood, sexual, perinatal	Blood, sexual (rare)	Blood, sexual	Fecal–oral, waterborne
Risk factors	Travel to endemic areas, MSM, food-/waterborne outbreaks, daycare settings	MSM, IVDU, blood transfusion	IVDU, blood transfusion	Requires coinfection with hepatitis B	Contaminated water, zoonotic exposure, pregnancy
Chronic infection	No	Yes	Yes	Requires coinfection with hepatitis B	Can occur
Vaccine preventable	Yes	Yes	No	Via hepatitis B vaccine	Vaccine under development

**Figure 7.1** Hepatitis A seroprevalence worldwide. (Source: <http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/hepatitis-a.htm>) (See insert for color representation of the figure.)

Committee on Immunization Practices (ACIP) recommended the hepatitis A vaccine routinely to all children in the United States [24]. In 2010, 1670 cases of HAV infection were reported in the United States, which represents a significant decrease from 13,397 cases in 2000 [17]. Worldwide, seroprevalence is classified into regions of high, intermediate, low, or very low HAV endemicity (Figure 7.1).

HAV is transmitted through the fecal–oral route. Transmission occurs through food and waterborne outbreaks and close personal contact with infected individuals.

Reported risk factors for HAV acquisition include international travel, close contact with an infected person, employee or child in a daycare center, food or water exposure during a common-source outbreak, illicit drug use, and men who have sex with men [40]. Fecal excretion of HAV can occur 14–21 days before the onset of jaundice and can continue until 8 days after symptom onset (Figure 7.2). Prolonged fecal shedding of HAV can occur, especially in infants and children; HAV RNA has been detected in the feces of infants up to 4 months after infection [59].

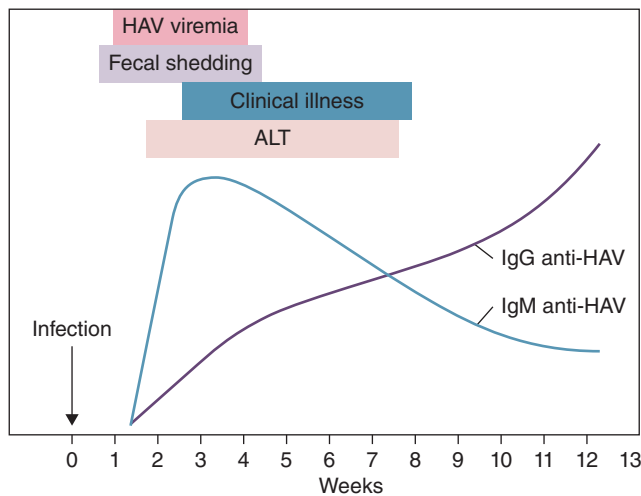


Figure 7.2 The clinical, virologic, and serologic course of acute hepatitis A virus (HAV) infection. ALT, alanine aminotransferase. (Reproduced with permission from Mandell, Douglas, and Bennetts *Principles and Practice of Infectious Diseases*, 7th Edition, 2010, M.P. Curry and S. Chopra, Acute Viral Hepatitis, 1577–1592, Churchill Livingstone/Elsevier, Philadelphia, PA.) (See insert for color representation of the figure.)

Acute HAV infection is diagnosed through detection of immunoglobulin M (IgM) antibody to hepatitis A virus. IgM anti-HAV is detectable 5–10 days after exposure (Figure 7.2). Past exposure can be identified by detection of immunoglobulin G (IgG) to HAV. HAV RNA can be detected in serum and stools of most persons during the acute phase of infection using nucleic acid amplification but this test is generally only used in research settings. Detection of HAV antigen in the stool (by enzyme immunoassay or HAV RNA PCR) does not necessarily indicate active infection; the period of infectivity is shorter than the period during which HAV RNA is detectable in feces.

Treatment is generally supportive during acute HAV infection. Post-exposure prophylaxis with the HAV vaccine and, in some instances, immunoglobulin, of exposed, susceptible contacts is recommended [5].

7.2.2 Hepatitis B virus

Hepatitis B virus (HBV) is a partially double stranded, enveloped, DNA virus in the *Hepadnavirus* family (Table 7.1); HBV is currently classified into eight main genotypes (named A–H). The onset of acute HBV infection is generally insidious, with anorexia, nausea, vomiting, abdominal distension, and absent to mild fever; arthralgias and rash may occur. Disease severity ranges from an asymptomatic transaminitis to fulminant hepatic necrosis (occurs in < 1%). The incubation period is 65 days (range: 28–160 days) [44]. The case-fatality rate is 1% but may be higher in older adults, pregnant women, and newborns. The risk of developing a chronic HBV infection decreases with increasing age at time of infection. Chronic infection occurs

in > 90% of perinatally infected infants, 10–20% of children infected between ages 1 and 5 years, and approximately 5% of immunocompetent older children and adults [43]. Over time, 15–40% of persons with chronic HBV infection will develop liver cirrhosis or hepatocellular carcinoma [20].

In 2010, 3350 cases of acute hepatitis B were reported to the CDC [17]. There are an estimated 800,000 to 1.4 million people living with chronic hepatitis B infection in the United States [75]. Globally, an estimated 240 million are chronically infected with hepatitis B and an estimated 600,000 persons worldwide die from hepatitis B-related liver disease annually [52] (Figure 7.3). The incidence of acute hepatitis B in the United States has declined dramatically over the past two decades with universal vaccination recommendations (from 11.5 acute cases per 100,000 population in 1985 to 1.1 per 100,000 in 2010) [17].

HBV is transmitted by percutaneous or mucosal exposure to blood or body fluids of an infected person, most often through injection drug use, sexual contact, from mother to child (perinatal transmission), or from unsafe blood transfusions. HBV is 50–100 times more infectious than HIV and can survive outside the body for at least 7 days [16].

The detection of hepatitis B surface antigen (HBsAg) indicates either acute or chronic active infection (Figure 7.4). The presence of IgM antibody to the hepatitis B core antigen (IgM anti-HBc) indicates acute infection (within past 6 months), while the IgG anti-HBc is nonspecific and can occur in acute, chronic, or resolved infection (Figure 7.4, Table 7.2). Antibody to HBV surface antigen (anti-HBs) develops after natural or vaccine exposure and generally indicates recovery and immunity from HBV infection. Hepatitis B E-antigen (HBeAg) is found during acute and chronic hepatitis B infection. The presence of HBeAg indicates that the virus is replicating and there is increased infectivity. Hepatitis B E antibody (anti-HBe) is a predictor of long-term clearance of HBV in patients on antiviral therapy (Figure 7.4). The US Preventative Services Task Force (PSTF) recommends performing an HBsAg test on all pregnant women at the first prenatal visit.

Treatment of chronic active HBV infection is indicated in some patients. The US Food and Drug Administration (FDA) approved therapies for hepatitis B, as of this writing, include interferon, lamivudine, adefovir, entecavir, tenofovir, and telbivudine [45].

HBV vaccine is recommended for all infants in the United States [47] and throughout the world [51]. The complete vaccine series induces protective antibody levels in more than 95% of infants, children, and young adults. Protection lasts at least 20 years and is possibly lifelong [47].

7.2.3 Hepatitis D virus

Hepatitis D virus (HDV), also known as “delta hepatitis,” is a unique RNA virus that can only infect and replicate in cells in the presence of HBV (Table 7.1). HDV, therefore,

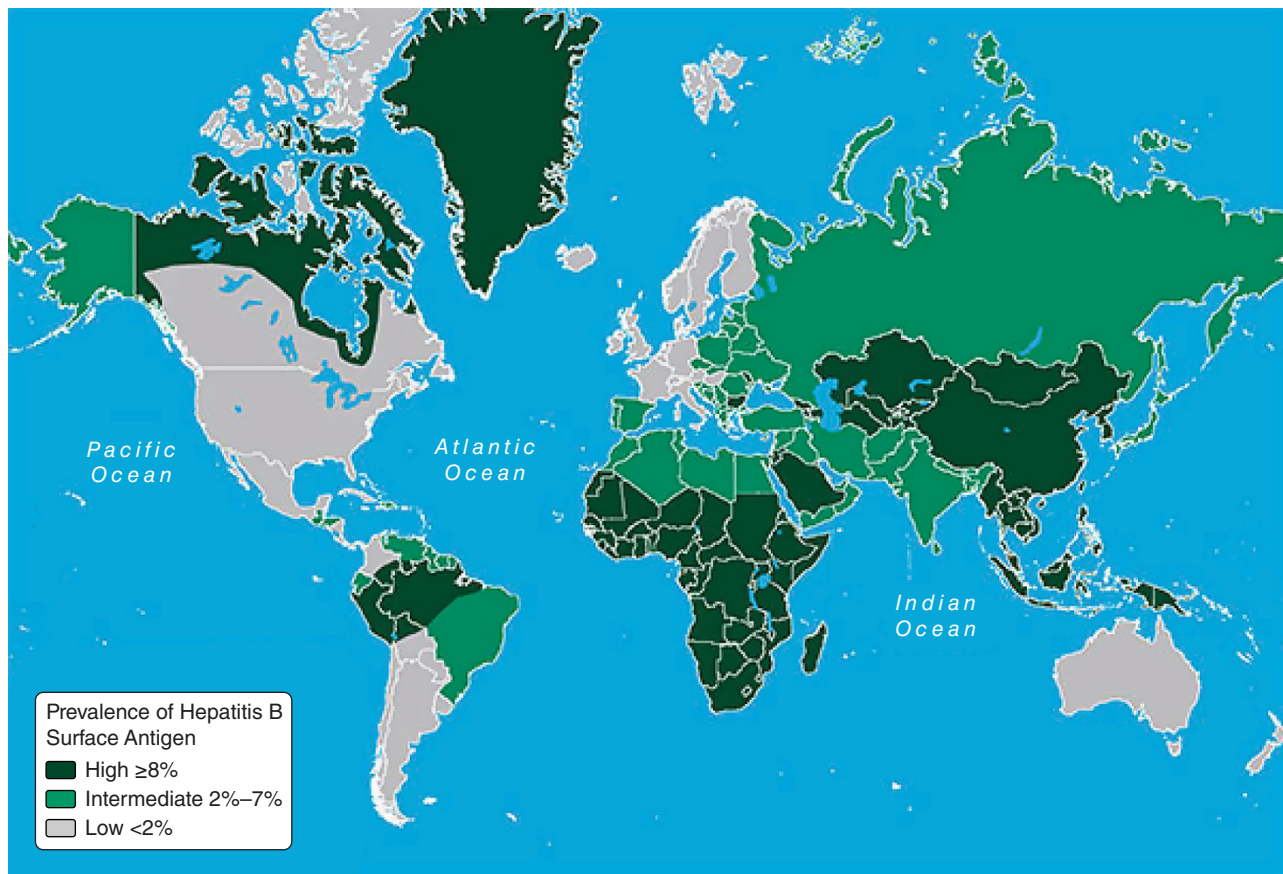


Figure 7.3 Hepatitis B seroprevalence worldwide. (Source: <http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/hepatitis-b.htm>) (See insert for color representation of the figure.)

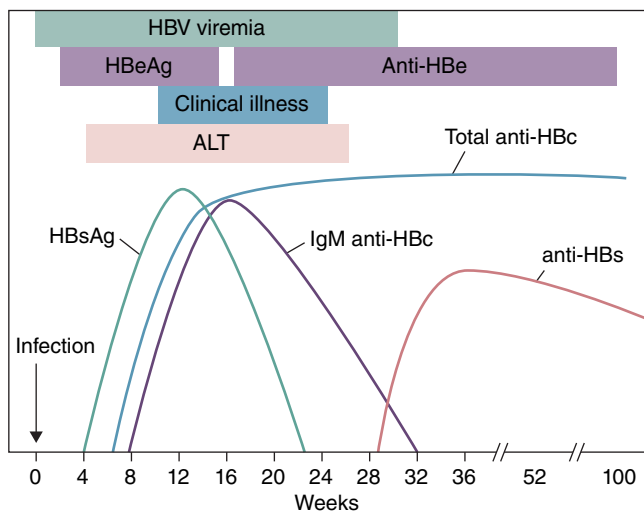


Figure 7.4 The clinical, virologic, and serologic course of acute hepatitis B virus (HBV) infection. ALT, alanine aminotransferase; HBc, hepatitis B core; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; IgG, immunoglobulin G; IgM, immunoglobulin M. (Reproduced with permission from Mandell, Douglas, and Bennetts *Principles and Practice of Infectious Diseases*, 7th Edition, 2010, M.P. Curry and S. Chopra, Acute Viral Hepatitis, 1577–1592, Churchill Livingstone/Elsevier, Philadelphia, PA.) (See insert for color representation of the figure.)

is acquired through either HBV–HDV coinfection or by HDV superinfection [57]. Persons who are coinfectd with hepatitis D may have more severe hepatic inflammation compared to HBV mono-infected persons [10].

Worldwide, approximately 15 million people are infected with HDV with higher prevalence in Venezuela, Brazil, Romania, and Italy and lower prevalence in the United States [58]. Both IgM and IgG antibodies to the hepatitis delta antigen (anti-HDV) are detectable during infection. HDV nucleic acid detection is available in research settings. Interferon alfa-2a is the only FDA-approved treatment for chronic hepatitis D, as of this writing. Vaccination against hepatitis B infection is an important tool to prevent HDV infection.

7.2.4 Hepatitis C virus

Hepatitis C virus (HCV) is an enveloped, single-strand RNA virus in the *Flaviviridae* family (Table 7.1). HCV infection is the most common chronic bloodborne infection in the United States; approximately 3.2 million Americans are chronically infected [11]. Worldwide, an estimated 170 million persons have chronic HCV infection [1] (Figure 7.5). Six HCV genotypes (named 1–6) are known to exist with variable geographic distribution.

Table 7.2 Interpretation of hepatitis B diagnostic studies

	Susceptible, Never exposed	Immune through natural exposure	Immune through vaccination	Acute infection	Chronic Infection	Indeterminate*
HBsAg	–	–	–	+	+	–
IgG Anti-HBc	–	+	–	+	+	+
IgM Anti-HBc	–	–	–	+	–	–
Anti-HBs	–	+	+	–	–	–

* Could indicate one of four scenarios including: (i) resolved infection, (ii) false positive anti-HBc, (iii) chronic infection at low level, and (iv) resolving acute infection.

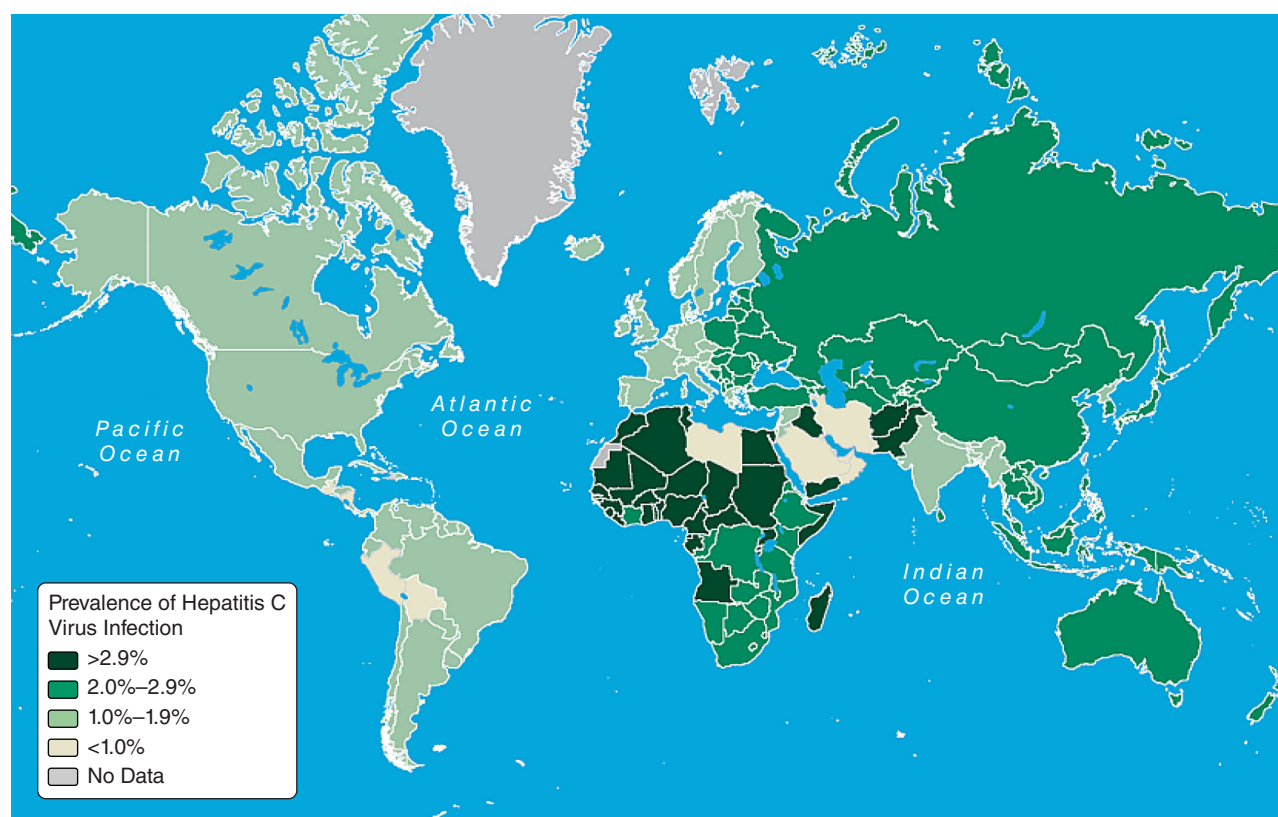


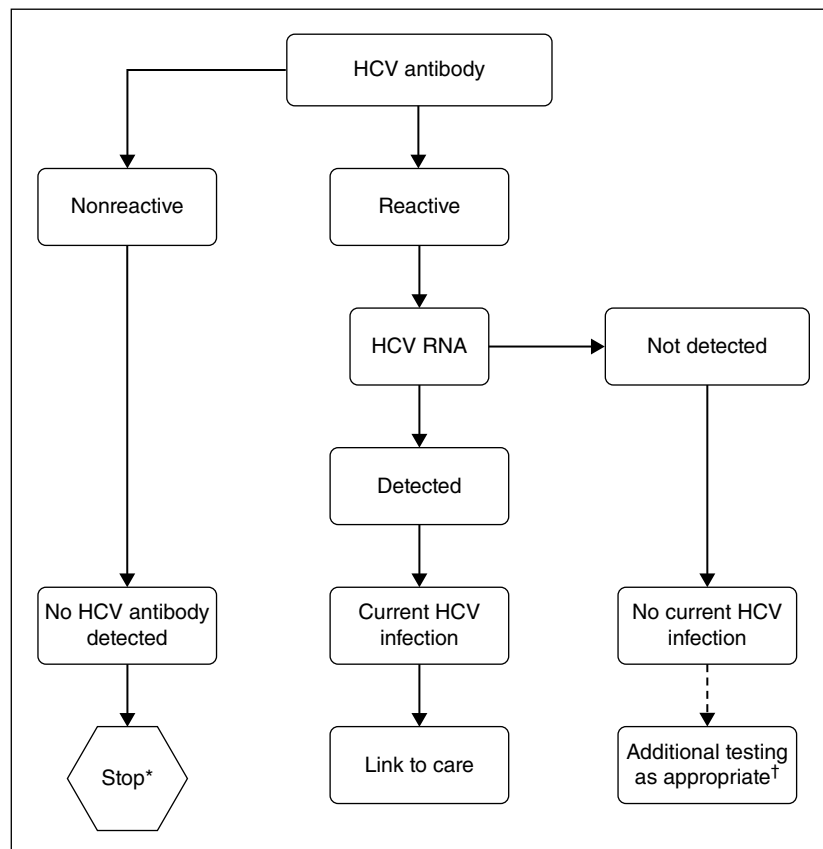
Figure 7.5 Hepatitis C seroprevalence worldwide. (Source: <http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/hepatitis-c.htm>) (See insert for color representation of the figure.)

Genotype 1 is most common in the United States, while genotype 4 is seen most frequently in Egypt [55]. Most acute HCV infections are asymptomatic, with less than 20% of persons developing jaundice. Chronic HCV infection develops in 70–85% of HCV-infected persons; 60–70% of chronically infected persons have intermittently elevated liver transaminase levels [8,34,70]. Chronically infected persons serve as a source of transmission to others and are at risk for cirrhosis, hepatocellular carcinoma, or other extrahepatic manifestations of HCV, even decades after infection.

HCV is most efficiently transmitted through large or repeated percutaneous exposure to infected blood. Although much less frequent, occupational, perinatal, and sexual exposures can result in the transmission of HCV.

Since implementation of routine blood donor screening in 1992, transmission of HCV via blood products is now extremely rare in the developed world. The use of contaminated needles for injection drug use is currently the most common method of HCV transmission in the United States [6]. However, healthcare settings are increasingly recognized as potential sources for HCV transmission, specifically through hemodialysis, unsafe injection practices, reuse of needles and fingerstick devices, and other lapses in infection control practices [2,25,30,48,67].

Detection of antibody to HCV can indicate past exposure to HCV with viral clearance, chronic active infection, or, rarely, a falsely positive test (Figure 7.6). Previously, recombinant immunoblot assays were recommended to distinguish viral clearance from a false-positive



* For persons who might have been exposed to HCV within the past 6 months, testing for HCV RNA or follow-up testing for HCV antibody is recommended. For persons who are immunocompromised, testing for HCV RNA can be considered.

† To differentiate past, resolved HCV infection from biologic false positivity for HCV antibody, testing with another HCV antibody assay can be considered. Repeat HCV RNA testing if person tested is suspected to have had HCV exposure within the past 6 months or has clinical evidence of HCV disease, or if there is concern regarding the handling or storage of the test specimen.

Figure 7.6 HCV. The recommended testing sequence for identifying current hepatitis C virus (HCV) infection. (Source: <http://www.cdc.gov/mmwr/pdf/wk/mm62e0507a2.pdf>)

HCV antibody result, however, this assay is no longer commercially available [7]. HCV RNA PCR assay is now recommended to distinguish past from current HCV infection [4]. Routine prenatal HCV screening is not recommended, however, antibody screening is appropriate for women with risk factors for infection.

While no HCV vaccine exists, the infection can be cleared in some patients with antiviral therapies. Pegylated interferon (PegIFN) combined with ribavirin (RBV) was the standard treatment for many years but newer, direct-acting antiviral (DAA) drugs are now recommended and have been shown to increase the likelihood of achieving a sustained virologic response [27].

A sustained virologic response (SVR) is defined as undetectable HCV RNA 3 to 6 months after completing antiviral treatment (considered a virologic “cure”). Three categories describe patients who underwent antiviral treatment for HCV but failed to achieve SVR: null responders (HCV RNA did not decline by at least 2 log IU/mL at treatment week 12), partial responders (HCV

RNA decline by 2 log IU/mL by week 12 but HCV RNA was still detectable at treatment week 24), and relapsers (HCV RNA was undetectable but then reappeared after treatment ended). The impact of antiviral resistance-associated variants (RAV) on treatment outcomes is an area of active study. Testing for the presence of RAVs either at baseline or in patients who have experienced a treatment failure is a practice that is currently evolving.

The current guidelines for the treatment of Hepatitis C emphasize both the need for a genotype determination prior to treatment decisions as well as precise HCV RNA determinations throughout treatment regimens.

7.2.5 Hepatitis E virus

Hepatitis E virus (HEV) is a single-strand RNA, nonenveloped virus in the *Hepeviridae* family (Table 7.1). HEV appears to be transmitted through the ingestion of fecal matter, even in microscopic amounts. HEV infection manifests in two clinically and epidemiologically distinct forms of diseases [66]. One form causes waterborne

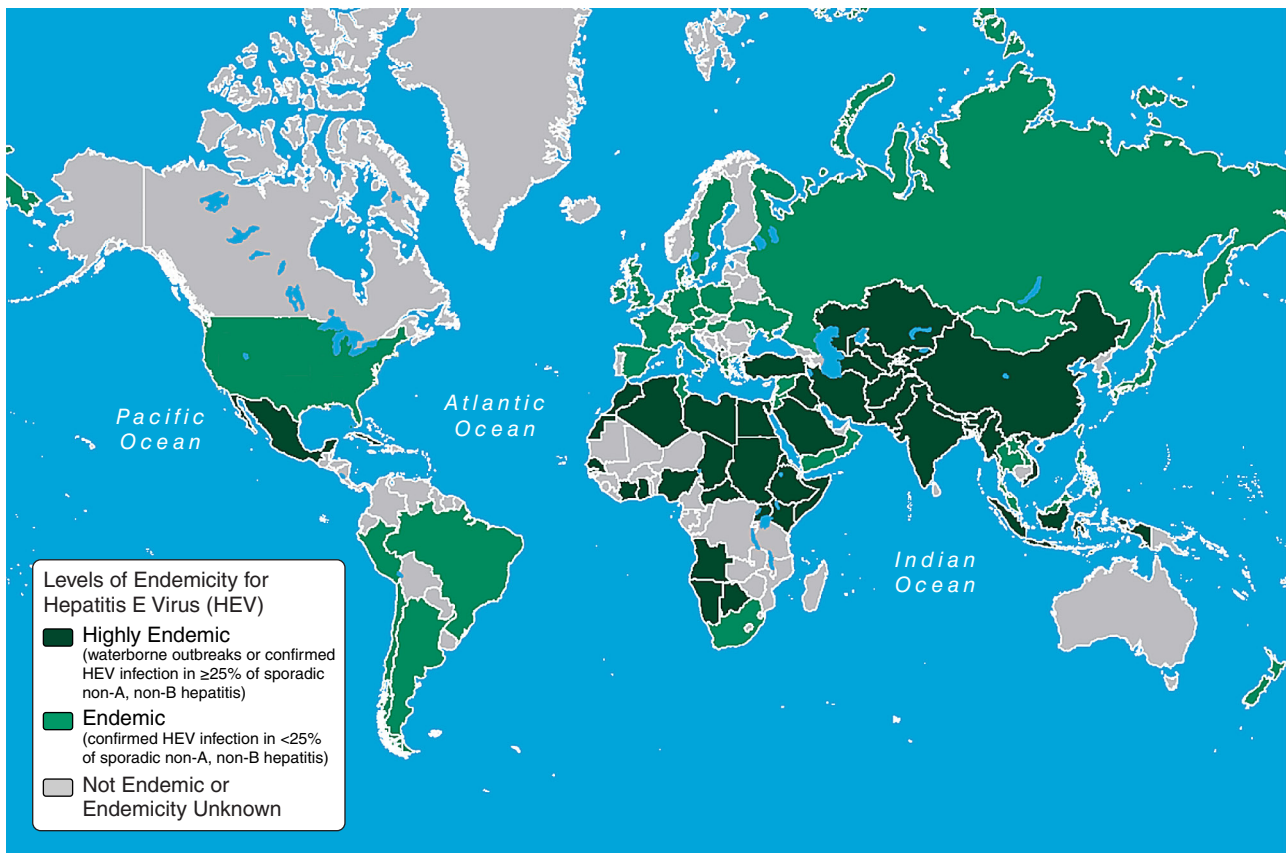


Figure 7.7 Hepatitis E seroprevalence worldwide. (Source: <http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/hepatitis-e.htm>) (See insert for color representation of the figure.)

outbreaks in developing countries, with acute onset of disease, and is associated with genotypes 1 and 2. Such large HEV outbreaks have been reported from India [49], China [13], Mexico [69], Sudan [28], and Uganda [65] (Figure 7.7). The second form, often associated with genotypes 3 and 4, causes a mild clinical presentation often with extrahepatic manifestations in developed countries. Transmission of this second form of hepatitis E may be from foodborne and zoonotic exposures, specifically swine. HEV genotype 3 has been detected in pork meant for human consumption in several countries [33,62].

Mortality rates during HEV epidemics range from 0.2 to 4% and death from HEV infection is more common in infants less than 2 years of age and pregnant women [38]. HEV genotype 3 can cause chronic infections in immunosuppressed patients, especially those who have HIV infection or have undergone transplantation [22,36,50]. Reported extrahepatic manifestations of HEV include arthritis, pancreatitis, aplastic anemia, and neurologic complications like polyradiculopathy, Guillian-Barre syndrome, Bell's palsy, peripheral neuropathy, ataxia, and mental confusion [35].

HEV RNA can be detected from serum and stool just prior to the onset of clinical symptoms but it does

not persist long in the blood (approximately 3 weeks) (Figure 7.8). HEV RNA may be detected in the stool for an additional 2 weeks. HEV IgM and IgG antibodies are generally detected at the time of symptom onset. Levels of IgM usually wane after 3–12 months, however, IgG antibodies may persist for years [19]. The duration of viral shedding is variable as is the presence of antibodies. Tests for HEV antibodies are available commercially, but none are approved for use by the US FDA at the time of this writing. IgG immunoblot assays can have low specificity [31]. Because of the variable specificity of serologic assays, serosurveys have documented widely variable prevalence estimates of HEV throughout the world [21,26]. HEV RNA PCR assays are available in research settings.

Most cases of acute HEV infection are self-limited and do not require treatment, however, patients with underlying chronic liver disease and HEV genotype 3 infection may require treatment with ribavirin.

7.3 Commercial methods

Hepatitis virus laboratory tests for HAV, HBV, and HCV rely heavily on immunoassay platforms for antibody and/or antigen detections, and other tests are

available (Table 7.3). Most commercial methods developed and approved for hepatitis viruses were intended for blood-donor screening in the same way as HIV [73]. In fact, hepatitis viral tests are listed together with HIV under viral marker surveys by the College of American Pathologists (CAP), and under the Transfusion Medicine Resource Committee (www.cap.org).

Overall qualitative tests are most commonly used for hepatitis viruses. In general, chemiluminescence is a very commonly used platform, and some laboratories also use enzyme immunoassay, but fluorescent EIA is used less frequently [74]. A number of commercial systems on automated platforms are available and are listed in Table 7.4.

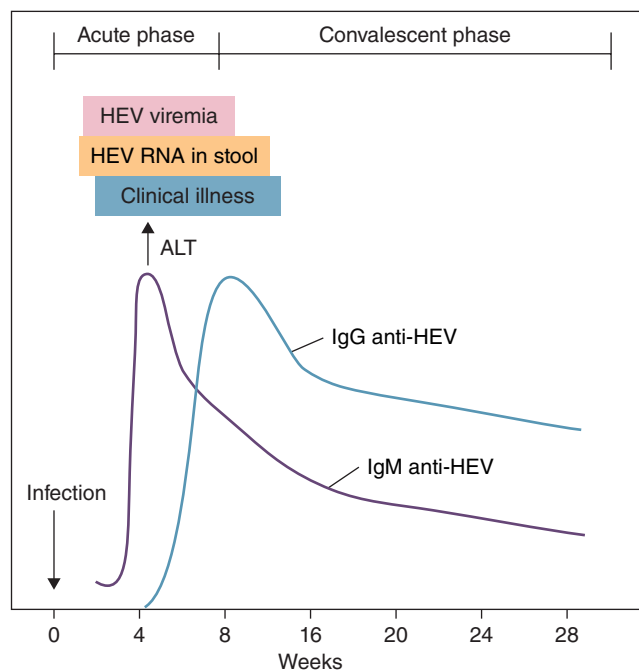


Figure 7.8 Time course of clinical and serologic events during acute hepatitis E virus (HEV) infection. ALT, alanine aminotransferase; IgG, immunoglobulin G; IgM, immunoglobulin M. (Reproduced with permission from Mandell, Douglas, and Bennetts *Principles and Practice of Infectious Diseases*, 7th Edition, 2010, M.P. Curry and S. Chopra, Acute Viral Hepatitis, 1577–1592, Churchill Livingstone/Elsevier, Philadelphia, PA.) (See insert for color representation of the figure.)

Routine blood-donor screening uses qualitative nucleic acid amplification test (NAAT) methods such as transcription mediated amplification (TMA) technology or the Procleix test (Hologic Gen-Probe). Multiplex NAAT was developed for screening for HBV DNA, hepatitis C virus (HCV) RNA, and the human immunodeficiency virus type 1 (HIV-1) RNA [77].

7.3.1 Hepatitis A virus

Methods for Anti-HAV (IgM) testing include chemiluminescence, enzyme immunoassay, or fluorescent EIA (Tables 7.3 and 7.4). A multicenter clinical study was conducted on sera from European and United States subjects for the evaluation of antihepatitis A virus enzyme immunoassays from Bio-Rad Laboratories by comparison with DiaSorin tests, and demonstrated good performance with overall agreement in detecting total antibodies and IgM, respectively [9]. There are not many commercial nucleic acid methods for HAV. The TaqScreen DPX test (DPX test, Roche Diagnostics) is a multiplex nucleic acid test assay for the simultaneous detection and quantitation of parvovirus B19 (B19V) DNA and the detection of hepatitis A virus (HAV) RNA. The test showed good reproducibility and was able to detect the HAV genotypes [42].

7.3.2 Hepatitis B virus

The common test platforms for Anti-HBc (IgM) are chemiluminescence, enzyme immunoassay, and fluorescent EIA. HBsAg can be confirmed using the Neutralization assay. The tests for HBeAg are based on chemiluminescence, or enzyme immunoassay. Assays for Anti-HBe are chemiluminescence, enzyme immunoassay, or fluorescent EIA (Tables 7.2–7.4).

Amino acid changes within the major antigenic determinant of the HBsAg may modify the antigenic properties of the protein and may impact the sensitivity of diagnostic assays. Modifications in the design of an assay can improve the ability to detect HBV mutants, by a commercial HBsAg test (Enzygnost HBsAg 5.0 and 6.0; Siemens) [12].

HBV transmission with serologically undetectable HBsAg despite the presence of circulating HBV DNA is called occult HBV infection (OBI), which is caused either

Table 7.3 Available diagnostic methods for hepatitis viruses

Diagnostic methods	Hepatitis A	Hepatitis B	Hepatitis C	Hepatitis D	Hepatitis E
Serology	Total, IgG, IgM anti-HAV	Anti-HBV, core, surface	Anti-HCV	Anti-HDV IgM, IgG	Anti-HEV, IgM*, IgG*
Antigen detection	None	Yes, surface antigen	HCV core antigen* (Europe)	None	None
Nucleic acid amplification	Research settings	Yes	Yes	Research settings	Research settings
Genotype	Research settings	Yes	Yes	No	Research settings
Rapid test	Yes	Yes	Yes	No	No

* Available commercially but not FDA approved as of this writing.

Table 7.4 Type of commercially available automated antibody or antigen detection systems*

Method	Detection method	Automated system (company)	High throughput/open platform	Full automation/random access
Colometric	Enzyme colometric	Evolis (Bio-Rad)	Yes/Yes	Yes/No
		Triturus (Grifols)	Yes/Yes	Yes/No
Chemiluminescence (CL) immunoassay	CLIA	ADVIA Centaur (Siemens)	Yes/No	Yes/Yes
		Architect (Abbott)	Yes/No	Yes/Yes
		Immolute (Siemens)	Yes/No	Yes/Yes
		Liaison (DiaSorin)	Yes/No	Yes/Yes
	Enhanced CLIA	VITROS (Ortho)	Yes/No	Yes/Yes
	Electro-CLIA (ECL)	Cobas/Elecsys (Roche)	Yes/No	Yes/Yes
Fluorescence	Fluorescence	AxSYM (Abbott)	Yes/No	Yes/Yes
		VIDAS (bioMérieux)	No/No	Yes/Yes
		Nexgen Four (Adaltis)	Yes/Yes	Yes/No
Lateral flow diffusion (rapid)	Lateral flow diffusion	OraQuick HCV (OraSure)	No/No	No/No

* Many products may include antibody detection assays such as anti-HAV, anti-HCV, anti-HBs, and anti-HBc. Throughput is generally high from 80 to 400 tests per hour. Other tests and instruments may be available or are in development at the time of this writing.

Table 7.5 Nucleic acid amplification (NAAT) methods for hepatitis viruses

	Product	Technology*	FDA	Automation	Sensitivity (IU/mL)	Company
RNA Quantitation/viral load	Versant HCV 3.0 bDNA (440)	bDNA, signal amplification	Yes	Semi-automated	615–7,690,000	Siemens
	COBAS AmpliCor HCV Monitor 2.0	PCR, target amplification	Yes	Manual or semi-automated	600–600,000	Roche Molecular
	COBAS AmpliPrep/COBAS TagMan HCV	TagMan real-time PCR	Yes	Semi-automated	43–69,000,000	Roche Molecular
	RealTime HCV m2000 system	Real-time PCR	Yes	Semi-automated	12–100,000,000	Abbott Molecular
	RealTime HBV m2000 system	Real-time PCR	Yes	Semi-automated	10–100,000,000	Abbott Molecular
	Versant HBV 3.0 bDNA (440)	bDNA	No	Semi-automated		Siemens
	HCV SuperQuant PCR	PCR				LabCorp (National Genetics Institute)
	HBV SuperQuant PCR	PCR				LabCorp
RNA qualitative	APTIMA HIV-1/HCV	TMA, target amplification	Yes	Automated	100	Hologic (Gen-Probe)
	APTIMA HCV/VERSANT HCV	TMA	Yes	Manual	10	Hologic/Siemens
	COBAS AmpliScreen HCV 2.0	PCR	Yes	Semi-automated		Roche Molecular
	COBAS AmpliCor HCV 2.0	PCR		Manual or semi-automated	50 in plasma 60 in serum	Roche Molecular

*PCR, polymerase chain reaction; TMA, transcription-mediated amplification; bDNA, branched DNA.

during the preseroconversion “window period” (WP), defined as the time between infection and detection of a serological marker, or during the late stages of infection. Implementation of HBV DNA screening has the potential to significantly reduce the WP and to reveal OBI or HBV carriage [79]. Two FDA-approved multiplex (MPX) NAAT systems are Procleix Ultrio by Hologic Gen-Probe (TIGRIS platform) and Cobas TaqScreen MPX by Roche Molecular Systems [64]. Although this is mainly for blood product and donor screening, the same approach might be used to confirm the infection.

Virological monitoring of HBV DNA is critical to the management of HBV infection. With several HBV DNA quantification or so-called “viral load” assays available, it is

important to use the most efficient testing system for virological monitoring in IU/mL. The available commercial assays are Roche COBAS TagMan (*in vitro* diagnostic (IVD) and other realtime), Abbott RealTime (IVD and analyte-specific reagents (ASR)), and Siemens Versant HBV 3.0 (Table 7.5). Unlike PCR, the Versant uses branched DNA, a signal amplification technology [71]. Other assays also may be available.

7.3.3 Hepatitis D virus

The tests and platforms for HDV are listed in Tables 7.3 and 7.4. In general, there are few commercial methods used for HDV testing and thus not discussed here in detail.

7.3.4 Hepatitis C virus

The tests for HCV are commonly based on the chemiluminescence platform, as described in Table 7.4. Molecular testing for HCV and genotype tests are listed in Tables 7.5 and 7.6. To compare the performance of three commercial automated assays for the detection of hepatitis C virus (HCV) antibodies, Monolisa anti-HCV Plus (Bio-Rad) version 2, AxSYM anti-HCV 3.0, and Vitros anti-HCV, were performed on 2020 hospitalized patients and showed specificities of 99.64% (Monolisa), 99.12% (AxSYM), and 99.33% (Vitros) [78]. More information can be found in Chapter 17.

One development in testing technologies for HCV is the rapid HCV antibody test. The OraQuick HCV Rapid Antibody Test (OraSure Technologies, Bethlehem, PA) is a rapid assay for the presumptive detection of HCV antibody in fingerstick capillary blood and venipuncture whole blood, which has received FDA approval for detection of HCV antibodies. This test can be performed in 20 min (<http://www.oraensure.com/products-infectious/products-infectious-oraquick-hcv.asp>). Its sensitivity and specificity are similar to those of FDA-approved, laboratory-conducted HCV antibody assays. A rapid assay for HCV by the OraQuick HCV Rapid Antibody Test was compared with four laboratory-based tests (three chemiluminescence assays and one enzyme immunoassay). Using the four seroconversion panels, the OraQuick HCV test showed results comparable to those of the laboratory-based assays [18].

The Chiron RIBA HCV 3.0 Strip Immunoblot Assay (Novartis Vaccines and Diagnostics, Emeryville, CA) that was formerly used for supplemental testing of blood samples after initial HCV antibody testing is no longer available. As a result, the only other FDA-approved confirmatory tests for HCV infection are those that detect HCV viremia (viral RNA by PCR) [4]. The supplemental testing for HCV antibody has been revised accordingly by CDC [3]. If testing is desired to distinguish between true positivity and biologic false positivity for HCV antibody, then, testing may be performed with a second HCV antibody assay approved by the FDA for diagnosis of HCV infection that is different from the assay used for initial

antibody testing. HCV antibody assays vary according to their antigens, test platforms, and performance characteristics, so biologic false positivity is unlikely to be exhibited by more than one test when multiple tests are used on a single specimen.

An FDA-approved NAAT assay intended for detection of HCV RNA in serum or plasma from blood of at-risk patients who test reactive for HCV antibody should be used (Table 7.5). There are several possible steps for NAAT after initial testing for HCV antibody: (i) blood from a subsequent venipuncture is submitted for HCV NAAT if the blood sample collected is reactive for HCV antibody during initial testing; (ii) from a single venipuncture, two specimens are collected in separate tubes: one tube for initial HCV antibody testing, and a second tube for HCV NAAT if the HCV antibody test is reactive; (c) the same sample of venipuncture blood used for initial HCV antibody testing, if reactive, is reflexed to HCV NAAT without another blood draw for NAAT; (iv) a separate venipuncture blood sample is submitted for HCV NAAT if initial test was performed by a Rapid Antibody Test [4].

Quantitative assays include real-time PCR in the unit of IU/mL, such as Roche COBAS TaqMan (IVD or other) and Abbott RealTime (IVD or ASR). Siemens Versant 3.0 (System 440 or other) by bDNA (IU/mL) is a non-PCR platform (Table 7.5). In addition to HCV RNA assay, there are commercial kits available for HCV genotyping and subtyping (Table 7.6).

Commercial assays such as the Abbott RealTime HCV assay and the Roche Cobas TaqMan HCV assay for HCV RNA quantification were used to investigate the prognostic relevance of HCV RNA viral loads at baseline, week 4, and week 12 in response to antiviral therapy. The high sensitivities and large dynamic ranges of the Abbott and Roche assays show that a single real-time quantitative PCR assay is fully adequate for clinical and therapeutic management of HCV [29]. When commercial HCV panels were used to evaluate linear range, sensitivity, and precision, the Roche COBAS TaqMan HCV test for research use only with samples processed on the MagNA Pure LC instrument (Roche RUO-MPLC) and Abbott analyte-specific reagents with QIAGEN sample processing (Abbott

Table 7.6 Genotyping methods for hepatitis viruses

Name of available test	FDA	Sensitivity and mutation detection	Company
TRUGENE HBV	No (RUO)	Sequencing	Siemens
TRUGENE HCV		Sequence	Siemens
Real-time HCV Genotype II m2000 system	Yes	RT-PCR, 5'UTR and NS5b 500 IU/mL (0.5 mL prep)	Abbott Molecular
VERSANT HCV genotype 2.0-LiPA	No (RUO)	Reverse hybridization with line probe, point mutation	Siemens
GeneSeq HBV or GeneSeq HCV	No		MonoGram Biosciences

ASR-Q) showed a sensitivity of 1.0 log(10) IU/mL with a linear dynamic range of 1.0 to 7.0 log(10) IU/ml [61]. In addition, tests including Roche COBAS TagMan (IVD) or COBAS Amplicor, and Abbott RealTime IVD can be used for HCV qualitative detection.

HCV genotype analysis can be performed by using a few commercial assays. Siemens Versant HCV Genotype Assay by LiPA is one platform and Siemens TruGene is another (Table 7.6). The Abbott RealTime HCV Genotype II is a new assay for HCV typing and subtyping, which uses the m2000/m24 System and ABI Sequencer. Abbott RealTime HCV Genotype has sensitivity at 500 IU/mL (0.5 mL prep) and good specificity with target region at 5'UTR and NS5b Genotype Detection 1a (NS5b), 1b (NS5b), 1, 2, 3, 4, 5, 6 (5'UTR). The Abbott automated RealTime HCV Genotype II assay is the first US FDA-approved HCV genotyping test. The results from HCV genotyping could provide relevant information to clinicians for targeted treatment to improve clinical outcomes (Table 7.6).

In addition to HCV antibody and molecular assays, the HCV core protein is another important marker for HCV infection and immunity [72]. The quantitative HCV core antigen (CAg) test can be used for the confirmation of viremia in patients with HCV. The utility and impact of HCV CAg detection via a commercial assay have been evaluated in diagnostic laboratory conditions [23]. The sensitivity and specificity of HCV CAg assay have been calculated as 75.8% and 95.1%, respectively. A viral load threshold of 1000 IU/mL has been recognized, above which the correlation with RNA became statistically significant and sensitivity increased to 90.9%. Detection of HCV core antigen could be an alternative to the NAAT test for HCV [23]. Another study tested anti-HCV (AxSYM, version 3.0; Abbott Diagnostics) and HCV Ag (Architect HCV Ag test; Abbott Diagnostics). For subjects with anti-HCV signal-to-cutoff ratios (S/CO) > 10 and/or HCV Ag > 3 fmol/L, HCV RNA data (Taqman HCV RNA; Roche Diagnostics) were further evaluated: HCV Ag correlated with HCV RNA. Using a combination of the values for anti-HCV (S/CO > 40) and HCV Ag (>3 fmol/L) as a cutoff to predict viremia, the sensitivity, specificity, accuracy, positive predictive value, and negative predictive value were 96.8%, 100%, 99.3%, 100%, and 99%, respectively [45,49].

Future research, development, validation, and cost-effectiveness studies on HCV will determine the best practices for detecting HCV viremia and for distinguishing between resolved HCV infection and biologic false positivity for HCV antibody in persons in whom HCV RNA is not detected [4].

7.3.5 Hepatitis E virus

Though not used widely commercially, the tests for HEV are listed in Table 7.4. Currently serological test formats are designed to detect IgG and IgM antibodies. Some

commercial anti-HEV assays had poor concordance, which warrants further study [54]. Other assays based on the HEV immunofluorescence test (HEV-IFT) were used for detection of anti-HEV IgG and IgA antibodies [53].

7.3.6 Approaches for global health

Due to a lack of comprehensive laboratory capacity in the setting of limited resources for specimen collection, storage, and transportation for hepatitis virus testing, novel approaches have been implemented recently. The use of alternative sample collections such as dried blood spots (DBS) could allow specimens to be handled without using refrigeration or freezing. A study was undertaken to optimize the efficiency of commercial EIAs for anti-HCV detection in DBS samples. The long-term stability of anti-HCV on DBS samples stored at three environmental conditions was also evaluated: at 2–8°C, 20–25°C, and –20°C. Commercial EIAs can be optimized for anti-HCV detection in DBS samples that are extremely stable at different conditions for a few weeks [46]. When DBS assays were evaluated, serological testing on plasma for HBV and HCV infections was used as the reference standard assay to control for the performance of DBS assays. Diagnosis using DBS may assist with implementation of national programs for HBV, HCV, and HIV screening and clinical care in middle- to low-income countries [37]. Paired serum/DBS samples were analyzed with the Abbott ARCHITECT HBsAg, anti-HBc, anti-HBs, anti-HCV, and HIV-1-p24-antigen/anti-HIV 1/2 assays as well as with the artus HBV LC PCR and VERSANT HCV RNA qualitative (transcription-mediated amplification – TMA) tests. The analytical sensitivity of HBsAg, anti-HBc, anti-HBs, anti-HCV, HIV-1-p24-antigen/anti-HIV 1/2, HBV DNA, and HCV RNA detections in DBS eluates was 98.6%, 97.1%, 97.5%, 97.8%, 100%, 93%, and 100%, respectively. It therefore seems possible to detect HBsAg, anti-HBc, anti-HBs, anti-HCV and HIV-1-p24 antigen/anti-HIV 1/2 with analytical systems in DBS eluates [60]. More clinical studies may need to be performed to optimize this testing modality.

Future developments could include a combination test for detection of antibody as well as antigen simultaneously, in the same way as the HIV fourth-generation test. The algorithm for confirmation could be fine-tuned accordingly. Real-time PCR arrays and multiplex assays capable of simultaneously detecting many human viral pathogens can be developed and used by viral array [56].

7.4 Summary

The commercial laboratory methods for hepatitis viruses have been developed and utilized in the setting of clinical laboratories as well as blood-donor screening. Routine utilization of immunology-based technologies for antibody/

antigen detection with the capacity of laboratory automation have made hepatitis assays, along with HIV, major components of clinical virology and immunology testing in the clinical laboratories. There have been many developments and implementations of molecular-based technologies and rapid test methods in hepatitis testing. In summary, commercial methods with improved automation for laboratory diagnosis of viral hepatitis and monitoring of antiviral treatment are available for both blood-donor screening and for routine patient care.

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Human Papillomaviruses

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8.1 Background

Human papillomaviruses (HPVs) are small (7900 bp), circular, double stranded DNA viruses belonging to the *Papillomaviridae* family, which is further divided into genera, species, types, and subtypes [34]. The HPV genome consists of eight open reading frames (ORF) encoding three oncogenes (E5, E6, and E7), two regulatory proteins (E1 and E2), and two capsid proteins (L1 and L2). These ORFs belong to either the early region (E: 4500 bp), the late region (L: 1500 bp), or the long control region (LCR: 1000 bp), a noncoding region located between the early and late regions [10]. To date, there are more than 150 HPV types based on a difference of at least 10% within the L1 ORF [34].

HPVs primarily infect the epithelial cells of the skin and mucosal surfaces and cause a range of infections from asymptomatic to benign skin warts to invasive cervical cancer [10]. Cutaneous HPVs (types 1, 2, 3, 4, 5, 7, 8, 10, 26–29, 41) are associated with various conditions including common warts, “butcher’s warts”, plantar warts, flat warts, and epidermodysplasia verruciformis, a condition most commonly found in patients with autosomal recessive mutations on chromosome 17 [13, 57].

Mucosal HPVs are classified as either low-risk (LR) or high-risk (HR) HPV types based on their oncogenic potential. Approximately 40 HPV types, both low- and high-risk HPVs, can infect mucosal membranes, resulting in warts (LR HPVs) or intraepithelial neoplasia and cancers (HR HPVs) [58, 81].

8.1.1 Low-risk HPV and related infections

Although multiple types exist, the two most common LR HPV types are HPV-6 and HPV-11. Both of these types are found in 90% of condyloma acuminata, a common type

of genital warts that affect both men (coronal sulcus, the glans penis, and penil shaft) and women (cervix and vulva) [49]. Other low-risk HPVs that have been associated with condylomata acuminata include HPVs 2, 40–45, 51–56, 61, 70, 72, and 81 and coinfections with intermediate- and high-risk HPV types are not uncommon [13, 49, 57].

HPV-6 and HPV-11 are also associated with infections of the oral mucosa, including recurrent respiratory papillomatosis (laryngeal papillomatosis), a benign infection of the upper respiratory tract thought to be transmitted from mother to child during delivery [73, 119].

8.1.2 High-risk HPV and related infections

Infections of the cervix with high-risk HPV types tends to be persistent and often result in precancerous cervical lesions and invasive cervical cancer [12]. The association between HPV and cervical cancer is well established. High-risk HPVs account for approximately 10% of cancer worldwide [90]. HPV-16 is the most common HPV genotype found in cervical cancer, encompassing 55–60% of all cases, followed by HPV-18, which is responsible for 10–15% of all cases, with other high-risk types (31, 33, 35, 39, 45, 51, 52, 56, 58, 66) accounting for the other 30% of cases [33, 81, 122]. Following acquisition and persistence of HPV infection, cervical cancer develops through progression of the infected cells into precancerous cells and eventually invasion of the cervix [103]. Cervical intraepithelial neoplasia (CIN) is a histological abnormality of the cervical squamous epithelium and is considered a precursor to cancer, although CIN can regress spontaneously within 1–2 years without treatment. CIN is graded from CIN1 to CIN3 based on the degree of invasion of the epithelium, with CIN1 representing mild dysplasia with invasion of one-third of the epithelium, CIN2 representing

moderate dysplasia with invasion of two-thirds of the epithelium, and CIN3 representing severe dysplasia with complete invasion of the epithelium [64, 124].

Similar to cervical cancer, high-risk HPV types are also associated with anal and oropharyngeal cancers. In a study of women with high-grade genital neoplasia, anal HPV DNA was detected in 51% of women, with 70% of infected women positive for a HR HPV subtype; 34% HPV-16 and HPV-18 and 36 % other HR HPV types including HPV-31, HPV-53, and HPV-59 [89]. Anal HPV infections are common in HIV positive men who have sex with men (MSM) [28, 85, 86]; in non-HIV infected MSM, high-risk HPV types can be detected in up to 75% of men screened, with multiple types being detected and HPV-16 being the most commonly detected genotype [21, 39].

About 25% of squamous cell carcinoma of the head and neck are associated with the presence of HPV. Approximately 60–70% of those are caused by high-risk HPV types, with HPV-16 being responsible for greater than 90% of the cases and other types, including HPV-18, 31, 33, and 35, accounting for the remaining cases [121]. In a recent study, Paolini et al. showed that both cutaneous and mucosal HPV infect the oral epithelium, but only mucosal HPV, particularly the high-risk HPV-16 type, is associated with cancer [87].

8.1.3 Testing utility and recommendations

The goal of screening is to identify women with pre-cancerous lesions, mainly CIN3, who are at high risk of developing cervical cancer. Screening guidelines for cervical cancer are established by the American Cancer Society (ACS), the American Society for Colposcopy and Cervical Pathology (ASCCP), and the American Society for Clinical Pathology (ASCP). The most recent guidelines recommend that women be screened starting at age 21 years, with frequency based on age; every 3 years by cytology for women 21–29 years of age; every 5 years by cytology and HPV testing or every 3 years by cytology alone for women 30–65 years of age; and no testing for women over 65 years of age and no history of positive HPV tests and abnormal cytology [101].

Women testing HPV positive by co-testing (i.e., HPV testing positive, cytology negative) should further be evaluated by either repeat co-testing within 12 months or immediate HPV genotyping for either HPV-16 alone or both HPV-16 and 18 followed by colposcopy for any positive results [101]. On the other hand, women with a negative HPV test but positive cytology should continue with routine surveillance screening as there is no evidence of decreased risk of cancer by increasing testing frequency in this subgroup of women [101, 128].

Although HPV testing has increased sensitivity over cytology for the detection of CIN2 and CIN3 lesions [3, 66, 68, 96], the lower specificity and inability to always ensure

specimen adequacy had resulted in a recommendation that HPV testing alone not be used for cervical cancer screening [101]. In the latest screening guidelines (2012), HPV co-testing with cytology or cytology alone remains the recommended methods for cervical cancer screening. However, in April 2014, the Cobas 4800 HPV (Roche Diagnostics, Indianapolis, IN) became the first molecular HPV test to be approved by the US Food and Drug Administration (FDA) as a first-line primary test for screening in women 25 years and older. The addition of this claim to a molecular HPV test will certainly impact the next set of recommendations for cervical cancer screening.

8.2 Commercial assays

8.2.1 Non-nucleic acid amplification tests

8.2.1.1 Cytology-based assays

Both low- and high-risk HPV types can cause abnormal cervical cytology. Abnormal cervical cytology diagnoses are reported as atypical squamous cells (ASC), low-grade squamous intraepithelial lesions (LSIL) or high-grade squamous intraepithelial lesions (HSIL), and squamous cell carcinoma. The two most common subcategories of ASC are ASC of undetermined significance (ASC-US) and ASC cannot exclude HSIL (ASC-H) [124]. In addition to abnormality in squamous cells, cervical screening can also reveal abnormalities in glandular cells, which are divided in increasing order of severity from atypical glandular cells (AGC), endocervical adenocarcinoma *in situ* (AIS) and adenocarcinoma [124].

Conventional cytology, commonly known as the Papanicolaou (Pap) smear, has been in use since the 1950s [88]. Pap smears are performed by manually collecting a cervical sample using a cervix brush and directly transferring cells onto a glass slide for subsequent staining and analysis [61]. Pap smears remain the gold standard for cervical cancer. However, the test is laborious and prone to false-negative results due either to sampling errors or detection errors, with reported sensitivity varying from 30 to 87% [51].

Liquid-based cytology (LBC) was developed to improve the quality of specimen sampled for cervical screening and concomitantly allows co-testing by HPV DNA testing and cytology. Currently, two LBC devices have been approved by the US Food and Drug Administration (FDA) as of August, 2014, and are commercially available (Table 8.1). Other methods and products may also be available. The ThinPrep Pap Test (Hologic Inc., Marlborough, MA) was FDA approved in 1996 and works by using a brush, spatula, or broom-like device to collect cells from the cervix. The collected cells are then transferred from the collection device into a vial containing PreservCyt solution. In the laboratory, the PreservCyt sample is set-up on a ThinPrep 2000 Processor (Hologic Inc., Marlborough, MA),

Table 8.1 Liquid-based cytology devices

Name	Manufacturer	Country	Method	Automation	FDA status*
ThinPrep Pap	Hologic Inc.	USA	Filtration	ThinPrep 2000 Processor	Approved
SurePath Pap	Becton, Dickinson and Co.	USA	Centrifugation	BD PrepStain slide processor	Approved
CYTO-screen	Seroa	Monaco	Centrifugation	None	Not approved
Turbitec	Labonord	France	Centrifugation	None	Not approved (CE marked)
CellSolution	Menarini Diagnostics	Italy	Centrifugation	CellSolutions 30	Not approved
Shandon	ThermoFisher Scientific	USA	Centrifugation	None	Not approved
Papspin					
DNA-Citoliq	Digene	Brazil	Centrifugation	None	Not approved

* As of August, 2014.

which gently disperses the specimen, collects diagnostic cells on a filter and transfers them onto a glass slide for downstream analysis. The ThinPrep Pap test is also FDA approved for use in additional testing, including glandular lesions, HPV testing, and *Chlamydia trachomatis* and *Neisseria gonorrhoeae* testing.

The second LBC device to be FDA approved was the SurePath liquid-based Pap test in 1999 (Becton Dickinson, Franklin Lakes, NJ). Similar to the ThinPrep Pap test, the SurePath LBC uses a brush, a spatula or broom-like device to collect cervical cells, followed by transfer to a vial containing BD CytoRich Red preservative fluid. Once in the laboratory, the specimen is placed on the PrepStain slide processor where it is vortexed and transferred to a PrepStain Density reagent for enrichment by centrifugal sedimentation. The sediment obtained is resuspended and moved to a PrepStain settling chamber for transfer onto a glass slide for downstream processing, including a modified Papanicolaou stain procedure [9]. The BD SurePath Pap test is also FDA approved for out-of-vial testing of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.

Although there is clear improvement in the quality of the smear obtained using LBC compared to conventional Pap smear [112], studies on the superiority of LBC in providing increased diagnostic sensitivity have yielded conflicting results. In one meta-analysis study, the ThinPrep Pap test had double the rate of diagnosis for HSIL lesions compared to conventional Pap smear (0.71% incidence vs. 0.30% incidence) but no significant difference in the detection of atypical squamous cells of undetermined significance (ASCUS) [8]. Similar findings were obtained in a more recent study showing a significantly higher detection rate by SurePath for ASCUS (2.07% vs. 0.87%) and a slightly higher rate of detection for LSIL (0.27% vs. 0.22%) and HSIL (0.64 vs. 0.56) [6]. A meta-analysis and a randomized controlled trial from the Netherlands comparing the positivity rate of the ThinPrep Pap test to that of conventional cytology did not show a statistically significant difference between the two methods for the diagnosis of CIN, ASCUS, LSIL, or

HSIL although the number of adequate slides was greater by LBC [1, 105, 106].

Only a few reports of head-to-head comparison of the ThinPrep versus SurePath have been published [45, 127, 131]. Zhao and colleagues compared the two methods for rates of unsatisfactory smears, residual specimens available for additional testing, and accuracy in detection of significant cervical lesions [131]. The two methods performed similarly, except SurePath resulted in less unsatisfactory smears than ThinPrep (0.2% vs. 1.5%). A study by Wright et al. also showed equivalence for both ThinPrep and SurePath in terms of predictive value for CIN2, high-grade, and low-grade dyskaryosis [127]. A recent meta-analysis demonstrated that SurePath LBC yields fewer unsatisfactory smears than ThinPrep, although multiple factors including year of publications, country of origin, and criteria to determine adequacy might have affected the outcome of the analysis [45]. Reprocessing of unsatisfactory slides obtained using ThinPrep Pap with 10% glacial acetic acid was approved by the FDA and was shown in one study to decrease the unsatisfactory rate by 34% [62].

8.2.1.2 Immunohistochemistry assays

The most common target for HPV immunohistochemistry (IHC) assays is the cyclin-dependent kinase inhibitor p16 (INK4a), which participates in the control of the cell cycle by the retinoblastoma (Rb) protein. In cervical lesions due to high-risk HPV, the p16 protein accumulates due to lack of transcription inhibition from an inactivated Rb by the HPV E7 oncoprotein [83]. Detection of the overexpressed p16 is used as a surrogate marker of HPV infection and is accomplished by IHC. Specific monoclonal antibodies are commercially available as part of a kit from several manufacturers. Following a series of washes and incubations with secondary antibodies, horseradish peroxidase, and chromogenic substrates, positive p16 IHC are visualized as brown spots that can have either a diffuse or strong focal staining pattern [70].

The p16 IHC has been evaluated against other HPV detection methods including cytology, nucleic acid testing,

and *in situ* hybridization. Reported sensitivity and specificity of p16 IHC varied from 75 to 80% and 62 to 71% respectively, for detection of p16 in ASCUS cytology with underlying CIN2+ and CIN3+ [35, 78]. In other studies, the sensitivity and specificity of p16 IHC in LSIL ranged from 82.1% to 100% and 93.3% to 100% respectively [70, 123]. Szarewski et al. compared the performance of p16 IHC against several assays using CIN2 as a cutoff and showed the sensitivity and specificity of p16 IHC to increase from 59.1% for CIN2, to 83.0% for CIN2+ and 92.7% for CIN3+, with specificity of 68.7% and 65.8% for CIN2+ and CIN3+ to 92.2% [113]. In a subsequent study by the same group the sensitivity of the p16 IHC increased slightly for CIN2 (78.2% vs. 59.1%) with a decrease in the overall specificity of the assay (54.7%) [113, 114, 123].

8.2.1.3 *In situ* hybridization assays

In situ hybridization (ISH) allows for the direct visualization of target nucleic acids in tissues or cells through binding of labeled probes. Commercial reagents available for ISH HPV include the INFORM HPV II Family 6 Probe and the INFORM HPV III Family 16 Probe (Ventana Medical/Roche Diagnostics, Tucson, AZ) for cytological specimens (C) and tissues samples (B) and the GenPoint Catalyzed Signal Amplification System (DAKO, Carpinteria, CA).

The INFORM HPV II Family 6 Probe consists of a cocktail of HPV genomic probes directed against the most common low-risk HPV types (6 and 11) while the INFORM HPV III Family 16 consists of a cocktail of HPV genomic probes directed against the most common high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66). Additional reagents include the ISH Protease for digestion of proteins surrounding the HPV DNA and the iVIEW Blue Plus detection kit (Ventana Medical/Roche Diagnostics, Tucson, AZ), which uses biotin labeled anti-fluorescein antibody to detect the fluorescein labeled probes followed by addition of streptavidin, nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate, and alkaline phosphatase, and yields an intense blue color when probes hybridize to target DNA and finally the Ventana Red counterstain [70]. Positive HPV ISH pattern can be either episomal or integrative.

The GenPoint Catalyzed Signal Amplification System targets high-risk HPV genotypes, including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and code Y1443 as well as the low-risk HPV types including types 6 and 11. Detection is performed using GenPoint Tyramide signal amplification system for biotin labeled probes, peroxidase-conjugated streptavidin, diaaminbenzide as the chromogenic substrate, and hematoxylin as the counterstain [32, 67].

Although labor-intensive and prone to subjective interpretation, HPV ISH assays are highly specific and have the

advantage of detecting HPV DNA in the right histological context of HPV genome integration within the nucleus [11, 32]. In one study, the results obtained using ISH (DAKO), cytology, and polymerase chain reaction (PCR) were comparable, with concordance between ISH and PCR of greater than 90% for CIN3+ and HSIL lesions [67]. The sensitivity and specificity of ISH for detection of CIN2+ was 89.5% and 39.9% respectively, emphasizing the need for additional testing.

Kong et al. compared the performance of INFORM HPV assays, a research-use only (RUO) version of the DAKO ISH assay, and p16 IHC and PCR in a set of specimens that included LSIL, HSIL, and negative cervical specimens [70]. The specificity of all ISH assays was 100% with the sensitivity of DAKO, INFORM HPV II, and INFORM HPV III being 55.6%, 53.6%, and 69.2% respectively for SIL. In this study, compared to p16 IHC, all ISH assays were less sensitive (IHC sensitivity 82.1%).

8.2.2 Nucleic acid amplification tests

8.2.2.1 FDA-cleared assays

Currently, there are four USFDA-cleared assays for high-risk HPV testing including the Digene Hybrid capture assay 2 (HC2) HR (Qiagen Inc., Germantown, MD), the Cervista HR HPV (Hologic Inc., Bedford, MA), the APTIMA HPV assay (Hologic Gen-Probe Inc., San Diego, CA) and the Cobas 4800 HPV (Roche Diagnostics, Indianapolis, IN) (Table 8.2). These assays are approved for co-testing along with cytology and for patients with ASCUS on cytology [101]. The Roche Cobas 4800 HPV test is additionally approved as a first-line cervical cancer screening assay in women 25 years of age or older.

The first molecular assay approved by the USFDA for the detection of HPV infection was the Hybrid Capture assay (Digene Corporation, Gaithersburg, MD). The HC2 assay is a hybridization assay with signal amplification detection. Two sets of single-stranded RNA probes, one for low-risk HPV subtypes and one for high-/intermediate-risk HPV subtypes, are incubated with denatured DNA from specimens suspected of harboring HPV DNA. The resulting RNA/DNA hybrids are captured by an antibody conjugated to alkaline phosphatase enzyme and detected by addition of a chemiluminescent substrate that emits light when cleaved by the alkaline phosphatase enzyme. The reaction is measured using a luminometer and the results expressed as relative light unit (RLU). The first generation of the HC assay, the HC1 assay, was designed to detect 14 HPV subtypes including nine high-risk types (16, 18, 31, 33, 35, 45, 51, 52, and 56) and five low-risk types (6, 11, 42, 43, and 44) and had a sensitivity for the detection of high-grade SIL varying from 71.2 to 93% with a positive predictive value of approximately 17.8% [23, 27, 43]. The HC1 assay was subsequently replaced by the second-generation assay, the HC2 assay, which

Table 8.2 Commercial assays for HPV nucleic acids detection

Test name	Technology	Target	Internal control	HPV Genotypes	Specimen	Regulatory status*	Extraction	Detection
Aptima HPV (Hologic Gen-Probe, Inc.)	TC	mRNA	Exogenous noninfectious RNA target	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	PreservCyt solution	FDA CE	Automated target capture on TIGRIS DTS system	TIGRIS DTS System or Panther System
Cervista HPV HR (Hologic, Inc.)	TMA HPA Invader chemistry (isothermal signal amplification)	DNA	Endogenous (Human histone 2 gene)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	PreservCyt solution	FDA CE	Manual (Gen-find DNA extraction kit)	Fluorescent plate reader
Digene HC2 HR and LR (Qiagen, Inc.)	Hybridization HC Signal amplification	DNA	None	6, 11, 42, 43, 44, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68	PreservCyt solution hc2 DNA Collection Device Biopsies collected in STM	FDA CE	Manual	Luminometer
COBAS HPV Test (Roche Molecular Diagnostics)	Multiplex real-time PCR	DNA	β -globin	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	PreservCyt solution	FDA CE	Cobas x 480	Cobas z 480
Abbott RealTime High Risk HPV (Abbott Molecular)	Multiplex Real-time PCR	DNA	β -globin	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	PreservCyt Solution SurePath Preservative Fluid Abbott Cervi-Collect	FDA CE	m2000sp	m2000rt
Roche Amplicor HPV test (Roche Molecular)	PCR	DNA	β -globin	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	Specimen Collection Kit PreservCyt Solution	CE	Manual	Plate reader
The PreTect HPV-Proofer (Norchip AS)	NA hybridization Real-time multiplex NASBA	mRNA	Endogenous (U1A)	16, 18, 31, 33, 45	SurePath Preservative Fluid PreservCyt Solution SurePath Preservative Fluid PreTect TM	CE	Manual or automated	Fluorescence reader
NuclISENS EasyQ HPV (bioMérieux)	Multiplex NASBA	mRNA	Endogenous (U1A)	16, 18, 31, 33, 45	PreservCyt Solution	CE	NuclISENS MiniMag Easy Mag	NuclISENS EasyQ
HPV OncoTect E6/E7 mRNA (IncellDx)	Flow cytometry In situ hybridization	mRNA			PreservCyt Solution SurePath Preservative Fluid	CE	None	FAC Scan cytometer

* As of August, 2014.

STM: specimen transport medium; TC: target capture; TMA: transcription mediated amplification; HPA: hybrid protection assay; HC: hybrid capture; CE: Conformité Européenne; FDA: Food and Drug Administration.

detects additional HPV types including 39, 58, 59 and 68 and has a reported sensitivity up to 98.1% for detection of high-grade cervical lesions [19, 24, 25]. The HC2 assay differentiates between low- and high-risk HPV infection but does not provide specific HPV genotypes. Another formulation of the HC2, the digene HC2 High-Risk HPV DNA, is designed to detect 13 high-risk HPV types including the nine types detected by the HC2 LR and HR assay plus HPV types 39, 48, 59, and 68. Limitations of the HC2 assay include cross-reaction with untargeted low-risk HPV types (6, 11, 26, 30, 40, 42, 53, 54, 61, 67, 70–73, 81, 83, 84, 87, and 91), resulting in reduced test specificity [18, 52, 93]. Additional limitations of the HC2 assay include the requirement for large sample volume (at least 4 mL) and the need to retest any results falling within the gray zone [80].

The APTIMA HPV assay is a target capture assay designed to amplify the messenger RNA (mRNA) transcript of the E6/E7 gene by transcription-mediated amplification and detect the amplified product by hybridization protection assay [36]. The assay targets 14 high-risk HPV including 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. The assay does not discriminate among the 14 genotypes. The lower limit of detection of the APTIMA HPV test is between 17 and 488 copies/mL depending on the genotype and the assay can be fully automated on the TIGRIS DTS platform or the PANTHER System (Hologic Gen-Probe Inc., San Diego, CA) [36, 37, 50]. In a recent meta-analysis study, the pooled sensitivity of the APTIMA HPV assay was similar to that of HC2 assay for detection of underlying CIN2 or CIN3 in cytology specimens showing ASCUS (95.7–96.2% vs. 93.8–95.5%) and LSIL (91.0–96.7% vs. 95.5–98.8%) but the APTIMA assay had a significantly higher specificity than HC2 assay for both ASCUS (54.9–56.4% vs. 44.9–46.8%) and LSIL (38.7–42.5% vs. 27.8–28.6%) [2].

The Cervista HPV HR gained FDA approval in 2009. The assay used hybridization probes and signal amplification to detect 14 high-risk HPV types including 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. In a multicenter evaluation, the Cervista HPV HR test showed a lower limit of detection varying from 1250 to 7500 copies/mL depending on the genotype [30]. The clinical sensitivity and specificity of the Cervista HPV HR assay for the detection of CIN2 and CIN3 from ASCUS specimens was 92.8 and 44.2% for CIN2 and 100% and 43% for CIN3, respectively [42]. In a recent head-to-head comparison of the Cervista HPV HR assay to the HC 2 assay, the two assays showed comparable sensitivity in detecting HR HPV in specimens with negative cervical cytology, although the positive agreement between the two assays was only 70% [71]. In other studies, the Cervista HPV HR assay showed comparable sensitivity but increased specificity for the detection of CIN3 or cancer, which was

hypothesized to be due to the higher viral load cutoff for Cervista HPV HR (3.2 pg vs. 1.0 pg) and the lack of cross-reactivity of the assay with LR HPV types [7]. However, cross-reactivity of the Cervista HR is seen with low-risk HPV types 67 and 70, in addition to HPV types 6, 73, 84, and 91 [52].

In 2011, the FDA approved the Cobas 4800 HPV test (Roche Diagnostics, Indianapolis, IN) for the detection of 14 high-risk HPV including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. In 2014, the Cobas 4800 HPV received FDA approval for use as a first-line primary test for screening in women 25 years and older. The assay is fully automated and combines specimen extraction (PreservCyt) followed by multiplexed real-time PCR amplification and detection. The clinical sensitivity and specificity of the Cobas 4800 HPV test for detection of CIN2+ was comparable to that of the HC2 assay when tested on archived cervical scrapes [59] or ThinPrep specimens [72, 111]. In two recent studies, the agreement between the Cobas 4800 HPV test and HC2 was shown to be moderate (85.9%) [47] or high (93.8%) [125] but the results of the detection rate were conflicting, with one study showing higher detection rate for Cobas 4800 HPV and the other study showing higher rate for the HC2 assay [47, 125].

8.2.2.2 Non-FDA-cleared assays

The following section covers assays that have not yet been approved for use by the US FDA, as of this writing, but are undergoing evaluation, or have already been Conformité Européenne (CE) marked or cleared for use in Europe, Canada, and other countries outside of the United States (Table 8.2).

The Roche Amplicor HPV test includes a conventional PCR step followed by hybridization with a cocktail of probes and detection of HPV DNA on a plate reader at 450 nm. The assay targets the following 13 high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 without differentiating among them [116]. In studies comparing the Amplicor HPV test to the HC2 assay, the two tests showed comparable sensitivity and specificity for detection of HPV DNA with agreement ranging from 82.6–89.2% [14, 40, 100]. Similar to the HC2 assay, the Amplicor HPV test can cross-react with low-risk HPV types including HPV-6, 11, 42, 53, 54, 62, 71, 81 and 83, resulting in lower specificity [100].

The Abbott *RealTime* High Risk HPV assay is a multiplex real-time PCR assay that specifically detects HPV 16 and HPV 18 and also detects other high-risk HPV including HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. The assay includes a nucleic acid extraction step from specimens collected in PreservCyt, SurePath, or Abbott Cervi-Collect on the m2000sp followed by multiplex real-time PCR on the m2000 platform. A few studies have

compared the performance characteristics of the Abbott assay to the HC2 on archived specimens and showed good agreement (>90%) between the two methods in detecting HPV in cervical cancer and/or CIN 2 or CIN3 specimens and increased specificity of the Abbott assay over the HC2 [15, 65, 92, 120].

The BD Viper HPV test (Becton Dickinson, Franklin Lakes, NJ) is an automated nucleic acid amplification assay that detects the 14 high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. In addition, the assay provides specific typing information for seven genotypes: 16, 18, 31, 45, 51, 52 and 59 [16]. A report comparing the BD Viper HPV test to HC2 on randomly selected archived PreservCyt specimens showed an overall agreement of 87.7% and a positive agreement of 81.4% with no statistical difference in detection rate between the two assays [16]. Clinical trials for the BD Viper HPV assay to be performed on the BD Viper LT system test were completed in Europe in 2013 but the assay is not currently commercially available.

The PreTect HPV-Proofer assay (Norchip AS, Klokkearstua, Norway) is a multiplex assay that targets and amplifies E6/E7 mRNA using nucleic acid base amplification (NASBA), an isothermal rapid amplification method, and molecular beacons for real-time detection of the amplicons [79]. The proofer assay detects five high-risk HPV types including HPV 16, 18, 31, 33 and 45. In various performance evaluation studies, the sensitivity and specificity of the Proofer assay varied between 73.6 and 85.9% and 73.1 and 75.5% respectively for the detection of CIN2+, depending on the population tested [5, 95, 113]. However, compared to other HPV DNA and RNA assays, the Proofer assay showed lower analytical and clinical sensitivity for CIN2, CIN2+ and CIN3, mainly due the limited number of HPV genotypes detected by the assay [113, 114].

The NucliSENS EasyQ HPV assay (bioMérieux, Marcy l'Etoile, France) uses the PreTect HPV-Proofer

technology combined with bioMérieux NASBA technology to detect E6/E7 mRNA from the same five high-risk HPV types detected by the PreTect HPV Proofer assay (16, 18, 31, 33 and 45). In a head-to-head comparison of the two assays using cervical scraped specimens, the overall concordance was >90% [63]. When compared to the HC2 assay, the agreement between HC2 assay and NucliSENS EasyQ HPV was fair (kappa value=0.49) but the clinical specificity of the NucliSENS Easy Q HPV was significantly higher than that of the HC2 (63% vs. 49%) [54].

A third mRNA-based assay is the HPV OncoTect E6/E7 mRNA assay (IncellDx, Menlo Park, CA). This assay combines flow cytometry with quantification of mRNA to establish the quantity of overexpressed oncogenes in cervical cells [26, 91]. In a prospective study comparing the HPV OncoTect assay to cytology, the sensitivity of the HPV OncoTect was 88% and 89% for CIN2+ and 100% and 92% for CIN3+ for women 30 years or older and 30 years or younger respectively, with specificity >80% for both CIN2+ and CIN3+ in both groups of women [91]. When compared to HC2, the sensitivity of the HPV OncoTect was comparable for the two methods but the specificity for CIN2 lesions was significantly higher (85% vs. 35%) for the HPV OncoTect [26].

8.2.3 Molecular genotyping assays

Several of the HPV detection assays described above include a genotyping assay for HPV genotypes 16 and 18 and/or additional HPV genotypes (Table 8.3). The following sections focus on the genotyping component of these assays.

8.2.3.1 FDA-cleared assays

There are currently two FDA-approved assays for the genotyping of high-risk HPV types, the Cervista HPV 16/18 (Hologic Inc. Bedford, MA) and the Roche Cobas 4800 HPV (Roche Diagnostics, Indianapolis, IN) tests.

Table 8.3 Commercial assays for HPV genotyping

Manufacturer	Test Name	HPV Genotypes	Regulatory status*
Hologic Gen-Probe, Inc.	APTIMA HPV 16 18/45	16, 18/45	Not currently approved
Hologic, Inc.	Cervista HPV 16/18	16, 18	FDA/CE
Roche Molecular Diagnostics	Cobas 4800 HPV	16, 18	FDA/CE
Roche Molecular Diagnostics	Linear Array HPV	6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108	CE
Innogenetics	The INNO-LiPA HPV Genotyping Extra	6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 67, 68/73, 70, and 74	CE
Greiner Bio-One	The Papillocheck HPV-screening	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82, 53, 66, 6, 11, 40, 42, 43, 44/55, and 70	CE
Qiagen	Reverse hybridization probe assay	16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82	CE

* As of August, 2014.

The Cervista HPV 16/18 genotyping test is approved for use in conjunction with the Cervista HPV HR test and/or cytology to detect the presence of HPV-16 and HPV-18 in women over 30 years or in women with ASCUS cytology results. The positive and negative agreement between the assay and PCR and bidirectional sequencing of the L1 gene was 94.1% and 85.7% respectively [4]. The reported sensitivity and specificity of the Cervista HPV 16/18 assay for $\geq$ CIN2 was 68.8% and for $\geq$ CIN3 was 77.3% [42]. Cross-reactivity was observed with HPV type 31 and a small number of Cervista HPV HR assay negative were positive when tested for HPV-16 and HPV-18 specifically [42].

The Roche Cobas 4800 HPV assay is approved for concurrent detection of high-risk HPV and genotyping of HPV-16 and HPV-18. Performance of the genotyping portion of the assay has been evaluated against Roche HPV Linear array (LA) test (Roche Diagnostics, Indianapolis, IN), a CE approved genotyping assay for both low- and high-risk HPVs (described below). In one study, the LA assay showed higher sensitivity for the detection of coinfections of other high-risk HPVs with HPV-16/HPV-18 than the Cobas 4800 HPV assay [125], although the significance of detecting more than one genotype is unclear. In another study, comparing the prototype Cobas 4800 assay to the LA assay, the two assays performed equally well, with a total agreement of 94.7% [17]. The high concordance of the two assays was further confirmed in a study comparing the Cobas 4800 HPV assay to a combination of Roche Amplicor/LA assay with a reported concordance of 92.7% [77].

8.2.3.2 Non-FDA-cleared assays

There are several assays, as of the time of this writing, that have been CE marked or are available as RUO kits for the detection and genotyping of multiple HPV subtypes including both high-risk and low-risk HPVs.

The Roche Linear Array (LA) HPV genotyping test is CE marked for the detection and genotyping of the following 37 low and high-risk HPVs: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108 [109]. The LA test has been available since 2006 and consists of a manual extraction step (AmpliLute) followed by conventional PCR, hybridization of amplified DNA to the corresponding type-specific probes for detection and typing of the 37 HPV genotypes by colorimetry [53, 109].

Multiple studies have evaluated the performance of the LA tests against other methods [31, 53, 109, 117]. In one study, the LA test was compared to direct sequencing of DNA amplified with the MY09/11 primers and the reported agreement between the two methods was 91.2%, although the LA assay was better at detecting coinfections [54]. The main limitation of the assay is its manual extraction, which is time consuming and

prone to cross-contamination. Automated extraction using various platforms including the MagNA pure LC (Roche Diagnostics, Indianapolis, IN) [22, 110], the Cobas AmpliPrep (Roche Diagnostics, Indianapolis, IN) [44], and the EasyMag (bioMérieux, St Louis, MO) [38] have been evaluated as alternatives to manual extraction. Although the concordance ranged from 80–90% between manual and automated extraction, the manual method generally showed higher sensitivity and specificity for HPV detection and genotyping [22, 110].

The INNO-LiPA HPV Genotyping Extra (Innogenetics, Ghent, Belgium) is CE marked for the detection of 43 genotypes with the typing of the following 25 HPVs: HPV-6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 67, 68/73, 70, and 74 [69, 117]. The LiPA assay is based on amplification of short PCR fragments (SPF), only 65 bp long in the L1 gene, with multiple sets of primers, followed by reverse hybridization of the amplified DNA with HPV specific probes immobilized on nitrocellulose membrane strips, incubation with alkaline phosphatase streptavidin and substrate and visual detection of positive results [69].

Van Hamont et al. compared the performance of the LA assay with that of the LiPa assay [117]. The LA assay can genotype more HPV subtypes than the LiPa, therefore the analysis was restricted to those types common to both assays. The overall agreement based on testing of 160 cervical scraping samples was 80.6%, with 11.2% samples showing concordant results for some but not all genotypes present in the samples. When tested on archived, paraffin-embedded biopsies, the sensitivity of the LiPa assay was higher than that of the LA assay (57% vs. 40%), although the LA assay yielded more genotypic information than the LiPa (40% vs. 83%) [115]. In another study, agreement between the two assays when tested on a variety of specimen types including cutaneous biopsies, laryngeal biopsies, and cervical specimens was very good for HPV genotypes 16 and 31 (kappa: 0.9) and good (kappa: 0.7–0.8) for HPV genotypes 6, 18, 53 and 66 [48].

The Papillocheck HPV-screening (Greiner Bio-One, Frickenhausen, Germany) uses DNA chip technology to detect and genotype 24 different high-risk (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) and low-risk (types 6, 11, 40, 42, 43, 44/55, 53, 66, and 70) HPV genotypes [29]. The assay consists of automated DNA extraction from cervical samples, amplification of a 350 bp fragment of the HPV E1 gene, hybridization of the amplified products with HPV-specific probes immobilized on a DNA chip, incubation with a Cy5-dUTP labeled detection probe and detection of HPV by scanning the DNA chip at 532/635 nm using a CheckScanner [29, 60]. The performance of the Papillocheck assay has been compared to that of the LA assay using cervical specimens previously tested with the HC2-HR assay [29]. The overall agreement for genotypes

detected by both assays was 92.5%, with 67.4% of specimens showing absolute agreement and 25.1% showing partial agreement (i.e., compatible genotypes). Similar results of high agreement (93%) were obtained in a study using a clinical cutoff of CIN 2+ and comparing genotyping by the Papillocheck assay to that of the LA assay [55]. One study compared the performance of the Papillocheck assay with both the LA and the LiPa assays using cervical specimens previously tested with the HC2 assay [104]. The results were widely varied depending on the HPV genotype and the concentration of HPV DNA present. The PapilloCheck assay showed higher sensitivity for HPV types 53, 56 and 33, the LiPa assay was most sensitive for HPV 18, 31, 53, 36 and 35, and the LA assay had better sensitivity for HPV 59 [104].

The reverse hybridization (RH) probe assay (Qiagen, Valencia, CA) is a recent genotyping assay. The procedure includes the following several steps: amplification by PCR of a 150 bp sequence in the L1 gene of the HPV genome using the GP5+/6+ primer sets, reverse line blot hybridization using type-specific probes, incubation with alkaline phosphatase streptavidin and substrate, and finally, visual detection of positive results [108]. The RH assay detects and types the following 18 high-risk HPV genotypes: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82 [74]. The RH assay performance was evaluated in one study against both the LA and the LiPa assays, using both plasmid samples for several HPV types and DNA extracts from archived clinical samples (cervical cells and paraffin-embedded cancer tissues) [108], and in another study against LA using cervical samples previously tested by the HC2 assay [74]. Overall HPV-type-specific agreement between the three methods was good with the RH assay showing lower sensitivity for HPV types 53, 68, and 82 and not being able to detect HPV-6 and HPV-11, which are included in the other two genotyping assays [74, 108].

8.3 Current challenges and future directions

8.3.1 Current challenges

The association of HPV with the development of cancers other than cervical cancers has been established, including penile cancer, anal cancer, and oropharyngeal cancers [86, 87, 89, 121]. Recommendations for HPV testing are now emerging for diagnosis of cancers other than cervical cancers. Current diagnosis of noncervical HPV-associated lesions relies mainly on histopathology and cytology, with sensitivity varying widely among studies but generally agreed low negative predictive value for LSIL or HSIL [75, 130].

Commercial assays currently available are only indicated for the detection of HPVs in cervical specimens. The National Comprehensive Cancer Network, The College of

American Pathologists, “Ear, Nose and Throat” (ENT)-UK and the Royal Colleges of Pathologists recommend HPV testing for head and neck squamous cell carcinomas, although the ideal tests or combination of tests remains to be established [126, 130]. Although there are no national guidelines, some organizations such as The Northwest Pennsylvania Rural AIDS Alliance, the Cleveland Clinic, and other organizations have proposed guidelines for screening and testing for anal cancer, which includes HPV testing [41]. However, the role of HPV DNA testing in the diagnosis of anal cancer remains unclear as the prevalence of HPV is high in target populations (i.e., HIV-positive MSM and other immune-compromised transplant patients), resulting in low specificity for anal intraepithelial neoplasia (AIN2+) [99].

Off-label testing of commercial assays has been performed with varying results. The HC2 assay has been evaluated for use in testing of anal samples and showed high sensitivity but low specificity and the authors of the study recommended that the assay be used as an adjunct test, similar to the recommendation for cervical cancer [98]. The NucliSENS EasyQ mRNA test was recently evaluated for detecting anal dysplasia in HIV-positive men having sex with men and showed similar sensitivity and higher specificity than a DNA-based assay [107].

Although HPV-associated anal and oropharyngeal cancers represent a small subset, they often are more aggressive and would benefit from large clinical trials to validate the use of current commercial assays for use in diagnostic algorithms.

In 2006, the FDA licensed the first HPV vaccine, a quadrivalent vaccine (Gardasil, Merck & Company, Inc.) targeting HPV-6, HPV-11, HPV-16, and HPV-18, followed in 2009 by the approval of a bivalent vaccine targeting HPV-16 and HPV-18 (Cervarix, GlaxoSmithKline). The quadrivalent vaccine is currently approved for prevention of vulvar and vaginal cancer, and vaccination of girls and women aged 9–26 years for prevention of HPV-associated genital warts, cervical and anal cancer, as well as vaccination of boys and men aged 9–26 years for prevention of genital warts and anal cancer. The bivalent vaccine is currently approved for prevention of cervical cancer in women aged 9–25 years [102]. Vaccination is now recommended by the Advisory Committee on Immunization Practices (ACIP) for routine immunization of girls and boys aged 11 and 12 years and catch-up immunization of girls aged 13–23 years and boys 13–21 years, using the quadrivalent vaccine [41, 76]. Post-vaccination reports have confirmed findings from clinical trials showing a positive impact of HPV vaccination on decreasing the rate of cervical lesions due to HPV-16/18 [82, 94]. Long-term effects of vaccination on HPV screening and testing guidelines remain to be seen, although modeling studies suggest a change in the screening procedure, with HPV

DNA testing being used as a screening test followed by Pap smear and cytology for confirmation of HPV-DNA-positive samples as an alternative algorithm [46].

8.3.2 Future directions

Several of the commercial assays described in this chapter are currently in clinical trials for FDA approval. Furthermore, prospective studies to validate additional specimen types for the assays that are already FDA approved or CE marked are needed to increase laboratories options of commercial tests available for screening and testing of HPV-associated cancers. Novel methodologies for detecting and genotyping both low- and high-risk HPVs are being developed and evaluated and include assays based on liquid bead microarray [20], Templex PCR in combination with liquid bead microarray [56], solid microarray [97], novel fluorescent quantitative probes [129], near-infrared Raman microspectroscopy [118], and vibrational spectroscopy [84]. The impetus for the development of these novel methods into commercial assays will most likely be driven by the post-vaccination prevalence of various HPV genotypes, as well as their applicability in accurately identifying patients at increased risk of developing HPV-associated cancers.

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Human Immunodeficiency Virus

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9.1 Introduction

Currently, a number and variety of techniques and instrumentation are available for use in the diagnosis and management of patients infected with human immunodeficiency virus (HIV) (Table 9.1). The selection of which tests to perform depends greatly on the patient population and clinical scenario and the intended use of each individual assay (Table 9.2). Serological screening and confirmatory assays designed for antibody detection or a combination of antibodies and p24 antigen are most commonly used for the laboratory diagnosis of HIV infection. Tests that simultaneously measure antibody and p24 antigen, assays that detect p24 antigen alone, and molecular amplification assays for identification of viral DNA or RNA allow for the detection of acute HIV infection in newborn babies and other individuals at risk and aid in shortening the diagnostic “window-period” between infection and seroconversion. The additional use of quantitative nucleic acid-based assays provides invaluable information about the prognosis and response to therapy, while phenotypic, genotypic, and tropotypic tests are commonly used to detect antiretroviral drug resistance. The choice of whether to incorporate each of these methods into the clinical laboratory will depend on the patient population and clinical needs as well as the number of specimens to be tested; the cost, turnaround time, and ease of testing; and the resources and capabilities of the individual laboratory.

The emphasis of this chapter is on the many and varied technologies that are currently available commercially for the diagnosis and monitoring of HIV infections, and is not meant to duplicate other extensive reviews of HIV. Important characteristics of the assays are described, and the key advantages and disadvantages are presented. Due to competition among manufacturers and strict regulation

worldwide, many excellent commercial diagnostic products have been developed that are well standardized and of high sensitivity and specificity. Most of the commercial assays are Conformité Européenne (CE) marked and are compliant with the European Union and have received regulatory approval for widespread clinical use in Europe, Asia, Japan, Canada, or Australia, while a smaller number have been registered in the United States and have been approved by the US Food and Drug Administration (FDA) for diagnostic use. The rapid pace of development of HIV-related tests has led to an ever changing market, with the introduction of many new assays and the discontinued use of others. A number of manufacturers and vendors are new to the field, while others have merged or left the market place. Consequently, some products have been transferred from one vendor to another, and many of the kit names have changed. Some products are also made by one company and distributed and sold under multiple brand names, making it more difficult to compile a comprehensive list. The information presented in this chapter about each commercial assay was obtained either from the published literature or from the individual manufacturers through their websites, written materials, and/or personal communications with company representatives. While every attempt has been made to include any and all manufacturers of HIV-related test products, users are advised that the amount of material is great and not every available test, device, product, or instrument may be included. Additional tests, devices, instruments, and products may be available elsewhere and the reader must thoroughly evaluate the availability and regulatory compliance of all products in their country or region. Individuals should contact the manufacturers listed in the Appendix of this manual for a more comprehensive description and the current list price and availability of a particular product.

Table 9.1 Characteristics of available virological methods for the diagnosis and monitoring of HIV infection

Test	Specimen required	Cost	Technical difficulty	Turn-around (days)	Diagnostic value
Screening immunoassays*	Serum or plasma	Low	Low	1–2	High in patients > 15–18 months of age
Standard p24 antigen†	Serum or plasma	Low	Low	1–2	Low in all groups
ICD p24 antigen†	Serum or plasma	Low	Low	1–2	Moderate to high in patients > 1 month of age
Qualitative proviral DNA PCR‡	PBMC	Moderate	Moderate	1–2	High in all groups
Qualitative RNA NAAT§	Plasma, serum	Moderate	Moderate	1–2	High in all groups
Viral culture¶	PBMC	High	High	14–28	High in all groups
Quantitative RNA NAAT	Plasma	Moderate	Moderate	1–2	High in all groups
Phenotypic drug susceptibility assays	Patient's viral isolate	High	High	14–42	High in all groups
Genotypic drug susceptibility assays	Plasma	High	High	1–2	High in all groups

ICD, immune complex dissociation; PCR, polymerase chain reaction; NAAT, nucleic acid amplification test; PBMC, peripheral blood mononuclear cells.

* Screening immunoassays currently include antibody-only and antigen/antibody combination laboratory-based and rapid, less-sophisticated tests.

† Individual p24 antigen-only assays are not routinely used in most clinical settings; used primarily in resource-poor countries and in research settings.

‡ Qualitative proviral DNA PCR has limited commercial availability; assay also can be performed on whole blood, dried blood spots, plasma, other body fluids, and tissues.

§ Qualitative RNA NAATs are commercially available and FDA licensed, and have largely replaced qualitative proviral DNA tests.

¶ Viral culture is primarily used in support of HIV research studies and is seldom needed today in a clinical setting; assay also can be performed on plasma, other body fluids, and tissues.

Table 9.2 Use of laboratory tests for HIV diagnosis and management

Clinical situation	Recommended test(s)
Blood donor screening	Antibody screening immunoassay (EIA or CLA) and confirmatory test (WB or IFA), qualitative RNA NAAT
Routine screening/diagnosis of HIV infection	Antibody-only and antigen/antibody combination EIA or CLA laboratory-based and rapid, less-sophisticated screening tests and confirmatory/differentiation assays
Acute HIV infection	EIA or CLA antigen/antibody combination immunoassays, qualitative RNA NAAT
Infant (≤18 months of age) born to HIV-infected mother	Qualitative proviral DNA PCR or qualitative RNA NAAT
Indeterminate HIV-1 WB	Repeat HIV-1 antibody screening immunoassay and WB in ~2–3 weeks, perform HIV-2-specific antibody immunoassay and WB, qualitative RNA or proviral DNA NAAT
Indeterminate HIV-1/HIV-2 antibody differentiation assay	Qualitative RNA NAAT
Prognosis	Quantitative RNA NAAT
Response to therapy	Quantitative RNA NAAT
Antiretroviral drug resistance	Phenotypic, genotypic, tropotypic resistance assays

EIA, enzyme immunoassay; CLA, chemiluminescent assay; WB, Western blot; IFA, immunofluorescence assay; NAAT, nucleic acid amplification assay; PCR, polymerase chain reaction.

9.2 Markers of HIV infection

HIV is classified into two major types, HIV type 1 (HIV-1) and type 2 (HIV-2). HIV-1 is responsible for the majority of HIV infections worldwide and is divided into four groups, designated M (major), O (outlier), N (non-O), and P. Group M is the most prevalent and is further subdivided into subtypes or clades designated A–D, F–H, J, and K. Coinfection with different subtypes of HIV-1 can give rise to circulating recombinant forms (CRFs).

Following infection with HIV-1, different HIV markers of diagnostic significance appear at various times in the blood of an infected individual, including viral RNA, p24 antigen, and circulating antibodies [16, 37, 99, 100, 122]. Measurable HIV-1 RNA is the first marker to be detected

by using qualitative and/or quantitative nucleic acid amplification tests. This normally occurs within 10–11 days of an individual being infected and the levels of viral RNA rise rapidly and become markedly elevated over the next few weeks before decreasing to baseline levels that remain detectable over time. HIV-1 p24 antigen is the next marker to rise in an infected individual and can be detected within 4–10 days after the initial detection of HIV-1 RNA by using assays that detect p24 antigen alone or by using HIV antigen/antibody combination assays. The production of p24 antigen is transient and declines to low or undetectable levels with the appearance of antibodies to this protein. This is due to the formation of immune complexes that interfere with p24 antigen

detection. Finally, HIV antibodies appear as the infection with HIV results in the induction of a humoral antibody response specific to viral proteins, with the production of immunoglobulin M (IgM), IgG, and IgA. The structural proteins of HIV-1 are the targets for the majority of the circulating antibodies directed against the virus. These include the envelope (*env*) proteins (surface glycoprotein [gp 120], transmembrane glycoprotein [gp 41], and their precursor glycoprotein [gp160]), polymerase (*pol*) proteins (reverse transcriptase [p65], endonuclease-integrase [p31], and protease [p10]), and core (*gag*) proteins (matrix protein [p18], internal capsid protein [p24], nucleocapsid protein [p7], and their precursor protein [p55]). Antibodies to HIV-1 are detectable in most persons within 4–12 weeks after infection and in virtually all patients within 6 months. Variable levels of immunoglobulin M (IgM) antibodies appear first, quickly reaching a peak and declining over the following weeks. HIV-specific IgM antibodies are detectable 3–5 days after the initial appearance of p24 antigen and 10–13 days after viral RNA is detected. About 1 week later, IgG antibody levels rise significantly, reach a plateau within a few months, and remain high for many years. The time at which IgG antibodies can be detected will vary depending upon the sensitivity of the serologic immunoassay used for screening and may be as short as 4–7 days and as long as 18–38 days or more after the initial detection of viral RNA. In some individuals, antibody to core proteins (p24 and/or p18) and sometimes polymerase proteins (p65 and/or p31) may drop significantly, and, in rare cases, even become undetectable with progression to HIV disease. Also, initiation of antiretroviral therapy prior to seroconversion can result in a delayed and decreased antibody response or a lack of a detectable antibody response to HIV infection. Elevated levels of HIV-specific serum IgA antibody can be found in infants as early as 3 weeks after birth and may persist for an extended time following acute infection.

9.3 HIV screening

Since 1989, tests for the detection of HIV-specific antibodies have been at the forefront for the diagnosis of HIV infection. They have been effectively used to determine if an individual has been exposed to HIV, to screen blood and plasma donations, and for epidemiological surveillance. During this time, the standard testing algorithm used in the United States and in many parts of the world involved a two-stage testing strategy. In this algorithm, HIV Infection is first identified by using an enzyme immunoassay (EIA), in which an enzyme and a substrate is used to produce a colorimetric signal to screen specimens for the detection of HIV-specific antibodies. If specimens are nonreactive for HIV antibodies when initially tested by EIA, no further testing is performed and the individual

is considered to be uninfected. Specimens with initially reactive results by EIA are then repeated in duplicate using the same EIA. If one or both of the duplicate samples of the specimen are reactive by EIA, the final EIA result is considered positive. If the two repeat samples of the specimen are nonreactive, the specimen is considered negative for HIV antibodies by EIA. Repeatedly reactive specimens are then confirmed by Western blot or immunofluorescence assays (IFA) to assure the specificity of the EIA result. Initially, immunoassays and confirmatory tests were designed to detect antibodies only to HIV-1. In 1992, the Centers for Disease Control and Prevention (CDC) recommended specific screening for both HIV-1 and HIV-2 antibodies as the prevalence of HIV-2 increased worldwide, and also recommended the use of more specific confirmatory tests to verify the presence of antibodies against HIV-2 if the HIV-1 Western blot was negative or indeterminate and the clinical situation warranted testing for HIV-2. In 2004, it was recommended by the CDC that all specimens determined to be reactive by rapid HIV-specific antibody assays also be confirmed with either a Western blot or IFA specific for antibodies to HIV-1. For over 25 years, a determination of positive antibody reactivity by EIA followed by confirmation using one of the confirmatory tests remained the most convincing laboratory evidence for a diagnosis of HIV infection. However, there are shortcomings with this approach, including delays in turnaround time for HIV test results, lack of differentiation of HIV-1 from HIV-2 infection, and the inability to detect acute primary HIV infections.

More recently, there have been significant advances in HIV diagnostics that have led to the development of newer screening immunoassays with improved speed and performance and enhanced sensitivity and specificity, immunoassays that can distinguish and differentiate antibody responses to HIV-1 and HIV-2, and antigen- and nucleic-acid-based assays for earlier detection of HIV infection. In June of 2014, based on these changes in technology, the CDC and Association of Public Health Laboratories recommended a new algorithm for the diagnosis of HIV infection in adults and children > 24 months of age [19]. In the new algorithm, testing begins with a fourth-generation combination screening immunoassay that detects HIV-1 and HIV-2 antibodies and HIV-1 p24 antigen to test for established infections with either virus and acute infection with HIV-1. This is followed by a rapid antibody test that differentiates HIV-1 and HIV-2 antibodies for confirmation of specimens that are reactive on the antigen/antibody combination immunoassay. Specimens that are nonreactive or indeterminate on the rapid HIV-1/HIV-2 antibody differentiation immunoassay are then tested by an HIV-1-specific nucleic acid amplification assay for resolution of the results. It is also recommended that this same testing algorithm be used on

serum or plasma specimens submitted for testing after a reactive preliminary result from any rapid HIV test and should be considered on specimens that are nonreactive by rapid HIV tests since laboratory-based antigen/antibody combination immunoassays detect HIV infection earlier than any of the rapid HIV tests, including commercially available rapid HIV-1/HIV-2 antigen/antibody combination test. The newly recommended algorithm offers distinct advantages over the previously used algorithm, including identification of acute HIV infection as much as 3–4 weeks earlier, more accurate diagnosis of HIV-2 infection, a decrease in indeterminate results and reporting times for most test results, and improved linkage to care and use of antiretroviral therapy [18, 19, 30, 99, 112, 122, 176]. Also, the HIV-1 Western blot and HIV-1 IFA are no longer part of the recommended algorithm.

Coincident with the changes in the testing algorithm, there have also been changes in the population for whom HIV testing is recommended. In 2006, the CDC recommended expansion from an approach of targeted testing of high-risk groups to a strategy that incorporates routine, universal screening of all patients aged 13–64 years [12]. The US Preventive Services Task Force (USPSTF) has since recommended that all adolescents and adults aged 15–65 years be screened for HIV infection and that screening be performed on all pregnant women, including those who present in labor who are untested and whose HIV status is unknown, and younger adolescents and older adults who are at increased risk for HIV infection [109].

9.4 Laboratory-based immunoassays

A wide selection of laboratory-based immunoassay kits that detect HIV-specific antibodies have been developed over the years and made available from a variety of commercial manufacturers (Table 9.3). The assays offer the distinct advantage of using highly standardized and stable immunoreagents that provide accurate and objective results. They normally require minimal training and equipment and are applicable to large numbers of specimens at a reasonable cost. The different test kits vary in their configurations, the number and type of antigens used to capture HIV-specific antibodies, the specimen sources that can be tested, and whether the assays are designed for detecting antibodies to either HIV-1 or HIV-2 or for the simultaneous detection of antibodies to both viruses or for the combined detection of both antibodies and antigens. Many of the newer immunoassays now use chemiluminescence, fluorescence, phosphorescence, and electrochemiluminescence measurements instead of the enzyme/substrate detection system used by the more traditional EIAs.

Since being approved for diagnostic use in 1985, immunoassays for the detection of HIV-specific antibodies have

evolved through four generations of change and improvement, with the source of antigen(s) used and the assay design being significant factors in determining the overall sensitivity and specificity of the tests (see reference [24] for a review). With each new generation of HIV immunoassay, the rate of false-positive results was decreased and the time to positivity was reduced, with a corresponding improvement in the detection of early infection.

The original first-generation kits used viral proteins prepared from lysates of HIV-1-infected human T-cell lines as the source of antigens that were bound to a solid phase for capturing HIV-specific antibodies from a patient's specimen. The antigen–antibody complexes that form were then detected with the addition of an enzyme-labeled, anti-human antibody that binds to the complexes and reacts with a chromogenic substrate to produce a color change. The intensity of the color generated was measured in a spectrophotometer and compared with a set of positive and negative controls performed with each batch of specimens. Horseradish peroxidase and alkaline phosphatase were the most common enzyme labels, and the surfaces of microwell plates and polystyrene beads were used as the solid-phase carriers. With first-generation assays, specimens had to be significantly diluted to overcome cross-reactivity with cellular proteins that contaminated the prepared lysates.

Scientific and technological advances over the years led to the incorporation of recombinant antigens and synthetic peptides into second- and third-generation immunoassays to improve their sensitivity and specificity over traditional tests based on whole viral lysates. The assay format of second-generation assays, however, was similar to that for first-generation tests and, like first-generation assays, detected only IgG antibody to HIV-1. Third-generation assays underwent a more radical design change adding antigens to detect antibodies to HIV-1 and HIV-2 and using an immunometric antigen sandwich format in which antibodies in the specimen are sandwiched between HIV-specific antigens attached to the solid phase and to HIV antigens that are conjugated to indicator molecules and added to the reaction. This format has the distinct advantage of efficiently and simultaneously detecting IgG and IgM class antibodies to both virus types. Third-generation assays also have a greater sensitivity for detecting HIV antibodies in the early stages of infection, thereby shortening the time to seroconversion. The time to positivity for first- and second-generation assays has been estimated to be approximately 35–45 days and 25–35 days, respectively, following infection, while the time to positivity for third-generation assays is approximately 20–30 days [19, 24].

Over the years, the availability of commercial first- and second-generation laboratory-based HIV screening

Table 9.3 Screening immunoassays for the detection of HIV antibodies and p24 antigen

Manufacturer (alphabetical)	Product name	Assay format	Sample	Detection	Assay generation	Selected references
Abbott	ARCHITECT HIV Ag/Ab Combo Assay	Automated CMIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	21, 36, 90, 106, 124
Acon Laboratories	Foresight HIV 1/2/O Antibody	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2	Third	NF
Avioq	Foresight HIV 1/2/O Antigen Antibody	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	NF
Avioq	AVIOQ HIV-1 Microelisa System	Microplate EIA	Serum, plasma, oral fluid, DBS	IgG and IgM Ab to HIV-1 group M	Second	89
Biokit	Bioelisa HIV 1+2 3.0	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2	Third	NF
bioMérieux	Bioelisa HIV 1+2 Ag/Ab	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	NF
	VIDAS HIV Duo Quick	Automated SPR ELFA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	90, 105
	VIDAS HIV Duo Ultra	Automated SPR ELFA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag (differentiates Ag/Ab signal)	Fourth	90, 105, 175
Bio-Rad	Vironostika HIV Uni-Form II Ag/Ab	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	1, 90, 167, 168
	GS HIV-1/HIV-2 PLUS O EIA	Microplate EIA	Serum, plasma, cadaveric serum	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2	Third	87
	GS HIV Combo Ag/Ab EIA	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	8, 106, 112
DiaSorin	Murex HIV-1.2.O	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2	Third	62
	Murex HIV Ag/Ab Combination	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	1, 83, 90
	LIAISON XL Murex HIV Ab/Ag	Automated CLIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	4, 86
J Mitra & Co., Ltd.	MICROELISA HIV	Microplate EIA	Serum, plasma	IgG Ab to HIV-1 groups M and O	Second	NF
	MICROELISA HIV Ag & Ab	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	NF
Maxim Biomedical, Inc.	HIV-1 EIA	Microplate EIA	Serum, plasma, oral fluids, urine	IgG Ab to HIV-1 group M	Second	3, 120
Ortho Clinical Diagnostics	VITROS EC/ECiQ Anti-HIV 1+2	Automated immunometric assay	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2	Third	106
Roche Diagnostics	Elecsys HIV combi PT 4 th Gen	Automated ECLA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	111, 160
Siemens Healthcare	Enzygnost® Anti-HIV 1/2 plus	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2	Third	1
	Enzygnost HIV Integral 4 Assay	Microplate EIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	2, 90
	ADVIA Centaur HIV 1/O/2 Enhanced (EHV)	Automated CMIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2	Third	85, 106, 142
	ADVIA Centaur HIV Ag/Ab Combo Assay	Automated CMIA	Serum, plasma	IgG and IgM Ab to HIV-1, including groups O and M, and HIV-2; HIV-1 p24 Ag	Fourth	132, 166

CMIA, chemiluminescent microparticle immunoassay; EIA, enzyme immunoassay; SPR ELFA, solid-phase receptor enzyme-linked fluorescent assay; CLIA, chemiluminescence immunoassay; ECLA, electrochemiluminescence assay; IgG, immunoglobulin G; Ab, antibody; Ag, antigen; IgM, immunoglobulin M; NF, none found.

immunoassays in the United States and international markets has diminished to the point that few, if any, kits remain, while some third-generation assays continue to be sold.

More recently, a number of fourth-generation screening immunoassays have been developed and commercialized for the simultaneous detection of HIV-1 p24 antigen and antibodies to HIV-1 (including group O) and HIV-2 (Table 9.4). These combination assays use synthetic peptide or recombinant protein antigens in the same antigen–antibody–antigen sandwich format as third-generation assays to detect IgG and IgM antibodies to HIV-1 and HIV-2, and also include monoclonal antibodies to capture and detect HIV-1 p24 antigen. The format of these assays usually does not allow for the differentiation of antibody reactivity from antigen reactivity, although, more recently, several assays have been designed to discriminate between antibody and antigen detection. Fourth-generation assays provide an increase in sensitivity over tests that only detect antibody and allow a reduction in the window period between infection with HIV-1 and detectable seroconversion. The time to positivity for fourth-generation assays has been estimated to be 15–20 days following infection [19, 24].

In general, the different generations and formats of laboratory-based immunoassays used over the years to screen specimens have been considered to be sensitive and specific for the detection of antibodies to HIV. The predictive value of a positive result, however, can vary considerably and depends on the prevalence of HIV infection in the population being tested. Few false-positive results are observed when testing high-risk populations, while a high rate of false-positive results may occur in populations with low prevalence of HIV infection, such as blood donors. When using first-generation laboratory-based assays, biological false-positive reactions were mainly due to reactivity of antibodies to human leukocyte antigen (HLA) proteins expressed by the T-cell lines used to prepare the viral lysates. With second-, third-, and fourth-generation assays, the use of recombinant antigens or synthetic peptides significantly decreased the number of false-positive results but has not completely eliminated them. False-positives may be the result of passive immunoglobulin administration, receipt of an experimental HIV vaccine, recent exposure to certain vaccine preparations (e.g., influenza vaccine), or the patient having cross-reactive antibodies to contaminating bacterial or yeast proteins used in recombinant antigen-based immunoassays. Therefore, the immunoassay, although an excellent test for screening, should not be used as the sole test for making a diagnosis of HIV infection. A positive confirmation test is also necessary to exclude false-positive results.

A negative antibody screening immunoassay result normally means HIV infection is unlikely. However, false-negative results may occur and may be due to immunosuppressive therapy, replacement transfusion,

severe hypogammaglobulinemia (B-cell dysfunction and defective antibody synthesis), genetic diversity of the virus itself, and testing too early or too late in the course of illness. Although it is possible that levels of HIV-specific antibodies may fall during advanced disease, they rarely drop below the detection limits of current immunoassays. Group O HIV-1 strains have been described that have evolved to the extent that antibodies to this group are not detected by some immunoassays on the market. Group O HIV-1 strains have been seen predominantly in Africa and Europe, although a small number of infections with this group have also been identified in the US. Immunoassay kits using viral lysates as the antigen source are more efficient at detecting this highly divergent group than kits that use recombinant antigens or synthetic peptides. Most current commercial immunoassays have been reformulated to contain specific antigens to group O in order to recognize infection with this group. Similarly, most commercial tests are now designed to detect antibodies to HIV-1 and HIV-2 to effectively screen for both virus types. Separate HIV-2 screening and supplemental tests are also commercially available and should be considered if it is suspected that HIV-2 infection is probable. Specific HIV-2 antibody testing should be considered for persons from West Africa, where the virus is endemic, individuals who have received blood transfusions from or have had sexual relations with someone from this region, and children of women at risk of infection or known to be infected with HIV-2.

Lastly, laboratory-based immunoassays have the greatest potential for automation, and a number of semi-automated and fully automated immunoassay analyzers are now commercially available for the performance of HIV serological assays. The majority of the automated immunoassay analyzers provide walk-away simplicity to perform assays from sample processing through interpretation of results (see Chapter 17).

9.5 Rapid, less-sophisticated immunoassays

Rapid (10–20 min) and less sophisticated immunoassays for the detection of HIV antibodies have also been developed by a number of manufacturers (Table 9.4). The tests offer the distinct advantages of lower costs and same-day results and are packaged as ready-to-use kits with all reagents and materials included. The kits are designed to be performed either in the laboratory or at the point of care (POC) where the specimen was collected using a single step or a few simple steps and self-contained, disposable devices. The performance of these rapid assays requires no specialized equipment and only limited technical expertise; some of the kits have the added advantage of possessing stabilized biochemicals that guarantee a long shelf life when stored at

Table 9.4 Rapid and/or simple immunoassays for the detection of HIV antibodies and p24 antigen

Manufacturer (alphabetical)	Product name	Assay type	Sample	Time to result (min)	Detection	Selected references
Alere	Determine HIV-1/2	LF	Serum, plasma, whole blood	15	HIV-1/HIV-2 Ab	169, 172
	Determine HIV-1/2 Ag/Ab Combo	LF	Serum, plasma, whole blood	20	HIV-1/HIV-2 Ab, HIV-1 p24 Ag	73, 100, 136
	Clearview COMPLETE HIV 1/2	LF	Serum, plasma, whole blood	15	HIV-1/ HIV-2 Ab	NF
	Clearview HIV 1/2 STAT-PAK	LF	Serum, plasma, whole blood	15	HIV-1/HIV-2 Ab	NF
	DoubleCheckGold HIV 1&2	LF	Serum, plasma	15	HIV-1/HIV-2 Ab	104
	DoubleCheckGold HIV 1&2 Whole Blood	LF	Serum, plasma, whole blood	15	HIV-1/HIV-2 Ab	NF
	DoubleCheckGold Ultra HIV (single reagent)	LF	Serum, plasma, whole blood	10	HIV-1/HIV-2 Ab	NF
	ImmunoComb HIV 12 BiSpot	ID	Serum, plasma	37	HIV-1/HIV-2 (differentiates)	NF
	ImmunoComb HIV 12 TriSpot	ID	Serum, plasma	85	HIV-1 and HIV-2 Ab (differentiates), HIV-1 p24 Ag	NF
bioMérieux	SD BIOLINE HIV-1/2 3.0	LF	Serum, plasma, whole blood	10–20	HIV-1 and HIV-2 Ab (differentiates)	59, 91, 173
Biorad	INSTI HIV-1 Antibody Test	FT	Plasma, whole blood	<2	HIV-1 Ab	149
	Biorad HIV-1&2	MPEIA	Serum, plasma, whole blood, saliva	25	HIV-1/HIV-2 Ab	NF
	VIKIA HIV 1/2	LF	Serum, plasma, whole blood	30	HIV-1/HIV-2 Ab	47, 141
	Multispot HIV-1/HIV-2 Rapid Test	FT	Serum, plasma	20	HIV-1/HIV-2 Ab (differentiates)	118, 119
	Genie III HIV-1/HIV-2 Assay	LF	Serum, plasma, whole blood	10–30	HIV-1/HIV-2 Ab (differentiates)	59
Chembio Diagnostic Systems	HIV 1/2 STAT-PAK Assay	LF	Serum, plasma, whole blood	15	HIV-1/HIV-2 Ab	34
	HIV 1/2 STAT-PAK DIPSTICK Assay	LF	Serum, plasma, whole blood	15–20	HIV-1/HIV-2 Ab	91
	STAT-VIEW HIV 1/2 Assay	LF	Serum, plasma, whole blood	15–20	HIV-1/HIV-2 Ab	NF
	DPP HIV 1/2 Assay	LF	Serum, plasma, whole blood, oral fluids	15	HIV-1/HIV-2 Ab	17, 63
	DPP HIV-Syphilis Assay	LF	Serum, plasma, whole blood	15	HIV-1/HIV-2 Ab; <i>Treponema pallidum</i> Ab	55
CORE Diagnostics	Core HIV 1&2	LF	Serum, plasma, whole blood	15–30	HIV-1/HIV-2 Ab	NF
	ImmunoFlow HIV 1-HIV 2	LF	Serum	15–30	HIV-1/HIV-2 Ab (differentiates)	59
Fujirebio	Serodia HIV-1/2	PA	Serum, plasma	10	HIV-1/HIV-2 Ab	32, 107, 130, 171
Hema Diagnostic Systems	Rapid 1-2-3 Hema Dipstick	LF	Serum, plasma, whole blood	15	HIV-1/HIV-2 Ab	NF
	Rapid 1-2-3 Hema EZ	LF	Serum, plasma, whole blood	15	HIV-1/HIV-2 Ab	NF
	Rapid 1-2-3 Hema Express	LF	Serum, plasma, whole blood	15	HIV-1/HIV-2 Ab	NF
J. Mitra & Co., Ltd	DIAGNOS HIV BI-DOT	FT	Serum, plasma	3	HIV-1/HIV-2 Ab	NF
	HIV TRI-DOT	FT	Serum, plasma	3	HIV-1/HIV-2 Ab (differentiates)	75, 156
MedMira Laboratories	HIV Tri-Dot + Ag	FT	Serum, plasma	5–15	HIV-1/HIV-2 Ab, HIV-1 p24 Ag	26
OraSure Technologies	Reveal G3 Rapid HIV-1 Antibody Test	FT	Serum, plasma	<2	HIV-1 Ab	NF
	OraQuick ADVANCE Rapid HIV-1/2 Antibody Test	LF	Plasma, oral fluid, whole blood	20	HIV-1/HIV-2 Ab	29, 58, 135, 182, 184
	OraQuick In-Home HIV Test	LF	Oral fluid	20	HIV-1/HIV-2 Ab	70, 77, 96
QualPro Diagnostics (Tulip Group)	Retroscreen HIV	LF	Serum, plasma	15	HIV-1/HIV-2 Ab (differentiates)	NF
Sayvon Diagnostics Ltd	HIVSav 1/2/O Rapid SeroTest	LF	Serum, plasma, whole blood	10	HIV-1/HIV-2 Ab	10, 134
Span Diagnostics Ltd	CombAids-RS Advantage	ID	Serum, plasma, whole blood	10	HIV-1/HIV-2 Ab	62, 102
	Signal HIV	FT	Serum, plasma	10	HIV-1/HIV-2 Ab	NF
	Signal HIV BLINK	FT	Serum, plasma	<2	HIV-1/HIV-2 Ab	NF
	Signal HIV 3D	FT	Serum, plasma	10	HIV-1/HIV-2 Ab (differentiates)	NF
Trinity Biotech	Uni-Gold HIV	LF	Serum, plasma, whole blood	10	HIV-1/HIV-2 Ab	91
	Uni-Gold HIV-Recombinant HIV-1/2	LF	Serum, plasma, whole blood	10	HIV-1/HIV-2 Ab	34, 172, 182

LF, lateral flow; FT, flow through; MPEIA, magnetic particle EIA; ID, immunodot; PA, particle agglutination; Ab, antibody; Ag, antigen; NF, none found.

room temperature. Rapid HIV tests have the major disadvantages of low throughput and subjective reading of the test results (see references 10, 11, 134 for a review).

Currently, there are four basic formats for these rapid HIV tests, including membrane flow-through immunoconcentration devices, lateral-flow immunochromatographic strips, particle agglutination, and immunodot comb assays. In membrane flow-through devices, HIV antigens immobilized on a membrane capture and concentrate HIV-specific antibodies on the surface of the device as the specimen flows through and is absorbed into an absorbent pad. This is followed by the sequential addition of enzyme-labeled anti-human antibody and a colorless substrate. Enzymatic hydrolysis of the substrate leads to a colorimetric result that is read visually as a dot or line that forms on the membrane. Many of the membrane flow-through kits include procedural controls to verify the satisfactory performance of the assay. Lateral-flow immunochromatographic strips, or so-called dipsticks, are the most recent addition to the development of rapid and simple assays for HIV. The specimen is applied to an absorbent pad and migrates by capillary action along a solid-phase strip, where it combines with HIV antigens and detector reagents to produce a visual line on the strip when HIV-specific antibodies are present. A procedural control is normally included on the strip and is also indicated by a visible line. Immunodot comb assays use a solid plastic comb with teeth that are sensitized at several spots with different HIV antigens and control material. Patient specimens are placed in individual wells that accommodate single teeth of the comb. The comb is then transferred from specimen wells to reagent wells, and the teeth are saturated by the different solutions; spots that form at reactive positions on the individual teeth indicate positive results. Lastly, in particle agglutination assays, HIV-specific antibodies in a patient's specimen will visually agglutinate or aggregate when cross-linked with particles (e.g., latex, red blood cells, or gelatin) that are coated and sensitized with HIV antigens.

Most of the described rapid tests use multiple recombinant and/or synthetic peptides to detect antibodies to HIV-1 and HIV-2, and some even include specific proteins to efficiently detect HIV-1 group O and the various subtypes of HIV-1 group M, and to differentiate antibody responses to either HIV-1 or HIV-2. Some rapid assays can detect both IgG and IgM responses, while most detect only IgG; many of the assays have procedural controls that ensure adequate test performance and control for specimen adequacy by the detection of human immunoglobulin molecules. Serum and plasma are the specimens of choice for the majority of the rapid devices, although many of the assays have also been adapted to allow the use of fingerstick or venipuncture whole blood or oral fluids. These assays

have sensitivities and specificities similar to those of the more traditional second-generation laboratory-based antibody-only immunoassays when performed and read by properly trained personnel, although some assays perform better than others. Predictive values comparable to those of the standard combination of laboratory-based immunoassay and Western blot testing can be obtained using multi-test algorithms comprised of a combination of two or more rapid tests. Some countries outside of the United States now use these combinations of rapid tests as a less expensive and more rapid alternative to using laboratory-based immunoassays and Western blotting for blood screening, diagnostic testing, and epidemiological surveillance.

The rapid HIV tests are commonly used in developing countries, where resources and facilities may make it impractical or even impossible to perform the more technically demanding laboratory-based immunoassays that require time, sophisticated equipment, and performance in a clinical laboratory. The tests are intended for use in emergency departments, hospital clinics, sexually transmitted diseases clinics, family planning clinics, and HIV outreach programs. In these settings, rapid testing may provide same visit results to individuals seeking HIV testing, as many persons, including those infected with HIV, never return to receive their results following the delay in reporting that may occur when using traditional testing. The rapid availability of test results may assist in providing more timely essential medical and prevention services to these individuals. Rapid tests also may be useful in assessing the risk of HIV transmission following exposure to possibly HIV-contaminated materials or in screening pregnant women presenting for delivery with unknown HIV serostatus. It has been shown that antiretroviral therapy reduces occupational transmission of HIV after percutaneous exposures and reduces vertical transmission when used in the intra- or postpartum periods.

In 2012, the FDA approved the OraQuick In-Home HIV Test (Orasure Technologies, Inc., Bethlehem, PA) as the first rapid home-use, over-the-counter kit that is performed by the consumer and does not require sending a sample to a laboratory for analysis [70, 77, 96]. The test is a single-use immunoassay to detect antibodies to HIV-1 and HIV-2 in oral fluid specimens, and provides a test result in 20–40 min. The assay has the potential to identify large numbers of previously undiagnosed HIV infections and is targeted to people who otherwise would be reluctant to visit their physician or a health-care facility to be tested. Positive test results using this test must be confirmed by follow-up laboratory-based testing and the test can be falsely negative until at least 3 months after infection with HIV. Prior to this test, the Home Access Express HIV-1 Test System (Home Access Health Corp., Hoffman Estates, IL) was FDA-approved in

1996 and was actually a home sample collection system in which dried blood spots were submitted by mail to a laboratory for HIV antibody screening and confirmation [39]. More recently in 2013, the FDA approved the first rapid POC test that detects both HIV-1/2 antibodies and HIV-1 p24 antigen (Determine HIV-1/2 Ag/Ab Combo Test, Alere, Waltham, MA). The test is a fourth-generation assay that is performed in three simple steps and takes 20 min to complete. The assay can be performed on fingerstick or venipuncture whole blood, serum, or plasma samples and can detect HIV infection earlier than first-, second-, or third-generation assays that detect only antibody to HIV. Similar to laboratory-based, fourth-generation combination assays, this test displays excellent performance for detecting established HIV infection, but appears to have a much lower sensitivity when compared to laboratory-based assays for detecting acute HIV infection [73, 100, 136]. At least two other rapid POC assays that detect both HIV-1/2 antibodies and HIV-1 p24 antigen have been developed and may be commercially available internationally (see Table 9.4).

9.6 Specimen matrices for HIV screening

Serum and plasma are the most widely used specimens in both the laboratory-based and rapid, less-sophisticated immunoassays. However, a number of these assays have been validated for use with other specimen types, including fingerstick and venipuncture whole blood, oral fluids, dried blood spots, cadaveric blood, urine, and, to a lesser extent, cerebrospinal fluid. The approval by the FDA of oral fluids [57, 97, 126], dried blood spots [7, 9, 71, 72, 79, 138], and urine [97, 98, 165] specimens for HIV antibody provides fast, convenient, and relatively noninvasive alternatives in specimen collection. Particular attention has been given to the value of oral fluids and urine specimens for the diagnosis of infection with HIV. Screening assays primarily intended for serum or plasma have been modified for use with oral fluid and urine specimens, and extremely sensitive assays specifically designed for these specimen types have also been developed and FDA approved. When such changes are made and assay protocols are optimized to accept oral-fluid or urine specimens, the sensitivities and specificities of these assays for the detection of HIV-specific antibodies are equal to, or only slightly less than, those when testing serum or plasma. Several commercial devices have also been developed specifically for the collection of oral mucosal transudate specimens, and the FDA has approved one of the devices (OraSure; OraSure Technologies) for diagnostic use. The devices provide a homogeneous specimen rich in plasma-derived IgG and IgM that is passively transferred to the mouth across the

mucosa and through the gingival crevices (for a detailed description of the devices, see reference 57).

Testing oral fluids, urine, fingerstick whole blood, or dried blood spots for antibody to HIV has wide application in the management of patients and epidemiological surveillance, particularly under circumstances in which collecting serum or plasma is less practical. The collection of these specimens does not require laboratory personnel with special training; patients can easily obtain the sample themselves. HIV antibodies in urine, oral fluids, and dried blood spots are stable for extended times at room temperature, and specimens can be mailed or shipped without degradation. Collection of oral fluids or urine also increases compliance and alleviates the fear that patients may experience when having their blood drawn. It reduces the potential danger to the health professional through blood exposure and may benefit more challenging populations whose blood may be difficult to obtain, including children, hemophiliacs, obese people, and the elderly and infirm. The use of oral fluids or urine may permit improved access for the surveillance of intravenous drug users, homeless persons, sex industry workers, and persons in developing countries. Finally, collection of these specimens may afford a greater opportunity to screen for HIV antibodies in POC settings, physicians' and dentists' offices, public health institutions, and community outreach programs.

9.7 Confirmatory and supplemental tests

The Western blot assay has been the principle supplemental test used worldwide to confirm the specificity of positive results obtained from HIV screening immunoassays. Western blot assays are essentially solid-phase immunoassays that use immobilized viral antigens to detect antibodies to specific viral proteins. The major advantage of Western blot assays over the screening immunoassays is that the specific interaction of antibody and antigen can be directly visualized. These assays have the disadvantages, however, of being technically demanding, relatively expensive, and subject to interpretation. A number of Western blot assays have been commercially developed for detecting antibodies to either HIV-1 or HIV-2 (Table 9.5). Only a few have been FDA approved as confirmatory tests. Most Western blot tests are similar to first-generation immunoassays in that viral lysates are used as the source of HIV antigens. Several commercial manufacturers have developed immunoblot assays that utilize recombinant HIV proteins and/or synthetic peptides that are applied in separate lines or bands to nitrocellulose membranes. These assays can differentiate between HIV-1 and HIV-2 infection and are more sensitive and specific than conventional Western blot assays. In the United States, due to

Table 9.5 Confirmatory/supplemental tests for the detection of HIV antibodies

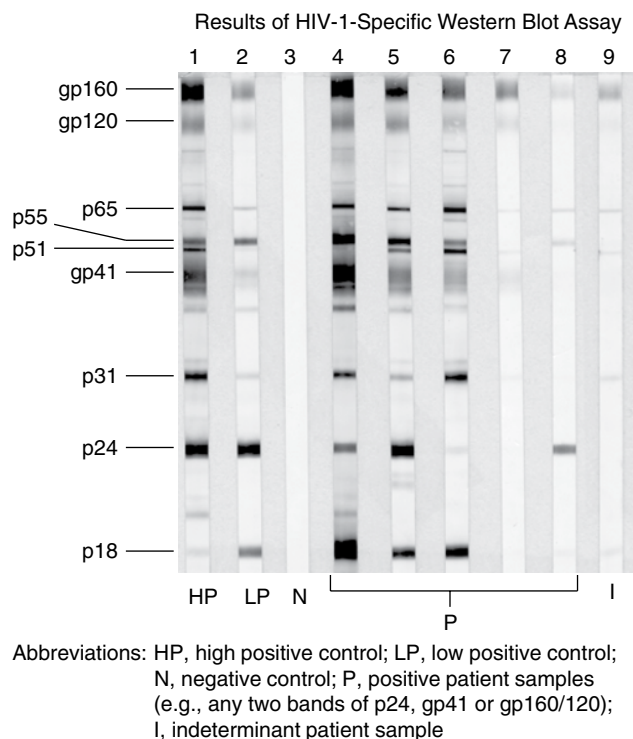
Manufacturer (alphabetical)	Product name	Assay type	Sample	Detection	Antigens	Selected references
Alere	ImmunoComb HIV 12	ID	Serum, plasma	HIV-1/HIV-2	RP + SP	NF
Biokit S.A.	CombFirm					
	Bioblot HIV 1 Plus	WB	Serum, plasma	HIV-1, indicates suspected HIV-2	VL + SP	NF
Bionor	Bionor HIV-1 & 2 ConfirmatoryMPEIA Test		Serum, plasma, whole blood	HIV-1/HIV-2	RP + SP	153
Bio-Rad	Genetic Systems HIV-1 Western Blot	WB	Serum, plasma, DBS	HIV-1	VL	115
	Multispot HIV-1/HIV-2 Rapid Test	FT	Serum, plasma	HIV-1/HIV-2	RP + SP	18, 123, 133
	Geenius HIV 1/2 Supplemental Assay	LF	Serum, plasma, or whole blood	HIV-1/HIV-2	RP + SP	93, 108
DiaSorin (MP Biomedicals)	HIV-1 Blot 1.3	WB	Serum, plasma	HIV-1	VL	NF
	HIV Blot 2.2	WB/LIA	Serum, plasma	HIV-1/HIV-2	VL + SP	NF
	HIV-2 Blot 1.2	WB	Serum, plasma	HIV-2	VL	NF
Fujirebio (Innogenetics)	Inno-Lia HIV/II Score	LIA	Serum, plasma	HIV-1/HIV-2	RP + SP	144, 145
Maxim Biomedical, Inc.	Cambridge Biotech HIV-1 Western Blot	WB	Serum, plasma, urine, oral fluids, DBS	HIV-1	VL	67, 103, 164, 165
OraSure Technologies	OraSure HIV-1 Western Blot	WB	Oral fluids	HIV-1	VL	44
Sanochemia Pharmazeutika	Fluorgonost HIV-1 IFA	IFA	Serum, plasma, DBS	HIV-1	IC	61, 157

ID, immunodot; WB, Western blot; MPEIA, magnetic particle enzyme immunoassay; FT, flow-through device; LF, lateral flow immunochromatographic device; LIA, line immunoassays; IFA, immunofluorescence assay; DBS, dried blood spots; VL, viral lysate; SP, synthetic peptides; RP, recombinant proteins; IC, infected Pall T-cells; NF, none found.

the low prevalence of HIV-2, Western blotting is usually performed for the confirmation of antibodies specific to HIV-1. HIV-2-specific Western blotting is not routinely done on all specimens that are positive by HIV screening immunoassays but is performed only when warranted by the clinical situation.

In the Western blot assay, whole-virus lysates of inactivated and disrupted viral proteins are separated by electrophoresis according to their molecular weight or relative mobility as they migrate through a polyacrylamide gel in the presence of sodium dodecyl sulfate. The resolved protein bands are then transblotted (transferred) to a sheet of nitrocellulose paper. The nitrocellulose paper is then cut into strips that are reacted with serum specimens. If HIV-1-specific antibodies are present in the serum, binding of the antibodies occurs in bands corresponding to the presence of the separated viral proteins. The bands are directly visualized by using an enzyme-labeled anti-human antibody followed by a chromogenic substrate (Figure 9.1).

If results of a repeatedly reactive screening test are confirmed by a positive Western blot, a diagnosis of HIV-1 infection can be made. Specificity for detecting HIV-1 antibodies has been reported to approach 100% with the combined use of an HIV screening immunoassay and a Western blot test. A negative Western blot assay result on a serum that is repeatedly reactive by an HIV immunoassay suggests a false-positive screening result. Specimens producing banding patterns that do not meet

**Figure 9.1** Results of HIV-1-specific Western blot assay.

the criteria for a positive result are classified as indeterminate. Indeterminate results can be observed in both the lysate-based Western blots and the immunoblot assays. Indeterminate HIV-1 Western blots may represent evidence of recent infection in high-risk persons exposed

to HIV-1, infection with HIV-2, or false reactions in persons at low risk for HIV-1 infection. In this situation, it is recommended that the immunoassay and Western blot assay be repeated at intervals over time (at least beyond 6 months). Individuals at low risk for HIV-1 infection with persistently indeterminate HIV-1 Western blots are most likely uninfected [68]. The most common types of indeterminate Western blots in someone who is uninfected include a p24 band only, a p18 band only, p24 and p55 bands, or a p55 band only (e.g., *gag*-derived proteins) in the absence of antibodies to the envelope glycoproteins gp41, gp120, and gp160 [27]. The sensitivity of a Western blot assay is approximately equal to that of the first- and second-generation immunoassay formats but is less than that of the third- and fourth-generation antigen sandwich immunoassays. The Western blot assay should not be used as a screening test, since its specificity is less than that of an immunoassay, with a significant number of indeterminate blots seen in low-risk populations.

An immunofluorescence assay is a very useful and inexpensive alternative to performing Western blot assay for confirmation of HIV-specific antibody responses [61, 157]. Although several IFA kits have been developed and used in various countries over the years, only one (Fluorognost HIV-1 IFA, Sanochemia Pharmazeutika, Vienna, Austria) remains commercially available and has received approval from the FDA for use in the United States. The Fluorognost HIV-1 IFA has also been FDA approved as a screening test in hospital laboratories, medical clinics, physicians' offices, emergency care situations, and blood banks or other settings where immunoassays are not practical or available.

The Fluorognost HIV-1 IFA is based on the specific binding of HIV-1 antibodies in a specimen to HIV-1 antigens expressed on the surface of immortalized human T-cells fixed to glass slides. Specific antibody-antigen complexes are then detected using an antihuman antibody conjugated with fluorescein isothiocyanate. Uninfected cells of the same type are used as negative controls. The stained slides are carefully examined and compared to infected and uninfected cells reacted with positive and negative control sera. A test is considered positive based on the percentage of fluorescent cells (40–60%) and the pattern (cytoplasmic versus peripheral staining) and intensity of fluorescence obtained when read with a fluorescence microscope. The assay offers the advantages of speed and simplicity, and the kit contains all of the necessary reagents, including substrate slides, labeled secondary antibody, wash buffer, mounting fluid, and positive and negative control sera. The major disadvantages of the IFA kit are that it requires a fluorescence microscope and dark-room for examining slides and that extensive training and critical evaluation is needed to accurately read and interpret the test results. In addition, IFA does not distinguish

reactivity to specific HIV proteins, as the Western blot assay allows. Therefore, some sera that may nonspecifically react with infected cells may be incorrectly classified as positive.

Although Western blots and, to a much lesser extent, IFAs are still in use as supplemental assays for the confirmation of positive screening tests for HIV-specific antibodies, they are being largely replaced by rapid immunoassays like the Multispot HIV-1/HIV-2 Rapid Test (Bio-Rad Laboratories, Redmond, WA) and the Geenius HIV 1/2 Supplemental Assay (Bio-Rad Laboratories) that are easy-to-use devices capable of confirming and differentiating antibody responses to either HIV-1 or HIV-2 in serum, plasma, or fingerstick and venipuncture whole blood in less than 30 min [18, 93, 108, 123, 133]. HIV-1 Western blots are labor and time intensive, may produce indeterminate results and delay reporting, and may be misinterpreted as positive in patients with HIV-2 infection due to antigenic cross-reactivity. Use of rapid assays in the newly recommended testing algorithm substantially decreases the time required to confirm an HIV infection and aids in the resolution of indeterminate Western blot results and correct identification of HIV-2 infections.

9.8 Serological testing of neonates

The antibody tests currently on the market are not useful for the diagnosis of HIV in infants less than 18 months of age born to HIV-infected mothers. These newborns possess passively transmitted maternal IgG antibodies that may persist for up to 15–18 months (median of 10–12 months) after birth. As such, a positive HIV serology in this population only indicates exposure and possible infection. Additional testing is required to determine if such newborns are truly infected. Evaluation of the patient's clinical status and the results of sequential antibody tests performed beyond 18 months of age can be diagnostic, although results from this approach occur too late for decisions regarding specific antiretroviral therapy or therapy for opportunistic infections. Children who remain antibody positive (seroconverter) beyond 18 months are considered to be infected, while children who remain asymptomatic and become antibody negative (seroreverter) are not infected. Rare instances of asymptomatic infants who have seroreverted and have positive results from molecular assays for viral RNA or DNA or who subsequently produced antibodies to HIV have been reported. Moreover, some children have hypogammaglobulinemia and remain seronegative despite clinical symptoms suggesting HIV infection. Detection of HIV-specific IgM and IgA antibodies may be useful for diagnosis in the newborn period, but such assays are not readily available and are generally less sensitive and specific than other methods during the first 3 months of

life. Although IgM and IgA assays are appealing because of the relatively simple technology, current assays have problems with false-positive results, and IgM is not always produced at detectable levels in HIV-infected newborns. IgA persists for a longer time than IgM in infants but can be difficult to detect before 3–6 months of age, making this test less useful for rapid diagnosis in the newborn. Early diagnosis of HIV infection in neonates requires direct detection of the virus using p24 antigen assays and molecular assays for HIV DNA or RNA.

9.9 p24 Antigen detection

A variety of commercial assays are available for the detection of HIV-1 p24 antigen (Table 9.6). The kits are simple and inexpensive to use, and are relatively sensitive and specific. In a typical p24 antigen assay, a monoclonal antibody bound to a solid support is used to capture HIV-1 p24 antigen from the sample. A second antibody specific to p24 antigen is then added and, if p24 antigen is present in the sample, the antibody will bind to the antigen captured on the solid phase to form an antibody–antigen–antibody sandwich. The antibody–antigen–antibody complexes are then detected by incubating with a third antibody labeled with an enzyme followed by a colorless substrate that is cleaved by the enzyme to give off a color reaction. The intensity of the color formed is proportional to the amount of HIV-1 p24 antigen present in the sample. Some manufacturers directly conjugate the second antibody with an enzyme label and do not use the third antibody as part of the detection system. Similar to the HIV antibody assays described above, patient specimens with initially reactive results for HIV-1 p24 antigen must be retested in duplicate using the same EIA. Repeatedly reactive specimens must be further tested to confirm the

results. This is accomplished by using a neutralization assay in which a neutralizing antibody specific to HIV-1 p24 antigen is incubated with the specimen and binds the p24 antigen in immune complexes, thus preventing its detection upon performance of the test.

Products that detect HIV-1 p24 antigen may be useful for the early diagnosis of HIV in persons at risk, as p24 antigen levels are high early in infected individuals, usually before antibodies to HIV can be detected. HIV-1 p24 antigen can also be detected during the later stages of illness when increasing failure of the immune system and increasing levels of viral replication are observed. Other uses for p24 antigen assays include monitoring the effect of antiviral therapy, assessing the prognosis for disease progression, diagnosis of HIV infection in infants born to HIV-infected mothers, and in research for the detection of HIV growth in viral culture. It has been observed that HIV infection is detectable by p24 antigen tests approximately 6 days earlier than with antibody tests [16, 37]. The window period is estimated to be 22 days for the detection of HIV-specific antibody using current EIAs and approximately 16 days for tests that detect p24 antigen. The detection of p24 antigen in newborn infants has been problematic. Only low levels of circulating p24 antigen are present in the sera of newborns during the first months of life, and excess maternal HIV-specific antibodies complex to any free p24 antigen, rendering it undetectable in the antigen assay. Similarly, p24 antigen is only detected in a minority of asymptomatic HIV-infected adults due mostly to immune complex formation. The detection of HIV p24 antigen in infants and asymptomatic HIV-infected individuals can be enhanced by pretreatment of serum with acid, base, or heat to dissociate immune complexes. Appropriate reagents are supplied with

Table 9.6 Enzyme immunoassays for the detection of HIV-1 p24 antigen

Manufacturer (alphabetical)	Product name	Sample	Test format	Qualitative/quantitative results	Confirmatory/ICD reagents	Selected references
Advanced Bioscience Laboratories (ABLinc)	HIV-1 p24 Antigen Capture Assay	Viral culture supernatants	Microplate sandwich EIA	Y/Y	N/N	NF
bioMérieux	VIDAS HIV p24 II	Serum, plasma	Automated SPR sandwich ELFA	Y/Y	Y/N	40
Fujirebio (Innogenetics)	Innotest HIV Antigen mAb	Serum, plasma, viral culture supernatants	Microplate sandwich EIA	Y/Y	Y/Y	40, 41
PerkinElmer	Alliance HIV-1 p24 Antigen ELISA	Serum, plasma, viral culture supernatants, DBS	Microplate sandwich EIA	Y/Y	Y/Y	NF
Quanterix Corporation	Simoa p24 Antigen	Serum, plasma	Microplate paramagnetic bead-based capture and single molecule fluorescence array technology	Y/Y	Y/N	NF
ZeptoMetrix	Retro-Tek HIV-1 p24 Antigen ELISA	Serum, plasma, viral culture supernatants	Microplate sandwich EIA	Y/Y	Y/Y	64

ICD, immune complex dissociation; EIA, enzyme immunoassay; SPR, solid phase receptacle; ELFA, enzyme linked fluorescent assay; ELISA, enzyme linked immunosorbent assay; DBS, dried blood spots; Y, Yes; N, No; NF, none found.

many of the current commercial p24 antigen assays to allow for immune complex dissociation (ICD) before completion of the testing (Table 9.6); ICD p24 antigen assays are sensitive and specific for the diagnosis of HIV in infants, although they are often negative in infected infants younger than 1 month of age. Of additional concern for the use of p24 antigen assays is the fact that, even with dissociation of immune complexes, these assays are not sufficiently sensitive for testing asymptomatic individuals at risk of HIV-1 infection or for monitoring those already infected following seroconversion. As a result, assays that detect p24 antigen alone have limited use in the United States. They are mainly used in research settings or in resource-poor areas of the world due to their low cost and easy of performance, and have been largely replaced by more sensitive molecular assays for diagnosis of HIV-1 and by quantitative molecular RNA assays for monitoring prognosis and antiviral therapy.

More recently, the Northwestern Global Health Foundation (Evanston, IL) has developed a rapid and simple ultrasensitive p24 antigen assay. The intended use of the Lynx p24 Antigen Assay is for the detection of p24 antigen in HIV-infected infants at the POC in resource-limited settings. The test uses a lateral flow strip and separate preanalytical devices to separate plasma from approximately 80 μ L of heelstick whole blood and disrupt immune complexes that have formed. The results are read following a 30-min incubation and the total turnaround time for the assay is 40 min. At the time of this writing, the assay is not yet available on the commercial market.

9.10 Qualitative molecular detection

The polymerase chain reaction (PCR) has proved to be a rapid and effective method for the early detection of HIV infection in virtually all HIV-infected individuals. The method can be used to detect integrated HIV proviral DNA obtained from purified peripheral blood mononuclear cells (PBMCs) or whole blood or HIV RNA in plasma or serum after the RNA is converted to cDNA by reverse transcription [25, 66, 183]. Over the years, the use of qualitative PCR for the detection of HIV DNA from purified PBMCs or whole blood has been the primary test for diagnosis of HIV infection in children under 18 months of age born to HIV-infected mothers, and has been used for the resolution of indeterminate serologies and testing high-risk individuals for acute HIV infection prior to seroconversion. Conserved sequences in the *gag* or *pol* genes are routinely used in DNA PCR assays for detecting the proviral HIV-1 DNA. The assay can be performed on small quantities of whole blood or dried blood spots, and low amounts of target nucleic acid can be detected. The sensitivity and specificity of DNA PCR are in the high 90th percentile and can approach 100%

when procedures and conditions for testing are carefully monitored and controlled [28, 67]. The sensitivity of PCR in the first 6 months of life depends on the virus load and the timing of infection and has been estimated to be 55% at birth, > 90% by 2–4 weeks of age, and 100% at 3–6 months. Infants infected *in utero* may be identified at birth or in the first week, while several weeks or months may pass before infection acquired intrapartum can be detected. Despite the importance of using DNA PCR to detect HIV in certain clinical situations, the availability of commercial qualitative PCR assays is still limited. Roche Molecular Diagnostics (Indianapolis, IN) has recently developed the fully automated COBAS AmpliPrep/COBAS TaqMan HIV-1 Qualitative Test for the detection of HIV-1 proviral DNA and RNA in plasma, anticoagulated fresh whole blood, and dried blood spots [20, 95, 180]. This assay is for research use only and has not been FDA approved as a diagnostic test at the time of this writing.

Proviral DNA-based molecular assays are now being replaced by newer HIV-1 RNA assays that have been developed and commercialized (Table 9.7). The APTIMA HIV-1 RNA qualitative assay (Hologic Gen-Probe Inc., San Diego, CA) is the first nucleic acid amplification assay approved by the FDA for the diagnosis of HIV-1 infection in plasma or serum. The assay is based on transcription-mediated amplification and targets the 5' long terminal repeat and the *pol* gene regions of the HIV-1 genome. The manufacturer reports detection rates of 98.5% for 30 RNA copies/mL, 82.6% for 10 copies/mL, 42.5% for 3 copies/mL, and 19.4% for 1 copy/mL, and the assay is designed to detect all major groups (e.g., M, N, and O) and subtypes of HIV-1. The test is intended for use in clinical situations in which HIV-1 antibodies may not yet be present, including the diagnosis of acute or primary infection in symptomatic patients and routine screening for defined patient populations, and in settings in which the presence of antibodies may not reflect disease status, such as screening of newborns of HIV-infected mothers [38, 113, 117, 154]. It is also used for detection of HIV-1 in alternative specimen types [113, 116] and in support of the new testing algorithm described above [129]. Abbott Molecular (Des Plaines, IL) also has recently developed a qualitative real-time PCR assay for the detection of HIV-1 RNA from human plasma and dried blood spots [20, 60, 113]. Qualitative RNA-based assays are considered to be comparable, if not slightly better, in detecting HIV infection at birth than corresponding assays that detect proviral DNA in newborns, with the exception that a negative HIV-1 RNA test may occur if either mother or infant are receiving highly active antiretroviral therapy at the time of testing. Also, HIV-1 RNA qualitative assays are sensitive for the detection of all major HIV-1 genetic variants, while the sensitivity of HIV DNA-based assays may vary for non-clade-B strains of HIV.

Table 9.7 Molecular assays for the qualitative detection of HIV

Manufacturer (alphabetical)	Product name	Sample	Amplification method	Gene region	Analytical sensitivity (copies/mL)	Intended use	Selected references
Abbott Molecular Grifols Diagnostic Solutions, Inc.	RealTime HIV-1 Qualitative	Plasma, DBS	RT-PCR (target)	<i>pol</i> integrase	110 (plasma) 2500 (DBS)	Diagnosis	20, 60, 113
	Procleix Ultrio Assay	Plasma, serum	TMA	<i>pol</i> and LTR	28.8 (multiplex) 32.2 (discriminatory)	Blood screening	80, 94, 101
	Procleix Ultrio Plus Assay	Plasma, serum	TMA	<i>pol</i> and LTR	16.6 (multiplex) 11.8 (discriminatory)	Blood screening	92
	Procleix Ultrio Elite Assay	Plasma, serum	TMA	<i>pol</i> and LTR	Compatible with Ultrio Plus Assay	Blood screening	NF
Hologic GenProbe, Inc.	APTIMA HIV-1 RNA Qualitative Assay	Plasma, serum	TMA (target)	<i>pol</i> and LTR	30 (98%), 10 (82.6%) 3 (42.5%), 1 (19.4%)	Diagnosis	38, 113, 116, 117, 129, 154
Roche Diagnostics	COBAS AmpliPrep/COBAS TaqMan HIV-1 Qualitative Test	Plasma, DBS	RT-PCR (target)	<i>gag</i> (v1.0) <i>gag</i> and LTR (v2.0)	500 (plasma, v1.0) 1100 (DBS, v1.0) 20 (Plasma, v2.0) 300 (DBS, 2.0)	Diagnosis	20, 95, 181
	COBAS TaqScreen MPX Test	Plasma	RT-PCR (target)	<i>gag</i> (v1.0)	49 IU/ml (HIV-1, group M) 89 (HIV-1, group O) 59 (HIV-2)	Blood screening	94, 121, 143, 178
	COBAS TaqScreen MPX Test, v2.0	Plasma	RT-PCR (target)	<i>gag</i> and LTR (v2.0)	46.2 IU/ml (HIV-1, group M) 18.3 (HIV-1, group O) 56.2 (HIV-2)	Blood screening	NF

DBS, dried blood spots; RT-PCR, reverse transcriptase-polymerase chain reaction; *pol*, polymerase; TMA, transcription-mediated amplification; LTR, long terminal repeat; IU, international units.

The American Academy of Pediatrics and Panel on Antiretroviral Therapy and Medical Management of HIV-Infected Children recommends that infants born to HIV-infected women be tested by either HIV-1 DNA or HIV-1 RNA nucleic acid tests [5, 125]. In infants with known perinatal HIV exposure, testing is recommended at ages 14–21 days, 1–2 months, and 4–6 months. Testing at birth should be considered for infants at high risk of HIV infection, and should be considered 2–4 weeks after stopping antiretroviral prophylaxis for infants receiving combination antiretroviral drugs and having a previously negative test result while on prophylaxis [15]. A positive RNA or DNA test result in an infant should be confirmed as soon as possible by repeating the test on a second specimen. Exclusion of HIV infection in infants is based on two or more negative molecular test results, with one obtained at age ≥ 1 month and one at age ≥ 4 months. Additional monitoring is recommended for infants that are breastfed since HIV can be transmitted through breast milk.

Lastly, multiplexed qualitative molecular assays have been developed by Hologic Gen-Probe, Inc. (Procleix Ultrio Assay, Procleix Ultrio Plus Assay, and Procleix Ultrio Elite Assay) and Roche Molecular (COBAS TaqScreen MPX Test, and COBAS TaqScreen MPX Test, v2.0) and are commercially available for the simultaneous detection of HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV) in plasma from blood, organ, and tissue donors [80, 92, 94, 101, 121, 143, 178]. These tests are intended to be used for screening individual blood

donors, including donors of whole blood and blood components, source plasma and other living donors, and to screen individual organ and tissue donors when specimens are obtained while the donor's heart is still beating, and in blood specimens from cadaveric (nonheart beating) organ and tissue donors. The assays have also been designed for testing of pooled samples. The Procleix Ultrio and Ultrio Plus Assays use transcription-mediated amplification to simultaneously detect multiple groups and subtypes of HIV-1 RNA as well as HBV DNA and HCV RNA; the Procleix Ultrio Elite Assay also detects HIV-2 along with the other viruses. For these assays, separate discriminatory tests are performed for full identification of individual viral targets. Similar to the Procleix Ultrio Elite Assay, the two COBAS TaqScreen MPX Tests from Roche Molecular detect various groups and subtypes of HIV-1 as well as HIV-2, HBV, and HCV from individual or pooled samples. The assays involve a multiplex, real-time PCR and version 2.0 of the assay has improved sensitivity compared to the original version of the test and simultaneously detects and differentiates, thus removing the need for discriminatory testing for identification of individual targets.

9.11 Quantification of HIV RNA

Our knowledge of HIV-1 infection has been expanded by using laboratory parameters that correlate with the stage of disease progression and the degree of viral replication.

However, methods used for monitoring HIV-1 infection, such as CD4+ cell counts and levels of p24 antigen, neopterin, β_2 -microglobulin, and interferon, involve surrogate markers that do not always correlate with the disease state or the antiviral activity of therapeutic regimens. The development of molecular assays to quantitate the levels of HIV RNA (e.g., viral load) in infected patients has provided one of the most valuable tools to assess the progression of HIV disease, monitor the impact of antiviral therapy and guide treatment decisions, predict treatment failure and the emergence of drug-resistant viruses, and facilitate our understanding of the natural history and pathogenesis of this virus. The quantitation of high levels of HIV-1 RNA has been related to vertical transmission of the virus from mother to fetus, a more rapid progression to AIDS and death in both adult and pediatric patients, and the emergence of drug resistance during prolonged antiretroviral therapy. Over the years, molecular methods such as quantitative competitive PCR, conventional endpoint PCR, nucleic acid sequence-based amplification, branched DNA (bDNA) technology, transcription-mediated amplification, and, most recently, real-time PCR have been developed for the accurate quantification of HIV-1 RNA in plasma samples from infected individuals (see reference 152 for a review). Real-time PCR assays have led to significant advancements in the performance of quantitative HIV-1 assays and offer the distinct advantages of being extremely sensitive with reliable quantification of low levels of virus and having high precision and reproducibility over a broad dynamic range. Also, several of the assays have been placed on semi- or fully automated systems that significantly decrease the turnaround time and chances for human error and carry-over contamination, while enhancing the workflow and increasing throughput. The different assays for measuring viral loads vary with respect to the principles of the methods, the lower limit of detection, the dynamic range, the accuracy in quantifying the various genetic variants of HIV-1, throughput, methodology, and cost. A comparative summary of the important characteristics of the different commercial molecular assays is provided in Table 9.8. Such tests may influence the choice of initial therapy and tailor treatment regimens to reduce the risk of viral mutation and subsequent viral resistance. Sustained decreases in patient viral load during treatment suggests an appropriate antiviral effect, while a return of the viral load to pretreatment levels is indicative of drug failure and the possible need for alternative therapy. Measurement of plasma HIV-1 RNA levels or viral loads is the strongest predictor of clinical outcome and the predominant test used by clinicians to monitor HIV-infected patients on antiretroviral therapy. The monitoring of the HIV-1 viral load is essential for making appropriate

patient management decisions regarding the initiation and alteration of therapy.

There has been tremendous interest in recent years in the development of more rapid and less-sophisticated molecular assays to monitor HIV viral loads at the POC in resource-limited countries to facilitate patient care and management in areas that lack the infrastructure and access to the more technologically demanding, laboratory-based quantitative molecular systems described above. To this end, there are a number of near-patient platforms in development that have small footprints and are portable, easy to use, and robust under the most extreme of environmental conditions (see reference 148 for a review). They have been designed to be low-cost and have few moving parts and require minimal training, and are capable of operating on a battery when necessary. Several instruments and point-of-care HIV viral load assays of high quality and performance are well along in the development process and show considerable promise for sample-to-result reporting, including the Simple Amplification Based Assay (SAMBRA) from the Diagnostics Development Unit at the University of Cambridge, which will be manufactured by Diagnostics for the Real World, Ltd (Sunnyvale, CA), the q HIV 1/2 system from Alere, Inc. (Waltham, MA), the Roche (IQuum, Inc., Marlborough, MA) COBAS laboratory-in-a-tube (Liat) platform, and the EOSCAPE HIV Rapid RNA Assay System from Wave 80 Biosciences (San Francisco, CA). Many other point-of-care HIV viral load platforms are in the early stages of development for future use.

Lastly, the ExaVir Load assay developed by CaviDi AB (Uppsala, Sweden) provides a low-cost alternative for performing viral load measurements and is particularly useful in resource-poor settings where dedicated instruments, technical expertise, and sophisticated laboratory facilities are limited and make it less practical to perform molecular-based viral load assays [50, 51, 82, 139, 155]. The ExaVir Load assay is based on an EIA microwell format and quantifies the activity of the enzyme reverse transcriptase (RT) as a marker of retroviral replication, allowing for the detection of all types and subtypes of HIV without modification of the test. A serially diluted standard, with a known concentration of recombinant HIV-1 RT activity, is used to quantify the RT in the tested samples. In the procedure, virus particles are separated from other components of the plasma sample, the virions are lysed, and the resulting lysates are added to an RT assay solution that contains an RNA template, a primer, and an RT substrate. If the lysate contains any RT, the enzyme will synthesize a DNA strand, which is then detected by the addition of an alkaline phosphatase-labeled antibody followed by a colorimetric substrate. The RT activity is

Table 9.8 Comparison of commercial molecular quantitative HIV-1 RNA assays

Characteristics		Manufacturer (alphabetical)						
	Abbott Molecular	bioMérieux, Inc.	Hologic Gen-Probe	Roche Diagnostics		Siemens Healthcare Diagnostics		
Kit name	RealTime TaqMan HIV-1	NuclISENS EasyQ HIV-1 v2.0	APTIMA HIV-1 Quant Dx	Amplicor HIV-1 Monitor, v1.5	COBAS AmpliPrep/ COBAS TaqMan HIV-1 Test v1.0, v2.0	COBAS TaqMan HIV-1 Test, v2.0 for use with High Pure System	VERSANT HIV-1 RNA 3.0 Assay (bDNA)	VERSANT HIV-1 RNA 1.0 Assay (kPCR)
Amplification method	RT-PCR (target)	NASBA (target)	TMA (target)	RT-PCR (target)	RT-PCR (target)	RT-PCR (target)	bDNA (signal)	kPCR (target)
Gene region	<i>pol</i> integrase	<i>gag</i>	<i>pol</i> and LTR	<i>gag</i>	<i>gag</i> (v1.0)	<i>gag</i> and LTR	<i>pol</i>	<i>pol</i> (integrase)
Sample	Plasma (EDTA, ACD-A)	Plasma (EDTA), whole blood (EDTA-DBS)	Plasma (EDTA, ACD-A)	Plasma (EDTA, ACD-A)	Plasma (EDTA)	Plasma (EDTA)	EDTA	EDTA, ACD-A
Sample input (μL)	1000, 600, 500, 200	1000, 500, 100	500	200 (standard) 500 (ultrasensitive)	1000	1000	1000	500
Kit size (batch size)	96 tests (≥24)	48 tests (≥8)	100 tests (random access)	24 tests (≥12)	48 (≥24) 96 tests (≥24)	48 (≥24)	96 tests (≥18)	96 tests (≥24)
Turn-around time	1 or 2 days	1 or 2 days	1 or 2 days	1 or 2 days	1 or 2 days	1 or 2 days	2 days	1 or 2 days
Standards/controls	1 internal control 2 external calibrators 2 external controls	1 internal calibrator	1 internal calibrator 3 external controls	1 internal QS 3 external controls	1 internal QS 3 external controls	1 internal QS 3 external controls	6 external standards 3 external controls	1 internal control 2 calibrators 3 external controls
Upper limit in copies/mL	1.0 × 10 ⁷	1.0 × 10 ⁷	1.0 × 10 ⁷	7.5 × 10 ⁵ (standard) 1.0 × 10 ⁵ (ultrasensitive)	1.0 × 10 ⁷ (v1.0 and v2.0)	1.0 × 10 ⁷	5 × 10 ⁵	1.1 × 10 ⁷
Lower limit in copies/mL (sample input volumes)	40 (1000, 600 μL); 75 (500 μL); 150 (200 μL)	Plasma: 10 (1000 μL), 50 (500 μL), 292 (100 μL); DBS: 802 (100 μL)	30	400 (standard) 50 (ultrasensitive)	48 (v1.0) 20 (v2.0)	34	75	37
HIV-1 groups and subtypes	All group M subtypes, group O, group N, and recombinants	All group M subtypes, and some recombinants	All group M subtypes, group O, group N, and recombinants	All group M subtypes	All group M subtypes (v1.0); all group M subtypes and group O (v2.0)	All group M subtypes and group O (v2.0)	All group M subtypes and group O	All group M subtypes and group O
Selected references	14, 48, 76, 146, 147, 150, 151, 158, 159	22, 48, 110, 147, 170, 180	NF	6, 14, 43, 65, 110, 158, 159	14, 22, 76, 110, 127, 147, 150, 161, 179	NF	6, 14, 22, 33, 43, 46, 158, 159	140, 163

RT-PCR, reverse transcriptase-polymerase chain reaction; bDNA, branched DNA; NASBA, nucleic acid sequence-based amplification; TMA, transcription-mediated amplification; EDTA, ethylenediaminetetraacetic acid; ACD, acid citrate dextrose; DBS, dried blood spots; QS, quantitation standard; NF, none found.

measured in femtograms of RT/mL and the values are converted to RNA copies equivalents/mL; the measureable range for the assay is 200 to 6×10^5 RNA copies equivalents/mL. A total of 30 samples can be analyzed from one kit and up to 60 tests can be performed with a turnaround time of 48 h. The assay requires 1.0 mL of EDTA-anticoagulated plasma, has a hands-on time of 5 h, and is CE marked for *in vitro* diagnostic use.

9.12 Phenotypic and genotypic assays for drug resistance

A variety of antiretroviral drugs have been developed and approved for the treatment of HIV-1 infection. They are divided into multiple classes based on their mode of inhibition of viral replication and include the nucleoside and non-nucleoside viral RNA-dependent DNA polymerase (reverse transcriptase) inhibitors, the viral protease inhibitors, fusion inhibitors, entry inhibitors, and integrase inhibitors. Therapy with these antiretroviral agents is beneficial to prolong survival, to reduce the incidence and severity of opportunistic infections in patients with advanced HIV-1 disease, and to delay disease progression in asymptomatic HIV-1-infected patients.

The development of HIV resistance to selected antiretroviral drugs has been well documented in patients receiving antiretroviral therapy. The emergence of HIV antiviral-drug resistance is thought to be a function of the

length of therapy, stage of disease or virus burden, and a high spontaneous mutation rate in the viral genome during active replication of the virus. Prolonged therapy and advancing disease increase the likelihood of developing viral resistance. Both *in vitro* and *in vivo* resistance to various antiretroviral drugs is due to the accumulation of specific mutations in the coding regions of the viral genome leading to single amino acid substitutions in the viral-protein targets. The extent of resistance appears to be correlated with the number of mutations, although individual mutations can be interactive, causing changes in the resistance or susceptibility of HIV isolates.

The emergence of antiviral-drug resistance has led to a clinical need for *in vitro* antiviral susceptibility testing. Laboratory confirmation of drug resistance is essential for defining the mechanisms of antiviral resistance, for determining the frequency with which drug-resistant viral mutants emerge in clinical practice, to help guide initial antiretroviral therapy, to predict treatment failure and identify cross-resistance to other antiviral agents, for the institution of the most appropriate alternative therapy, and when evaluating new antiviral agents [53, 56]. To this end, both phenotypic and genotypic antiviral susceptibility assays have been developed for the determination of HIV-1 drug resistance (Table 9.9). Genotypic drug resistance testing should be performed in all treatment naïve patients at the time of HIV diagnosis and before initiation of therapy, and in all patients before changing therapy because of treatment

Table 9.9 Commercial phenotypic and genotypic assays for detection of HIV-1 resistance to antiretroviral drugs

Assay type	Manufacturer (alphabetical)	Product name	Technology	Drug susceptibility measurement(s)	Selected references
Phenotypic	Monogram Biosciences	PhenoSense GT	Culture-based, hybrid virus assay combined with gene sequencing	Combined phenotype and genotype; protease and RT inhibitors; viral loads ≥ 500 copies/mL	84, 128
		PhenoSense	Culture-based, hybrid virus assay	Protease and RT inhibitors; viral loads ≥ 500 copies/mL	137, 174, 185
		PhenoSense Integrase	Culture-based, hybrid virus assay	Integrase inhibitors; viral loads ≥ 500 copies/mL	45
		PhenoSense Entry Trofile	Culture-based, hybrid virus assay	Entry inhibitors; viral loads ≥ 1000 copies/mL	NF
		Trofile DNA	Viral RNA co-receptor tropism assay Proviral DNA co-receptor tropism assay	CCR5 antagonist; viral loads ≥ 1000 copies/mL CCR5 antagonist; undetectable viral loads	13, 88, 177 74, 131
Genotypic	Abbott Molecular Monogram Biosciences	ViroSeq HIV-1	Electrophoretic, sequencing-based assay	Protease and RT mutations; viral loads ≥ 2000 copies/mL	23, 35, 72, 162
		GenoSure PRLme	Electrophoretic, sequencing-based assay	Protease, RT, and integrase inhibitors; viral loads ≥ 500 copies/mL	NF
		GenoSure MG	Electrophoretic, sequencing-based assay	Protease and RT inhibitors; viral loads ≥ 500 copies/mL	NF
	Siemens Healthcare Diagnostics	Trugene HIV-1	Electrophoretic, sequencing-based assay	Protease and RT mutations; viral loads ≥ 1000 copies/mL	42, 49, 52, 81

failure. Phenotypic assays should be used in addition to genotypic assays for complex drug resistance patterns that may arise because of successive treatment failures.

9.12.1 Phenotypic assays

Conventional phenotypic assays for susceptibility testing of HIV-1 directly measure the amount of drug needed to inhibit or suppress viral replication. Historically, the susceptibility of a particular virus isolate was traditionally measured in cell culture using primary PBMCs from HIV-1-negative donors [69]. Virus replication in the presence or absence of an antiretroviral drug was monitored by quantitating the HIV-1 p24 antigen by EIA. In this system, the susceptibility of a virus isolate is expressed as the drug concentration required to inhibit virus replication by 50% (IC_{50}) relative to a no-drug control. The results are compared to the IC_{50} values obtained from resistant and susceptible control strains that are tested with each batch of patient isolates. The disadvantages of this assay are many and include the fact that it is costly, labor-intensive, and highly variable and has a turnaround time of a number of weeks. The procedure also requires that infected PBMCs from the patient be cocultivated with donor PBMCs to produce a stock of the clinical isolate to be tested. This may lead to the selection of subpopulations of the original HIV-1 isolate that are not representative of the total virus population. Comparison of phenotypic *in vitro* susceptibility data among different laboratories is also valid only if the same assay, host cell, viral inoculum, and range of drug concentrations are used. The definition of a sensitive or resistant isolate may differ for each assay system and for different laboratories performing the same assay. More recently, recombinant-virus phenotypic susceptibility assays have been developed for HIV-1 testing [31, 54, 78, 114] and are viewed as major advances in phenotypic resistance testing. In these assays, total RNA is extracted from a patient's plasma sample, and the reverse transcriptase, protease, and integrase genes are amplified by PCR. The amplified viral sequences are then inserted into an HIV-1 molecular vector that has these sequences deleted but contains the remainder of the HIV-1 genome. The chimeric virus that is generated can replicate efficiently in CD4+ T lymphocytes, eliminating the need for initial isolation of the virus in primary coculture and the subsequent development of high-titer virus stocks in order to complete the phenotypic testing. Since the newly formed chimeric virus contains the reverse transcriptase, protease, and integrase gene sequences from the wild-type virus, the recombinant virus can be used in a phenotypic resistance assay to directly measure the susceptibility of the patient's isolate to virtually any antiretroviral drug. The recombinant-virus assays are also highly automated and improve both the throughput and reproducibility of the traditional phenotypic assay.

Recombinant-virus phenotypic assays are available on a fee-for-service basis through the reference laboratory Monogram Biosciences-LabCorp (San Francisco, CA). The PhenoSense assay directly measures the drug susceptibility of recombinant virus to RT and protease inhibitors [137, 174, 185], while the PhenoSense Integrase and PhenoSense Entry assays measure drug susceptibility to integrase inhibitors [45] and entry inhibitors, respectively. The PhenoSense GT directly measures the drug susceptibility of recombinant virus to RT and protease inhibitors combined with a genotypic assessment of the drug susceptibility by sequencing [84, 128]. The same laboratory offers two phenotypic coreceptor tropism assays (Trofile and TrofileDNA) in patients with detectable viral loads ≥ 1000 copies/mL (Trofile) and for patients with undetectable viral loads (TrofileDNA) to determine if a patient's virus is likely to respond to CCR5 antagonist therapy [13, 74, 88, 131, 177].

9.12.2 Genotypic assays

Genotypic assays have also been developed and used routinely to identify specific gene mutations that may lead to resistance when patients are treated with antiretroviral drugs (Table 9.9) [31]. These assays normally involve using PCR for amplification of the reverse transcriptase and protease genes of HIV-1 (the two most common targets for antiretroviral drugs) and direct sequencing of the amplified products. Direct sequencing is considered to be the "gold standard" for detecting HIV-1 drug resistance mutations. The method is based on a modification of the Sanger dideoxynucleotide chain terminator chemistry, using specific sequencing primers to produce DNA amplicons labeled with four different fluorescent dyes, one specific for each nucleotide. The DNA is then separated by electrophoresis, and an argon laser excites the fluorescence-labeled nucleotides. The nucleotides are identified one by one as they pass through the laser detector. Specialized software is used to analyze the sequence data and is displayed graphically as an electropherogram or a linear string of one-letter nucleotide bases or codes. To identify the presence of drug resistance mutations, the generated base sequences are then compared to a genomic database containing a consensus sequence of wild-type HIV-1 and sequence variations of well-characterized resistance mutations that correlate with phenotypic drug resistance. Two commercial FDA-approved kits and accompanying instrumentation have been developed and evaluated for PCR amplification and automated, high-speed sequencing to detect HIV-1 resistance mutations. These include the Abbott Molecular (Des Plaines, IL) ViroSeg HIV-1 kit and analyzers [23, 35, 72, 162] and the Siemens Healthcare Diagnostics (Tarrytown, NY) TruGene HIV-1 kit and OpenGene System [42, 49, 52, 81]. These assays only detect mutations in the RT and

Table 9.10 Advantages and disadvantages of HIV-1 phenotypic and genotypic antiviral resistance assays

Assay type	Advantages	Disadvantages
Phenotypic	<p>Direct measure of viral susceptibility to any given drug</p> <p>Identifies drug-resistant viruses with novel mutations to given drugs</p> <p>Measures amount of drug needed to inhibit viral replication</p> <p>Can test samples with viral loads of 500 RNA copies/ml or more and can evaluate non-B clade HIV-1 strains</p>	<p>Assays are complex, costly, highly variable, and labor-intensive</p> <p>Tests for the effect of one drug at a time. Difficult to test effect of drug combinations</p> <p>Turn-around time of 2–6 weeks, depending on assay</p> <p>May not detect minority drug-resistant species at levels < 10–30% of total viral population or minority species with low phenotypic resistance</p> <p>Clinical relevance of testing not well established for each drug</p> <p>Testing likely to remain centralized in large, highly specialized reference laboratories</p> <p>Thresholds for defining drug susceptibility are arbitrary and not standardized</p>
Genotypic	<p>Detects actual viral mutations associated with drug resistance</p> <p>Turn-around time of 1–2 days</p> <p>Less costly, higher throughput and more reproducible</p> <p>Clinical utility is more widely established</p> <p>Less technically demanding and complex</p> <p>May allow for earlier detection of emergence of drug resistance</p> <p>Can determine effect of multiple mutations on resistance to one drug or combinations of drug</p>	<p>Indirect measure of viral susceptibility to drug</p> <p>Only detects known drug-resistant mutations; interpretation requires prior knowledge of mutational changes</p> <p>Results may be confounded by presence of mutations that have no bearing on drug resistance</p> <p>May not detect minority drug-resistant species at levels < 10–30% of total viral population or minority species with low phenotypic resistance</p> <p>Reproducible results are not achieved with samples below 500–2000 RNA copies/mL</p> <p>Complexity of assays still make them less than routine for most clinical laboratories</p>

protease genes associated with drug resistance; separate assays to detect mutations associated with resistance to integrase, fusion, and CCR5 inhibitors are available only from Monogram Biosciences-LabCorp reference laboratory.

9.12.3 Summary

A compilation of the advantages and disadvantages of HIV-1 phenotypic and genotypic antiviral resistance assays is summarized in Table 9.10. Phenotypic assays offer the advantage of being a direct measure of viral susceptibility to any antiretroviral drug and can provide data on the concentration of drug needed to inhibit viral replication. Genotypic assays offer the distinct advantages of speed and efficiency in analyzing large numbers of virus isolates and may allow earlier detection of the emergence of drug resistance than phenotypic assays. The clinical relevance of genotypic testing has also been established, while data on the usefulness of phenotypic assays is limited. Current genotypic assays, however, detect only known drug-resistant mutations, and phenotypic assays are still required to identify drug-resistant viruses with novel mutations for antiviral resistance. Neither assay

may detect mutants present at low frequency nor distinguish minority species with low phenotypic resistance. In general, phenotypic assays are more cumbersome and technically demanding than genotypic assays, and their complexity prohibits their use by most clinical laboratories. The availability of commercial kits and automated instrumentation for genotypic assays make them more accessible and desirable to use. The performance and accuracy of genotypic assays, however, still depend on the use of highly skilled medical technologists and scientists, accurate interpretation of the results obtained, and stringent quality management of the reagents and protocols.

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Chlamydia

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10.1 Introduction

Chlamydia trachomatis genital infection causes the most common bacterial sexually transmitted infection (STI) in the world, and chlamydial infection of the eyes causes trachoma, the leading cause of preventable blindness. The high prevalence and incidence of urogenital chlamydial infections are primarily due to a high rate of transmission during unprotected sexual activities, long duration of infection, and to the absence of overt clinical symptoms that would restrict or discourage sexual activity. Despite lack of clinical symptoms, *C. trachomatis* infection can lead to serious long-term health problems, or sequelae, including pelvic inflammatory disease, ectopic pregnancy, infertility, and chronic pelvic pain. Moreover, in the absence of robust protective immunity, which can be explained at least partially by the existence of multiple serologically distinct *C. trachomatis* strains, or serovars, reinfection is relatively common, which leads to an increase in sequelae severity.

In light of these critical findings on the epidemiology, natural history, and clinical presentation of *C. trachomatis* infections, and based on the fact that chlamydial infection can be successfully treated with antibiotics, public health agencies and many healthcare providers have recommended timely screening of sexually active people at high risk for infection as the most effective way of reducing the transmission and morbidity associated with chlamydial infection [6, 9]. Historically, these recommendations have led to development of numerous commercial laboratory methods for detection of *C. trachomatis*, including cell-culture-based methods, Giemsa staining of chlamydial inclusions, direct fluorescent antibody (DFA), micro-immunofluorescence (MIF), enzyme immunoassay (EIA), nucleic acid probe hybridization, and nucleic acid amplification tests (NAATs) (Table 10.1). For example,

during the past three decades, the US Food and Drug Administration (FDA) has cleared the use of over 150 medical devices intended for detection of chlamydial infection.

Based on the results of numerous research and comparative clinical studies performed during the past few decades, it has become evident that the commercial NAATs offer significant advantages compared to other laboratory diagnostic methods. NAATs are: (i) highly sensitive and specific; (ii) compatible with the use of non-invasive specimens, such as urine and vaginal swabs; (iii) suitable for automation and rapid turn-around time; and (iv) increasingly cost effective. Noticeably, in recent years (2008–2012), there have been only six devices cleared by the FDA for the detection of *C. trachomatis*, all of them associated with improvements of the NAATs technology. Moreover, these new devices represent primarily improvements in the automation of previously FDA-cleared NAATs methods, which indicates that this technology has entered a mature stage of acceptance and commercialization. Indeed, a survey conducted by the Centers for Diseases Control and Prevention (CDC) among public health laboratories in the United States on the volume and type of testing for chlamydial STIs has shown a dramatic increase in the use of commercial NAATs in the past decade (Table 10.1).

Consistent with the current scientific, technological, and commercial development of laboratory detection methods for *C. trachomatis* infection, and according to the current recommendations by public health agencies and healthcare providers, this updated chapter, focuses primarily on commercially available NAATs. The significance of using highly sensitive and specific laboratory tests for detection of all infections agents, particularly for those that are not associated with strong or specific acute clinical symptoms, as is the case with the urogenital chlamydial infections, cannot be overemphasized.

Table 10.1 Volume and type of *C. trachomatis* (CT) tests performed by public health laboratories* in the United States in 2007 [11]

Type of test	Laboratorys reporting testing (%)	Total tests reported (%)	Urine tests (%)	Vaginal swab tests (%)
Any CT testing	87 of 94(92.6)	3,290,390	582,265(17.7)	424,316(12.9)
Non-NAAT		524,947(16.0)	30(0.0)	108,550(25.6)
Culture	10(11.5)	2379(0.1)	–	2(0.0)
DFA	3(3.4)	245(0.0)	–	–
EIA	2(2.3)	62,420(1.9)	30(0.0)	–
Hybrid Capture 2 (Digene)	1(1.1)	47,402(1.4)	–	–
PACE 2 (Gen-Probe)	13(14.9)	109,681(3.3)	–	17,510(4.1)
PACE 2C (Gen-Probe)	11(12.6)	302,820(9.2)	–	91,038(21.4)
NAAT		2,684,278(81.6)	578,882(99.4)	312,284(73.6)
SDA (Becton Dickinson)	28(32.2)	844,093(25.7)	242,422(41.6)	265,684(62.6)
Aptima Combo 2 (Gen-Probe)	51(58.6)	1,747,651(53.1)	303,558(52.1)	45,074(10.6)
Aptima CT (Gen-Probe)	8(9.2)	46,643(1.4)	13,813(2.4)	1526(0.4)
PCR COBAS Amplicor (Roche)	2(2.3)	34,802(1.1)	8,000(1.4)	–
PCR Amplicor MWP (Roche)	1(1.1)	11,089(0.3)	11,089(1.9)	–
Serology	84(0.0)			
CF	0(0.0)	0(0.0)		
IFA	2(2.3)	67(0.0)		
MIF	1(1.1)	7(0.0)		
Serology – other	1(1.1)	10(0.0)		
Other antigen tests	5(5.7)	81,081(2.4)	3353(0.6)	3482(0.8)

* 153 public health laboratories (PHLs) were invited to participate in the survey. The survey was completed by 94 of 153 of PHLs, for an overall response rate of 61.4%. Very low percentages are represented in this table as 0.0%.

In the absence of clear clinical symptoms, the use of less sensitive commercial methods would generate a higher level of false-negative results, which could lead to increased risk for chronic disease and sequelae and increase potential for *C. trachomatis* transmission.

10.2 Epidemiology

National surveys conducted in the United States from 1999 to 2008 showed a 6.8% overall prevalence of *C. trachomatis* infection among sexually active females aged 14–19 years (4.4% among non-Hispanic whites and 16.2% among non-Hispanic blacks) [10]. In the general population, represented by men and women aged 14–39, the *Chlamydia* prevalence during 2007–2008 was approximately 1.6% (2.2% among females and 1.1% among males); however, the prevalence among non-Hispanic black persons was 6.7% [13]. Based on these and other studies, it was estimated that approximately 2.8 million *Chlamydia* infections occur annually in the United States, with the highest prevalence in persons aged ≤ 25 years [10].

In Europe, *Chlamydia* is also the most frequently reported STI. For example, in the United Kingdom it was reported that 10.3% of women and 13.3% of men ≤ 25 years of age were infected; the prevalence in Switzerland ranged from 2.8% in women to 1.2% in men, and in France the prevalence was 1.5% in the general population and 3% among 18–24 year old individuals [3]. Globally, the estimated annual number of *C. trachomatis* infections, affecting primarily sexually active young adults, is

over 90 million. For example, in a recent international study evaluating the incidence and correlates of symptomatic and asymptomatic chlamydial infection in 18,014 participants, age 18 or older, from five different countries (China, India, Peru, Russia, and Zimbabwe), the authors found an overall prevalence of 3.4% in males and 7.8% in females [14]. However, it is important to note that *Chlamydia* prevalence estimates vary considerably among different age groups and settings. For example, a systematic review and analysis of prevalence studies in the United Kingdom found a prevalence of 8.1% in the under 20 year old age group, 5.2% in 20–24 year olds, and 2.6% in 25–29 year olds, in a general practice surgical population. However, among under 20 year olds, estimates were 17.3% in genitourinary medicine clinics, 12.6% in antenatal clinics, 12.3% in termination of pregnancy clinics, 10.7% in youth clinics, 10.0% in family planning clinics, and 8.1% in general practice, compared to 5.0% in population-based studies [1].

The high prevalence of chlamydial infections can be explained by a relatively high transmission rate during unprotected sexual activities, poor immunity to reinfection, and mild or absence of clinical symptoms that would discourage sexual activity. Therefore, screening and treatment is essential for reducing the prevalence of chlamydial infection and the associated morbidity. However, in most regions of the world, including developed countries, only a small fraction of *C. trachomatis* infections are detected and treated, which leaves a large reservoir of transmissible infections.

In the United States, *Chlamydia* was only made a nationally notifiable disease in 1995, and since then it has become the most reported notifiable disease [7]. For example, in 2009, 1.2 million cases of *Chlamydia* were reported, four times more than the next notifiable disease on the list, which was gonorrhea [8]. Among women aged 20–24 years, the reported rate in 2009 was approximately 3300 cases per 100,000 people. Among men aged 20–24 years, the rate was less than half, approximately 1200 cases per 100,000. The lower reported rates in men are likely to be the result of reduced screening as compared to women. Therefore, it is expected that the apparent disparity in the rate of chlamydial infection between women and men will decrease as screening of sexually active young men and of those at high risk for infection will become more frequent.

Although in the United States and other developed countries the number of treated chlamydial cases has increased steadily over the past few decades, the number of reported infections has also increased. This paradox can be explained by the increase in the testing coverage and the use of NAATs, which detects more infected people as compared to other laboratory detection methods. For example, the prevalence of chlamydial infection in persons attending family planning clinics in Philadelphia, Pennsylvania has increased by 46% when NAATs replaced less sensitive laboratory diagnostic methods [15]. Similarly, a transition from less sensitive tests to NAATs in a short period (2005–2006) resulted in a 53% increase in *Chlamydia* prevalence in a study associated with the National Job Training Program [30].

Monitoring STD *Chlamydia* infections in various populations and predicting epidemiologic trends has been challenging during the transitory periods of changing testing technology and screening coverage. However, because a higher percentage of *C. trachomatis* infections are detected and treated, overall, it has been estimated that the disease burden as well as the transmission rate are declining, which is the ultimate goal of the screening programs.

In pregnant women, the prevalence of *Chlamydia* infection is relatively low. However, *Chlamydia* infection can lead to complications for the mother during pregnancy, and the rate of transmission to infants during vaginal delivery is very high, up to 65% in some studies [32]. Therefore, CDC recommends screening all pregnant women at their first prenatal visit and additionally during the third trimester for all women who were positive at the initial visit or were at risk for new infection [9].

10.3 Biology

Chlamydia trachomatis has a unique and complex developmental cycle, which led to their original misclassification as protozoa, and later as viruses [12]. However, since the 1960s it has been known that *C. trachomatis*

and other chlamydiae (e.g. *C. pneumoniae*, *C. psittaci*, and *C. pecorum*) infecting humans and various animal species are nonmotile, Gram-negative bacteria with an obligate intracellular developmental cycle.

Infection with *C. trachomatis* strains, or serovars, can cause trachoma, inclusion conjunctivitis, lymphogranuloma venereum (LGV), urogenital/rectal diseases and, in babies, respiratory tract infections. Interestingly, because of the lack of distinct clinical symptoms, chlamydial infection was not recognized as a sexually transmitted disease until 1976 [31]. Based on tropism and disease features, *C. trachomatis* serovars can be separated into three disease-associated groups, trachoma serovars (A–C), urogenital/rectal serovars (D–K), and LGV (L1–L3). The serovars traditionally have been identified based on serological markers specific for the major outer membrane protein (MOMP), which is the most abundant protein on the surface of the chlamydial cellular membrane. Currently, *C. trachomatis* strains are usually identified using genotyping methods that detect genetic variation in the *ompA* gene, which codes for MOMP, or in other variable chlamydial genes [28].

Chlamydiae have a unique life cycle consisting of two major developmental stages [24]. The extracellular infectious form, called the elementary body (EB), which is metabolically inert, enters the host cell by endocytosis and remains enclosed in a vacuole formed by the host membrane. Within the vacuole, the EB develops into a metabolically active, but noninfectious, reticulate body (RB), which grows and undergoes multiple rounds of division by binary fission. Throughout their intracellular developmental cycle, the chlamydial pathogens remain enclosed with distinctive vacuoles, called inclusions, which often fuse with each other, and can be identified by light or fluorescence microscopy, as they occupy much of the cell's cytoplasm. After multiple rounds of division, which produce up to a thousand or more chlamydial progenies per inclusion, the RBs differentiate into new, infectious EBs. The EBs are released from the host cell by lysis or exocytosis and can infect new host cells in the same or, if transmitted, different individuals. In cell culture, the chlamydial developmental cycle usually takes 48–72 h, depending on the *C. trachomatis* strain and the host cells.

Most *C. trachomatis* strains isolated from patients carry multiple copies of an extrachromosomal dsDNA (i.e. plasmid), which codes for several proteins [29]. The plasmid confers *C. trachomatis* the ability to synthesize glycogen and it might also code for virulence factors. From a diagnostic perspective, the presence of a chlamydial plasmid is very important, as some of the NAATs specifically target DNA sequences located on this multicopy plasmid, which can increase their sensitivity. Interestingly, a new *C. trachomatis* variant (i.e., mutant), which lacks the plasmid targeted sequence of some of the

NAATs, was recently discovered at relatively high prevalence in a population with a high rate of screening [25]. Apparently, due to broad screening and treatment, most of the circulating wild-type strains in the population were reduced whereas, in the absence of detection and treatment, the mutated variant spread very quickly. This isolated incident, which was promptly resolved, is strong testimony to the efficiency of *C. trachomatis* screening programs, and points to the importance of choosing NAATs gene targets that are essential for the life cycle of the infectious agents or for developing tests that target multiple sequences.

10.4 Natural history

Despite major advances in understanding the biology and pathogenicity of *C. trachomatis*, which was facilitated by sequencing of the chlamydial genome more than a decade ago [36], the course of untreated infections and that of the development of associated pathology and disease features remain poorly understood [18–20].

Chlamydia trachomatis can be easily transmitted during vaginal, anal, and oral sex. *Chlamydia* can also be transmitted from an infected mother to the baby during vaginal childbirth [32]. *Chlamydia trachomatis* can initiate infection at multiple mucosal sites, including the genital tract, rectum, throat, lungs, and eyes. In women, for example, the infection usually starts in the cervix and urethra and can spread to the fallopian tubes as well as to other mucosal tissues, such as the rectum. Culture studies have shown that among women infected with *C. trachomatis*, 50–60% are infected at both the cervix and urethra, 30% have only cervical infections, and 5–30% have only urethral infections [33]. In men, chlamydial infections are usually urethral, but can affect the sperm ducts of the testicles, as well as the rectum, causing proctitis. In babies, chlamydia usually causes eye and respiratory tract infections. The LGV strains, which are prevalent in parts of Africa, Asia, and South America, are more invasive than other urogenital serovars, leading to ulcers and inflammation and swelling of the inguinal lymph nodes.

Untreated *C. trachomatis* infections in women can last for months, or even years. It is estimated that only about half of urogenital chlamydial infections are resolved within 1 year after infection. This prolonged period of infection as well as the high rate of *C. trachomatis* transmission during unprotected sexual activity, which can be as high as 75%, indicate that the chlamydial pathogen is highly successful in evading the human immune system during transmission and the initial acute infection, and it can establish a chronic infection that can lead to enhanced immunopathogenesis and serious sequelae.

Antibiotic treatment of *C. trachomatis* infections usually help to resolve them quickly; although rare cases of apparent persistent infections in treated patients have been reported [34]. It is important to emphasize that it might take up to 4 weeks for the clearance of all traces of chlamydial nucleic acids, which is relevant for ‘test-of-cure’ using NAATs [17].

Similar to the natural history of *C. trachomatis* infection of the eye, which causes trachoma and eventually blindness, the duration and the number of urogenital reinfections are strong determining factors for the severity of STI immunopathology and sequelae. However, most if not all untreated *C. trachomatis* urogenital infections are eventually cleared in immuno-competent patients, suggesting that vaccine development is feasible [2, 26]. Until the development of a successful vaccine, a strong screening program using highly sensitive and patient-friendly diagnostic approaches, followed by timely and effective treatment, remain the primary mode for limiting the spread of chlamydial infection among people at high risk.

10.5 Clinical symptoms and sequelae

Approximately half the urogenital *C. trachomatis* infections in both men and women are asymptomatic during the course of infection, which, in the absence of treatment, can last for several months or longer. When present, clinical symptoms, which might develop within 1–3 weeks after transmission, are relatively mild, and often and are indistinguishable from those caused by other infectious agents such as *Neisseria gonorrhoeae*.

In women, the symptoms usually consist of vaginal discharge and dysuria. Post-coital bleeding is often reported. Symptoms of chlamydial pelvic inflammatory disease (PID), which may be subtle, include pelvic, uterine, or adnexal pain. Both symptomatic and asymptomatic chlamydial infections are an important cause of PID and ectopic pregnancy. Interestingly, chlamydial infections can apparently cause more severe tubal immunopathology than other agents in spite of the absence of overt symptoms. This is most probably due to the persistence of chlamydial infections compared with more acute infectious agents such as *N. gonorrhoeae*. Also, even in the absence of PID, silent and untreated salpingitis can lead to infertility. Similarly, multiple studies have shown associations between previous chlamydial infection, both symptomatic and asymptomatic, and ectopic pregnancy. Pregnant women with urogenital *C. trachomatis* infections are at increased risk for adverse outcomes of pregnancy, including preterm delivery and, in rare cases, stillbirth and neonatal death.

Chlamydia trachomatis is the most common cause of neonatal conjunctivitis and one of the most common

causes of pneumonia in early infancy. Symptoms of conjunctivitis usually develop within 2 weeks of delivery, and if the infection is untreated, chlamydial pneumonia can develop at 4–17 weeks after delivery. Infants with chlamydial pneumonia are at increased risk for later pulmonary dysfunction and possibly for chronic respiratory disease.

Among heterosexual men, chlamydial infections are usually urethral and up to 50% are asymptomatic. Nongonococcal urethritis is the most common clinical syndrome seen in men in the United States; 30–50% of cases of nongonococcal urethritis are caused by *C. trachomatis*. Epididymitis caused by *C. trachomatis* infection of the sperm ducts of the testicles does occur but is rare. In STD clinic populations, 4–8% of homosexual men have chlamydial infections of the rectum, most of which are asymptomatic. When they do occur, symptoms of rectal infection in men and women who practice receptive anal intercourse may include rectal discharge and pain during defecation. In homosexual men with chlamydial proctitis, the same symptoms are found, together with tenesmus, diarrhea, and rectal bleeding.

Lymphogranuloma venereum is a systemic disease caused by *C. trachomatis* serovars L1–L3. LGV is uncommon in the United States and Europe, but prevalent in parts of Africa, Asia, and South America and it occurs in both men and women. The LGV serovars of *C. trachomatis* are more invasive than other genital serovars, resulting in infection of the epithelial layers and underlying soft tissue. The primary symptom is a painless genital ulcer or papule. In homosexual men and women who practice receptive anal intercourse, the ulcer is often accompanied by proctocolitis with symptoms resembling inflammatory bowel disease.

The most common manifestation of the secondary stage of LGV is inflammation and swelling of the inguinal lymph nodes. The secondary stage of infection is characterized by systemic symptoms including fever, malaise, chills, anorexia, myalgia, and arthralgia. Untreated infections can lead to late complications including ulceration and hypertrophy of the genitalia, arthritis, and fistula formation involving the rectum, bladder, vagina, or vulva.

10.6 Treatment

Because of the high incidence of infection, annual *Chlamydia* screening and treatment is recommended in the United States and some other developed countries for all sexually active women age 25 years or younger as well as for people at high risk for infection, including sexual partners of those who test positive for *C. trachomatis* infection [9].

Chlamydia trachomatis is susceptible to broad-spectrum antibiotics, including macrolides, quinolones, tetracyclines, rifampin, and sulfonamides [22]. However, because its cell wall lacks the usual peptidoglycan composition found in the most common bacterial species, *C. trachomatis* is only partially susceptible to β -lactam antibiotics, such as penicillin.

For treatment of uncomplicated genital *C. trachomatis* infection in adolescent and adult men and women, CDC recommends a single dose of Azithromycin (1 g), or a 7-day course of Doxycycline (100 mg orally twice a day) [9, 22]. Alternative regimens include Erythromycin base, Erythromycin ethylsuccinate, Levofloxacin, and Ofloxacin. For treatment of *C. trachomatis* infection in pregnant women CDC recommends one dose of Azithromycin (1 g) or a 7-day course of Amoxicillin (500 mg three times a day), and for treatment of LGV infection treatment with Doxycycline (100 mg, twice a day for 21 days).

Although resistance to the common antibiotics used against *C. trachomatis* has been detected and characterized, it has been rarely documented in infected patients [34]. The apparent lack of antibiotic resistance in *C. trachomatis* as compared to other bacterial pathogens is probably due to its intracellular secluded developmental cycle, which does not favor exchanges of genetic material among chlamydial cells. However, with the continuous large-scale screening and treatment, the potential for antibiotic resistance selection remains of high concern; therefore, close monitoring of persistent infections is highly recommended as they might represent cases of antibiotic resistance.

10.7 Laboratory testing for *C. trachomatis*

Similar to laboratory diagnosis of many other infectious agents, there are two major approaches for the detection of *C. trachomatis* infection: (i) direct detection of the organism or of its components, such as nucleic acids or proteins, in specimens collected from the site of infection; (ii) indirect detection by serological testing using systemic immunological markers, such as antibodies. Although serological testing has been a valuable approach for surveillance, prevalence, and disease-association studies, because of poor sensitivity and specificity, it is not recommended for diagnostic testing.

Until the development of NAATs technology, the gold standard for laboratory diagnosis of chlamydial infection was the growth and detection of *C. trachomatis* in cell culture [4]. Cell culture has a specificity that approaches 100%, but compared with NAATs its sensitivity is less than 85%. Moreover, cell culture requires a high level of laboratory technical expertise, a stringent cold chain of

Table 10.2 Non-NAATs assays for *Chlamydia* detection

Assay	Specimen Type(s)	Mean TAT	Advantages	Disadvantages
Culture	Endocervical, urethral Nasopharyngeal (infants) Conjunctival, vaginal, rectal	2–4 days	Required in legal cases High specificity (98–100%)	Long TAT Sensitivity 40–70% compared to NAATs
Enzyme immunoassay	Manufacturer dependent Endocervical, urethral Ocular	Same day	Organism available for typing, susceptibilities Automated for large scale testing Kit based for small volume screening Rapid TAT (hours), relatively low in expense Moderate skill level Specimen may be held for up to 7 days prior to test Can detect non-viable organisms	Labor intensive, expensive Sensitivity of 60–70% compared to NAATs Specificity approximately 95% compared to culture Requires confirmation of positives in low prevalence populations
Direct fluorescent antibody	Manufacturer dependent Urethra, endocervical Rectal, conjunctival, ocular Nasopharyngeal (pediatrics)	Same day	Approved for use with different specimens Can assess quality of specimen. Rapid TAT: 1–2 h Relatively inexpensive	Labor intensive, moderately high skill level Specialty immunofluorescence microscope Sensitivity approximately 50–70% compared to NAATs Specificity approximately 95% compared to Culture
Direct nucleic acid probe	Endocervical, urethra Conjunctival	Same day	Can detect non-viable organisms* Large scale screening Detects non-viable organisms Rapid TAT: 3–5 h Relatively inexpensive, moderate skill level Can test for <i>N. gonorrhoeae</i> in same sample	Sensitivity approximately 60–75% compared to NAATs Specificity approximately 95% compared to culture Limited specimen type for testing

TAT, turn-around time.

* May also be considered a disadvantage.

transportation of specimens, and a lengthy period (3–7 days) for generating results; these features are not compatible with broad and competitive commercial development. However, at the time of this writing, in the United States, culture is the only test acceptable for medico-legal purposes, which includes testing for chlamydial infection in cases of suspected sexual abuse in children [21]. Also, culture has the advantage that it isolates viable organisms that can be used for additional applications or studies, such as antimicrobial susceptibility testing and genome sequencing. Therefore, it is likely that culture will remain a valuable laboratory technology for studying *Chlamydia*, but is of limited use for routine diagnostic screening.

Cell culture, DFA, MIF, EIA, and nucleic acids probe hybridization technologies for the detection of *C. trachomatis* infection including collection, transport, and the quality-assurance parameters have been described in detail in the previous edition of this book and other references [4, 27] (Table 10.2). A more recent compilation of some of the commercially available technologies is also available [35]. However, as shown in dozens of studies completed in the past few decades, because of improved

performance characteristics of NAATs, non-NAATs methods are used less frequently for routine chlamydial laboratory diagnosis and testing [5, 23, 37].

An intrinsic problem with laboratory diagnostic testing is the delay between the collection of the specimens, and testing and receiving the results for making treatment decisions. This is a significant issue because a delay in treatment could lead to increased chlamydial pathogenicity and sequelae, as well as to new transmission cases. This delay is of particular concern in populations, such as adolescents, who have a low rate of return for treatment. A potential solution would be the development of highly sensitive and specific rapid diagnostic tests that are performed at the “point of care”, so that patients are tested and treated during the same visit. Although promising, the current rapid technologies are not commonly used for routine testing [16]. Again, using tests with low sensitivity is highly problematic, because in the absence of distinct clinical symptoms, infected individuals who receive false-negative results are unlikely to be treated, which could lead to increased sequelae and spread of infection.



Figure 10.1 Cobas System. (Courtesy of Roche Diagnostics Corporation.) (See insert for color representation of the figure.)



Figure 10.2 The m2000 System. (Courtesy of Abbott.) (See insert for color representation of the figure.)

In addition to being highly sensitive and specific, NAATs have the advantage of being compatible with fully automated laboratory platforms that are being developed for the diagnosis of other infectious and genetic diseases. As mentioned in the introductory section, most of the devices cleared by the FDA for *Chlamydia* testing in the past few years are improvements in the automation of previously FDA-cleared NAATs methods. This development reflects the superiority of NAATs, which have entered a mature stage of acceptance, automation, and commercialization. Also, because NAATs are used for the detection of other infectious STIs, which have overlapping clinical symptoms with *C. trachomatis* (CT), such as infection with *N. gonorrhoeae* (NG), and are found in the same clinical specimens, often the commercial testing assays and kits are designed for simultaneous detection of more than one infectious agent, which is a substantial advantage.

NAATs are based on the amplification and detection of DNA or RNA targets specific for the infectious agent. The



Figure 10.3 The GeneXpert platform. (Courtesy of Cepheid.) (See insert for color representation of the figure.)



Figure 10.4 The Tigris DTS System. (Courtesy of Hologic, Inc.) (See insert for color representation of the figure.)



Figure 10.5 The BD Viper System. (Courtesy and © Becton, Dickinson and Company.) (See insert for color representation of the figure.)

current commercially available NAATs for the detection of *Chlamydia* and other STIs such as gonorrhea are based on three amplification methods: polymerase chain reaction (PCR), transcription-mediated amplification (TMA), and strand displacement amplification (SDA). All NAATs technologies follow a similar overall protocol: (i) preparation of nucleic acids from the clinical specimen for amplification; (ii) amplification of nucleic acids sequences specific for *C. trachomatis*; (iii) detection of amplified

Table 10.3 Comparison of the most recent FDA-cleared NAATs diagnostic assays/devices for *C. trachomatis* (FDA clearance date)

	Gen-Probe Aptima Combo 2 (January 22, 2007)	BD ProbeTec CT Qx (June 2, 2009)	Abbott RealTime CT/NG (May 28, 2010)	Roche Cobas CT/NG (January 24, 2012)	Cepheid Xpert CT/NG (December 27, 2012)
Assay type and intent	Qualitative <i>in vitro</i> diagnostic test for direct detection of <i>Chlamydia trachomatis</i> and/or <i>Neisseria gonorrhoeae</i> in patient specimens	Qualitative <i>in vitro</i> diagnostic test for direct detection of <i>Chlamydia trachomatis</i> in patient specimens	Qualitative <i>in vitro</i> diagnostic test for direct detection of <i>Chlamydia trachomatis</i> and/or <i>Neisseria gonorrhoeae</i> in patient specimens	Qualitative <i>in vitro</i> diagnostic test for direct detection of <i>Chlamydia trachomatis</i> and/or <i>Neisseria gonorrhoeae</i> in patient specimens	Qualitative <i>in vitro</i> diagnostic test for direct detection of <i>Chlamydia trachomatis</i> and/or <i>Neisseria gonorrhoeae</i> in patient specimens
Amplification technology	Ribosomal RNA transcription mediated amplification (TMA) CT ribosomal RNA	Strand displacement DNA amplification (SDA) CT cryptic plasmid DNA	Real-time polymerase chain reaction (PCR)	Real-time polymerase chain reaction (PCR)	Real-time polymerase chain reaction (PCR)
CT analyte targets	CT ribosomal RNA	CT cryptic plasmid DNA	CT cryptic plasmid DNA	CT cryptic plasmid DNA and CT <i>ompA</i> gene	CT genomic DNA
Detection chemistry	Photon measurement from selectively hybridized chemiluminescent probes reported as relative light units (RLU)	Fluorescent dye labeled probes using fluorescence resonance energy transfer (FRET)	Real-time fluorescence detection	Paired reporter and quencher fluorescence labeled probes (TaqMan Technology) using fluorescence resonance energy transfer (FRET)	Real-time fluorescence detection
Specimen type	Urine (male and female) Endocervical swabs Vaginal swabs (clinician or patient-collected) Male urethral swabs Cervical specimens in PreservCyt media	Urine (male and female) Endocervical swabs Male urethral swabs Vaginal swabs (patient-collected) Cervical specimens in PreservCyt and SurePath media	Urine (male and female) Endocervical swabs Vaginal swabs (clinician or patient-collected) Male urethral swabs	Urine (male) Vaginal swabs (patient-collected)	Urine (male and female) Endocervical swabs Vaginal swabs (patient-collected)
Result analysis	Determined by a cutoff based on the total fluorescence unit and kinetic curve type	Determined by relating MOTA score (signal strength to pre-determined cutoff values	Based on PCR cycle threshold (Ct) analysis	Based on PCR cycle threshold (Ct) analysis	Based on PCR cycle threshold (Ct) analysis

product; (iv) generating results. These amplification methods have been used and validated for laboratory detection of *C. trachomatis* and *N. gonorrhoeae* in both research and clinical settings for more than two decades. However, the format of the commercial NAATs, including the type of clinical specimens and their degree of automation, has changed dramatically during this time. A highly relevant development in NAATs technology has been the use of noninvasive specimens, which are equal or more sensitive than the traditional invasive cervical and urethral swabs. This has led to an increase in the willingness of people to be tested for *C. trachomatis* and other STIs, facilitating the screening process. Currently, CDC recommends the use of vaginal swabs from women and first-catch urine from men.

The most recent FDA-cleared PCR tests for the detection of *C. trachomatis* in the United States are: Cobas CT/NG Test, performed on the Cobas 4800 PCR system, provided by Roche Molecular Systems, Inc. (Pleasanton, CA, U.S.) (Figure 10.1), Abbott Real Time CT/NG assay,

performed on the m2000 System, provided by Abbott Laboratories (Abbott Park, IL, U.S.) (Figure 10.2), and the Xpert CT/NG, performed on the GeneXpert modular cartridge-based platform, provided by Cepheid (Sunnyvale, CA, U.S.) (Figure 10.3). The TMA technology is used in the Gen-Probe Aptima Combo assay performed on the Tigris DTS and Panther Systems provided by Gen-Probe Incorporated (San Diego, CA, U.S.) (Figure 10.4). The SDA technology is used in the Becton Dickinson (BD) ProbeTec ET Assays for CT/NG, performed on the BD Viper System and provided by Becton Dickinson (Franklin Lakes, NJ, U.S.) (Figure 10.5).

A comparison of the most recent FDA-cleared NAATs diagnostic assays/devices for *C. trachomatis* detection is presented in Table 10.3. As for all commercially available and FDA-cleared diagnostic technologies, it is essential that the assays are performed strictly according to the manufacturer package inserts instructions, including those for collection, transport, storage, and preparation of the specimens for testing.

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Rickettsiae and Tick-borne Diseases

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11.1 Introduction

Tick-borne diseases (TBDs) are increasing in prevalence and represent a growing occurrence of arthropod-borne infectious diseases. Possible reasons for this phenomenon include shifts in the distribution of animal reservoirs and tick vectors, as well as more human presence in areas where there is increased contact with ticks and their associated pathogens. From a public health point of view, the complex interactions of ticks, pathogens, hosts, and the environments involved in this changing pattern of disease are not completely understood [14, 49, 50, 58, 59]. However, the reporting of these illnesses and diseases to health department agencies allows for the gathering of information and statistics, in support of establishing trends related to these illnesses [11].

Tick-borne illnesses occur in distinct geographic locations, which is a key factor in determining specific tick-borne infections to be considered in diagnosis. Patients with TBD often present with nonspecific symptoms, including various rashes or skin lesions, which are often accompanied by fever and influenza-like symptoms [4]. Travel history, recent locations, and potential for tick bite are important. However, individuals are usually unaware of tick exposure and less than 50% present with a positive history of a tick bite [12, 26, 33]. Nonetheless, the absence of a tick-bite history should not exclude a diagnosis given the correct clinical presentation. Severe illness and mortality can occur, if there are delays in diagnosis and treatment.

Since the organisms transmitted by ticks are infrequently encountered in clinical specimens, diagnosis of tick-borne diseases can pose a challenge in that the etiologic agents of these illnesses can be both difficult and hazardous to culture in laboratories. Therefore, clinical specimens for culture, molecular analysis, and some serologic assays are

commonly referred for diagnosis at specialized laboratories utilizing specific testing methodologies [4]. This chapter discusses the most common agents associated with TBD and details the testing methodologies currently available for laboratory diagnosis.

11.2 Overview of tick-borne diseases

11.2.1 Ticks as vectors for infectious disease transmission

Ticks are excellent vectors for disease transmission, with more than 800 recognized species [7, 27]. These arthropods, of the class *Arachnida*, are second only to mosquitoes in worldwide transmission as vectors of human disease. Of the recognized families of ticks, only hard ticks (*Ixodidae*) and soft ticks (*Argasidae*) are of medical importance. TBD are transmitted by tick vectors such as the deer tick, dog tick, wood tick, and lone star tick [12]. In the United States, ticks are the most common vectors of vector-borne diseases. Ticks can carry and transmit a vast number of pathogens, such as bacteria (including rickettsiae), spirochetes, protozoa, viruses, nematodes, and toxins. A single tick bite can transmit multiple pathogens, which can also lead to atypical presentations of classic tick-borne diseases [27].

11.2.2 Rickettsial infections

11.2.2.1 Epidemiology and clinical manifestations

Species of *Rickettsia* comprise a genera of small, obligately intracellular Gram-negative bacteria within the family *Rickettsiaceae*. The genus is divided by their phylogenetic clustering into the spotted fever group (SFG) and typhus group (TG). The SFG contains bacteria that are generally recognized as human pathogens, including *R. rickettsii*, *R. conorii*, and *R. africae*, which are respectively

the causative agents of Rocky Mountain spotted fever (RMSF), Mediterranean spotted fever (also referred to as boutonneuse fever) and African tick-borne fever [2, 12, 85, 89]. The TG consists of only two members, namely *R. prowazekii* and *R. typhi*. As part of their life cycle, *Rickettsia* spp. reside in an arthropod host, during which they are maintained by transovarian transmission and/or cycles and horizontally transmitted to vertebrate hosts. It is important to note that the SFG organisms are transmitted by ticks, whereas those of the TG are transmitted by fleas, lice, and mites [8, 49, 64]. Due to the TBD focus of this chapter, only rickettsial diseases transmitted by ticks will be further described.

RMSF can become a life-threatening illness for previously healthy individuals. Approximately 500 cases of RMSF are documented annually, with *Dermacentor andersoni* and *Dermacentor variabilis* being the most common vectors for this disease [17, 82]. In the United States, these infections occur from April through October. An average of 7 days after arthropod bite inoculation of *R. rickettsii*, patients with RMSF develop fever, severe headache, malaise, and myalgia, frequently accompanied by nausea, vomiting, and abdominal pain, and sometimes cough [12, 32, 40]. The major diagnostic sign of infection is a characteristic maculopapular rash that typically appears on the palms and soles of 83–90% of infected patients, following 3–5 days of illness [32]. The organism infects endothelial cells, frequently leading to increased vascular permeability and focal hemorrhages. In severe cases, noncardiogenic pulmonary edema and rickettsial encephalitis with coma and seizures are grave conditions that often presage death [37, 84].

Rickettsia parkeri, transmitted by *Amblyomma maculatum* (Gulf Coast tick), occurs in the southeastern region of the United States. *Rickettsia parkeri* causes a mild illness at the tick inoculation site eschar, fever, headache, myalgia, usually a maculopapular or vesiculopapular rash, less frequently tender regional lymphadenitis, and no reported deaths [17, 61, 63].

In southern Europe, *Rhipicephalus sanguineus* is the primary tick vector of *R. conorii*, which causes Mediterranean spotted fever. Disease is usually a benign self-limited exanthematous febrile illness, however, it can have a fatal outcome in patients who are elderly or have underlying diseases or other risk factors [2, 29, 71]. Fatal cases more frequently have acute renal failure, hyperbilirubinemia, obtundation, tachypnea, petechial rash, gastrointestinal symptoms, and coagulopathy [18].

African tick bite fever (ATBF) is broadly distributed across most of the African continent. The disease pathogen, *R. africae*, has been detected in *Amblyomma* spp. ticks [2]. Of note for the epidemiology of ATBF is the acquisition of the disease by North American tourists, hunters, or deployed military staff returning from areas

where *R. africae* is endemic [31, 52, 60]. The disease begins approximately 5–7 days following tick bite, with sudden onset of fever, fatigue, headache, and myalgia. Inoculation eschars are reported inconsistently and are identified in roughly 50–100% of cases. However, the occurrence of multiple eschars has been reported to frequently occur. Additional clinical manifestations include regional lymphadenopathy and generalized maculopapular or papulovesicular rash. No cases of fatal disease resulting from these bacteria have been reported, although more severe manifestations, including myocarditis and neuropathy, are sometimes described [28, 63].

11.2.2.2 Laboratory diagnosis and diagnostic challenges

For most clinical microbiology laboratories, assays for antibodies to rickettsiae are the only tests performed. These assays are utilized principally for serologic confirmation of the diagnosis in convalescence. Such testing is not useful in situations of life threatening, or acutely incapacitating, rickettsial disease. For serologic diagnosis, blood is collected as early in the course of disease as possible, a second sample is collected after 1 or 2 weeks, and if a fourfold rise in antibodies has not occurred, a third sample is collected 3 or 4 weeks after onset. The serum may be stored for several days at 4°C but should be stored frozen at –20°C or lower for longer periods to avoid degradation of the antibodies. Serologic assays focus on the indirect immunofluorescence assay (IFA). Other approaches include indirect immunoperoxidase assay, latex agglutination, enzyme immunoassay (EIA), line blot, Western immunoblotting, and rapid lateral flow assays [35, 38–42, 70, 83]. Only some of these assays are available as commercial kits or developed for use in reference laboratories.

The IFA contains all the rickettsial heat-labile protein antigens and group-shared lipopolysaccharide antigen and, thus, provides group-reactive serology. The IFA reagents are available commercially for SFG and TG rickettsiae from Scimedx Corp., Denville, NJ; Focus Technologies, Cypress, CA; Fuller Laboratories, Fullerton, CA. In cases of RMSF, IFA detects antibodies at a titer of ≥ 64 , usually in the second week of illness. For boutonneuse fever, an IFA titer of ≥ 40 occurs in 46% of patients between days 5 and 9 of illness, in 90% of patients between 20 and 29 days, and in 100% of patients thereafter [12].

Indirect immunoperoxidase assays for boutonneuse fever (research-use only, RUO) and presumably other rickettsioses yield results similar to those of IFA when the IgG diagnostic titer is set at 128 and that of IgM is set at 32 [42]. Advantages include the use of a light microscope rather than a fluorescence microscope and the production of a permanent slide result. Latex agglutination test reagents are available commercially from Scimedx Corp.: only for *R. rickettsii* in the United States. Latex beads

coated with an extracted rickettsial protein–carbohydrate complex containing rickettsial lipopolysaccharide are agglutinated mainly by IgM antibodies, with reports of a sensitivity of 71–94% and a specificity of 96–99% [35]. A diagnostic titer of 128 is often detected early in the second week of illness.

The EIAs have been developed in various formats, including antigens coating microtiter wells or immobilized on nitrocellulose or other sheets for use in the commercial reference laboratory setting. Dot-EIA kits are commercially available in the United States from Scimedx Corp., for detecting antibodies against *R. rickettsia* and *R. conorii*. Standard EIAs for detecting IgG or IgM antibodies against SFG rickettsiae are also available from Panbio Diagnostics (Brisbane, Australia) internationally, but are not currently available for purchase in the United States. The utilization of these tests for paired sera from populations with clinical (fever, headache, or rash) and epidemiologic (vector exposure) features consistent with rickettsiosis would most likely yield useful information.

The most useful methods for establishing a diagnosis during the acute stage of illness when therapeutic decisions are critical include immunohistochemical and molecular diagnostic testing. Such testing is presently available in only a few reference laboratories. The diagnoses of RMSF, *R. parkeri* infection, boutonneuse fever, and African tick-bite fever, have been established by immunohistochemical detection of rickettsiae in formalin-fixed, paraffin-embedded sections of biopsy specimens of rash and eschar lesions [37, 40, 83]. Monoclonal antibodies that are specific for lipopolysaccharides of SFG rickettsiae have been used to detect rickettsiae by immunohistochemical staining. These antibodies are commercially available through Fuller Laboratories (Fullerton, CA, USA). The sensitivity and specificity of immunohistochemical detection of *R. rickettsii* in cutaneous biopsy specimens are 70 and 100%, respectively. Eschar biopsies yield sensitive specimens for the diagnosis of SFG rickettsioses [40, 83].

Polymerase chain reaction (PCR) has been applied to the amplification of the DNA of *R. rickettsii*, *R. parkeri*, *R. conorii*, *R. prowazekii*, *R. africae*, and other species. This methodology provides a higher diagnostic yield when applied to biopsy specimens of rickettsia-infected lesions [18, 43]. If whole blood, plasma, buffy coat, or tissue cannot be processed for PCR within several days, it should be stored at –20°C or lower. Serum has a lower sensitivity for PCR, but is often diagnostic in fatal cases [48, 58]. Nested PCR applied to skin biopsy specimens, particularly of eschars prior to treatment, has a sensitivity of 78% [30]. For all pathogenic *Rickettsia* spp., the 17 kDa lipoprotein gene is a target. The *gltA*, *rrs*, *groEL*, *ompA*, and *ompB* genes have also been amplified diagnostically, with the *Rickettsia* being identified through either restriction fragment length polymorphism analysis using

AluI and *XbaI* or sequencing of the PCR product. Recent advances in technology such as real time PCR allow for increased sensitivity in the detection of rickettsiae, during the early or acute phase of disease before the development of antibody [55, 69, 72, 77, 86].

11.2.3 Other tick-borne diseases

11.2.3.1 Bacterial agents

11.2.3.1.1 Lyme disease

Epidemiology and clinical manifestations Lyme disease or Lyme borreliosis (LB) is a multisystem disease, and is the most common vector-borne disease in the United States, with an annual incidence of 20–100/100,000. Cases occur throughout the year, with peak incidence occurring in the summer. The highest incidence of disease in the United States occurs in the northeastern and midwestern states [11, 34]. *Borrelia burgdorferi sensu lato*, the Gram-negative spirochete that causes this disease, is divided into three separate species, which are pathogenic in humans: *B. burgdorferi sensu stricto* (United States and Europe), *B. garinii*, and *B. afzelii* (Europe and Japan). Lyme borreliosis usually presents with fever, headache, malaise, fatigue, and weight loss. There are three stages of clinical Lyme disease (primary, secondary, and late persistent), although patients do not necessarily progress through all stages [6, 16, 87]. Early localized disease occurs 3–30 days after a tick bite. Approximately 60% of patients develop erythema migrans, which is a skin lesion at the site of the *Ixodes* tick bite and is the best clinical marker of Lyme disease [16, 87]. The lesion begins as a macule, which expands to form an annular erythema with partial central clearing. Involvement of the heart, joints, and central nervous system may occur in the late stages of disease. Arthritis of the knees is the most common long-term disease manifestation [6, 53, 88].

Diagnosis and diagnostic challenges The Centers for Disease Control and Prevention (CDC), Infectious Diseases Society of America (IDSA), and the Public Health Agency of Canada recommend serologic antibody testing as the initial diagnostic test of choice for Lyme disease [1, 11, 88]. However, such testing should be reserved for patients with a clinical picture highly suggestive of Lyme disease, as it can have a poor positive predictive value even in endemic areas. Both steps can be done using the same blood sample. The first step is a screening enzyme immunoassay (EIA) or indirect fluorescent-antibody (IFA), which is followed by confirmatory Western immunoblot testing for positive or equivocal EIA or IFA result [19, 57]. Both commercial and reference laboratory assays are available for this purpose. For an up-to-date listing of US FDA-cleared assays, refer to <http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/pmn.cfm>; type “LSR” at “Product Code” prompt. The EIA assays incorporate the

testing of serum for the presence of antibodies reacting to whole-cell sonicates of low-passage *B. burgdorferi* antigens immobilized to a solid surface. Some commercial assays incorporate variations to the antigen preparation through utilization of the addition of recombinant proteins or purified antigens (Vidas Lyme IgG and IgM EIA, bio-Mérieux, Durham, NC; ImmunoWELL Borrelia (Lyme), GenBio, San Diego, CA; PreVue *B. burgdorferi*, Chembio Diagnostics Systems, Stoneybrook, NY) and synthetic C6 peptide, derived from the VlsE protein of *B. burgdorferi* (Immunetics, Inc., Boston, MA). Outside the United States, *Borrelia burgdorferi* IgG and IgM enzyme-linked immunosorbent assay (ELISA) test kits are available through Immunolab GMBH, Kassel, Germany. Western blot analysis detects *B. burgdorferi* IgM and IgG antibodies. Amongst commercial sources of these Western blot tests are Marblots (Trinity Biotech, Jonestown, NY) and Virablots (Viramed Biotech, Oceanside, CA) [19, 53, 88].

It should be noted that variability of *B. burgdorferi* antigen and stage of disease may affect serological results [1, 16]. Serologic testing lacks sensitivity in early Lyme disease, as IgM antibodies typically do not develop until 3–4 weeks after infection onset. The sensitivity of IgM blots is approximately 40% in acute phase, and 60–80% during convalescence. Sensitivity increases for patients with disseminated disease [19]. If a patient with suspected early Lyme disease has a negative single serology, serologic evidence of infection is best obtained by testing of paired acute- and convalescent-phase serum samples. IgG antibodies develop at 6–8 weeks following illness onset. Therefore, serum samples from persons with disseminated or late-stage Lyme disease almost always have a strong IgG response to *B. burgdorferi* antigens [34, 88].

PCR testing is recognized by the IDSA as having the highest sensitivity for Lyme disease in synovial fluid samples from patients with untreated late Lyme arthritis, as well as those with untreated neurologic disease [56, 87].

11.2.3.1.2 Tick-borne relapsing fever

Epidemiology and clinical manifestations Tick-borne relapsing fever (TBRF) is a spirochete disease caused by at least 16 different *Borrelia* spp. and is present worldwide [7, 27]. The vector is the soft-bodied argasid tick of the *Ornithodoros* genus, of which rodents are commonly infected [7, 75]. Those found in the United States occur in the western mountains and high deserts and plains of the southwest, with fewer than 30 cases diagnosed annually [22]. In the United States there are two forms of relapsing fever: epidemic louse-borne disease caused by *B. recurrentis* and the endemic tick-borne form caused by various organisms including *B. hermsii*, *B. turicatae*, and *B. parkeri*. Louse-borne relapsing fever occurs under conditions of poor hygiene, crowding and

poverty, where organisms are maintained by passage between lice and humans. TBRF is transmitted via the saliva during the attachment of ticks. Clinically, in both types of relapsing fever, after an incubation period of 2–15 days, there is a massive spirochetemia accompanied by high fever (usually above 39°C), headaches, myalgias, arthralgias, nausea, chills, and weakness. Jaundice occurs in 7% of patients, and the case fatality rate is 2–5% [7]. The initial febrile episode usually ends suddenly and patients defervesce after 3 to 7 days, when an immune response is mounted and the spirochetemia disappears. Several days to weeks later, the disease can recur with a less severe course. The relapses are due to variation of surface antigens of the organism, resulting in temporary evasion of the immune system [7, 22, 27].

Diagnosis and diagnostic challenges A definitive diagnosis is made by blood smear examination during a febrile period. Direct examination by dark-field microscopy or staining of blood specimens with Giemsa or Wright's stain may be performed to detect *Borrelia* spp. Either method provides a sensitivity of 70%. The organisms can usually be seen in the blood from patients with relapsing fever, but not from those with Lyme disease. The Warthin–Starry silver stain or monoclonal antibodies can be used to stain tissues. Nucleic acid amplification techniques have high sensitivity when used with tissue specimens. Therefore, skin biopsy specimens, from the periphery of the erythema are recommended. Cultures of joint fluid are recommended for patients with arthritic symptoms. The medium of choice is Barbour–Stoenner–Kelly broth (Sigma, St. Louis, MO) and the culture should be maintained in a microaerophilic environment at 30–33°C for 4–6 weeks. While useful in detection of TBRF causing borreliae, culture has low sensitivity for diagnosis of *B. burgdorferi* associated with Lyme disease. Serologic assays for TBRF are not readily available, and diagnostic value of these assays is limited due to antigenic variation shown by etiologic borreliae agents [1, 7, 22, 27, 75].

11.2.3.1.3 Tularemia

Epidemiology and clinical manifestations Tularemia is caused by *Francisella tularensis*, a small aerobic and pleomorphic Gram-negative coccobacillus that has worldwide distribution, and can be transmitted by organism inhalation, direct inoculation, or by tick vectors. In the United States, cases of tularemia have been reported in all states, except Hawaii, with most occurring during the summer months [25, 36, 66]. Tick-borne cases of tularemia are known to be spread by three tick species: *A. americanum*, *Dermacentor andersoni*, and *Dermacentor variabilis*. *Francisella tularensis* is a highly potent human pathogen. Irrespective of acquisition by inhalation or inoculation of skin or mucous membranes, the infective dose may be as low as 10 organisms. Tularemia is an acute febrile invasive disease characterized pathogenically by rapid intracellular replication into high bacterial densities, accumulation of phagocytes, and extensive tissue necrosis. The clinical presentation of this infection is abrupt, with fever,

chills, headache, and generalized pain. There are several distinct clinical forms including ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, and typhoidal. The most common form of presentation in the United States is the ulceroglandular form, resulting from tick bites and contact with infected animals (e.g., squirrels, rabbits, rats, and other rodents) [25]. Following an incubation period of 3–10 days, a papule forms at the site of infection, eventually ulcerates, and is accompanied by regional lymphadenopathy [15]. Due to the rapid development of the infection, successful treatment of disease is dependent on early clinical suspicion and initiation of appropriate antibiotics. In general, mortality may be associated with 2% of cases, even with antibiotic treatment [36].

Diagnosis and diagnostic challenges In routine clinical work, culture of specimens in tularemia-suspected cases is infrequently undertaken due to *F. tularensis* being fastidious and relatively slow growing. However, *F. tularensis* grows favorably on cysteine supplemented agar media such as enriched chocolate agar, cysteine heart agar with 9% heated sheep red blood cells (CHAB) agar, or GC Agar II with 1% hemoglobin and 1% isovitalax, with cultures incubated in a 5% CO₂ environment at 37°C. Within 2–4 days of incubation on CHAB agar, green opalescent shiny colonies appear. When contaminating flora is likely to occur, the use of media containing antibiotics (e.g., CHAB-A) is recommended. Gram stain of isolated colonies reveals weakly staining, primarily single Gram-negative coccobacilli. The identity of colonies is most simply tested by slide agglutination or by direct fluorescent antibody staining. When culture work is performed, it must be done under biosafety level-3 conditions, as *F. tularensis* is one of the more frequently reported laboratory-acquired infections among workers handling infectious specimens and cultures. It is also important to note that even under optimized growth conditions, cultures from patients with documented tularemia infections are often negative [15, 36].

Serologic testing has been the most consistently utilized method for diagnosis of tularemia. Typical diagnostic criteria involve an upper-limit cut-off for a single test (e.g., > 1:160) or a fourfold rise in titers between acute and convalescent samples, to confirm diagnosis. A large case study described the use of tube and slide agglutination reactions for diagnosis of tularemia from 1949 to 1979 [26]. These agglutination tests, including microagglutination, demonstrate excellent sensitivity and specificity. Commercial *F. tularensis* antigens are available to be used with standard tube agglutination tests. BBL (Becton Dickinson, Franklin Lakes, N.J.) offers a latex agglutination test that has been used to identify individuals with antibodies to *F. tularensis*. With this assay, dilutions greater than 1:20 are considered specific and significant. Although agglutination tests are frequently used for diagnosis, there have been isolated strains of *F. tularensis* that have failed to agglutinate commercially available *F. tularensis* antigens [13]. ELISA has also been utilized to measure and differentiate

antibodies to *F. tularensis* [10]. The advantages of serologic assays are the relative simplicity of testing, high sensitivity, and rarity of false-negative results in patients with symptoms for an extended duration. Disadvantages of serology include: (i) false-negative results in early infection; (ii) frequent need for convalescent serum to confirm diagnosis; (iii) possible cross-reactivity with other organisms (e.g., *Brucella*, *Salmonella*, *Yersinia*, and *Legionella* antigens); and (iv) high titers of IgM and IgG antibodies may persist for longer than a decade after infection, limiting the value of a single positive result [25, 36].

PCR has been described and successfully applied to wound specimens of patients acquiring tularemia, and prospects for application on other human specimens (blood, respiratory, exudate) are promising. Besides allowing diagnostics at high sensitivity and specificity, the PCR technology also facilitates the identification of cases of tularemia presenting with aberrant signs and symptoms [25, 36, 76]. PCR using primers for the 17 kDa lipoprotein *F. tularensis* gene *tul4* was 77% sensitive and culture was 63% sensitive for the diagnosis of ulceroglandular tularemia, as summarized in a review of three reports from Sweden [78]. Real-time PCR assays with multiple targets offer promise for improved sensitivity and specificity and for field use. Although promising, PCR has disadvantages: (i) false-positive and false-negative results; (ii) expense of the test may inhibit routine usage in resource-limited clinical microbiology laboratories; (iii) inability to perform antimicrobial susceptibility testing on the identified organism; (iv) lack of testing standardization between laboratories [76].

Specimen collection and transport When tularemia is clinically suspected, submission of patient specimens should be preceded by a contact with the receiving laboratory. The laboratory may need to undertake preparatory measures, including safe and efficient sampling and transportation guidance and safe diagnostic procedures. Due to a varying readiness to accept specimens for culture of *F. tularensis*, knowledge of media optimal for transportation and culture is limited. For wound samples, conventional transport systems are less suitable for preservation of *F. tularensis* than for other fastidious Gram-negative bacteria such as *Neisseria*. In a comparative study of four transportation devices, Amies agar with charcoal or Thayer-Martin medium modified by the addition of antibiotics were superior for optimal transport of gonococci [68].

11.2.3.1.4 *Erlichiosis*

Epidemiology and clinical manifestations “Ehrlichiosis” is a generic name for infections caused by Gram-negative, obligate intracellular bacteria of the family *Anaplasmataceae* [21]. These are primarily in the genera *Ehrlichia* and *Anaplasma*, which cause the majority of human infections. Upon transmission, the organisms reside within the reticuloendothelial system and produce a clinical syndrome

indistinguishable from that of RMSF. Three distinct species comprise the majority of these infections: *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Anaplasma phagocytophilum*. Circulating leukocytes are the targets of these organisms, and diseases associated with them are often named according to the type of leukocyte each infects. *Ehrlichia chaffeensis* is transmitted primarily by the lone star tick, *Amblyomma americanum* (can also be transmitted by *Dermacentor variabilis* tick), and infects monocytes and causes human monocytic ehrlichiosis (HME). The white-tailed deer is a major host of all stages of lone star ticks and is an important natural reservoir for *E. chaffeensis*. *Anaplasma phagocytophilum*, which is transmitted by *Ixodes persulcatus* group ticks, infects granulocytes and causes human granulocytic anaplasmosis (HGA). HME occurs most commonly in the mid-Atlantic, southeastern and south-central United States. *Ehrlichia ewingii* are also transmitted by the lone star tick and share a geographic distribution that overlaps HME and RMSF [9,62]. HGA occurs internationally with areas of endemicity including the United States (northeastern, mid-Atlantic, upper midwest and Pacific northwest states), Europe, and Asia (China, Siberian Russia, and Korea) [20]. The annual incidence of ehrlichiosis in the United States is reported to be 1.7/1,000,000, with the peak infectious season occurring from April through September [21]. Risk factors include: (i) exposure to regions where the tick vector is endemic; (ii) older age (>60 years); (iii) immunocompromised state; and (iv) asplenia. Incubation period ranges from 1 to 2 weeks. Common symptoms are fever, headache, and myalgias. Leukopenia, thrombocytopenia, and elevated liver function tests can be seen in 50–90% of patients. Rashes occur in approximately one-third of patients with HME, and 2–11% of patients with HGA. Additional findings of acute respiratory distress syndrome (ARDS), meningitis, pancarditis, renal failure, and disseminated intravascular coagulation (DIC) have been reported. Fatality rates have been reported at 2.7% for HME and 0.7% for HGA [20, 21, 27].

Diagnosis and diagnostic challenges PCR is the diagnostic method of choice for ehrlichiosis because organisms reside within circulating leukocytes and are therefore easily accessible for testing. Tests are available from CDC, selected state health laboratories, and a limited number of research and commercial laboratories. Conventional PCR tests have no specified standard, and diagnostic sensitivity and specificity varies amongst assays [7, 20, 32, 54]. Antibiotic treatment, namely with doxycycline, can decrease the sensitivity of PCR [20]. In studies of *A. phagocytophilum* infection, PCR was estimated as 60–70% sensitive, and for diagnosis of infection with *E. chaffeensis*, PCR was estimated to be 52–56% to 87% sensitive [12, 20, 21, 63].

Microscopic examination of blood smears stained with Wright–Giemsa stain provides for observation of morulae in the cytoplasm of infected circulating leukocytes of 1–20% of patients with HME and 20–80% of patients with HGA [7, 12, 17, 20, 21] during the first week of infection,

which is highly suggestive of ehrlichial or anaplasma infection. However, blood smear examination is insensitive and should be performed by an experienced microscopist [20, 21].

Serologic assays are commonly available through multiple commercial and state public health laboratories. Indirect IFA assay is most commonly used. Sensitivity of the IFA assay is substantially dependent on the timing of collection of the sample. As the illness progresses to 7–10 days, the sensitivity of IFA serology increases. The IFA is estimated to be 94–100% sensitive after 14 days of illness onset, and that sensitivity is increased if paired samples are tested [20, 21]. Paired serum specimens taken early (i.e., acute) and later (i.e., convalescent) in the disease course represent the preferred specimens for evaluation. Typically, these specimens should be taken at least 2–3 weeks apart to examine for a fourfold or greater increase in antibody titer [20, 21, 54]. Although IFA remains the principle serological assay for the diagnosis of ehrlichial infections, no standardized antigens, conjugates, or agreement on what constitutes a positive result exists amongst various testing laboratories. Individual laboratories should be consulted regarding their test threshold levels. ELISA is also available for serological studies, however, tests used in ehrlichial diagnosis are qualitative and cannot be used effectively to monitor increases or decreases in antibody titer [12, 20, 21].

Another approach to diagnosing ehrlichial infections is immunohistochemical (IHC) staining of antigens in formalin-fixed, paraffin-embedded biopsy or autopsy tissues. This test can be particularly useful to diagnose fatal infections in those patients for whom diagnostic levels of antibodies have not developed before death. Immunostaining for *E. chaffeensis* and *A. phagocytophilum* is also offered by the CDC, as well as certain university-based hospitals and commercial laboratories in the United States [12, 21, 54].

Another diagnostic alternative is the recovery of *E. chaffeensis* and *A. phagocytophilum* in culture; because these agents are obligate intracellular pathogens, they must be isolated by using cell culture techniques that are typically more labor-intensive and time-consuming than serologic, molecular, or IHC assays. As a result, culture is rarely used for diagnosis, and other methods are typically used to confirm infection [20].

11.2.3.2 Viral agents

11.2.3.2.1 Colorado tick fever virus

Epidemiology and clinical manifestations Colorado tick fever virus (CTFV) is a member of the *Coltivirus* genus, family *Reoviridae*. The primary vector for CTFV is the wood tick, *Dermacentor andersoni*. The enzootic cycle of CTFV involves larval and nymphal stages of *D. andersoni* and small mammals. Humans are incidental hosts, and become infected following the bite of an adult tick. Human cases of Colorado

tick fever overlap with the range of *D. andersoni* (western United States and southwestern Canada, at elevations above 1200 m). Most cases of Colorado tick fever are reported during the summer months, when ticks are seasonally active. The overall incidence of Colorado tick fever is higher in males than females. Most studies report highest incidence in young adults in the United States, although a recent study of cases in three states from 1995 to 2003 reported highest incidence in persons aged 61–70 years [5].

Following an incubation period of usually 2–5 days, symptoms appear abruptly. In the study reported by Brackney et al. the most common reported symptoms were fever (98%), headache (78%), and myalgia (75%) [5]. Other symptoms include pharyngitis, fatigue, rash, nausea, and vomiting. Neurologic signs occur in 5–10% of cases, most often children, and include nuchal rigidity and photophobia [73]. Recurrence of fever following a period of clinical improvement (saddleback fever) is common. Symptoms lasting 3 weeks or longer were reported in half the cases in the study by Goodpasture et al., although adults may experience fatigue for months [33]. Permanent sequelae and death are extremely rare.

Diagnosis and diagnostic challenges The primary means of diagnosis of Colorado tick fever are serologic tests. Serum is the indicated specimen source. Enzyme-linked immunosorbent assay and the plaque-reduction neutralization test (PRNT) are sensitive when performed on convalescent phase samples. A study by Attoui et al. demonstrated that ELISAs, including IgM capture ELISA, had poor sensitivity when performed on sera collected within 4 days of symptom onset, but showed high sensitivity when performed on sera collected between 13 and 49 days after onset [3]. In another study, neutralizing antibodies were not detected until nearly 3 weeks after onset of symptoms [46]. Reverse transcriptase PCR (RT-PCR) has been used to detect CTFV. RT-PCR sensitivity was 100% when performed on sera collected within 1 week of onset of symptoms, and dropped to 50% when performed on sera collected between 15 days and 6 weeks after onset of symptoms [46]. CTFV has been isolated from blood and cerebrospinal fluid in cell lines such as Vero and baby hamster kidney, BHK-21 [73]. Laboratory tests for CTFV are available at limited state public health laboratories in the western United States, the CDC, and commercial reference laboratories. There are no commercially available reagents or kits for the diagnosis of Colorado tick fever at the time of this writing.

11.2.3.2.2 Powassan virus

Epidemiology and clinical manifestations Powassan virus (POWV) is a member of the genus *Flavivirus*, family *Flaviviridae*. POWV consists of two lineages associated with human infections: lineage I (prototype POWV) and lineage II [deer tick virus (DTV)] [23, 67]. The lineages are antigenically related, and distinguishable using nucleotide

sequencing. POWV is widely distributed in the Northern Hemisphere, including eastern Canada, northeastern and north-central United States, and eastern Russia. In North America, lineage I is found primarily in eastern Canada and northeastern United States, while lineage II is found additionally in the north-central United States [23]. The enzootic cycle of lineage I includes *Ixodes cookei* and medium-sized mammals such as woodchucks. The enzootic cycle of lineage II consists of *Ixodes scapularis* (deer tick) and white-footed mice [24]. To date, less than 50 cases of POWV illness have been reported in North America [24]. As with other tick-borne diseases, human infections peak during the summer months when ticks are most active. The incidence may be increasing, in part due to increased surveillance for West Nile virus encephalitis, resulting in the incidental identification of POWV cases [24].

POWV causes encephalitis in humans. Following an incubation period of 8–34 days, cases may present with a prodromal stage characterized by sore throat, headache, and confusion. The clinical course usually progresses to include nausea and vomiting, seizures, paralysis, coma, and respiratory failure secondary to neurologic impairment. The case fatality rate is approximately 10%. Long-term neurologic sequelae, such as upper limb paralysis, cognitive loss, and recurrent headaches, are present in at least 50% of survivors [24].

Laboratory diagnosis and diagnostic challenges Serologic methods such as IgM-capture ELISA, PRNT, and hemagglutination inhibition are the primary means of diagnosis of Powassan encephalitis. These tests can be performed on serum or cerebrospinal fluid. ELISAs will cross-react with antibodies of other flaviviruses, whereas PRNT is specific for POWV. Laboratory-developed RT-PCR assays, performed post-mortem on brain tissue, have been described [79]. Testing is available at selected public health laboratories, including the CDC. There are no commercially available reagents or kits for the diagnosis of Powassan encephalitis at the time of this writing.

11.2.3.3 Parasitic agents

11.2.3.3.1 Babesia

Epidemiology and clinical manifestations The genus *Babesia* is comprised of a number of species. In the United States, clinically significant species include the widely distributed *B. microti*, *B. duncani* in the Pacific Northwest, and *B. divergens* and the *B. divergens*-like strain MO1 in the central states. In Europe, clinically important species include *B. divergens*, the *B. divergens*-like strain EU-1, and to a lesser extent *B. microti*. In Japan, *B. microti* or *B. microti*-like strains have been linked to human disease [81]. *Babesia* spp. are transmitted by the bite of ixodid ticks. In the United States, cases of babesiosis overlap with the range of *I. scapularis*. Correspondingly, most cases occur in the northeast and upper midwest United States. *Ixodes ricinus* is the primary vector in Europe. Most human

cases are reported during the summer months, when both tick activity and human outdoor activities increase. Transfusion-transmitted babesiosis also occurs [47].

Babesiosis usually presents as a nonspecific, viral-like illness following an incubation period of 1–6 weeks. Three syndromes are described: (i) asymptomatic (about one-third of all infections) [81]; (ii) a mild to moderate viral-like illness characterized by fever, chills, fatigue, headache, and arthralgia, that can last for weeks to months; and (iii) severe disease with death or persistent relapsing illness. The severe syndrome is more likely to occur in patients with underlying immunosuppression (AIDS, splenectomy, malignancy). Among immunocompromised patients, mortality can reach 20% despite specific treatment. Among patients hospitalized with severe babesiosis, mortality rates of 5–9% have been reported. Evidence suggests that *B. divergens* and *B. duncani* cause more severe infection than *B. microti* [81].

Laboratory diagnosis and diagnostic challenges Babesiosis is usually diagnosed by detection of the parasite in thin and thick blood films stained by the Giemsa or Wright's stain. Thin films should be examined at a magnification of 1000× to avoid missing the small *Babesia* ring forms. Parasitemia can be < 1% early in illness, further decreasing the sensitivity of microscopy. The small, delicate ring forms of *Babesia* can be confused with those of *Plasmodium falciparum*, necessitating a thorough patient history.

The indirect IFA test is widely available in reference laboratories for the detection of anti-*B. microti* IgG and IgM in serum. Serologic testing is important after the first week of illness, when blood smears often are negative [44]. An IFA test that detects anti-*B. microti* IgG was shown to be sensitive and specific in a multisite study. Against a reference standard consisting of a clinical diagnosis, and either a positive blood smear or culture in hamsters, IFA sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 88–96, 90–100, 69–100, and 96–99%, respectively [44]. In another study, IgM IFA was also shown to be highly sensitive and specific when compared to a fourfold rise in IgG titer or a positive PCR. IgM IFA sensitivity, specificity, PPV, and NPV were 91, 99, 86, and 99%, respectively [45]. Although there are no FDA-cleared *Babesia* IFA kits commercially available in the United States, RUO kits and individual reagents are commercially available (Focus Diagnostics, Cypress, CA; Fuller Laboratories, Fullerton, CA). Laboratories using these reagents for diagnostic purposes must first establish the performance characteristics for their patient population.

Real-time PCR has been shown to offer sensitive and specific detection of *B. microti*, detecting lower levels of circulating parasites than can be detected by microscopic examination of blood films [80]. This improvement in analytical sensitivity is potentially most beneficial in chronic

infections when parasitemia is low or intermittent. There are no commercially available PCR reagents or kits for the diagnosis of babesiosis at the time of this writing.

Specimen collection, transport, and storage Peripheral blood collected in EDTA is the preferred specimen for microscopic examination of blood parasites. The same specimen can also be used for PCR [80]. Thin and thick films should be prepared ideally within 60 min, at most within several hours of blood collection. Serologic testing for babesiosis is performed from serum, collected and stored under routine conditions.

11.3 Newly emerging tick-borne disease

11.3.1 Novel phlebovirus

In June 2009, two cases of an influenza-like illness with diarrhea, thrombocytopenia, and leukopenia were reported from northwestern Missouri, United States. Both cases were men who resided in rural areas, and reported tick bites. Cell cultures of blood leukocytes yielded a novel phlebovirus of the *Bunyaviridae* family, tentatively named the Heartland virus [51]. The Heartland virus was later isolated from *Amblyomma americanum* (lone star) ticks collected from rural areas within or near the patients' farms [74]. Phylogenetic analysis of the viral nonstructural protein sequence showed that the tick strains and human strains were highly similar. Case-based surveillance identified six additional cases during 2012–2013 [65]. All patients participated in outdoor activities, and five reported tick bites during a 2-week period prior to their illness. Virus was identified by detection of RNA from blood or tissue by a laboratory-developed reverse-transcriptase-PCR, or by detection of virus-specific plaque reduction neutralization antibodies [65]. Being a recently described virus, there are no commercially available diagnostic tests at the time of this writing.

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Mycoplasma

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12.1 Introduction and clinical considerations

12.1.1 Mycoplasmas and ureaplasmas as human pathogens

Mycoplasmas and ureaplasmas are the smallest free-living microorganisms. Their simplicity is reflected by their fastidious nature and complex cultivation requirements. They demand special techniques for their laboratory detection and characterization that are often not available in hospital microbiology laboratories. Their fastidious nature and lack of appreciation for their importance as human pathogens has led to them being ignored by many diagnostic laboratories. This situation has changed somewhat in recent years because of greater appreciation for their clinical importance, improved methods for detection, including nonculture-based molecular assays such as polymerase chain reaction (PCR), availability of commercially manufactured growth media, serological assays, and products sold as complete diagnostic kits. However, the number and types of products available for detection and characterization of mycoplasmas is still very limited in comparison to products sold commercially for other types of bacteria. A number of products available for use in mycoplasma detection and characterization have never been subjected to rigorous external evaluation so that recommendations for their use cannot be made based on objective evidence of performance.

Readers interested in an in-depth discussion of the taxonomy, disease associations, and biological characteristics of mycoplasmas and ureaplasmas of humans are referred to other reference texts [50] for more detailed information on these aspects of human mycoplasmas. Only a brief synopsis of this information is included here.

Class *Mollicutes* ("soft skin"), includes the bacteria commonly referred to as mycoplasmas ("fungus-form"). The class is comprised of four orders, five families, eight

genera, and about 200 known species. Sixteen species have been isolated from humans, excluding occasional animal mycoplasmas that have been detected in humans from time to time, but are generally considered transient colonizers. *Mollicutes* are the smallest life-forms capable of an independent existence. They permanently lack cell walls and peptidoglycan precursors and require a complex enriched medium containing serum, amino acid precursors, nucleotides, yeast extract, and other supplements.

Mycoplasma pneumoniae is the best known and most intensely studied mycoplasma that occurs in humans. This organism causes pharyngitis, tracheobronchitis, and pneumonia in persons of all age groups. Some people may experience extrapulmonary complications including skin rashes, hemolytic anemia, arthritis, meningoencephalitis, peripheral neuropathy, and pericarditis. *Mycoplasma pneumoniae* has been detected by culture or PCR from extrapulmonary sites such as synovial fluid and cerebrospinal fluid, pericardial fluid, and skin lesions. Clinical manifestations are not sufficiently unique to allow differentiation from infections caused by other common microorganisms.

Ureaplasma urealyticum, *U. parvum*, and *M. hominis* are commensal organisms in the lower genital tract in many sexually active adults. Their common occurrence in healthy persons has lead to difficulty in accepting these organisms as independent causes of disease. Nevertheless, there is evidence that these organisms play etiologic roles in some conditions. *Ureaplasma* spp., particularly *U. urealyticum*, and *M. genitalium* are causes of nongonococcal urethritis in men. *Mycoplasma hominis* causes a small portion of cases of pyelonephritis and has been isolated from endometrium and fallopian tubes of women with salpingitis. *Mycoplasma genitalium* also plays a significant role in female cervicitis and salpingitis. Ureaplasmas can cause placental inflammation and may invade the

amniotic sac early in pregnancy in the presence of intact fetal membranes, causing persistent infection and adverse pregnancy outcome, including premature birth. *M. hominis* and *Ureaplasma* spp. have been isolated from the blood of women with postpartum or postabortal fever. Congenital pneumonia, bacteremia, progression to chronic lung disease of prematurity, and even death have occurred in very low birthweight infants and have been attributed to ureaplasma infection of the lower respiratory tract. Both *M. hominis* and *Ureaplasma* spp. have been isolated from maternal and umbilical-cord blood, as well as the blood of neonates. Both organisms can also invade the cerebrospinal fluid of neonates.

Mycoplasmas and ureaplasmas can be important pathogens in individuals with immunodeficiencies and in other immunosuppressed persons such as organ transplant recipients. They can cause invasive disease of the joints and respiratory tract with bacteremic dissemination, especially individuals with antibody deficiencies. *M. hominis* has been isolated in pure culture from the bloodstream after renal transplantation, trauma, and genitourinary manipulations, and it has also been found in women with postpartum endometritis and in wound infections. *Mycoplasma fermentans*, *Ureaplasma* spp., and *M. hominis* have been detected in synovial fluid of persons with rheumatoid arthritis and other inflammatory arthritides, although their precise contribution to these diseases is still uncertain. There is also some evidence that *Ureaplasma* spp. are involved in reactive arthritis. *Mycoplasma fermentans* has been detected in adults with an acute influenza-like illness, and in bronchoalveolar lavages, peripheral blood lymphocytes, and bone marrow from patients with AIDS.

12.1.2 Indications for diagnostic testing

Due to the relatively mild clinical course of most respiratory tract infections caused by *M. pneumoniae*, lack of widespread availability, and considerable expense of rapid diagnostic tests, many clinicians choose to treat empirically when infection with this organism is strongly suspected. However, laboratory testing would almost certainly become more common in the United States if rapid molecular-based assays become more readily available as they have in Europe. It is reasonable to obtain laboratory testing for *M. pneumoniae* by molecular-based procedures when respiratory illness in an adult or child is of sufficient severity to warrant hospitalization and no other causative microorganism is readily apparent. Testing should also be considered if there is an unsatisfactory clinical response to macrolide treatment because this may indicate the presence of a macrolide-resistant strain; in patients with an underlying comorbid condition or immunodeficiency that would make severe and disseminated disease more likely; and when there are significant extrapulmonary symptoms present. Measurement of serum antibodies, though

widely used, has limited value for laboratory diagnosis of *M. pneumoniae* infection if direct detection methods such as PCR are available, especially in the first few days of illness. Some infections that are well documented by seroconversion are negative by the PCR assay, so a single test may not always yield the microbiological diagnosis.

Lack of commercial molecular-based tests for detection of *M. genitalium* in the United States has greatly limited interest among physicians in seeking this mycoplasma in clinical specimens since it cannot be satisfactorily isolated by culture and no serological tests are available. Given the growing evidence for the importance of *M. genitalium* in a variety of urogenital conditions and the likelihood of venereal transmission to other individuals, use of molecular-based assays would undoubtedly increase if they become available. If *M. genitalium* is detected in lower urogenital tract specimens from cervix, urethra, endometrium, or fallopian tube from a symptomatic patient, it should be considered clinically significant. Unlike *M. hominis* and *Ureaplasma* spp., *M. genitalium* is less likely to be carried by asymptomatic persons.

Since *M. hominis* and *Ureaplasma* spp. are commonly found in the lower urogenital tracts of healthy adults, and healthy infants may be transiently colonized on the skin or in the upper respiratory tract with organisms of maternal origin, a positive PCR or culture result from specimens from these sites can usually be expected and has little clinical value. Positive PCR or culture results for *Ureaplasma* spp. from the urethra in men with urethritis, from tracheal aspirates of neonates with respiratory distress, from the bloodstream or cerebrospinal fluid in neonates with pleocytosis, or from sterile extragenital sites should be considered diagnostic of clinically significant infection. Likewise, detection of *M. hominis* in any quantity from sterile body fluids or tissues of neonates or adults is significantly associated with disease. *Ureaplasma* spp. and *M. hominis* can be relatively common opportunistic pathogens causing systemic infections involving various body sites in immunosuppressed people. There are many reports of systemic infections that were caused by *M. hominis* or *Ureaplasma* spp. for which diagnosis was delayed simply because no one attempted to perform any diagnostic laboratory tests. Even though PCR may be more sensitive than culture for detection of ureaplasmas and *M. hominis*, it is less widely available since there are no commercial PCR assays available in the United States and it requires submission to a reference laboratory with molecular diagnostic facilities. Culture for these organisms is available in reference laboratories and many larger hospital laboratories and usually requires about 2–5 days for detection of growth.

No specific recommendations can be made for laboratory diagnosis of *M. fermentans* since its role in human disease is much less established than the other species previously discussed. PCR is the best diagnostic approach since

optimum cultivation conditions are not well described and a PCR assay would be necessary anyway to identify the organism to species level. Some reference laboratories offer their own PCR assay to detect *M. fermentans*.

12.2 Culture-based diagnosis

Successful cultivation of mycoplasmas and ureaplasmas from clinical specimens involves careful consideration of the type of specimen to be obtained, the type of patient suspected of having infection, and organisms sought. For respiratory specimens, if *M. pneumoniae* is the only organism of interest, it is sufficient to inoculate a single general-purpose medium type such as SP4 broth and agar, using a broth-to-agar technique [45]. Culture is less likely to be used than PCR because of its lower sensitivity and slow turnaround time, up to 6 weeks. However, culture is sometimes useful to obtain clinical isolates of *M. pneumoniae* for epidemiological purposes or to measure *in vitro* antimicrobial susceptibilities. Likewise, if only *M. hominis* or *Ureaplasma* spp. are of interest, it is sufficient to set up cultures using a single broth and agar formulation such as 10B broth and A8 agar. All of these media are available from various commercial sources. Mycoplasmas of human origin grow best in an atmosphere supplemented with 5–10% CO₂ at 37°C. If the specimen to be cultured is from an extragenital or extrapulmonary site, is a sterile body fluid or tissue, and/or the patient is immunosuppressed, any mycoplasma of human origin or accidental infection with a mycoplasma of animal origin should be considered. Many animal mycoplasmas are not reliably detected in culture using protocols described for the more common species, and alternative nonculture-based tests such as PCR assays should also be performed, if suitable reagents are available. However, isolation may occasionally be successful, usually after prolonged incubation in culture. SP4 broth and agar, containing both glucose and arginine, should also be inoculated and incubated under anaerobic conditions.

Gram stains can assist in excluding the presence of other bacteria from a clinical specimen, even though mycoplasmas cannot be visualized since they lack cell walls. In order to stain mycoplasmas in clinical specimens or cultures, a DNA fluorochrome stain such as Hoechst 33258 can be used. These types of stains are commercially available from several vendors.

Procedures for performance and interpretation of mycoplasma cultures and identification of species are available in other reference texts [45,50]. Therefore, only brief comments will be presented here. *Ureaplasma* spp. can be easily identified to the genus level by the characteristic brown granular colonial morphology that occurs due to urease activity on an agar such as A8 that contains a CaCl₂ indicator. A number of different techniques are available to identify an unknown large colony mycoplasma definitively

to species level. Using a combination of biochemical reactions, colony morphologies, and growth rates, a preliminary characterization of the most frequently encountered isolates in clinical settings often can be achieved [50]. From a practical standpoint, PCR assays available through specialty or reference laboratories have now replaced older phenotypic methods for definitive mycoplasmal species identification from culture-positive clinical specimens and differentiation of *U. urealyticum* from *U. parvum*, as well as providing an alternative to primary culture-based detection of the organisms from clinical specimens. Culture of *Ureaplasma* spp. and *M. hominis* is still widely used because of its relative simplicity, rapid turnaround time, low cost, and because it does not require laboratory facilities and staff experienced with molecular-based techniques.

12.2.1 Specimens appropriate for culture

Respiratory tract specimens, including nasopharyngeal and throat swabs, sputum, pleural fluid, bronchoalveolar lavage fluid, endotracheal aspirates, and lung tissue obtained by biopsy are all acceptable specimens for cultural detection of *M. pneumoniae*. Cultures of cerebrospinal fluid, other sterile body fluids, or tissues can also be performed for detection of *M. pneumoniae* if extrapulmonary manifestations are present. However, PCR will provide a more sensitive and rapid means for detection that is particularly important for extrapulmonary sites. Urethral swabs in men are preferred over urine for detection of *M. hominis* and *Ureaplasma* spp. Prostatic secretions, semen, and urinary calculi can be cultured. For females, urine and cervical or vaginal swabs are acceptable. Avoid specimens that are contaminated by lubricants or antiseptics. Urine samples from females are most meaningful when obtained by catheter or suprapubic aspiration and if numbers of organisms are quantitated. Endometrial tissue, tubal samples, or Pouch of Douglas fluid can be obtained to confirm mycoplasmal etiology of pelvic inflammatory disease or post-partum fever. For women with clinical amnionitis, amniotic fluid, blood, and placenta should be cultured. Culture of nasopharyngeal, throat, and endotracheal or gastric secretions of neonates is appropriate, especially if birthweight is < 1000 g., and there is clinical, radiographic, laboratory, or other evidence of pneumonia.

Extragenital or extrapulmonary specimens submitted for culture should reflect the site of infection and disease process. Ureaplasmas and mycoplasmas should always be sought from synovial fluid in the setting of acute arthritis in hypogammaglobulinemia. Other sterile fluids, including peritoneal fluid, pericardial fluid, cerebrospinal fluid, and blood are suitable for culture if clinical conditions warrant. Bone chips from patients with chronic osteomyelitis without a proven bacterial etiology are also appropriate for culture as are wound aspirates and tissue collected by biopsy or autopsy. *Mollicutes* are inhibited by sodium

polyanethol sulfonate (SPS) present in most commercial blood culture media, but the effect can be overcome by adding 1% gelatin. Commercial blood culture media designed for use in automated instruments may support growth of *M. hominis*, but the instruments usually do not flag the bottles containing this organism as positive. Successful isolation of *M. hominis* and *Ureaplasma* spp. from blood can be achieved by inoculating ≥ 10 mL directly into liquid mycoplasmal growth media in at least a 1:10 ratio. Smaller volumes can be used for neonates or children. There is sometimes difficulty in detecting a color change in liquid media in the presence of large amounts of blood due to hemolysis, and there may be a slight color change immediately after introducing blood into liquid media. Serial dilution of the original specimen and subcultures to agar will help distinguish such nonspecific color changes.

12.2.2 Specimen collection and transport

Due to their lack of a cell wall, mycoplasmas are extremely sensitive to adverse environmental conditions, particularly to drying and heat. Therefore, a suitable transport system to preserve specimen viability until cultures can be inoculated is essential under most conditions. If shipping is to occur, it is imperative to have a transport system containing some type of cryoprotectant in order to prevent lysis of the organisms during freezing. Specimens should be inoculated at bedside whenever possible, using appropriate transport, or mycoplasma culture medium. Liquid media including SP4 glucose, arginine or urea broths, or 10B arginine/urea broths, manufactured and sold in the United States by various commercial vendors can serve as transport as well as growth media. 2 SP (10% v/v heat inactivated fetal calf serum with 0.2 M sucrose in 0.02 M phosphate buffer, pH 7.2), and trypticase soy broth with 0.5% v/v bovine albumin are also acceptable transport media. Specialized commercial liquid transport media such as A3B have been designed by deletion of some of the growth supplements present in other growth media so that increase in pH caused by urea hydrolysis by ureaplasmas will be delayed and result in less toxicity to the organisms during transport. Some transport media also contain buffers to maintain a neutral pH. Laboratories may choose to stock a single universal transport medium that can be used for mycoplasmas, ureaplasmas, chlamydiae, and viruses. Most universal transport media contain inhibitors to prevent bacterial or fungal overgrowth from specimens obtained from nonsterile sites that have their own indigenous microbial flora. Avoid using any transport system that contains gentamicin or another aminoglycoside since these antibiotics can be inhibitory to mycoplasmas and ureaplasmas. The M4, M5, and M6, UTM, and BD UVT media are examples of universal transport systems available from various suppliers. Additional specialized transport media for genital mycoplasmas are also available in Europe from various suppliers.

Fluids or tissues do not require special transport media if cultures can be inoculated within 1 h, provided the specimens are protected from drying. Tissues can be placed in a sterile container, which can be delivered to the laboratory immediately. Otherwise, all specimens should be placed in transport media if delay in culture inoculation is anticipated. When swabs are obtained, care must be taken to sample the desired site vigorously to obtain as many cells as possible since mycoplasmas are cell-associated. Flocked swabs can be used to obtain more cells from the specimen. Dacron, or polyester swabs with aluminum or plastic shafts are preferred. Wooden shaft cotton swabs should be avoided because of potential inhibitory effects. Specimens should be refrigerated if immediate transportation to the laboratory is not possible. If specimens must be shipped and/or if the storage time is likely to exceed 24–48 h prior to processing, the specimen in transport medium with cryoprotectant should ideally be frozen and shipped on dry ice to prevent loss of viability. ThermoFisher transport media are reported by their manufacturer to maintain mycoplasma viability for up to 48 h when stored at 2–8°C. Becton Dickinson Diagnostics provides data on their internet web site to support storage at 2–25°C for up to 48 h using their UVT transport media while maintaining viability. Mollicutes can be stored for long periods in appropriate growth or transport media at –70°C or in liquid nitrogen. Storage at –20°C for even short periods can result in loss of viability. Frozen specimens may be shipped with dry ice to a reference laboratory if necessary. When specimens are to be examined, they should be thawed rapidly in a 37°C water bath. In order to maximize yield of mycoplasmas from clinical specimens, serial ten-fold dilution in liquid media to 10^{-3} to remove inhibitors and inoculation of broth dilutions to appropriate agar has been suggested [45,50]. Tissues should be minced prior to dilution.

12.2.3 Commercial growth media and diagnostic kits

No single medium formulation is ideal for all pertinent species due to their different properties, optimum pH, and substrate requirements. Mycoplasmas of importance for human infections utilize glucose and/or arginine as metabolic substrates, whereas ureaplasmas utilize urea. The various medium formulations designed for their cultivation must provide these substances along with other essential ingredients, including serum as a cholesterol source, yeast extract, and a pH indicator for detection of growth when the substrate is metabolized. SP4 broth and agar can be used for both *M. pneumoniae* and *M. hominis* [45]. These media can also be used to cultivate other fastidious and slow-growing species. Shepard's formulations of U9 or 10B broth have been used successfully for cultivation of *Ureaplasma* spp., with A7 or A8 agar as the corresponding solid media

[45]. Other media such as bromothymol Blue (B broth), Hayflick's medium, Boston broth, pleuropneumonia-like organisms (PPLO) medium, and several others have also been used for detection of mycoplasmas and/or ureaplasmas in clinical specimens. Detailed description of essential components of growth medium, formulations, instructions for preparation of various nonproprietary types, and incubation conditions for different mycoplasmas can be found in other reference texts [45].

Multiple vendors including ThermoFisher and Hardy Diagnostics in the United States market various formulations of growth media, including 10B broth, A7 and A8 agars for cultivation of *M. hominis* and *Ureaplasma* spp., 10B broth with arginine and arginine broth for *M. hominis*; and SP4 broth and agar for *M. pneumoniae*, *M. hominis*, and other *Mycoplasma* spp. The PPLO broth and agar can be used for general purpose cultivation of *Mycoplasma* spp. Since the broth does not contain a pH indicator, it is not possible to detect growth by direct observation of the liquid medium. Some manufacturers will produce customized orders of various sizes for most of their media, with or without inhibitors, or special additives, as desired by the purchaser. Most broths and transport media can be obtained in lyophilized form to prolong shelf-life. For laboratories that prefer to prepare their own media, individual media components described in other reference texts [45] can be obtained from various commercial sources.

Mycoplasma Experience manufactures and sells a variety of diagnostic products for use in detecting mycoplasmas of veterinary importance, primarily marketing their products in the United Kingdom and Europe. However, they also produce their own line of liquid and solid media suitable for use in detecting human mycoplasmas and ureaplasmas. Mycoplasma Experience manufactures their own broth formulations containing specific biochemical substrates, e.g., for glucose or arginine metabolism, to aid in preliminary characterization of species. Media can be purchased as ready to use broths and agar plates. Liquid media is also available frozen in various volumes. Frozen agar supplements that can be added to agar are also available for purchase. Quality control strains of *M. pneumoniae* and *U. urealyticum* are available in 96-well microtiter plates.

Several French companies produce a variety of liquid and solid media for urogenital mycoplasma and ureaplasma detection, with many of the same names and formulations as those sold in the United States. Additionally, there are some kits that can be used for detection as well as presumptive species identification. Some of the products are the companies' versions of the traditional media initially described by mycoplasma researchers, while others are proprietary products of their own design. The diagnostic kits have been marketed primarily in Europe, but some have now been introduced into the United States. The



Figure 12.1 MYCOSCREEN PLUS (ELITech). MYCOSCREEN PLUS is a commercial kit for specimen transport, enumeration, and identification of *Ureaplasma* spp. and *M. hominis* in the urogenital tract. The kit consists of UMMt liquid transport medium and UMMlyo lyophilized growth medium in microplate wells. Detection of growth of the organisms is based on color change in liquid medium containing urea and arginine. Species identification is based on differential inhibition by erythromycin and lincomycin. This product is available in the United States. (Photograph courtesy of ELITechGroup, Inc.) (See insert for color representation of the figure.)



Figure 12.2 MYCOFAST US (ELITech). MYCOFAST US is a commercial kit for the detection, identification, and enumeration of *Ureaplasma* spp. and *M. hominis* in clinical specimens. The kit consists of microplates containing lyophilized growth substrates and antimicrobials. Detection of growth of the organisms is based on color change in liquid medium containing urea and arginine. Species identification is based on differential inhibition by erythromycin, trimethoprim/sulfamethoxazole, and lincomycin. This product is available in the United States. (Photograph courtesy of ELITechGroup, Inc.) (See insert for color representation of the figure.)

ELITech products MYCOSCREEN PLUS and MYCOFAST US are now distributed in the United States by Wescor, Inc. (Figures 12.1 and 12.2).

Diagnostic kits typically consist of strips with wells containing specific dried or lyophilized substrates and inhibitors. Specimens are placed in a suspension-transport medium that is used to inoculate wells. The detection, identification, and quantitation of organisms are based

on the color change of specific wells containing substrates and inhibitors. Some products also contain an antimicrobial susceptibility testing component. Descriptions of some of the commercial diagnostic kits along with their manufacturers are provided in Table 12.1. Some commercially sold diagnostic products have undergone external validation in comparison to one another and with other non-proprietary products in clinical trials that have been published in peer-reviewed journals. Citations, when available, are also indicated in Table 12.1. Some products included in earlier published evaluations are now sold by different companies than when the original evaluations were performed. Other commercial products from various manufacturers and countries exist, but to our knowledge, have not been subjected to rigorous comparison with standard methods of testing. None of these diagnostic kits has undergone complete evaluation and received approval by the United States Food and Drug Administration (FDA) as of this writing.

Abele-Horn et al. [1] evaluated the MYCOSCREEN broth and agar (ELITech), arginine broth and A7 agar (bioMérieux), U9 broth, arginine broth, and A7 agar (Bio-Rad), and mycoplasma broth and A7 agar (Biotest AG) in 298 clinical specimens and concluded that the commercial media were generally satisfactory for organism detection and identification when compared to unspecified noncommercial media. These authors also evaluated diagnostic kits for quantitative organism detection and identification, which included the Mycoplasma IST (bioMérieux), Mycoplasma Duo (Bio-Rad), and the Mycoplasma "All-In" (no longer available). Performance of these kits was generally satisfactory, but when quantitation is desired, standard methods were better. Cheah et al. [8] compared the Mycoplasma Duo kit with PCR for detection of *Ureaplasma* spp. in endotracheal aspirates from 60 premature neonates. The overall agreement for 13 total *Ureaplasma*-positive infants between the two tests was 96%. Evans et al. [16] reported that the Mycoplasma Duo kit actually detected more positive urogenital and placental specimens than traditional culture using A7 agar.

Clegg et al. [10] compared the Mycoplasma IST kit with arginine broth, 10C broth, and A7 agar to determine the prevalence of *M. hominis* and *Ureaplasma* spp. in 100 vaginal specimens. The Mycoplasma IST kit gave a sensitivity and specificity of 92.9% and 86.7% for *M. hominis* versus 97.4% and 72.7% for *Ureaplasma* spp., respectively. They noted that false-positive reactions may occur in the Mycoplasma IST kit if contaminating bacteria are present. Therefore, organism identification and purity should be verified by colony morphology on agar plates. Vasquez and colleagues [44] evaluated Mycoplasma IST and MYCOFAST All-In for detection of *M. hominis* and *Ureaplasma* spp. against isolation on A7 agar (bioMérieux), testing 123 vaginal and cervical swabs in women

and urethral swabs in men. The sensitivity was 100% for *M. hominis* and *Ureaplasma* spp. for Mycoplasma IST versus 29.2% and 50% for MYCOFAST All-In. Specificity was lower for *Ureaplasma* spp., (59.7%) for Mycoplasma IST than in MYCOFAST All-In (100%).

Rastawiki et al. [38] compared PCR with the Mycoplasma IST 2 against culture for the detection of *Ureaplasma* spp. and *M. hominis* in 173 genitourinary samples. Their study showed 100% sensitivity and 94% and 90% specificity, respectively for detection of *Ureaplasma* spp. using PCR and Mycoplasma IST 2. *Mycoplasma hominis* was detected in 26 samples only by Mycoplasma IST.

Lower rates of organism detection by some commercial kits in comparison to standard methods has been attributed to the fact that standard methods usually include recommendations for serial dilution of the original specimen to remove inhibitors that may be present. Some of the kits and media such as those described above have a shelf life of several months, making them attractive for use in laboratories that have only occasional needs for performing mycoplasma cultures, but they are considerably more expensive to purchase than media and reagents prepared in-house. Commercial products have been designed for use with specimens from the urogenital tract, but some are now recommended for use in detecting ureaplasmas in gastric and/or endotracheal secretions from neonates.

The very limited independent evaluations of some of the commercial media suggest that in the hands of experienced laboratorians, these media may perform in a satisfactory manner for routine detection of urogenital mycoplasma and ureaplasma infections. Some products have never been evaluated against standard noncommercial media, so their performance characteristics are largely unknown. The commercial kits for organism detection, quantitation, and identification have also undergone relatively limited comparative studies, but most appear to perform in a generally satisfactory manner.

For self-prepared media, as well as ready-to-use commercial media, rigorous quality control is crucial for detection of fastidious mollicutes. New lots or batches of broth are considered satisfactory if the numbers of organisms that grow are within 10- to 100-fold of the reference batch. Agar plates should support growth of at least 90% of the colonies that are supported by the reference media. Lyophilized cultures of American Type Culture Collection (ATCC) type strains can be used for laboratory quality control procedures for mycoplasma media. Low-passage clinical isolates of *Mycoplasma* and *Ureaplasma* spp. should also be included in quality control testing. Almost any formulation of mycoplasma growth medium that is prepared correctly can be expected to grow prototype laboratory-adapted strains. The real challenge is the ability to isolate mycoplasmas and ureaplasmas from clinical specimens, hence the need for inclusion of known

Table 12.1 Commercial detection and susceptibility test kits for *Ureaplasma* spp. (Us) and *M. hominis* (Mh). The information included in this table was current as of the time of writing

Product name (Company, number of tests)	Parameters				
	Samples	Format	Detection, identification and enumeration	Susceptibility testing*	Antibiotics tested
Mycoplasma IST2 (bioMérieux, 25)	Cervico-vaginal, urethral swabs, semen	Microplates of 22 wells	Yes Us ≥ 10 ⁴ CFU/mL Mh ≥ 10 ⁴ CFU/mL	Yes (categorization S, I, R)	Tetracycline, doxycycline, azithromycin, clarithromycin, josamycin, clindamycin, pristinamycin, ofloxacin, ciprofloxacin
Mycoplasma Duo (Bio-Rad, 20)	Vaginal, endocervical, urethral swabs, semen, urine	Microplates of 6 wells	Yes Us ≥ 10 ⁴ CCU/mL Mh ≥ 10 ⁴ CCU/mL	No	NA
SIR Mycoplasma (Bio-Rad, 10)	Broth culture of Us or Mh from well X of Mycoplasma Duo or from A3 broth inoculated with the sample and enriched with mycoplasmas	Microplates of 16 wells	No	Yes (categorization S, I, R)	Tetracycline, doxycycline, erythromycin, azithromycin, josamycin, clindamycin, pristinamycin, ofloxacin
Mycokit-NUM (Eurobio, 24)	Vaginal, endocervical, urethral swabs	Microplates of 16 wells	Yes Us ≥ 10 ⁴ CCU/mL Mh ≥ 10 ⁴ CCU/mL	No	NA
Mycokit-ATB (Eurobio, 6)	Broth culture of Us or Mh A3 broth inoculated with the sample and enriched with mycoplasmas	Microplates of 16 wells	No	Yes (categorization S, I, R)	Fusidic acid, doxycycline, erythromycin, lincomycin, minocycline, pristinamycin, josamycin
MYCOSCREEN Plus (ELITech, 16 or 32)	Cervico-vaginal, urethral swabs, semen, urine	Microplates of 6 wells Liquid medium UMMt	Yes Us ≥ 10 ⁴ CCU/mL Mh ≥ 10 ⁴ CCU/mL (to be read on the microplate for urethral and cervicovaginal swabs) Us or Mh ≥ 10 ² CCU/mL if the UMMt medium is positive	No	NA
MYCOFAST Evolution 2 (ELITech, 12 or 30)	Cervico-vaginal, urethral, endotracheal swabs, semen, urine	Microplates of 20 wells	Yes Us ≥ 10 ⁴ CCU/mL for urethral swabs, ≥ 10 ³ CCU/mL for urines, semen, and endotracheal swabs Mh ≥ 10 ⁴ CCU/mL	Yes (categorization S, I, R)	Doxycycline, roxythromycin, ofloxacin
MYCOFAST Evolution 3 (ELITech, 12 or 30)	Cervico-vaginal, urethral, endotracheal, semen, urine	Microplates of 20 wells	Yes Us ≥ 10 ⁴ CCU/mL for urethral swabs, ≥ 10 ³ CCU/mL for urines, semen, and endotracheal swabs Mh ≥ 10 ⁴ CCU/mL	Yes (categorization S, I, R)	Doxycycline, azithromycin, roxithromycin, josamycin, pristinamycin, ofloxacin, ciprofloxacin
MYCOFAST US (ELITech, 30)	Cervico-vaginal, urethral, endotracheal, semen, urine, gastric secretions	Microplates of 20 wells	Yes Us 10 ³ to ≥ 10 ⁵ CCU/mL Mh ≥ 10 ⁴ CCU/mL	No	NA
MYCOFAST Revolution (ELITech, 50 microplates for screening, 25 microplates for numbering and susceptibility testing)	Cervico-vaginal, urethral, endotracheal, gastric, semen, urine	Microplates of 6 wells for screening Microplates of 20 wells for numbering and susceptibility testing	Yes Us ≥ 10 ⁴ CCU/mL for urethral swabs, ≥ 10 ³ CCU/mL for urines, semen, and endotracheal swabs Mh ≥ 10 ⁴ CCU/mL	Yes (categorization S, R)	Tetracycline, erythromycin, clindamycin, levofloxacin, moxifloxacin: at the breakpoint concentrations recommended by the CLSI guidelines

CFU, colony-forming unit; CCU, color-change unit; NA, not applicable/available; CLSI, Clinical and Laboratory Standards Institute.

* S, I, R: Susceptible, intermediate, or resistant.

positive specimens or recent clinical isolates as part of the quality control process. Testing inhibitory properties of media against growth of various other organisms likely present in specimens from nonsterile sites may also be worthwhile to prevent loss of mycoplasmas due to overgrowth of contaminating organisms. It is also important to watch carefully for the presence of contaminating bacteria that can cause false-positive results.

M. hominis is unique among the mycoplasmal species that occur in humans in that it may occasionally be detected growing in clinical specimens inoculated to bacteriologic media such as Columbia, chocolate, or even trypticase soy agar with 5% sheep blood after 3–5 days of incubation, usually in 5–10% CO₂ or anaerobic conditions. Many accidental detections of *M. hominis* infections of the urogenital tract or systemic sites have been reported because of the organism's ability to survive and grow under conditions not specifically designed for its cultivation. Laboratories that encounter pinpoint colonies that do not Gram stain should consider the possibility of *M. hominis* and attempt to establish the identity of the organism as a mycoplasma by subculture to appropriate mycoplasmal medium and proceeding accordingly, or placing the organism in suitable transport medium and referring it to a reference laboratory. Availability of frozen or freeze-dried mycoplasma media from commercial sources facilitates such transport or subculture that may be needed on an infrequent basis for low-volume laboratories that do not offer complete diagnostic service for mycoplasmas.

12.3 Serological diagnosis

Serological diagnosis of *M. pneumoniae* respiratory infections has been extremely important in the past because it was the only test many laboratories offered. However, serology has many drawbacks including: (i) the fact that it takes several days after infection before measurable antibody is produced; (ii) immunosuppressed persons are often unable to mount an antibody response; its retrospective nature requires acute and convalescent sera for optimum diagnostic accuracy; and (iv) the erratic nature of the humoral immune response in many immunologically normal persons. The prominent historical role of serology is mainly because of the relative lack of sensitivity and time-consuming nature of culture, as well as the carrier state that may occur in an unknown percentage of persons in the absence of acute infection. Serology is now becoming less important as rapid molecular-based assays become more widely available for direct detection of acute mycoplasmal infection and the limitations and inaccuracies of many commercial serological assays are becoming more apparent.

M. pneumoniae possesses both protein and glycolipid antigens that elicit antibody responses in infected individuals with a functioning humoral immune system that can be detected after about 1 week of illness, peaking at

3–6 weeks, followed by a gradual decline over months to years. Elevation of *M. pneumoniae*-specific IgM alone is often interpreted as evidence of acute infection since this antibody typically appears approximately 2 weeks before IgG antibody. This approach has the theoretical advantage in that only a single specimen, taken approximately 7–10 days after infection, is required. The presence of IgM is considered most significant in pediatric populations when there have been fewer opportunities for repeated exposures. However, children may produce IgM erratically and the antibodies can persist for several months after an acute infection. Infants younger than 6 months of age may not produce a measurable IgM response [27]. Additionally, adults who have been infected repeatedly over a period of years may not respond to mycoplasma antigens with a brisk IgM response. In these cases, reinfection may lead directly to an IgG response; so the absence of a positive IgM test does not rule out an acute infection. A single IgG measurement is also not advisable due to the presence of a high background of IgG antibodies in many healthy persons and its persistence after clinical resolution of infection. One publication reported that the percentage of individuals with radiologically proven pneumonia and diagnostic complement fixation (CF) titers who demonstrated a positive IgG response in the acute phase by various assays was less than 50%. When convalescent sera were tested, the number of IgG-positive specimens rose to 82% [40]. This same study also found that only 14 of 27 (52%) acute-phase sera tested positive by various IgM assays, rising to 88% when convalescent sera were tested. In view of these considerations, it is advisable to test simultaneously for both IgM and IgG in paired specimens collected 2–3 weeks apart for the most accurate diagnosis of recent or current *M. pneumoniae* infection, especially in adults. A fourfold or greater rise in antibody titer indicates a current or recent infection [49].

IgA antibodies are produced early in the course of disease, rise quickly to peak levels, and decrease earlier than IgM or IgG. In theory, a single early specimen could detect an acute infection even after multiple reinfections. However, Narita et al. [33] found no advantage of IgA in detecting acute *M. pneumoniae* infection in children. Further research into IgA responses in adult and pediatric populations and assessment of antibody levels in other body fluids such as urine is warranted.

12.3.1 Cold agglutinins

Cold agglutinins are found in about 30–50% of *M. pneumoniae* infections. They are IgM antibodies to the I-antigen of erythrocytes, and are detected by agglutination of type O, Rh-negative erythrocytes at 4°C. They are produced 1–2 weeks after initial infection, and persist for several weeks. A positive test result is not specific for mycoplasmal infection since other bacteria, viruses, or even collagen vascular

diseases and other hematological conditions can also induce cold agglutinins and a negative test does not exclude mycoplasmal infection since they are often absent. Better tests are available that have eliminated the need for this assay.

12.3.2 Complement fixation

Historically, the CF test gained early popularity for measuring the serological response to *M. pneumoniae* among laboratories that routinely ran CF tests for viral agents. However, mycoplasmas are much more antigenically complex than viruses, leading to nonspecific reactions. The glycolipids of *M. genitalium* are highly cross-reactive with *M. pneumoniae* due to shared lipid antigens and cause problems for CF tests. Sera from patients with bacterial meningitis also tend to have high CF titers. Cross-reactions of CF antigens with other organ-specific antigens unrelated to microorganisms may also occur. The CF tests measure IgM and, to a lesser extent, IgG. Therefore, one cannot differentiate between antibody classes using this technique, and recent infection in adults who may have minimal IgM response may be missed. Confirmation of CF test results with Western immunoblots can aid in interpretation, but greatly adds to the time and expense of testing. In view of the many limitations of CF tests and their time-consuming, labor-intensive nature, they have largely been replaced by improved commercial methods using alternative technologies.

Numerous publications have appeared since the early 1980s describing a number of different techniques for measuring antibody response to *M. pneumoniae* infection, but many of the assays have never been developed for commercial distribution. Only tests that can be purchased as kits will be discussed in detail. Several of the existing commercial kits have been evaluated in clinical studies, using CF as the reference method. However, in most instances it is not as accurate a test as some of the newer assay formats, considering its lack of antibody class distinction and tendency for cross-reactivity with other microorganisms. Thus, CF is not really suitable as a reference standard. Appreciation of this fact makes interpretation of the specificity and sensitivity results of comparative studies using CF as the reference standard somewhat problematic. More recent studies evaluating commercial kits have merely compared one method or product versus another. Results and conclusions from some studies were based upon assay of a single acute-phase serum sample, while others have used paired specimens. Often, there was no clear-cut designation of what constituted a true infection, so comparisons and extrapolations from multiple studies becomes rather complex and not always feasible.

12.3.3 Commercial serological assay formats

A variety of different antigen preparations have been used for development of serological assays for measurement of antibodies directed against *M. pneumoniae*. These have

included whole organisms, protein fractions, glycoprotein fractions, and recombinant antigens. Assay formats adapted for commercial distribution include indirect immunofluorescence (IFA), particle agglutination (PA), and enzyme-linked immunoassay (EIA); some of the serology kits sold commercially may be marketed under different names in various countries and by different companies. A summary of some of the major commercial serologic test kits and their various assay formats that are approved for diagnostic testing by the FDA is provided in Table 12.2 along with their Clinical and Laboratory Improvement Amendments (CLIA) complexity classification and pertinent references for comparative clinical studies, where available. Many additional commercial assays are available in various European and Asian countries.

12.3.4 Indirect immunofluorescence assays

Zeus Scientific distributes separate IFA tests for IgM and IgG. These kits have been studied in comparison to other methods and have been shown to provide accurate, quantitative serologic data [2,17]. The Zeus IFA test is relatively simple, requiring about 2 h to perform, but its interpretation is subjective and it requires a fluorescent microscope, which adds to expense and limits availability. Results can be affected by the presence of rheumatoid factor and high *M. pneumoniae*-specific IgG antibody levels, requiring additional procedures to validate IgM results in these settings.

12.3.5 Particulate antigen-antibody assays

Particulate agglutination (PA) tests consist of particles such as latex or gelatin, coated with *M. pneumoniae*-specific antigen. The antigen-coated particles are incubated with test serum, and if the serum contains specific antibodies, the particles agglutinate, resulting in a visible reaction. The PA tests use a mixture of *M. pneumoniae* antigens to detect both IgG and IgM simultaneously and can be adapted for use in a qualitative or semi-quantitative format [3,4,15,21,25,30]. Commercial kits utilizing PA technology have gained more widespread use outside of the United States, where EIAs predominate. Lieberman et al. [25] evaluated the Serodia Myco II (Fujirebio) in comparison to the Serodia *Mycoplasma pneumoniae* antibody-capture EIA kit and concluded that the antibody-capture EIA was better than PA for diagnosis of current *M. pneumoniae* infection when testing a single serum sample, but stressed that testing of acute and convalescent sera is required for precise diagnosis, even with the EIA. Barker et al. [4] also compared the Serodia Myco II PA kit with IgM-specific antibody-capture EIA and IFA. They determined that this PA test was not as specific for detection of IgM as the other tests. Aubert and colleagues [3] felt that PA tests performed about as well as EIAs and IFAs, but Matas et al. [30] reported the Meridian ImmunoCard EIA was able to detect lower levels of IgM antibodies than

Table 12.2 Major types of commercial serologic test kits used for *M. pneumoniae*. The information included in this table was current as of the time of writing

Assay format	Antibodies measured	Equipment	Description	Commercial kits (CLIA complexity classification)	References
Microtiter plate EIA	IgM, IgG, or IgA separately	Spectrophotometer/ EIA reader	EIAs are typically performed using a microtiter plate format in which antigens are adsorbed onto the polystyrene surface. Dilutions of sera are added to the wells and incubated. Antibodies bound to the solid-phase antigen are visualized by using enzyme-labeled conjugates directed against the primary antibody and substrate read in a spectrophotometer. The amount of conjugate reacting is proportional to the levels of antibody present. EIAs have the advantages of requiring very small serum volumes (<100 µL), are adaptable to testing large or small numbers of specimens, and can be made isotype specific. Membrane EIAs are rapid, qualitative, point-of-care procedures designed for testing single serum specimens. A permeable membrane or filter paper is impregnated with antigen to which serum is added, followed by addition of anti-human IgG or IgM enzyme conjugate. Development of color after enzyme substrate is added constitutes a positive test. Lipemic or hemolyzed serum samples will interfere with results. PA tests utilize latex or gelatin as carrier particles coated with antigen that are incubated with test serum. If specific antibodies are present, the particles agglutinate, resulting in a visible reaction. PA products provide qualitative results that can be visualized on a card or quantitative data read in a microtiter plate. PA assays do not offer any advantages over other techniques except possibly their ease and simplicity of performance.	GenBio (Remel) ImmunoWELL <i>Mycoplasma pneumoniae</i> Antibody IgM or IgG (high) Zeus Mycoplasma IgG and IGM ELISA Test System (high) Wampole Mycoplasma IgG ELISA (high) Trinity Biotech Captia Mycoplasma IgG or IgM ELISA (high)	9, 40, 42 40 NA NA
Membrane EIA	IgM or IgG separately	None		Meridian ImmunoCard – qualitative (moderate) Remel IgG and IgM Antibody Test System (moderate)	2, 30, 40, 41 17, 40–42
PA	IgG or IgM separately or simultaneously	None		Shared Systems <i>M. pneumoniae</i> Rapid Latex – qualitative or semi-quantitative (moderate)	NA
IFA	IgG or IgM separately	Fluorescence microscope	Antigen is fixed to glass slides. Specific antibody is detected in dilutions of test serum after staining with anti-human IgM or IgG fluorochrome conjugate. Results can be affected by the presence of rheumatoid factor and high <i>M. pneumoniae</i> -specific IgG levels. Additional procedures to validate IgM results are needed in these settings. IFA provides quantitative data but interpretation is very subjective	Zeus <i>M. pneumoniae</i> IgG or IgM IFA (High)	2, 17

EIA, enzyme immunoassay; IFA, indirect immunofluorescence; PA, particle agglutination; CLIA, Clinical Laboratory Improvement Amendment; NA, not available.

either the Serodia Myco II or CF tests. Based on evidence presented to date, PA assays do not offer any advantages over other techniques such as EIAs or IFAs, except possibly their ease and simplicity for performance that may allow for a moderate complexity designation by CLIA for some kits, meaning that more laboratories can perform the test.

12.3.6 Enzyme-linked immunosorbent assays

EIAs, first developed in the 1970s, have become widely adapted in clinical laboratories, and are the most common commercial mycoplasma serology tests utilized in the United States. They are amenable to a variety of assay conditions, detect very small amounts of antibody, and can be made isotype-specific. Crude multiantigen preparations, purified proteins, μ -capture approaches, purified glycolipids, and synthetic peptides have all been used as targets. Most EIAs are sold as 96-well microtiter plate formats, although some can be obtained as break-away microwell strips which allow smaller numbers of sera to be tested economically.

Two EIAs are sold as qualitative membrane-based procedures for the detection of single test specimens. These are truly rapid EIAs (10 min or less) and are simple to perform. The Meridian ImmunoCard (Figure 12.3) is an IgM-only assay that is simple to read, and is especially useful for testing pediatric samples. Typical of IgM-only assays based on *M. pneumoniae* protein antigens, the specificity may be somewhat compromised in patients with autoimmune disease. Moreover, there are limitations in interpreting the



Figure 12.3 ImmunoCard (Meridian Bioscience, Inc.). The Meridian ImmunoCard is a qualitative, membrane-based EIA that can be performed in 10 min or less to detect the presence of IgM antibody directed against *M. pneumoniae*. A positive test is indicated by development of a blue color in both of the upper reaction ports. (Photograph courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)

results of IgM-only assays when testing sera from adults, and the use of a single test rather than paired sera, as previously discussed. Other studies using sera from confirmed *M. pneumoniae* cases have also shown that the ImmunoCard performed better than the CF test [2,30,41].

The *M. pneumoniae* IgG/IgM Antibody Test System (originally marketed by Remel, now ThermoFisher) is a qualitative membrane-based assay that detects IgM and IgG simultaneously and has shown good sensitivity and specificity when compared to other tests, including IFA, the GenBio ImmunoWell EIA, Meridian ImmunoCard, and CF [17,41,42]. Although the manufacturers and some investigators have endorsed the use of a single assay by this product for diagnosis of acute *M. pneumoniae* infections in young persons, acute and convalescent sera are necessary for greatest accuracy. However, the value of the single rapid EIAs is lost if paired specimens are required. Numerous other microtiter well-format EIA kits are sold in various countries.

It is apparent that problems exist with essentially all of the serological tests that have undergone comparative evaluations. For others that have not undergone such comparisons, nothing is known about their diagnostic accuracies beyond what the manufacturer states in package inserts. Csango et al. [12] concluded in their evaluation of four commercial EIAs sold in Europe that a substantial proportion of healthy blood donors were serologically positive for IgA, IgM, and/or IgG, suggesting the likelihood that these tests result in considerable overdiagnosis of acute *M. pneumoniae* infections. Beersma and coworkers [7] published a comprehensive European study, evaluating 12 commercial *M. pneumoniae* serology tests on paired sera that included EIAs and PA formats as well as CF. They used a positive real-time PCR (RT-PCR) test as the reference standard and also included PCR-negative patients with and without lower respiratory disease as controls. They found a wide range of results with the various assays noting, as have others, that single acute-phase titers were often negative in PCR-proven infections, and appropriately concluded that there are few commercial serologic assays with appropriate performances in terms of sensitivity and specificity, placing a greater importance of PCR rather than serology for the diagnosis of *M. pneumoniae* infections in defined groups of patients. Nilsson et al. [34] reported that only 21% of patients with confirmed *M. pneumoniae* infection were positive by serology when tested during the first symptomatic week, versus 56% during the second week, and 100% during the third week. These data emphasize the value of PCR over serology, particularly during the first few days of illness.

12.3.7 Genital mycoplasma assays

The common occurrence of genital mycoplasmas as commensals in the lower urogenital tracts of healthy persons makes interpretation of antibody titers difficult, and

the mere existence of antibodies alone cannot be considered significant. However, if invasive extragenital disease occurs, elevation of antibody titers is often apparent. Serologic methods for *M. hominis*, *M. genitalium*, and *Ureaplasma* spp. have been developed for research purposes and include IFAs, EIAs, microimmunofluorescence, and metabolic inhibition. No single serologic test has proven satisfactory in identification of genital mycoplasmal infections and most assays for *M. hominis* and *Ureaplasma* spp. have not been evaluated for cross-reactions that could potentially occur with sera containing antibodies to *M. genitalium*. PCR-based studies performed at the Centers for Disease Control and Prevention showed that respiratory specimens rarely contain *M. genitalium*. However, this does not preclude the potential for cross-reacting antibodies arising from a genital infection with this organism. No commercial serologic kits for detection of antibodies to urogenital mycoplasmas are produced and sold commercially in the United States. Some serologic assays for *M. hominis* and *Ureaplasma* spp. based on the metabolic inhibition technique (ApoH-technologies, ELITech) are sold in Europe. No external clinical evaluations have been reported for these products at the time of this writing and serologic approach to diagnosis is not recommended for genital mycoplasmas at present.

12.4 Molecular detection systems

Culture is well adapted to species such as *M. hominis* and *Ureaplasma* sp. which can be isolated easily and rapidly from clinical specimens and has the advantages of being able to provide quantitative results and an isolate for antimicrobial susceptibility testing. However, it is not satisfactory for detection of fastidious and/or extremely slow-growing organisms such as *M. genitalium* and, to some degree, *M. pneumoniae*. Therefore, alternative rapid methods have been devised based on knowledge concerning characterization of epitopes and DNA sequences.

Prior to development of nucleic acid amplification tests (NAATs), several types of nonamplified rapid methods were developed for antigenic detection of *M. pneumoniae*. The utility and general acceptance of DNA hybridization probes has been limited by low sensitivity, typically requiring 10^3 – 10^6 CFU for detection, low specificity, due to the antigenic similarity between *M. pneumoniae* and *M. genitalium*, and by nonspecific reactions with other bacteria. Kits were previously commercialized in the United States by Gen-Probe or by other companies in Europe, but are no longer available. The sensitivity of NAATs is superior to that of the traditional antigen detection procedures and they are now considered as “the new gold standard for diagnosis” [20].

12.4.1 Commercial molecular assays for *M. pneumoniae*

The use of PCR is more often accepted as a rapid diagnostic test for *M. pneumoniae*, since culture is slow and insensitive and serology is retrospective. PCR is also particularly valuable when attempting to detect *M. pneumoniae* in extrapulmonary sites where culture is very rarely successful [51]. Moreover, PCR has been shown to be more sensitive than serology in the diagnosis of early *M. pneumoniae* infection [34]. Numerous in-house traditional and RT-PCR assays have been developed to detect *M. pneumoniae*, as summarized in recent reviews [27,51]. Many of them target the P1 adhesin gene, but various other targets have also been utilized [35,51]. However, proper validation and standardization are still lacking for many assays and quality control studies have revealed frequent deficiencies for some of them [28, 29].

Over the past decade, several commercialized kits have become available in Europe for the detection of *M. pneumoniae* alone or in combination with other respiratory tract bacteria or viruses, either in mono- or in multiplex formats. Table 12.3 summarizes some of the tests that have been developed for commercial distribution in various countries, mainly in Europe. One multiplex assay, the Biofire Diagnostics FilmArray RP detects nucleic acids in nasopharyngeal swabs for 20 respiratory tract pathogens, including *M. pneumoniae* [37]. This assay has received FDA clearance for use in the United States and is also available in Europe. Touati et al. [43] evaluated five commercial RT-PCR assays from Nanogen Advanced Diagnostics, Focus Diagnostics, Diagenode SA, Cepheid, and Minerva Biolabs, for detection of *M. pneumoniae* in respiratory tract specimens in comparison to an in-house RT-PCR assay. All kits allowed prompt and specific results, validated by the use of an internal control. The Pneumoplex assay, not available commercially as of September 2013, targeting seven respiratory pathogens yielded sensitivity and specificity of 100% and 98.5%, respectively, on 125 clinical bronchoalveolar lavage fluid samples [22]. Dumke and Jacobs [14] reported that the Venor Mp-QP (Minerva) and the artus LC PCR (Qiagen, currently research use only [RUO]) were able to demonstrate *M. pneumoniae* DNA in a concentration comparable to 1 CFU/ μ L when testing dilutions of type strains, as well as DNA extracted from respiratory tract specimens. They were also able to detect the different *M. pneumoniae* subtypes. Three multiplex NAAT tests, the Resplex (Qiagen, currently RUO), RespiFinder plus and RespiFinder Smart 21 (PathoFinder), were evaluated by examination of an external quality assessment panel of 48 respiratory tract specimens [28]. Large differences in amplification efficacy were found between the commercially available tests; acceptable results were obtained by using the PathoFinder assays, but lower sensitivities were reported for the Resplex assay.

Table 12.3 Commercial nucleic acid amplification tests for detection of *M. pneumoniae* available in Europe. The information included in this table was current as of the time of writing

Manufacturer	Kit	Technique	Detection procedure	Pathogens targeted	References
Affigene AID GmbH	Cp/Mp tracer AID CAP bacterial assay	MX-PCR MX-PCR	Real-time ICT	<i>M. pneumoniae</i> and <i>C. pneumoniae</i> <i>S. pneumoniae</i> , <i>H. influenzae</i> , <i>M. catarrhalis</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>L. pneumophila</i> , <i>B. pertussis</i> , <i>B. parapertussis</i> <i>M. pneumoniae</i> <i>M. pneumoniae</i>	NA NA
Becton Dickinson Bio-Evolution	<i>M. pneumoniae</i> BD ProbeTec ET PCR TpsReel <i>M. pneumoniae</i>	SDA PCR	Fluorescence Real-time	<i>M. pneumoniae</i> Influenza A H1N1, influenza A H1, influenza A H1-2009, influenza A H3, influenza B, RSV, human metapneumovirus, coronavirus NL63, OC43, 229E, HKU1, adenovirus, parainfluenza virus 1-4, bocavirus, rhino/enterovirus, <i>B. pertussis</i> , <i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i> , <i>Legionella</i> spp.	NA NA 37
Biofire Diagnostics, Inc. (recently acquired by bioMérieux)	FilmArray RP*	MX-PCR	Microarray	<i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i>	18 NA 43 43
bioMérieux/Argène bioMérieux/Argène Cepheid Diagenode	Chlamylege Chla/Mycopneumo r-gene <i>Mycoplasma pneumoniae</i> ASR <i>Mycoplasma pneumoniae</i> and <i>Chlamydia pneumoniae</i> Simplex <i>M. pneumoniae</i> ProPneumo1+ Venor MP	MX-PCR MX-PCR PCR PCR PCR MX-PCR PCR	Hybridization Real-time Real-time Real-time Real-time Real-time Agarose gel electrophoresis and real-time Real-time	<i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i> <i>M. pneumoniae</i> , <i>C. pneumoniae</i>	43 NA 14, 43
Focus Diagnostics Hologic-Gen Probe Minerva Biolabs	Mycoplasma pn. Q-PCR Alert RespiFinder 19 (previously RespiFinder Plus)	PCR MX-PCR PCR	Capillary electrophoresis	<i>M. pneumoniae</i> , <i>C. pneumoniae</i> , <i>L. pneumophila</i> , <i>B. pertussis</i> , influenza A, influenza A/H5N1, influenza B, RSV A and B, parainfluenza 1-4, rhinovirus, coronavirus NL63, OC43, 229E, human metapneumovirus, adenovirus <i>M. pneumoniae</i> , <i>C. pneumoniae</i> , <i>L. pneumophila</i> , <i>B. pertussis</i> , influenza A, influenza A/H1N1v, influenza B, RSV A and B, parainfluenza 1-4, rhino/enterovirus, coronavirus NL63, OC43, 229E, HKU1, human metapneumovirus, adenovirus <i>M. pneumoniae</i> , <i>C. pneumoniae</i> , <i>L. pneumophila</i> , <i>B. pertussis</i> , influenza A, influenza A/H1N1v, influenza B, RSV A and B, parainfluenza 1-4, rhino/enterovirus, coronavirus NL63, OC43, 229E, HKU1, human metapneumovirus, adenovirus <i>M. pneumoniae</i> , <i>C. pneumoniae</i>	36 NA
Nanogen Advanced Diagnostics Patho Finder	RespiFinder 22	MX-PCR	Capillary electrophoresis	<i>M. pneumoniae</i> , <i>C. pneumoniae</i> , <i>L. pneumophila</i> , <i>B. pertussis</i> , influenza A, influenza A/H1N1v, influenza B, RSV A and B, parainfluenza 1-4, rhinovirus, coronavirus NL63, OC43, 229E, human metapneumovirus, adenovirus <i>M. pneumoniae</i> , <i>C. pneumoniae</i> , <i>L. pneumophila</i> , <i>B. pertussis</i> , influenza A, influenza A/H1N1v, influenza B, RSV A and B, parainfluenza 1-4, rhino/enterovirus, coronavirus NL63, OC43, 229E, HKU1, human metapneumovirus, adenovirus <i>M. pneumoniae</i> , <i>C. pneumoniae</i>	43
Patho Finder	RespiFinder SMART 22	MX-PCR	Real-time	<i>M. pneumoniae</i> , <i>C. pneumoniae</i> , <i>L. pneumophila</i> , <i>B. pertussis</i> , influenza A, influenza A/H1N1v, influenza B, RSV A and B, parainfluenza 1-4, rhino/enterovirus, coronavirus NL63, OC43, 229E, HKU1, human metapneumovirus, adenovirus <i>M. pneumoniae</i> , <i>C. pneumoniae</i> , <i>L. pneumophila</i> , <i>B. pertussis</i> , influenza A, influenza A/H1N1v, influenza B, RSV A and B, parainfluenza 1-4, rhino/enterovirus, coronavirus NL63, OC43, 229E, HKU1, human metapneumovirus, adenovirus <i>M. pneumoniae</i> , <i>C. pneumoniae</i>	NA
Patho Finder	RealAccurate <i>M. pneumoniae</i> + <i>C. pneumoniae</i> duplex PCR	MX-PCR	Real-time	<i>M. pneumoniae</i> , <i>C. pneumoniae</i> , <i>L. pneumophila</i> , <i>B. pertussis</i> , influenza A, influenza A/H1N1v, influenza B, RSV A and B, parainfluenza 1-4, rhino/enterovirus, coronavirus NL63, OC43, 229E, HKU1, human metapneumovirus, adenovirus <i>M. pneumoniae</i> , <i>C. pneumoniae</i>	NA
Seegene	Seeplex PneumoBacter ACE	MX-PCR	Capillary electrophoresis	<i>S. pneumoniae</i> , <i>H. influenzae</i> , <i>C. pneumoniae</i> , <i>M. pneumoniae</i> , <i>L. pneumophila</i> , <i>B. pertussis</i>	NA

Source: Adapted from reference 19.

MX, multiplex; SDA, strand displacement assay; ASR, analyte specific reagent (not a complete diagnostic kit); ICT, intramolecular charge transfer; NA, not available.

* Available in the United States as well as Europe.

The Chlamylege multiplex PCR assay (Argene, Inc.) is a multiplex PCR assay used in conjunction with microplate hybridization with specific probes to detect *M. pneumoniae*, *Chlamydia pneumoniae*, and *Legionella* spp. This kit was evaluated with 154 clinical specimens from patients with respiratory infections, which included nine patients with *M. pneumoniae* infection, and in another study of 220 endotracheal aspirates from children, among which there were six specimens positive for *M. pneumoniae*. Results were compared to detection by serology and/or in-house PCR assays. Chlamylege correctly identified all *M. pneumoniae*-positive and negative specimens as well as two additional samples positive for *M. pneumoniae* in the second group [18].

Meridian Bioscience introduced the *illumigene* Mycoplasma Assay into the United States market in 2013 after it received FDA approval. This Loop Mediated Isothermal Amplification Assay (LAMP) shown in Figure 12.4 is based on Meridian's technology that is already available for detection of *Clostridium difficile* toxins in clinical specimens. This assay enables detection of *M. pneumoniae* in up to 10 clinical specimens that can be tested simultaneously within 1 h after extracted DNA is set up in the incubator/reader that is provided by the manufacturer. The availability of this instrumentation, which can easily be incorporated into clinical microbiology laboratories that do not have extensive molecular diagnostic facilities, may increase the frequency that microbiological diagnosis of *M. pneumoniae* respiratory tract infections is attempted. Our evaluation of this product indicated it had comparable sensitivity (100%) and specificity (99%) to culture.

NAATs will be successful in detecting mycoplasmas in clinical specimens only if there is an adequate DNA extraction beforehand. Lysis and proteinase K treatment usually yield PCR-detectable DNA for body fluids other than blood and transport systems containing material obtained from swabs. Potentially inhibitory specimens, including blood, tissues, and lower respiratory tract secretions can be purified using QIAamp DNA Blood Mini Kit (Qiagen) or other commercial kits available from various sources. Automated or semi-automated nucleic acid isolation methods, such as Qiagen BioRobot EZ1 (Qiagen), NucliSENS (bioMérieux), easyMAG (bioMérieux), or MagNAPure LC (Roche Applied Science) have been evaluated independently and they can also be used to prepare samples for *M. pneumoniae* PCR assays. Some automated extraction systems appear to work as well as manual systems. The NucliSens easyMAG extracted DNA and RNA more efficiently from clinical samples, with a higher recovery and/or fewer inhibitors than did QIAGEN extraction, by showing, on average, lower Ct values in the LightCycler RT-PCR assay [26].



Figure 12.4 *illumigene* (Meridian Bioscience, Inc.). The *illumigene* is a loop-mediated isothermal amplification assay (LAMP) that enables detection of *M. pneumoniae* in up to ten respiratory specimens within 1 h after extracted DNA is set up in the incubator/reader. (Photograph courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)

Further studies are required, including an extended number of clinical specimens, representing different *M. pneumoniae* subtypes obtained from different geographic locations with low organism concentrations. Comparison of PCR with other established modalities including culture, and using assays with different formats and gene targets are needed to verify accuracy and reproducibility of commercial NAATs. It is important to develop effective clinical and laboratory algorithms for the optimal use of those assays and to study their impact on patient care in different populations in various settings. The lack of comprehensive externally published comparative data for validation of the wide variety of PCR assays and other NAAT formats used for detection of human mycoplasmas and ureaplasmas remains a significant shortcoming.

Laboratories that use PCR for detection of *M. pneumoniae* should also subscribe to some sort of proficiency testing program such as that developed in Europe [28, 29], the College of American Pathologists (CAP), or use an alternative proficiency testing program by exchanging blinded specimens with other laboratories.

12.4.2 Commercial molecular assays for genital mycoplasmas and ureaplasmas

Development of commercial molecular assays is of considerable interest for *M. genitalium*, a species that is extremely fastidious and very difficult to grow from specimens. The interest in such assays is less evident for the other urogenital species, *M. hominis*, and *Ureaplasma* spp., which are easy to obtain by culture; however, some kits have been developed for these species. Table 12.4 summarizes the commercial tests developed for *M. genitalium*, *M. hominis*, and *Ureaplasma* spp. They are currently sold in Europe and various other countries, but not in the United States.

Table 12.4 Commercial nucleic acid amplification based tests for detection of urogenital mycoplasmas available in Europe. The information included in this table was current as of the time of writing

Manufacturer	Kit	Technique	Detection procedure	Pathogens detected	References
Bio-Rad	Dx CT/NG/MG assay	MX-PCR	Real-time	<i>C. trachomatis</i> , <i>N. gonorrhoeae</i> , <i>M. genitalium</i>	23
Diagenode	<i>Mycoplasma genitalium</i> real-time PCR	PCR	Real-time	<i>M. genitalium</i>	Le Roy et al., <i>STI and AIDS World Congress</i> , 2013
Patho Finder	STD Finder assay	MX-PCR	Agarose gel and capillary electrophoresis	<i>N. gonorrhoeae</i> , <i>C. trachomatis</i> , <i>T. vaginalis</i> , <i>M. genitalium</i> , <i>T. pallidum</i> , HSV-1 and HSV-2	32
Roche/TIB MolBiol	LightMix <i>Mycoplasma genitalium</i>	PCR	Real-time	<i>M. genitalium</i>	Le Roy et al., <i>STI and AIDS World Congress</i> , 2013
Sacace	<i>Mycoplasma genitalium</i> real-TM	MX-PCR	Real-time	<i>M. genitalium</i>	31
Seegene	Magicplex STI 1	MX-PCR	Real-time	<i>C. trachomatis</i> , <i>N. gonorrhoeae</i> , <i>M. genitalium</i> , <i>U. urealyticum</i> , <i>M. hominis</i>	NA
Seegene	Anyplex MG/UU/UP	MX-PCR	Real-time	<i>M. genitalium</i> , <i>U. urealyticum</i> , <i>U. parvum</i>	NA
Seegene	Anyplex TV/MH	MX-PCR	Real-time	<i>T. vaginalis</i> , <i>M. hominis</i>	NA
Seegene	Anyplex II STI-7	MX-PCR	Real-time	<i>C. trachomatis</i> , <i>N. gonorrhoeae</i> , <i>T. vaginalis</i> , <i>M. hominis</i> , <i>M. genitalium</i> , <i>U. urealyticum</i> , <i>U. parvum</i>	NA
Seegene	Seeplex Master ACE	MX-PCR	Capillary electrophoresis	<i>C. trachomatis</i> , <i>N. gonorrhoeae</i> , <i>T. vaginalis</i> , <i>M. hominis</i> , <i>M. genitalium</i> , <i>U. urealyticum</i> , <i>U. parvum</i>	NA
Seegene	Seeplex STD 4 ACE	MX-PCR	Capillary electrophoresis	<i>M. hominis</i> , <i>M. genitalium</i> , <i>U. urealyticum</i> , <i>U. parvum</i>	NA
Seegene	Seeplex STD 6 ACE	MX-PCR	Capillary electrophoresis	<i>C. trachomatis</i> , <i>N. gonorrhoeae</i> , <i>T. vaginalis</i> , <i>M. hominis</i> , <i>M. genitalium</i> , <i>U. urealyticum</i>	24

NA, not available.

Several commercialized kits have become available for *M. genitalium* in monoplex (Roche/TIB MOLBIOL, Diagenode, and Sacace Biotechnologies) or in multiplex format (PathoFinder, Seegene, and Bio-Rad) combined with other sexually transmitted disease pathogens. Most of them are based on RT-PCR. They have been successively evaluated by comparison to an in-house Taqman RT-PCR assay and can be used for the detection of *M. genitalium* in urogenital specimens [23,31]. Several multiplex PCR assays have been commercialized by Seegene to amplify *M. genitalium*, *U. urealyticum*, *U. parvum*, and *M. hominis* DNA from urogenital specimens, in some cases along with other sexually transmissible pathogens by multiplex ligation-dependent probe amplification and other formats [24].

12.5 Antimicrobial susceptibility testing

Historically, testing *in vitro* susceptibilities of antimicrobial agents against *M. pneumoniae* was not indicated except for evaluation of new drugs since there was no resistance to tetracyclines or fluoroquinolones, and macrolide

resistance was extremely uncommon. This situation has changed over the past decade with the emergence of high-level macrolide resistance that now affects the majority of *M. pneumoniae* isolates in some Asian countries [6]. This resistance, which has clinical significance in terms of patient outcomes and choice of antimicrobial therapy, is now being reported in Europe and the United States, but still remains relatively uncommon [6]. In view of tetracycline resistance among *M. hominis* and *Ureaplasma* spp. that may affect up to 50% of clinical isolates according to some sources [5,48], reports of macrolide and fluoroquinolone resistance [52,53], and concern for poor therapeutic response in systemic infections in immunosuppressed patients, it is worthwhile to perform *in vitro* susceptibility tests to guide patient management in some clinical settings. Depending on the species being tested, it can be useful to determine minimum inhibitory concentrations (MICs) for tetracyclines, macrolides, clindamycin, and fluoroquinolones.

Agar disk diffusion is not recommended for use in testing mycoplasmas for antimicrobial susceptibilities because the relatively slow growth of many species on

agar will allow widespread diffusion of the antimicrobial from the disk throughout the agar plate before visible growth can be detected, and because there are no data to correlate the zone diameters with MICs. Broth microdilution and agar dilution techniques, and the agar gradient (Etest) technique have been adapted for use with these organisms. When properly performed, with appropriate controls, these techniques can provide reliable susceptibility data to aid in treatment of infections caused by or associated with mycoplasmas and ureaplasmas. Standardized methods, including designated quality control reference strains with defined MIC ranges, media formulations for broth microdilution and agar dilution, as well as MIC breakpoints for selected antimicrobial agents have now been published by the Clinical and Laboratory Standards Institute (CLSI) [11].

Susceptibility testing kits using the broth microdilution technique, such as Mycoplasma IST2 (bioMérieux), SIR Mycoplasma (Bio-Rad), MYCOFAST *Evolution2*, *Evolution3* (ELITech), *RevolutionN* (ELITech), and Myco-Kit-ATB (Eurobio) are currently sold in Europe, but not in the United States. Some of these kits combine detection and identification in the same product, as described in previous sections. Table 12.1 summarizes some of these kits, which include reagents for detection, identification, and susceptibility testing of *Ureaplasma* spp. and *M. hominis*. They consist of microwells containing dried antimicrobials, generally in one or two concentrations corresponding to the thresholds proposed for conventional bacteria to classify the strains as susceptible, intermediate, or resistant. To date, only the *RevolutionN* kit shown in Figure 12.5 has adapted the antibiotics tested and their concentrations to the new mycoplasma susceptibility testing guidelines published by the CLSI, which include designations for susceptibility and resistance [11]. Inoculum size can influence MICs and direct inoculation with a clinical specimen without a defined inoculum or preculture is not advisable. SIR Mycoplasma is adapted for use with *Ureaplasma* spp. and *M. hominis* after a primary culture so that a standardized inoculum can be incorporated into the test system. Renaudin and Bébér [39] evaluated the SIR Mycoplasma using a defined inoculum and determined that this product gave results comparable to those obtained by broth microdilution. They considered it a reasonable choice for diagnostic laboratories. The Mycokit-ATB also includes procedures for standardization of the inoculum prior to determining antimicrobial susceptibilities.

The agar gradient diffusion technique (Etest, bioMérieux) technique for detection of tetracycline and fluoroquinolone susceptibilities in *M. hominis* and *Ureaplasma* spp. produces results comparable to both broth microdilution and agar dilution [13,46,47], though the procedures for performing the Etest MIC are currently not standardized. Performing susceptibilities using the Etest can be adapted

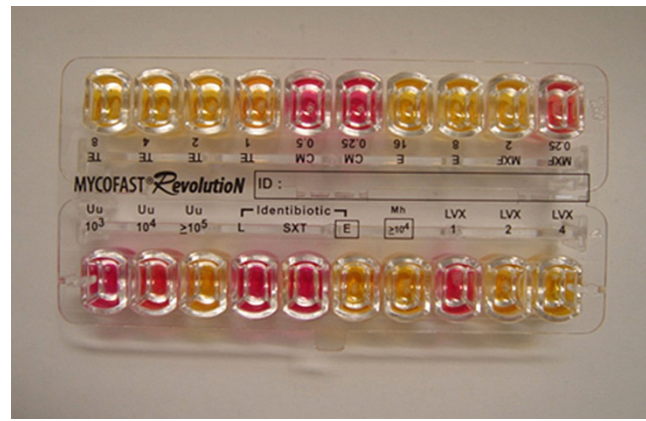


Figure 12.5 MYCOFAST RevolutionN (ELITech). Mycofast *RevolutionN* is a commercial kit for identification, enumeration, and antimicrobial susceptibility testing of *M. hominis* and *Ureaplasma* spp. that can be used directly with clinical specimens. The kit consists of microplates containing lyophilized growth substrates and antimicrobials. Detection of growth of the organisms is based on color change in liquid medium containing urea and arginine. Species identification is based on differential inhibition by erythromycin, trimethoprim/sulfamethoxazole, and lincomycin. Susceptibility testing is provided for levofloxacin, moxifloxacin, erythromycin, clindamycin, and tetracycline. This product is currently available in Europe, but not in the United States. (Photograph courtesy of ELITechGroup, Inc.) (See insert for color representation of the figure.)

from standardized methods used for other techniques. Two Etests can be placed on each standard sized petri plate. Agar plates are incubated until colonies are apparent in the periphery of the plate and an ellipse becomes apparent. The MIC is read under the microscope as the number on the Etest strip corresponding to the intersection of mycoplasma growth. Staining plates with Dienes Stain helps visualize the ellipse. The Etest has the advantages of the simplicity of agar-based testing, has an endpoint that does not shift over time, does not have a large inoculum effect, and can easily be adapted for testing single isolates. This technique is readily adaptable to laboratories not specializing in mycoplasma diagnosis that may encounter isolates needing susceptibility tests only on an occasional basis. Etests are commercially available, can be maintained frozen for 3–5 years, and have been shown to work well with commercial SP4 broth and agar [46]. For laboratories testing occasional clinical mycoplasma or ureaplasma isolates, the Etest is a practical technique since all of the materials can be purchased commercially and have a long shelf life.

12.6 Future directions

Availability of powerful molecular-based techniques designed to detect human mycoplasmas and ureaplasmas for research purposes and their entry into clinical diagnostic laboratories over the past decade has led to

increased interest in these organisms as bonafide agents of clinically significant human infection. This is particularly true for opportunistic infections caused by *M. hominis* and *Ureaplasma* spp. in neonates and immunosuppressed persons. The use of NAATs has also facilitated discovery and preliminary characterization of extremely fastidious species, most notably *M. genitalium*. PCR systems have now been developed for all mycoplasmas known to cause disease in humans. Practically, PCR technology is less valuable for routine diagnostic purposes in the case of the more rapidly growing and easily cultivable organisms, such as *M. hominis* and *Ureaplasma* spp., except in some specific cases where isolation by culture could be difficult. For instance, the PCR technique may detect urogenital mycoplasmas more easily in nonurogenital specimens such as synovial fluids and tissue biopsies.

Importance of detection of *M. pneumoniae* for diagnostic and management purposes has become better appreciated by some clinicians as a result of the mounting evidence implicating this organism as a serious cause of pneumonia requiring hospitalization, that it can affect persons in all age groups, including the elderly, and that it can be resistant to commonly used macrolide antibiotics. For *M. pneumoniae*, and especially for slow-growing, extremely fastidious species for which optimum cultivation techniques are not established, such as *M. genitalium*, the use of PCR assays is the most practical means of detecting their presence in clinical material. The development of commercially available and marketed assays for detection of *M. genitalium* in the United States should allow a broader use of PCR for diagnostic purposes and will help clinicians to make decisions about treatment.

The detection limit of the PCR is very low, corresponding to a single organism, or a single copy of the gene, when purified DNA is used. In contrast, culture requires having at least 1–10 viable organisms; larger numbers are probably needed when clinical specimens are considered. Comparison of the PCR technique with culture and/or serology, in the case of *M. pneumoniae*, has yielded varied results and large-scale experience with this procedure is still limited. Positive PCR results for *M. pneumoniae* in culture-negative persons without evidence of respiratory disease suggest inadequate specificity, persistence of the organism after infection, or its existence in asymptomatic carriers, making interpretation of such results difficult. Thus, even though the many benefits of the PCR for detection and speciation of mycoplasmas in humans are readily apparent, a positive result must still be correlated with clinical events. Further development and standardization of commercial PCR kits should bring about better standardization of the technique, and if available at a reasonable cost, PCR will become a major method for the diagnosis of mycoplasmal infections in the United States as it has in Europe over the past decade.

Thus, the future of mycoplasma diagnostics should ideally include further development and refinement of commercial NAAT kits for detection of *M. pneumoniae* that could be employed as a simultaneous screening device for the presence of respiratory infection for this organism and unrelated bacteria that cause pneumonias. At the present time, in the United States, the Biofire Filmarray RP may begin to address this need and the Meridian *illumigene* Mycoplasma DNA Assay may provide the means for laboratories that do not have extensive molecular diagnostic facilities and experienced personnel in this field to offer rapid detection of *M. pneumoniae* at a reasonable cost. Use of the PCR assay to detect and identify unknown or uncommon mycoplasmas is also likely to become important, but the utilization of such technology would be limited primarily to mycoplasma research or reference laboratories due to the infrequent occurrence of such organisms in clinically significant human infections and lack of commercial reagents to detect and identify them.

Given that culture remains the preferred diagnostic method for detection of *M. hominis* and *Ureaplasma* spp. in many laboratories, the most obvious need will be for reliable commercial products to be sold more widely and that media capable of detecting these organisms in all types of clinical specimens will be validated. More widespread availability of complete diagnostic kits may further increase the likelihood that hospital-based microbiology laboratories will begin offering diagnostic services for all mycoplasmas and ureaplasmas that infect humans.

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Commercial Methods for Identification and Susceptibility Testing of Fungi

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Rapid diagnosis of fungal pathogens is crucial for immunocompromised patients, for whom opportunistic infections can often be fatal. The advance of human immunodeficiency virus (HIV) disease, the widespread use of antibiotics, and the growing number of transplants performed each year all contribute to the increased incidence of fungal infections [17]. Most currently available commercial systems are relatively rapid (2–72 h) and widely used; however, in many cases, these methods still lack sensitivity and require substantial laboratory expertise to interpret supplementary tests, identify microscopic features of organisms, or correctly choose isolates for appropriate testing. Clearly, more efforts will be necessary to advance more rapid and sensitive test methods to diagnose fungal disease.

The methods described in this chapter are meant to represent those that are commercially available, US Food and Drug Administration (FDA) approved, and published in peer-reviewed journals. Some newer tests yet to be approved by the FDA are included since it is clear that this approval is forthcoming (e.g., MALDI-ToF/MS [matrix-assisted laser desorption ionization with time-of-flight mass spectrometry]). Interest in commercially available solutions for accurate, more rapid and less costly diagnosis has forced a change in the algorithms used for fungal diagnosis and increased the numbers of published comparisons and evaluations of commercial methods and systems. Certainly, publications of this nature will continue to appear at a relatively rapid pace, as the healthcare and clinical-diagnostics industries strive to improve patient care and reduce costs.

13.1 Direct examination and detection methods

Accurate and sensitive detection methods for fungal pathogens in clinical specimens are essential for a number of reasons. Many patients infected with fungi are

immunosuppressed, and disease in these patients can be severe and progress rapidly. Results from fungal cultures and serological testing often come too late to affect patient management. Direct microscopic examination has traditionally been performed by analysis of KOH preparations with and without Calcofluor white staining, but smears may lack sensitivity [140].

Despite these limitations, the direct recognition of fungi in clinical specimens remains the most rapid means of fungal diagnosis. Unfortunately, only a few pathogens have commercial products specifically designed to enhance their direct detection in clinical specimens. Most available methods target fungal cell-wall antigens or fungal enzymes.

13.1.1 *Cryptococcus* spp.

There are two species of pathogenic *Cryptococcus*: *Cryptococcus neoformans* – which has two varieties *C. neoformans* var. *neoformans* (serotype D) and var. *grubii* (serotype A) – and *Cryptococcus gattii* (serotype B and C) – formerly considered a variety of *C. neoformans* but has been assigned as a separate species in the genus *Cryptococcus* (Table 13.1). Using molecular typing *C. neoformans* var. *grubii* has been found to be the most common worldwide etiologic agent of opportunistic cryptococcosis in HIV-AIDS infected individuals. *Cryptococcus neoformans* var. *neoformans* is most common in Europe and *C. gattii*, originally described in Australia, has migrated to the Pacific Northwest United States and British Columbia and is increasingly reported from many countries and continents [285]. For the remaining discussion in this chapter, the *Cryptococcus* complex (*neoformans* var. *neoformans* and var. *grubii* and *gattii*) will be referred to as *C. neoformans*.

Due to its high specificity and increased sensitivity, the *Cryptococcus* antigen detection tests have, for the most

Table 13.1 Two varieties of *Cryptococcus neoformans* and the second pathogenic species, *C. gattii*

Characteristic	<i>C. neoformans</i> var. <i>grubii</i>	<i>C. neoformans</i> var. <i>neoformans</i>	<i>C. gattii</i>
Serotype (s)	A	D	B, C
Main geographic distribution	Worldwide (major agent in AIDS patients)	Northern Europe	Tropical and subtropical; also in British Columbia and the Pacific Northwest United States
CGB medium	No growth	No growth	Growth

Source: adapted from reference 260.

CGB, canavanine–glycine–bromothymol blue medium.

Table 13.2 CrAg method sensitivity [232]

Immunoassay method	Serotype sensitivity (ng CrAg/mL)			
	A	B	C	D
Immy LFA Strip	1	1	9	8
Immy Latex	28	47	380	62
Meridian CALAS Latex	19	37	940	54
Crypto-LA latex	38	64	1600	50
Meridian Premier ELISA	28	23	>2000	770

part, replaced the more historic India ink test and have become the “gold standard” for direct *Cryptococcus* testing [154, 214]. The cryptococcal glucuronoxylomannan (GXM) capsular antigen can be detected via latex agglutination (LA), enzyme immunoassay (EIA), or lateral-flow immunoassay (LFA). Due to the soluble nature of the cryptococcal antigen, most methods can detect antigen in serum or cerebrospinal fluid (CSF) even before viable organisms can be cultivated. Different cryptococcal serotypes (A, B, C, D) are known to be cross-reactive [203], and are detected by these assays. However, one study comparing the sensitivity of the LFA, LA, and EIA assays shows that the LFA is the most sensitive, with less sensitivity by other assays and a wide range of sensitivity for serotype C (Table 13.2; 232].

The amount of antigen detected is generally accepted to correlate with disease severity; however, titers may persist for some time after therapy is completed, especially for AIDS patients, whose titers may decline slowly even after clinical improvement has occurred. Results may be difficult to interpret when patients are immunocompromised, since some patients will never clear the antigen. Titers can be helpful for monitoring disease, but care must be taken to compare titers only when and if the specimens have been tested with the same test methods or kits during the same batch testing. For titers to have diagnostic meaning,

**Figure 13.1** Cryptococcal Antigen Latex Agglutination System. (Courtesy of Meridian Bioscience, Inc.)

there must be careful adherence to protocols, and samples must be collected and frozen over time for simultaneous batch testing.

13.1.1.1 Latex agglutination tests

Latex agglutination methods were the first tests developed and provide an immunochemical technique for detecting cryptococcal antigen in serum or CSF. Available kits include:

- Cryptococcal Antigen Latex Agglutination System (CALAS; Meridian Bioscience, Inc., Cincinnati, OH) (Figure 13.1)
- Immy Latex Cryptococcal Antigen Latex Agglutination Test (Immuno-Mycologics, Inc., Norman, OK)
- Crypto-LA (Alere North America, Orlando, FL)

Most test kits contain latex particles sensitized with anti-cryptococcal immunoglobulins. Normal rabbit immunoglobulins are used as the control to test for nonspecific latex agglutination. When the anticryptococcal immunoglobulin-sensitized latex particle comes in contact with the polysaccharide antigen of *C. neoformans* in the patient's specimen it forms a complex that results in visible agglutination. The reaction occurs on a slide included in the kit and the test is read by observation. There are both qualitative and semiquantitative protocols for this assay. In the semiquantitative procedure, the titer is the reciprocal of the last dilution to demonstrate agglutination [293].

Despite the sensitive and accurate nature of cryptococcal antigen testing, there are a few pitfalls related to the use of latex test kits. As with any assay, low concentrations of antigen can cause false-negative reactions to occur. False-negative reactions can also be caused by very high antigen concentrations that cause a prozone effect or evident in cases of immune complex formation. The latter can be avoided by the dissociation of such complexes with pronase pretreatment of sera [121, 289].

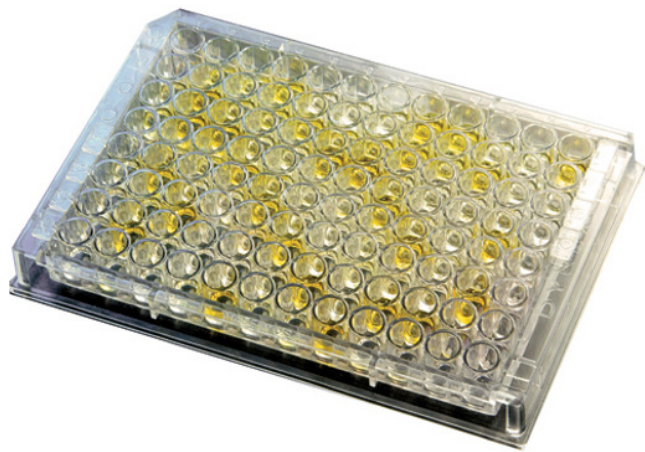


Figure 13.2 Enzyme linked immunoassay for cryptococcal antigen. (Courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)

In addition, false negatives have also been reported in specimens from patients infected with the nonencapsulated variants of *C. neoformans* [154]. Alternatively, false-positive reactions can be caused by the presence of *Trichosporon beigelii* [190], *Stomatococcus mucilaginosus* [45], *Capnocytophaga canimorsus* [314], and the use of BBL Port-a-Cul anaerobic transport device [319]. Rheumatoid factor may cause nonspecific agglutination [80, 140] as will the presence of some soaps and disinfectants [25], povidone-iodine from skin preparations [62], and syneresis fluid [136].

13.1.1.2 Enzyme immunoassays

The Premier Cryptococcal Antigen EIA kit (Figure 13.2; Meridian Bioscience, Inc.) uses anticryptococcal polyclonal antibodies adsorbed to plastic microwells, which can be used for batch assays or snapped off and used for smaller sample runs. The detection system is based on a sandwich EIA using a monoclonal peroxidase conjugate. If cryptococcal antigen is present in the sample, a complex is formed between the adsorbed antibody, the antigen, and the enzyme conjugate. After the wells are washed to remove unbound conjugate, a substrate solution is added, and color will develop in the presence of the bound enzyme and in proportion to the amount of cryptococcal antigen present. With a spectrophotometer, semiquantitative results can be generated, producing titers that can be used to monitor drug therapy or disease severity. Prozone effects, like those that interfere with latex-based testing, are generally not a problem. Cross-reactions can occur, causing false-positive reactions with the yeast *T. beigelii* [190] and with syneresis fluid [136]. Test endpoints are objective but are more costly to



Figure 13.3 Cryptococcal antigen Lateral Flow Assay. (Courtesy of IMMY.) (See insert for color representation of the figure.)

achieve than endpoint titers generated by the latex-based methods [79, 136].

The ALPHA Cryptococcal Antigen enzyme immunoassay (CrAg EIA; Immuno-Mycologics, Inc.) is a qualitative or semiquantitative test system for the detection of capsular polysaccharide antigens of *C. neoformans* in serum and cerebrospinal fluid (CSF). It is a direct immunoenzymatic sandwich microplate assay.

13.1.1.3 Lateral flow assay

The CrAg Lateral Flow Assay (LFA; Immuno-Mycologics, Inc.) is a qualitative or semiquantitative dipstick sandwich immunochromatographic assay, which can be used as a point of care test. It has recently been cleared by the FDA for testing serum and CSF (Figure 13.3). It incorporates two monoclonal antibodies against GXM to cover all serotypes of *C. neoformans* [115, 232]. The LFA reagents are stable at room temperature, have a 2-year shelf life, a rapid turnaround time, and can be performed with minimal laboratory support. The test is performed by adding 40 μ L of serum or CSF to a specimen diluent glycine buffered saline with blocking agent in a microfuge tube or microtiter well, the LFA strip is added and read after incubation at room temperature for 10 min. No pretreatment with pronase is required.

13.1.1.4 Comparative studies

In a comparison of the Premier EIA with the CALAS latex agglutination assays, 475 specimens were screened, including 120 positive specimens from serum and CSF. Serum samples that contained rheumatoid factor were tested to determine which methods would be affected and thus generate false-positive results. When the results were compared, the EIA was found to be virtually equivalent to

the latex method, with 99% sensitivity and 97% specificity [111]. Another report [105] compared the Premier EIA and the CALAS latex agglutination tests ability to detect cryptococcal capsular antigen in serum ($n = 471$, with 103 positives) and CSF ($n = 123$, with 18 positives). The methods were evaluated for the dual purposes of sample screening and quantitation. Good concordance (97.8%) was reported between the tests. However, 12 serum samples and one CSF sample had discrepant screening results. When used for semiquantitative assays for titer determination, the titers obtained with *Cryptococcus* antigen assays performed by EIA were generally higher than those obtained with latex [105].

The Premier EIA kit was compared to several latex agglutination kits by using CSF and serum specimens. Of the 182 CSF specimens, 19 had positive cultures, and of the 90 serum specimens, 30 cultures were positive. The latex agglutination kits used for comparison were CALAS and IMMY. For CSF testing, the sensitivities for all of the kits were comparable, ranging from 93 to 100%. The specificities were also comparable ranging from 93 to 98%. The specificities of the kits for serum testing were similar, ranging from 97 to 100%; both IMMY and Premier EIA had a sensitivity of 97% [293].

Pronase treatment is known to be beneficial for enhancing the sensitivities of polyclonal antibody-based latex agglutination methods [121, 131, 289]. While it was beneficial in reducing false-positive serum test results, studies have shown that pronase pretreatment of CSF is not efficacious, and it is not recommended [131].

A study performed in Cape Town, South Africa involved 62 patients with laboratory confirmed HIV-associated cryptococcal meningoencephalitis. Serum, plasma, and urine were tested with the CrAg LFA and an enzyme-linked immunosorbent assay (ELISA) using the same monoclonal antibodies used in the LFA test. All patients had GXM detected in all specimen types by the ELISA and 61/62 (one urine negative) by LFA with a sensitivity of 100% for serum and plasma and 98% for urine [147].

A prospective study performed in Sidney, Australia evaluated 92 patient's serum, CSF, and urine when available, by both CALAS and the CrAg LFA. Twenty-five of the patients were diagnosed with cryptococcosis either by culture (20), histology (three) or molecular methods (two). They found that the LFA and CALAS were equivalent when used qualitatively but suggest that further study is required to correlate titer between latex agglutination and the lateral flow assay [191]. In a study of archived sera from HIV-infected patients the LFA was compared to Premier EIA positive specimens with a 90% agreement at a 5 min reading and 96% agreement when read at 15 min [173]. Two EIA negative specimens were positive by LFA. Seventy percent of

urines from EIA serum positive patients were positive. The FDA-approved LFA kit is currently cleared for serum and cerebrospinal fluid and not urine.

13.1.2 *Candida* spp.

The presence of an unidentified heat-labile *Candida* antigen can be detected in serum by the Cand-Tek Latex Agglutination test (Ramco Laboratories, Stafford, TX). In an early study of 911 samples, the reproducibility of titer results was reported to be 90% (exact titer) to 100% (± 1 dilution) [40], but the sensitivity for detection of disseminated candidiasis was limited depending on titer used to define a positive (46–95%). The specificity for disseminated candidiasis ranged from 22 to 99% [40, 137, 218].

The Cand-Tek method may be best used as a measure of the severity of disease, since it has been suggested that the antigens detected by this assay accumulate in sera from patients with renal failure. In a study [32] that measured creatinine levels of 22 patients with proven disseminated candidiasis, the Cand-Tek assay yielded positive results for a significantly larger number of patients having disseminated candidiasis than those with simple local colonization. In addition, serum creatinine levels exhibited an increase parallel with that of Cand-Tek titers. Despite this association, results based on disease prevalence and a positive reaction cutoff of a 1:8 titer showed that a positive Cand-Tek assay result had only a 79% probability of being associated with disseminated candidiasis.

In an analysis of the Platelia *Candida* Ag Plus test (Bio-Rad, France (formally Pastorex); not currently available in United States) with the Cand-Tek antigenemia test, the specificities were 100 and 98.3%, respectively. The overall sensitivities of both tests were low, but with multiple testing, a positive antigen test result preceded other indications of candidemia for six of ten Platelia-positive patients and five of nine Cand-Tek positive patients. Multiple sampling increased the sensitivity of the Platelia *Candida* test to a high of 76.9%; however, this was not the case with the Cand-Tek assay [137].

The Cand-Tek test was compared to methods for the detection of enolase, mannan antigen (Pastorex *Candida* assay) and to the detection of β -glucan by the *Limulus* test. In 39 patients with candidemia, the sensitivity of the assays was 76.9%, 25.6%, 84.4%, and 71.2% for enolase, Cand-Tek, Pastorex *Candida*, and β -glucan assays respectively. Of 10 patients with superficial *Candida* colonization, 10 patients with deep infections, and 20 healthy subjects, it was found that two patients with superficial infections and one patient with invasive pulmonary aspergillosis were also positive with the Cand-Tek assay. None of the tests in this study appeared

satisfactory for the initial diagnosis of candidemia; however, the tests may be helpful in certain diagnostic situations [204].

A meta-analysis of 14 studies of mannan antigenemia determined by the Platelia *Candida* assay comprised of 453 patients and 767 controls showed an overall sensitivity of 62% (range 31–100%) and specificity of 93% (range 65–100%) [200]. These studies included patients with hematological malignancy and from intensive care units (ICU) that were classified by the 2008 European Organization for Research and Treatment of Cancer-Mycoses Study Group (EORTC-MSG) criteria [65].

13.1.3 *Aspergillus*

Invasive aspergillosis is a serious problem for all immunocompromised patients, especially those undergoing bone marrow transplantation or chemotherapy. The disease is severe, progresses rapidly, and is difficult to diagnose and treat. Fatality rates can approach 100% but can be reduced with early diagnosis and proper therapy. Unfortunately, blood and respiratory cultures may be negative during the early stages of disease [138].

13.1.3.1 Platelia *Aspergillus* EIA

The Bio-Rad Platelia *Aspergillus* EIA (Bio-Rad Laboratories, Redmond, WA) is a one-stage immunoenzymatic sandwich assay that uses the rat monoclonal antibody EBA-2 to detect circulating galactomannan antigen in human serum and bronchoalveolar lavage fluid (Figure 13.4). The galactomannan antigen is released in the circulation of patients or respiratory fluid during active invasive aspergillosis. Results are expressed in index values and can be obtained in 3 h. This method is now integrated into the EORTC-MSG criteria of definition and classification of invasive aspergillosis [303]. Platelia *Aspergillus* EIA allows the diagnosis of invasive aspergillosis at an early stage of the disease, often before the onset of clinical signs [182].

A modified pretreatment technique (MT) was used on consecutive negative Platelia *Aspergillus* EIA plasma samples from invasive *Aspergillus* patients in order to improve the sensitivity of the assay [195]. Fifty-two plasma samples were collected from healthy donors, and 174 plasma samples with a galactomannan index (GMI) below 0.5 were collected from 25 unclassifiable and 23 patients with invasive aspergillosis. The reactivity of pretreated samples was determined before (conventional technique [CT]) and after filtration concentration by using a filter with a 50-kDa cutoff (MT). For the MT, the sensitivity of the Platelia *Aspergillus* EIA increased from 42.9% (CT) to 78.6% (MT) using a cutoff for the GMI of 1.5 in the probable and proven group, whereas specificity slightly decreased from 98.7 to 96.1% in the control group. The tenfold concentration step increased the GMI as high as 121-fold. The MT resulted not only in positive reactivity in samples that tested negative with the CT but also in the earlier detection of antigen by 2–17 days [195].

13.1.3.2 Cross-reactivity

Soon after the introduction of the Platelia *Aspergillus* assay it was noted that cross-reactions occurred with patients on antibiotic therapy. The initial association was with piperacillin-tazobactam but was expanded to amoxicillin-clavulanate, cefepime and cefoperazone-sulbactam in both serum and bronchial alveolar lavage (BAL) specimens [26]. Nine of 11 hematological patients with disseminated *Fusarium* spp. infection tested at least twice for *Aspergillus* galactomannan (GM) had repeated positive results in the absence of *Aspergillus* isolation in culture. The centrifuged supernatants of 12 *Fusarium* isolates were tested by a GM enzyme-linked immunosorbent assay (EIA). All the isolates produced positive reactions when tested undiluted. These results show cross-reactivity of *Fusarium* spp. with *Aspergillus* GM that may constitute a drawback with respect to the specificity of the Platelia EIA [300]. In another study [323] cross-reactivity in the



Figure 13.4 Platelia *Aspergillus* Antigen assay kit. (Courtesy of Bio-Rad Laboratories, Inc.) (See insert for color representation of the figure.)

Platelia *Aspergillus* enzyme immunoassay was evaluated using 120 sera from patients with paracoccidioidomycosis, histoplasmosis, or cryptococcosis. At a cutoff value of 0.5, positivity rates were 50%, 67%, and 50%, respectively. An evaluation of HIV-infected patients in Taiwan found that *Penicillium marneffei* cross-reacts in the GM assay of patients with penicilliosis, cryptococcosis, and controls, 73.3%, 13.6%, and 9%, respectively ($P = 0.0001$). The use of the GM antigen assay may facilitate earlier diagnosis of *P. marneffei* infection for HIV-infected patients in areas of endemicity [141].

Twenty-three of 48 serum specimens positive for antigen in the second-generation *Histoplasma* antigen EIA (MiraVista Diagnostics, Indianapolis, IN) were positive in the Platelia *Aspergillus* EIA. Seven of 11 BAL fluid specimens that were positive in the *Histoplasma* antigen EIA were positive in the Platelia *Aspergillus* EIA. Ten control BAL fluid specimens that were negative in the *Histoplasma* antigen EIA were negative in the Platelia *Aspergillus* EIA. Care must be taken to exclude histoplasmosis for patients with positive Platelia *Aspergillus* EIA results [315].

13.1.4 1,3- β -D-glucan

The Fungitell assay (Associates of Cape Cod, East Falmouth, MA) is based on a modification of the *Limulus* Amebocyte Lysate assay. The Fungitell reagent is modified to eliminate Factor C (specific for endotoxin) and only react to 1,3- β -D-glucan (BDG) through the Factor G mediated side of the pathway. The reaction uses a protease zymogen-based colorimetric assay for the qualitative detection of BDG in the serum of patients with symptoms of, or medical conditions predisposing the patient to, invasive fungal infection. The serum concentration of BDG, a major cell-wall component of various medically important fungi can be used as an aid in the diagnosis of disseminated mycoses and fungemia such as *Candida* and *Aspergillus* [65, 215]. A positive result does not indicate which class of fungi may be causing infection. In addition the Fungitell assay does not detect certain fungal species such as the genus *Cryptococcus*, which produces low levels of BDG [205], and *Zygomycetes* [221], which are not known to produce BDG. The yeast phase of *Blastomyces dermatitidis* produces little BDG and may not be detected by the assay [118]. Difficulties have been reported in interpreting the results of this assay, since plasma glucan activity was found to be increased in hemodialysis patients [151, 153], and subjects who were undergoing chemotherapy for hematologic malignancies but who had no signs of invasive fungal disease [150]. Other false-positive reactions have also been observed in patients after surgery, perhaps due to the presence of BDG in surgical gauze or cellulose dialyzer tubing [216, 259].

A report investigating the interaction between intravenous ampicillin-sulbactam treatment and BDG assay did not observe any significant association of ampicillin-sulbactam administration and positive assays for BDG [199]. Fifteen patients without known risk factors for invasive fungal infections who received a daily dose of ampicillin-sulbactam monotherapy from different batches were included in the study. Thirteen patients had soft tissue infections; five of 13 patients who underwent surgery had surgical dressings. Serum samples were obtained both before and after antibiotic infusion on the first, third, seventh and tenth days of an ampicillin-sulbactam treatment course. The BDG was assayed using the Fungitell kit. A total of 37 of 117 serum samples were positive for BDG at a threshold of 80 pg/mL. After ruling out fungal infections and all known potential causes of false BDG positivity, environmental contamination remained a possible cause of BDG reactivity. False-positive reactions can also occur with patients receiving certain lots of piperacillin-tazobactam or unknown factors [198].

As with many other laboratory tests it is important to apply antigen testing to patient populations with a high probability of invasive fungal infection. Odabasi et al. evaluated 283 patients with acute myelogenous leukemia or myelodysplastic syndrome receiving antifungal prophylaxis by obtaining serial blood specimens for BDG testing. They report a median of 10 days for a positive BDG prior to diagnosis of an invasive fungal infection with a 100% negative predictive value and 90% specificity for a single positive test and 96% for two or more successive positive tests [220].

Onishi et al. in 2012 published a meta-analysis of the utility of the BDG assay in the diagnosis of *Pneumocystis jirovecii* pneumonia (PJP) and invasive fungal infections (IFI) by *Candida* spp. and *Aspergillus* spp. This is an extensive analysis that included 36 studies from many different countries (Japan > United States > others) with both HIV positive and negative patients. The majority of studies were on patients with a hematologic underlining disease. They found that the diagnostic accuracy is high for PJP and moderate for IFI. The sensitivity and specificity for PJP was 96% (confidence interval (CI) 92–98%) and 84% (CI 83–86%) and 80% (CI 77–82%) and 82% (CI 81–83%) for IFI [224]. Comparative studies of the direct stain method to molecular assays are addressed below in the section on molecular diagnostics.

13.1.5 Endemic mycoses

Antigen detection tests can be used as part of a diagnostic approach for histoplasmosis, blastomycosis, and coccidioidomycosis. There are three proprietary tests, two offered by reference laboratories: MiraVista Diagnostics

(Indianapolis, IN) and ARUP (Salt Lake City, UT) [47]. ARUP developed their assay using ASRs from Immuno-Mycologics, Inc. The reference laboratories have validated the assays in their respective laboratories. The only currently FDA-approved test is the ALPHA *Histoplasma* EIA test (Immuno-Mycologics, Inc.).

The ALPHA *Histoplasma* EIA test is a sandwich microplate assay which detects *Histoplasma* antigens in urine. Polyclonal rabbit anti-*Histoplasma* IgG antibodies are bound to microwell plates and capture antigen, which is detected after washing by biotinylated rabbit anti-*Histoplasma* IgG antibodies. As with all fungal antigen assays there is significant cross-reactivity. Antigens from *B. dermatitidis*, *C. immitis*, and *P. brasiliensis* were detected while antigens from *Aspergillus* spp., *Candida albicans*, and *C. neoformans* did not cross-react.

Many reports in the literature demonstrate the utility of antigen detection in specific patient populations and in cases where clinical suspicion of an infection by one of the systemic mycoses is high. However, there is significant cross-reactivity of each of the assays to the others and to other fungal organisms [7, 73, 165]. The authors of one recent publication regarding the specificity of the MiraVista reference test state that it is not possible to differentiate between histoplasmosis and blastomycosis by antigen detection alone [56].

13.2 Culture and detection

13.2.1 Fungal blood cultures

Increases in cases of fungemia have resulted from greater numbers of immunocompromised patients, the widespread use of invasive medical devices, and the use of broad-spectrum antibiotics. Mortality rates range from 38 to 100%, and early diagnosis is critical. While *Candida* spp. account for a large percentage of isolates, the range of different fungal species isolated is extensive [117]. Many publications have compared fungemia detection systems prior to the 1990s, and the reader may refer to reviews of those studies elsewhere [117]. Clearly, no system is 100% sensitive or amenable to use in every situation or health-care facility. A combination of different methodologies may provide the most sensitive culture, but this is not usually feasible. The shifting prevalence of commonly identified species and emerging pathogens may force the use of algorithms to provide guidelines for the most effective use of resources. Many of the blood-culture systems described here are also described and depicted elsewhere in this text in the sections describing bacterial blood-culture systems.

13.2.2 Manual systems

The Wampole Isolator System (Alere North America, Orlando, FL) lysis-centrifugation blood-culture method has long been regarded as one of the most sensitive systems

for the detection of fungemia. Blood-collection devices are available as 10 mL draws and as 1.5 mL draws for children [236]. The Isolator system (Table 13.3, Figure 13.5) uses saponin to lyse erythrocytes and leukocytes, releasing microorganisms from white blood cells and inactivating inhibitory serum factors and antimicrobial agents. In this system, the blood is lysed before organisms are concentrated by centrifugation. The sediment is subsequently inoculated to solid media [294]. Improvements to and automation of broth-based systems have made them an attractive possibility for the future, but continued studies performed with the Isolator system reconfirm its merit for the detection of fungemia, primarily for the detection of filamentous fungi.

The Isolator system has been proven to be more rapid and sensitive than detecting fungi in vented biphasic or broth media, especially for the recovery of *Histoplasma capsulatum* [117, 213, 294]. It was also found to be superior to the other broth-based systems [31]. In one retrospective study, 23,586 matched pairs of fungal blood cultures were compared, and the Isolator system enhanced the detection of fungi by as much as 30% compared to the Septi-Chek system (Becton, Dickinson and Co., Franklin Lakes, NJ; please check on the availability of this product in your region or country) [124]. Fungal recovery can be enhanced by as much as 15–50% in comparison to broth cultures, and time to recovery is generally decreased [117]; however, newer automated systems have compared more favorably than the traditional broth-based fungal cultures.

In one study the Isolator method had the highest recovery rate and shortest recovery time compared to both the BACTEC and Septi-Chek systems [294]. When the Isolator and BACTEC Plus 26 aerobic medium were compared to the BACTEC high-blood-volume fungal medium (HBV) for 93 test isolates, HBV proved to be comparable, except for the recovery of *H. capsulatum*, where the Isolator system was superior. In contrast, *Candida glabrata* was detected earlier in HBV than in the Isolator system. In another study the Isolator system proved to be superior to the BACTEC Plus 26 aerobic medium for recovery of *H. capsulatum*, *C. glabrata*, and other fungi when 86 cultures positive for fungi were tested [321]. The Isolator system also detected significantly more *Candida* spp. than the ESP 80A or the Septi-Chek system [54]. A significantly improved recovery rate was also documented for 171 isolates of *C. albicans* and *Candida parapsilosis* [159].

The Isolator system was also found to be superior, especially for *Candida* spp., *H. capsulatum*, and *C. neoformans*, to the BacT/Alert system. In 22 cases of candidemia, the Isolator system recovered 100% of the isolates, with a mean time to detection of 2.9 days (range, 0–9 days), while the BacT/Alert system recovered 68.2% of the isolates, with a comparable mean time to detection of 2.1 days (range,

Table 13.3 Comparison of Isolator method with other blood-culture methods and systems

Assay	Method	Comparison	n	Sensitivity (%)		Comments	Reference
				Isolator	Compared system		
Isolator 10	Lysis centrifugation	Bactec HBV	93			Isolator provides better detection of <i>H. capsulatum</i> ; HBV better for <i>C. glabrata</i>	321
		Bactec 26B	86			Isolator superior for <i>H. capsulatum</i> , <i>C. glabrata</i> , others	321
		Bactec 9240 with MYCO/F lytic bottle	24	79.2	70.8	Isolator provides advantage for <i>C. neoformans</i>	312
		BacT/Alert	22	100		Improved TTD with Isolator; <i>H. capsulatum</i> and <i>C. neoformans</i> from Isolator only	181
		BacT/Alert	22		68.2		181
		BacT/Alert for <i>C. neoformans</i>	53	88.7	43.4	Shorter TTD with Isolator; both systems costly	23
		BacT/Alert for <i>H. capsulatum</i>	18	100	22.2	Shorter TTD with Isolator, both systems costly	23
		ESP 80A (VersaTREK)	72	85	56	Isolator superior; detected significantly more <i>Candida spp.</i>	31,54
		Septi-Chek	72	85	57	Isolator superior; detected significantly more <i>Candida spp.</i>	54
		Conventional broth methods	20 (true positive)	70	90	False negatives in 30% (6 of 20) with Isolator vs. 20% with broth method	211
			42 (false positive)	97.6	2.4	Routine use of Isolator was not advocated	211
Isolator 1.5		Bactec NR660 Aerobic 6A	89	85.4	82	Comparable systems for <i>Candida spp.</i> in pediatric population; similar TTDs	236

TTD, time to detection.

**Figure 13.5** ISOSTAT system (Isolator). (Courtesy of Alere.)

0–5 days). *Histoplasma capsulatum* and *C. neoformans* were recovered only by the Isolator system [181].

When the small volume Isolator 1.5 was compared to the BACTEC NR660 system aerobic NR6A blood-culture

bottle for detection of fungal infections in pediatric patients, NR6A was found to be comparable to Isolator 1.5 in both yield and recovery time [236].

In another study with 53 isolates of *C. neoformans*, the Isolator system detected 47 while the BacT/Alert system detected 23. Of 18 isolates of *H. capsulatum*, the Isolator system detected 18 and the BacT/Alert system detected four. Time to detection was shorter for the Isolator system, 14 days for *H. capsulatum* and 6 days for *C. neoformans*, compared to the BacT/Alert with 18 and 11 days, respectively [23]. It should be noted that with current recommendations for holding automated blood cultures for 5 days without terminal subculture would have resulted in negative cultures for both *H. capsulatum* and *C. neoformans*.

In a 1998 comparison, the BACTEC 9240 system with MYCO/F lytic medium was compared to the Isolator system. Of 24 blood cultures positive for fungi, 12 were positive in both systems, seven were positive only in the Isolator system and five were positive only with the MYCO/F lytic bottle. Of 14 positive blood cultures containing *H. capsulatum*, seven were positive in both systems and seven were positive only in the Isolator system. For fungal isolates in this study, the mean times to detection were similar at 8–9 days, but the organisms were more quickly identifiable from the Isolator plates. For *C. neoformans*, the MYCO/F bottle may have an advantage. Of ten blood cultures positive for *C. neoformans*,

five were positive in both systems and five were positive in only the MYCO/F system. The mean time to detection was 4 days in the MYCO/F system and 7 days with the Isolator system [312].

Despite its widespread use, the Isolator system can produce false-positive results due to contamination events related to the multiple-step processing of Iso-STAT tubes; this is observed mostly with bacterial isolates and not with fungi. It is important to monitor false-positive rates in individual laboratories to identify the case definition of fungemia, and to determine the types of organisms isolated in each patient population [206, 207]

In one retrospective study, the utility of the Isolator system for the detection of fungal recovery was compared to that of culture in the BACTEC NR660 system. Strict definitions for both positive and false-positive blood cultures were given, specific to the study. Of 66 positive fungal blood cultures, 42 were considered to be due to contaminants, and all except one derived from the Isolator system. The use of these definitions produced summary results with 16 different species of fungal contaminants [58]. Similarly, relatively high contamination rates were reported in other studies and may detract from

the cost-effectiveness of a system that is very labor- and cost-intensive [106, 157, 210, 294]. Clearly, the utility and cost-effectiveness of the Isolator system for fungal blood cultures will vary with institutional blood-culture collections, patient population, processing, and contamination rates.

13.2.3 Continuously monitored automated blood-culture systems

A major advancement in the diagnosis of both bacteremia and fungemia was the development of the continuously monitored blood-culture systems. Each of the commercially available systems are effective but vary in the type of media and specimen types, other than blood, that are offered (Table 13.4).

13.2.3.1 BacT/Alert 3D

The BacT/Alert system (bioMérieux, Durham, NC) is a continuously monitored closed blood-culture system for which no special fungal media are currently available. The system is based on the photochromatic detection of CO₂ as organisms grow in the broth. In 2004 Horvath et al. [139] used blood-culture bottles with blood from

Table 13.4 Continuous monitor blood-culture system

Instrument	Bottle type	Media	Specimen type	Maximum volume (mL)
BacT/ALERT	Standard aerobic (SA)	40 mL suppl TSB	Blood or normally sterile body fluid	10
	Standard anaerobic (SN)	40 mL suppl TSB	Blood or normally sterile body fluid	10
	FAN aerobic (FA)	30 mL peptone-enriched TSB, supplemented with BHI solids and activated charcoal	Blood or normally sterile body fluid	10
	FAN anaerobic (FN)	40 mL peptone-enriched TSB, supplemented with BHI solids and activated charcoal	Blood or normally sterile body fluid	10
	Pediatric FAN (PF)	20 mL peptone-enriched TSB, supplemented with BHI solids and activated charcoal	Blood	4
BACTEC	Standard/10 aerobic/F	Enriched soybean-casein digest broth	Blood	10
	Standard anaerobic/F	Pre-reduced enriched soybean-casein digest broth	Blood	10
	Plus aerobic/F	Enriched soybean-casein digest broth with cationic resin	Blood	10
	Plus anaerobic/F	Pre-reduced enriched soybean-casein digest broth with cationic resin	Blood	10
	Lytic/10 anaerobic/F	Pre-reduced enriched soybean-casein digest broth	Blood	10
	Peds plus/F	Enriched soybean-casein digest broth with cationic resin	Blood	3
	Mycosis-IC/F	40 mL BHI broth, soybean-casein digest broth	Blood	10
VersaTREK	Myco/F lytic	40 mL supplemented 7H9 and brain heart infusion broth	Blood and body fluids	10
	REDOX 1 EZ Draw, aerobic with stir bar	40 mL	Blood and sterile body fluids	5
	REDOX 2 EZ Draw, anaerobic	40 mL	Blood and sterile body fluids	5
	REDOX 1, aerobic with stir bar	80 mL	Blood and sterile body fluids	10
	REDOX 2, anaerobic	80 mL	Blood and sterile body fluids	10

healthy donors and seeded with *Candida* spp. to compare recovery by the BacT/Alert (FA, SN, and MB+suppl) to the BACTEC 9240 (Plus Aerobic/F, Plus Anaerobic/F, and Myco/F Lytic bottles). Fifty clinical isolates, 10 of each species were used. Six bottles representing each medium received 10^3 CFU (colony-forming units) of each strain of *C. albicans*, *C. glabrata*, *Candida krusei*, *C. parapsilosis* or *Candida tropicalis* and incubated per manufacturers' instructions. Of the 300 bottles inoculated with *Candida* spp, 235 (78%) grew the corresponding strain. The remaining 65 bottles not flagged by the instruments by day 12 were subcultured and all of the corresponding organisms were grown. A breakdown of the number of positives detected by the instruments revealed that the BACTEC Myco/F, BacT/Alert FA, and BacT/Alert MB bottles recovered all isolates. This was in contrast to the BACTEC Plus Anaerobic (45 negatives), BacT/Alert SN (15 negatives) media, and the BacT/Alert SA (five negatives). Most laboratories do not include mycology media in the routine blood-culture protocol. When comparing only the aerobic and anaerobic media, the BacT/Alert performed better than the BACTEC in overall detection, time to detection, number of false-negatives, and missed episodes of simulated candidemia [139].

Although the BacT/Alert system had slightly diminished capacity for the detection of some fungi [181], use of the FAN aerobic media isolated significantly more yeasts than standard aerobic medium, with the same mean time to detection [313]. When compared to the standard anaerobic bottle, vented for aerobic use, the FAN bottle recovered significantly more *Candida* spp. [57]. In contrast, when the FAN anaerobe bottle was compared to standard aerobic medium, more *C. glabrata* isolates were recovered from the aerobic medium [117]. When the BacT/Alert system was compared to the BACTEC NR660 or 730 system, the BacT/Alert system had a noticeable but not statistically significant advantage in recovery of fungi; however, terminal subcultures of both systems added many of the fungi that otherwise would not have been recovered [322]. Saito et al. compared the FAN BacT/Alert media to standard media in a before-and-after study over a 4-year period. Although not focused on candidemia they did find that the FAN formulations had a higher percentage of positives for *Candida* spp. [267].

13.2.3.2 BACTEC

Various models of the BACTEC blood-cultures systems (Becton, Dickinson and Co., Sparks, MD) are used in clinical laboratories worldwide. Older models, based on the radiometric, interval-based detection of CO_2 , have been replaced by nonradiometric (NR) systems and, most recently, by the BACTEC 9000 and FX systems, which use fluorescent sensor technology to continuously monitor

CO_2 production. Barcode-scanning and data management systems are also included with these systems.

In a study of the BACTEC NR660 and Oxoid Signal systems (Remel), the BACTEC system proved to have the advantage in detection time. The NR660 system isolated 18 yeasts, with median times to detection of 39 h compared to 168 h for the Signal system [277]. In 1992, BACTEC PLUS high-volume resin (BP-HBV) and aerobic BP26 and anaerobic BP27 vials were compared to standard BACTEC aerobic NR6A and anaerobic NR7A vials. BACTEC PLUS vials enhanced the recovery rate for *C. albicans* for patients receiving antibiotics [184].

The BACTEC system has a slightly diminished capacity for detection of some fungi compared to the Isolator system. When BACTEC HBV was compared to the Isolator system and BACTEC BP26 with 93 isolates tested, HBV proved to be comparable, except for the recovery of *H. capsulatum*, where the Isolator system was superior. In contrast, *C. glabrata* was detected earlier in HBV than in the Isolator system. For HBV versus BP26, 68 positive fungal isolates were identified, and HBV was found to be superior for *C. glabrata* and all fungi combined. No difference was noted in the mean time to detection [321].

The BACTEC 9240 system was evaluated in comparison with the conventional BACTEC NR730 system using two media with the same formulation: NR26 for the BACTEC NR730 system and Aerobic Plus F for the BACTEC 9240 system. Simulated blood cultures were prepared for 41 strains belonging to 18 different fungal species, meant to represent fungi that are responsible for hematogenous dissemination. In a second phase of the study, two different media, HBV and NR26, were both compared on the BACTEC NR730 system. The differences between the two systems and the two media (NR26 and HBV) were not statistically significant, but use of HBV did improve the detection of fungi on the BACTEC NR730 system. In addition, the BACTEC 9240 system had a mean detection time that was significantly shorter than that of the BACTEC NR730 system [109].

In one of two 1998 studies, simulated blood cultures were used to compare the BACTEC 9240 medium (Mycosis IF/C) with the BACTEC standard medium, the Aerobic Plus F. In this study, 43 strains and 10 species of yeast were tested. The median time to detection was 29.03 ± 13.99 h for Mycosis IF/C compared to 73.92 ± 56.74 h for Aerobic Plus F medium. Mycosis IF/C reduced the time to detection [108]. In another study, specifically targeting *C. neoformans*, the MYCO/F medium appears to have an advantage over the Isolator system. Of ten blood cultures positive for *C. neoformans*, five were positive in both systems and five were positive in only the MYCO/F medium. The mean time to detection was 4 days in MYCO/F and 7 days with the Isolator system [312].

13.2.3.3 VersaTREK (ESP) System

The VersaTREK (Trek Diagnostic Systems, Inc. Cleveland, OH, now ThermoFisher, Waltham, MA) is the current evolution of what was originally the DIFCO ESP system, which was acquired by Trek Diagnostics and now marketed through ThermoFisher Scientific. The VersaTREK and its predecessor the DIFCO ESP blood-culture system detect changes in bottle headspace gas pressure due to oxygen consumption and CO₂ generated by microbes. In 1994, the ESP system with ESP 80A and 80N bottles was compared to the BACTEC NR660 system using BACTEC 6A/7A, 16A/17A, or pediatric bottles. In this study, ESP 80N anaerobic bottles detected significantly more *Candida* spp. [209]. In an assessment of neonatal sepsis, the ESP blood-culture system isolated 41 yeasts. By 48 h of incubation, 88% (36 of 41) of those isolates were identified as positive cultures. There was no difference in the time to positivity in pre-therapy and post-therapy blood cultures [114]. In a comparison of the ESP and the BacT/Alert systems, no significant difference was documented for 31 yeast isolates [327].

Differences in instruments and changes in media formulation make the early studies of this system less relevant. Only a few comparison studies using the current system have been published. Mirrett et al. performed a comparison of the VersaTREK, using REDOX 1 aerobic, REDOX 2 anaerobic media, to the standard aerobic (SA) and anaerobic (SN) media of the BacT/ALERT blood culture systems with patients suspected of having sepsis [202]. The fungal isolates were all *Candida* spp. (14 *C. parapsilosis*, 12 *C. glabrata*, nine *C. albicans*, four *C. tropicalis* and one *C. krusei*). No breakdown of the results for each of the species was provided, so the data are expressed as an aggregate of the total number of 40 isolates. Yields for *Candida* spp. were the same for both systems (23 both systems, eight VersaTREK only, nine BacT/ALERT only). This was also true when selecting only clinically significant isolates (14 both systems, six VersaTREK only, three BacT/ALERT). The mean times to detection were 35.8 h and 37.5 h respectively for VersaTREK and BacT/ALERT. They conclude that the systems are not significantly different in their ability to detect *Candida* spp. with the same time to detection.

A second study compared the VersaTREK REDOX 1 medium to the BACTEC FX Plus Aerobic/F medium using bottles seeded with 10–100 CFU of clinical isolates and ATCC reference strains of *C. albicans* (10), *C. glabrata* (4), *C. tropicalis* (3), *C. parapsilosis* (2), *C. krusei* (1), *C. neoformans* (3) and *C. laurentii* (1) [265]. All isolates tested susceptible using Sensititer YeastOne system (TREK) to amphotericin B, fluconazole, voriconazole, and caspofungin with the exception of *C. glabrata*, which were susceptible–dose dependent (S-DD) to fluconazole.

A total of 721 seeded blood-culture bottles were incubated with and without antifungal agents in each of the systems. *Candida krusei*, *C. neoformans* (3) and *C. laurentii* (1) failed to be detected, were negative on terminal subculture, and therefore not included in the analysis. In the absence of antifungals both systems had comparable recovery rates, with VersaTREK detecting 99.1% versus 97.4% for BACTEC ($p = 0.154$). VersaTREK had the advantage in time to detection (2.2 h faster, $p = 0.001$) in the absence of antifungals but the BACTEC system had a significant advantage when antifungals were present (10.8 h faster, $p = 0.001$).

13.3 Identification systems

13.3.1 Media for isolation and identification

Available media include:

- CHROMagar *Candida* (CHROMagar, Co. Paris, France and Becton, Dickinson and Co.)
- ChromID *Candida* (bioMérieux); *albicans*ID and *albicans*ID2 are previous versions; *Candida*ID2 (Figure 13.6)
- CandiSelect 4 (Bio-Rad); current version of CandiSelect (Figure 13.7)

CHROMagar is a chromogenic differential and selective medium for isolation and differentiation of *C. albicans*, *C. tropicalis*, and *C. krusei* directly from clinical specimens. The suggested incubation temperature is $35 \pm 2^\circ\text{C}$. Colony color and morphology are used for identification

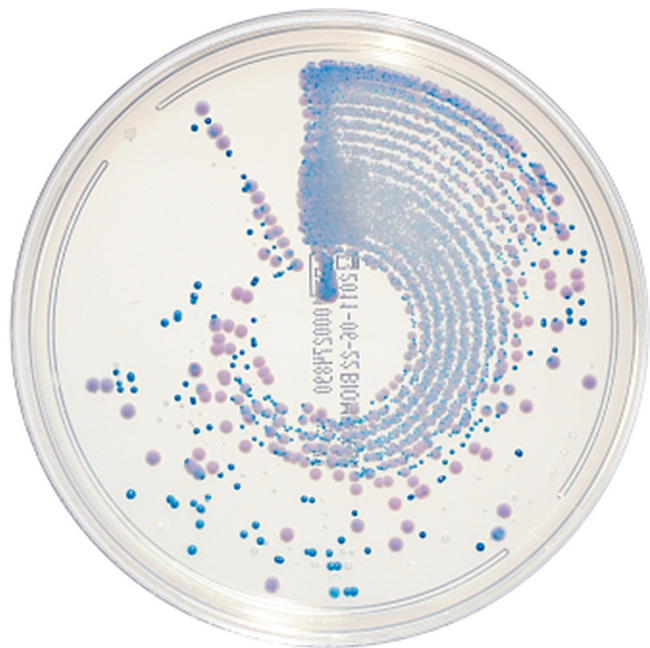


Figure 13.6 ChromID *Candida* agar. (Courtesy of bioMérieux.) (See insert for color representation of the figure.)

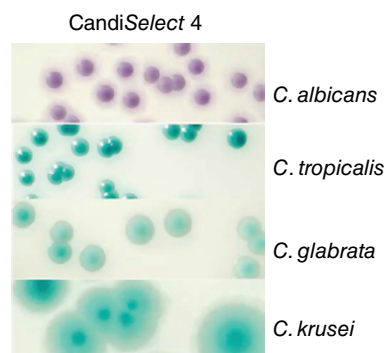


Figure 13.7 Different appearance of *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. krusei*. (Courtesy of Bio-Rad Laboratories, Inc.) (See insert for color representation of the figure.)

purposes. *Candida albicans* grows as light to medium green colonies with slight green halos due to the presence of the enzyme β -N-acetylgalactosaminidase [1]. *Candida tropicalis* grows as dark blue to metallic blue colonies. *Candida krusei*, the isolation and identification of which are important because of its resistance to the common antifungal fluconazole, grows as large light mauve to mauve colored crenated colonies with white edges. CHROMagar supports the growth of yeasts and most fungi while inhibiting most bacteria due to the presence of chloramphenicol.

In another study of CHROMagar [222], the presumptive identification of *C. albicans*, *C. krusei*, and *C. tropicalis* exceeded 99% for all three species when blinded reading tests were performed by four different personnel. Several occurrences of potential misinterpretation are noteworthy, although such instances are rare. For example, rare isolates of *C. norvegensis* produced colony morphology similar to that of *C. krusei*. In addition, some *Geotrichum* spp. and *Pichia* spp. formed colonies with a grey, blue, or green color or a dark halo in the agar. In addition, *Candida dubliniensis* has been reported to grow as dark-green-colored colonies upon primary isolation [160, 275]; however, the presence of this phenotype is variable. When compared to identification by DNA sequencing, only 56% of all *C. dubliniensis* isolates tested ($n=53$) displayed the green phenotype [297].

In one study [269], in which 1537 yeast isolates were tested and read after 48 h of incubation, sensitivity and specificity were 99% and 100% for *C. albicans*, 93.8% and 99.1% for *C. tropicalis*, and 100% and 100% for *C. krusei*. A large sample size makes this comparison noteworthy, with 970 *C. albicans*, 165 *C. parapsilosis*, 131 *C. glabrata*, 62 *C. guilliermondii*, 35 *C. krusei*, 32 *C. tropicalis*, 31 *Rhodotorula rubra*, and 23 *Trichosporon* spp. and a variety of other species. The accuracy of CHROMagar is reported to be similar to those of

germ-tube tests and chlamydospore development for the identification of *C. albicans* [268].

Additionally, CHROMagar was evaluated for 618 yeast isolates and 128 direct specimen inoculations. After 2 days at 37°C, the sensitivities for identifying species were as follows: *C. albicans*, 99.4% ($n=341$); *C. glabrata*, 98.9% ($n=99$); and *C. krusei* ($n=35$) and *C. tropicalis* ($n=73$) 100%. The specificities were 100%, 99.8%, 99.6%, and 99.6%, respectively. The authors also report CHROMagar to be useful for the identification and determination of mixed cultures [22]. In another study [21] detection rates for *C. albicans* were reported to be as much as 20% higher than those reported with Sabouraud-chloramphenicol plates ($n=951$), with 92.2% correct identification after 72 h. The specificity was reported to be 100%.

Another study [107] compared CHROMagar to two other commercial media for identification of *Candida* spp., *albicans* ID and CandiSelect (Sanofi Diagnostics Pasteur), both of which contain fluorogenic substrates in the agar. All were compared with isolation on traditional Sabouraud-chloramphenicol agar medium followed by standard yeast identification. A total of 192 clinical specimens were tested. For all yeast species, CHROMagar yielded the largest colonies. The identification rates for *C. albicans* were nearly equivalent at 86.59%, 83.79%, and 83.24%, respectively. At 72 h, *albicans* ID, Candi Select, and CHROMagar identified 92.75%, 91.3%, and 88.57% of the specimens, respectively, but at 24 h the sensitivities for growth and specific pigment were substantially lower at 56.6%, 37.68%, and 11.59%, respectively. Although CHROMagar did not identify *C. albicans* as quickly as the other two commercial identification media, the agar allowed correct identification of *C. tropicalis*, the main source of false-positives on most other chromogenic media. In addition, the authors report that morphology is easier to read with CHROMagar.

In 1998, two studies were reported. In one [1], 21 yeast isolates from neutropenic and AIDS patients were tested, both on CHROMagar and Sabouraud dextrose agar. The overall sensitivities for detecting *C. albicans* were the same for both media. The primary isolation on CHROMagar was found to be 100% sensitive and 100% specific for *C. albicans*. For identification purposes, CHROMagar proved to be the most economical and the least time-consuming method. The authors of the study suggest its use for blood-culture plating when yeasts are seen in microscopy or when early therapy is imperative. In another study [253], CHROMagar *Candida* was tested with a total of 262 yeast isolates: *C. albicans* (173); *C. tropicalis* (21); *C. krusei* (8); *C. glabrata* (49); and other yeasts (12). Compared to conventional identification *C. albicans* was correctly identified 98% of the time. In addition, CHROMagar helped identify 37 of 46 mixed cultures that were otherwise not

detected. The authors suggest that it is reasonable to include CHROMagar for identification of *C. albicans* and as an aid in identification of mixed cultures.

Recently CHROMagar performance for clinical specimens was compared with Candi Select 4 (current formulation of Candi Select, Bio-Rad), which identifies *C. albicans* by detecting hexosaminidase resulting in pink to purple-colored colonies [278]. Phosphatase activity was used to presumptively identify *C. tropicalis*, *C. glabrata*, and *C. krusei* based on turquoise colonies associated with a typical morphologic appearance (Figure 13.8). Chloramphenicol and gentamicin were included to suppress bacteria present in the specimen. A total of 502 cultures were positive, 37 of these were mixed, with a total of 542 yeast isolates. The species distribution was 392 *C. albicans*, 60 *C. glabrata*, 25 *C. tropicalis*, 12 *C. krusei*, and 53 others as identified by standard phenotypic methods. Correct identification of *C. albicans* was equal at 24 h (31.2%; 31.9%), 48 h (82.9%; 82.9%) and 72 h (92.1%; 91.1%). Presumptive identification of *C. glabrata*, *C. tropicalis*, and *C. krusei* was evaluated at 48 h. Percentage of strains with typical colonies was 80, 68, and 84.6% respectively for CandiSelect 4, and 75, 76, and 76.9% for CHROMagar.

Eraso et al. [82] tested 100 isolates each of *C. albicans* and *C. dubliniensis* on CHROMagar *Candida* (Paris, France), reformulated CHROMagar *Candida* (BD, BBL Cockeysville, MD), and *Candida* ID2 (now chromID *Candida*). They found that *Candida* ID2 gave the best resolution with a positive predictive value of 91.7% and negative predictive value of 100% for identifying *C. dubliniensis*.

13.3.1.1 Bismuth sulfite glucose glycine yeast agar

Several studies have evaluated bismuth sulfite glucose glycine yeast (BIGGY) agar (Remel Microbiology, Lenexa, KS) for isolation and identification of yeasts. One [325] comparing CHROMagar to BIGGY agar examined 169 *C. albicans*, 33 *C. tropicalis*, 24 *C. glabrata*, 18 *C. parapsilosis*, 12 *C. krusei* and other *Candida* and yeast. They found CHROMagar sensitivity and specificity for *C. albicans* of 99.4%, 100% and for BIGGY 87%, 78.2%. The sensitivity for *C. tropicalis*, *C. glabrata*, and *C. krusei* on CHROMagar was 97%, 90.9%, and 100% and on BIGGY agar 66.6% (*C. tropicalis*) and 100% (*C. krusei*). In a different study [326] by the same authors examining 215 yeast isolates on BIGGY agar, CHROMagar, and *albicans*ID2 found the same difference in sensitivity when comparing CHROMagar and BIGGY agar.

In 2007 a study compared BIGGY agar to *albicans* ID2 and Sabouraud agar with chloramphenicol for isolation and direct identification from vaginal cultures [143]. They did not find any difference in the number of yeasts isolated on each of these media. They found that BIGGY

agar had a sensitivity of 86.7% and specificity of 56.3% compared to *albicans* ID2 sensitivity of 80% and specificity of 64.6% for *C. albicans*.

An additional chromogenic agar, HardyCHROM *Candida* (Hardy Diagnostics), is available, however, as of this writing there are no publications indicating performance relative to the other chromogenic media.

13.3.1.2 Eosin methylene blue agar

In testing with 92 isolates, all *C. glabrata* isolates grew better on eosin-methylene blue agar plates than on blood agar plates at 24 h. The criterion of better growth on EMB agar is used as an identification method for *C. glabrata* and is combined with the germ tube test as a cost-effective method for identification of *C. glabrata* and *C. albicans* [16].

13.3.2 Rapid identification and screens

13.3.2.1 Identification of unique enzymes

Traditionally, *C. albicans* has been identified by the germ tube test. In 1987, Perry and Miller evaluated the use of 4-methylumbelliferyl-*N*-acetyl- β -galactosamine (NAG) to differentiate between *C. albicans* and *C. tropicalis* [233]. Later, the enzymes L-proline aminopeptidase and β -galactosaminidase further increased the specificity of the test [234]. *Candida albicans* produces both enzymes, while other yeasts produce only one or neither.

The MUREX *Candida albicans* Test Kit (Remel Microbiology) test uses the enzyme substrates *p*-nitro phenyl-*N*-acetyl- β -D-galactosamide (NAG) and L-proline- β -naphthylamide, incorporating them onto a paper disk in a kit-provided test tube. The disk is moistened with distilled or deionized water, and a heavy inoculum of yeast is placed on it with an inoculating loop. The tube is capped and incubated at 35–37°C for 30 min, after which 0.03% sodium hydroxide is added. If one or both of the enzymes are present, the substrates will be hydrolyzed and a colored reaction will be noted. *Candida albicans* is positive for both enzymes; other yeasts may be positive for one or the other.

The BactiCard™ *Candida* (Remel Microbiology) contains NAG and L-proline- β -naphthylamide; the latter is the same substrate found in the Murex system. These substrates are also dehydrated on a packaged test card, which is rehydrated prior to testing and inoculated with a thick paste of yeast cells. If the substrates are cleaved after 5 min of incubation at room temperature, one fluorescent reaction product, 4-methylumbelliferone, and one colorimetric reaction product will be produced. The fluorescent product is viewed under longwave UV light. The test can be performed on pure cultures that are 18–72 h old.

These tests were compared to the traditional germ tube test for presumptive identification of *C. albicans* [59]. The API 20 C AUX test (bioMérieux) and conventional

methods were used as the gold standards. A variety of yeasts were tested, including *C. albicans* (303), *C. glabrata* (153), *C. tropicalis* (70), *C. parapsilosis* (36), *Cryptococcus* spp. (5), *S. cerevisiae* (3), and other yeasts (13). Murex *C. albicans*, BactiCard, and the germ tube test identified 98.7%, 99.3%, and 94.7% of *C. albicans* isolates, respectively. One false-positive each was observed using the Murex and BactiCard™ *Candida* (Figure 13.8) tests, while two false-positives were seen with the traditional germ tube test. All of the enzymatic methods were noted to be rapid and accurate alternatives to germ tube testing of *C. albicans*. In another study [135] these tests were also used to test 133 yeast isolates in comparison with the germ tube test and the API 20 C AUX test and were found to correlate 100%. All of the assays generated some false-positive results for the non-*Candida* yeasts. The assays were compared for cost and other parameters.

The Murex *C. albicans* test was also compared with standard 2-h germ tube tests ($n = 502$). In this study, the number of *C. albicans* isolates tested was 316; the number of non-*albicans* yeasts tested was 186. Discrepant reactions were confirmed with the API 20 C AUX test and by chlamydospore production. Two yeast isolates, *C. albicans* and *Candida lusitanae*, gave incorrect results with the Murex test. Compared to the germ tube test in fetal bovine serum and Fetal Clone II, the sensitivity and specificity of the Murex test were 94.6% and 97.8%, respectively [100].

The AlbiQuick (Hardy Diagnostics, Santa Maria, CA; Figure 13.9) is an assay that measures the presence of



Figure 13.9 AlbiQuick. (Courtesy of Hardy Diagnostics.)

enzymes that will hydrolyze the proline-*p*-nitroanilide (PRO) and NAG compounds on a card with two reaction wells for the identification of *C. albicans*. No peer-reviewed studies have been reported as of this writing.

13.3.2.2 Methods for identification of *C. glabrata*

13.3.2.2.1 Rapid trehalose

In a 1996 study, a screening protocol for *C. glabrata* based on trehalose fermentation at 42°C for 24 h (Trehalose Fermentation Broth; Hardy Diagnostics) was compared to identifications by the API 20 C AUX and Microscan Rapid Yeast Identification Panel. Trehalose Fermentation Broth was found to have 97.8% sensitivity and 95.8% specificity when used for presumptive identification of *C. glabrata* [170]. A follow-up study [98] comparing three commercial methods for the rapid identification of *C. glabrata* included the 24 h Trehalose Fermentation Broth, the 3 h Remel Rapid Trehalose Assimilation Broth, and the 24 h Remel Yeast Fermentation Broth (Remel Laboratories) to the API 20C AUX. All of the methods were compared for accuracy, cost, and turnaround time. Sensitivities and specificities were 91.5 and 96.3% (Remel assimilation), 95 and 89% (Remel fermentation), and 96 and 100% respectively.

A rapid assimilation panel, GlabrataQuick (Hardy Diagnostics) includes maltose and sucrose in addition to trehalose, which reduces false-positive results (Figure 13.10).

13.3.2.3 Identification of *C. dubliniensis*

Identification of *C. dubliniensis* is a relatively new diagnostic dilemma, and few commercial systems have been tested. In one such study [112], 66 isolates of *C. dubliniensis* and 100 isolates of *C. albicans* were tested with the API 20 C AUX and the Vitek Yeast Biochemical

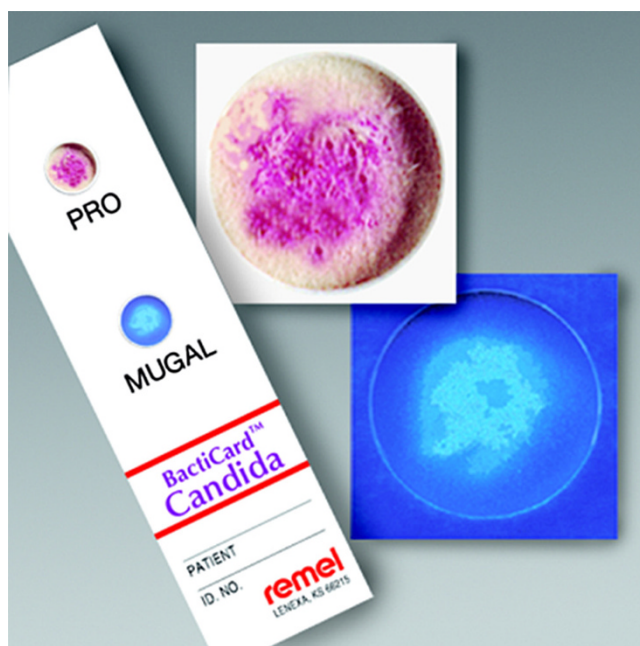


Figure 13.8 Thermo Scientific™ Remel™ BactiCard™ *Candida* Kit. (Courtesy of ThermoFisher Scientific, copying is prohibited.)



Figure 13.10 GlabrataQuick. (Courtesy of Hardy Diagnostics.) (See insert for color representation of the figure.)

Card (YBC) systems. The results were specifically focused on the abilities of the respective systems to identify xylose and α -methyl-D-glucoside (MDG) utilization by *C. albicans* and lack of utilization by *C. dubliniensis*. Growth at 45°C was also examined. The results suggested that clinical laboratories could use the lack of growth at 45°C and negative xylose utilization with either the API 20 C AUX or Vitek system to presumptively identify *C. dubliniensis*. Less correlation existed with a negative MDG test on either system, and misclassification may occur if this result is used, especially with the API 20 C AUX system. Ells et al. published a review of the reliability of methods for distinguishing *C. dubliniensis* from *C. albicans* [77].

Methyl blue Sabouraud agar contains 0.01% methyl blue as a supplement to Sabouraud agar [119]. This medium has been reported to distinguish between *C. albicans* and *C. dubliniensis* based on the yellow fluorescence of *C. albicans* under a Woods lamp [275].

13.3.2.4 Identification of *C. neoformans* and *C. gattii*

In 1962 Staib observed that when yeasts were grown on media containing *Guizotia abyssinica* seeds that *Cryptococcus neoformans* formed brown colonies while other yeasts were nonpigmented [287]. Subsequently it was determined that the seeds contain caffeic acid (3,4-dihydrocinnamic acid), which is the melanin-producing component. Phenol oxidase activity results in the brown pigment of *C. neoformans* and *C. gattii* colonies. Incubation requires up to 7 days before considering it negative. An attempt to circumvent this limitation was undertaken using a disk containing caffeic acid, which is placed on a glass slide, moistened with water or a nonglucose-containing agar, and inoculated with 5–6 colonies and incubated at 35°C in the dark for up to 4 h. The production of a brown pigment should be considered presumptive and the identification verified

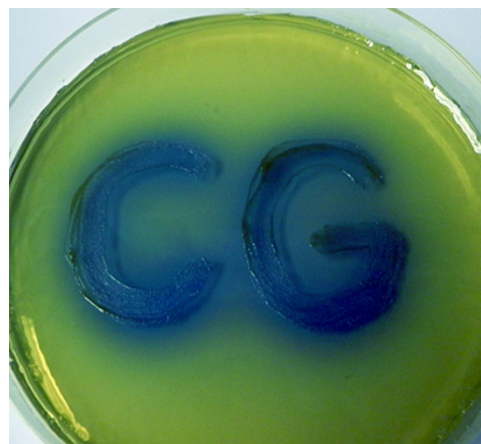


Figure 13.11 CGB agar for the differentiation of *C. gattii* (positive) from *C. neoformans*. (Courtesy of S. Moser.) (See insert for color representation of the figure.)

by another test. Also, it does not differentiate between *C. neoformans* and *C. gattii*. Both the caffeic agar and disks are available from Hardy Diagnostics and Remel.

The majority of clinical disease in immunocompromised patients is due to *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*. The recognition of *C. gattii* as an emerging pathogen in immune competent individuals makes the differentiation of the two species important. Kwon-Chung et al. in 1982 reported a selective medium which differentiated between *C. neoformans* and *C. gattii* based on the ability of *C. gattii* to grow in the presence of canavanine, and uses glycine as a sole carbon and nitrogen source resulting in a shift in pH detected by bromothymol blue (CGB agar; Figure 13.11) [168]. This did not receive broad acceptance in clinical diagnostic laboratories outside areas where *C. gattii* had been initially described, e.g., tropical and subtropical regions [167]. More recent studies utilizing mostly molecular methods have demonstrated a much wider distribution of *C. gattii* in North America [167], making it more important to differentiate the species. In 2009 Klein et al. [163] compared the ability of CGB to differentiate between *C. neoformans* and *C. gattii* and a number of other yeasts. They found that 100% of *C. gattii* grew and turned the media blue (positive) while 100% of *C. neoformans* did not. They also found that other *Cryptococci*, *Trichosporon* spp., and some *Candida* spp. also would give a positive reaction on CGB agar.

A recent study evaluated Remel rapid urea broth and BD BBL urease test broth, caffeic acid disk, CGB, and internal transcribed spacer (ITS) sequencing in 147 reference and clinical strains of *Cryptococcus* spp., *Candida* spp., *Trichosporon* spp., and other non-*Cryptococcus* species [192]. All of the *Cryptococci* were urease positive as well as *Rhodotorula* and *Trichosporon* spp. Addition of the caffeic acid result eliminated all of

the noncryptococci with 85 of 86 (99%) *C. neoformans* positive and 26 of 27 (96%) *C. gattii* positive. Utilizing these two rapid tests, identification of *C. neoformans/gattii* was made in 4 h. Within 48 h further separation of the species was achieved using CGB and/or ITS sequencing. A positive CGB was found in 25 of 27 (93%) of *C. gattii* and none of the *C. neoformans*. It required ITS sequence to find the urea positive, caffeic-acid positive, and CGB negative *C. gattii*. The CGB agar is available from Hardy Diagnostics and Remel.

13.3.3 Commercial kits for identification of yeasts and yeast-like organisms

The advantages of the nonautomated and automated identification systems discussed here include the fact that identification is based on databases that include a variety of substrate utilization patterns and that contain many yeast biotypes.

13.3.3.1 Uni-Yeast-Tek

The Uni-Yeast-Tek (Remel) system was the first widely used commercial system for yeast identification. It consists of a sealed multiwell plate, with each well containing medium for testing carbohydrate utilization, nitrate utilization, urease production, and morphology on cornmeal agar. The kit also includes supplementary tests, such as the C-N screen, glucose beef extract, and sucrose assimilation medium tubes. Evaluations of this product have shown it to be generally satisfactory for the identification of commonly isolated yeasts [254]. However, the system may require up to 7 days for complete identification.

13.3.3.2 API 20 C AUX

The API 20 C AUX (bioMérieux) test is a micromethod for carbohydrate assimilation of clinically important yeasts. Yeasts are inoculated into basal medium in test strips containing dehydrated substrates for assimilation testing. The API 20 C AUX system consists of a strip that contains 20 microcupules; all but two contain dehydrated substrates for determining substrate utilization profiles. As the yeasts utilize various substrates, the microcupules will appear cloudy (Figure 13.12). The reactions are compared to the first reaction cupule, which does not contain a carbohydrate substrate. The test strips are incubated at 30°C for 24–72 h. The results of assimilation reactions are read and converted to a seven-digit biotype profile number. In addition, the system requires that the microscopic morphology be documented on cornmeal agar containing 1% Tween 80 and that this information be used in conjunction with the substrate utilization data for yeast identification [254]. This yeast identification system is perhaps the most widely evaluated and well published of all the current

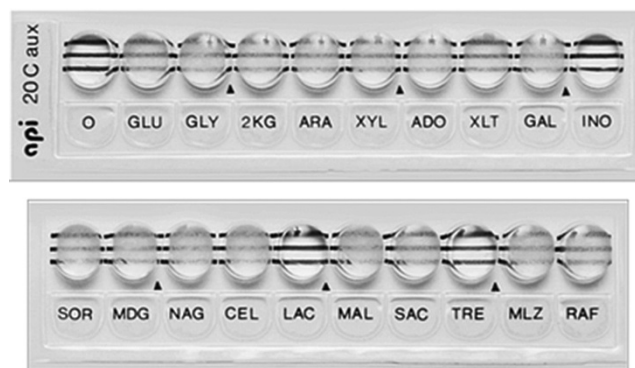


Figure 13.12 API 20 C strip for identification of yeast isolates. Close-up view of substrate utilization of a yeast isolate. Positive reactions appear cloudy in comparison to the negative control (0). (Courtesy of bioMérieux.)

commercial systems. It is frequently used as the gold standard against which comparisons are made.

In early studies [328] performed to compare the API 20 C AUX test with conventional methods, 98% correlation was documented ($n = 45$). The system was considered to be an acceptable substitute for conventional carbohydrate fermentation and assimilation tests. In a more recent study [276], 178 yeasts were tested. After 72 h, correct identification occurred for 86.5% of those isolates tested by the API 20 C AUX system compared to 86% with Auxacolor (Sanofi Diagnostics Pasteur), 68% with Mycotube (Roche Diagnostics), and 51.1% with Candifast (International Microbio, Milan, Italy). When 100 common yeasts were tested, a correct identification was obtained for 98% of the isolates with Vitek YBC (bioMérieux) and 100% with the API20 C AUX [126]. In another study, 123 common and 120 rare clinical yeast isolates were tested, and the API 20 C AUX test correctly identified 97 and 88%, respectively [256]. A further evaluation reported 90.8% of 120 yeast and yeast-like isolates were correctly identified by the API 20 C AUX test [132].

13.3.3.3 RapID Yeast Plus

The RapID™ Yeast Plus system (Innovative Diagnostic Systems; sold as IDS YeastPlus Panel from Remel/ThermoFisher) is a qualitative method that uses conventional and chromogenic substrates to identify carbohydrate utilization, hydrolysis of fatty acid esters, and constitutive enzymes of yeast and yeast-like organisms. It combines conventional tests and single-substrate chromogenic tests with 4-h incubation. A suspension of pure yeast culture is made in the RapID inoculation fluid, turbidity is standardized, and the suspension is used to rehydrate the biochemicals in the RapID plastic reaction panel (Figure 13.13). With a one-step inoculation, the suspension will disperse in the panel



Figure 13.13 Thermo Scientific™ RapID™ YEAST PLUS System. (Courtesy of ThermoFisher Scientific, copying is prohibited.)

by way of the plastic inoculating trough. After a 4-h incubation at 30°C, the RapID™ Yeast Plus system is interpreted. Some reactions require additional reagents to be added to the reaction panel. The resulting pattern of positive and negative reactions is used to compile a profile number based on the organism's individual reactions in the panel. The RapID™ Yeast Plus Code Compendium supplies species identification for the biochemical profile numbers, which are generated based on the results of biochemical reactions. The RapID™ Yeast Plus method correctly identified 94.1% of 304 clinical yeast isolates within 5 h [162].

In a study of 201 yeasts [311], when no further tests were performed, identification to the species level was 96%, 83%, and 86% for the RapID™ Yeast Plus system, the API 20 C AUX system and the Vitek YBC respectively. The API 20 C AUX system did not correctly identify any *C. krusei* isolate without supplemental testing, and this organism accounted for most of the differences seen between the API 20 C AUX system and the RapID™ Yeast Plus system.

In another study [135] the RapID™ and API 20 C AUX systems were compared with 57 *C. albicans* isolates, 26 *C. tropicalis* isolates, 23 *C. glabrata* isolates, and 27 isolates from other species. The RapID™ system correctly identified 125 (94%) of all yeasts tested and 99% (105 of 106) of the isolates of the three most common species, *C. albicans*, *C. tropicalis*, and *C. glabrata*. Discrepancies were resolved with Vitek 2 Yeast ID cards (bioMérieux), germ tube production, and microscopic morphology

and confirmed that the RapID™ system compared favorably with the API 20 C AUX system but had a more rapid identification process. In another study, the identification rate for yeasts tested with this system was 78–84% [38].

In a similar study, good correlation was observed between the two systems, with 95.7% agreement with the API 20 C AUX system for *Candida* and *Cryptococcus*. Lower agreement was noted with emerging *Candida* spp. and with other, yeast-like pathogens (79.1 and 75.2%) [85]. In another study, the RapID™ Yeast Plus system was compared with the Uni-Yeast-Tek system (Remel). A total of 117 fresh and frozen yeast isolates were tested. The bioMérieux Vitek system and morphology were used to resolve discrepant results. The RapID™ system correctly identified 96.6% of the isolates in 4 h [207].

13.3.4 Automated systems

13.3.4.1 MALDI-ToF/MS

Traditional microbiological techniques have long relied on the phenotypic characteristics of an organism including morphology, biochemical profiles, and culture patterns. Over the past several years, molecular techniques including genetic probes, polymerase chain reaction (PCR), and sequencing have become increasingly prevalent in microbial identification algorithms. Recently, MS has been adapted to determine the physical protein expression profile of an organism and compare it to known profiles in a database to identify organisms in patient samples. Mass spectrometry was previously



Figure 13.14 Bruker Biotyper. (Courtesy of Bruker Daltonics.)

used for bacterial identification in 1975 in a process that focused on lipid analysis. In the 1990s, MALDI-ToF/MS was used to identify surface proteins of bacteria. A change of matrix composition allowed the analysis of bacterial ribosomal proteins that is considered more reliable for microbial identification [274]. MALDI-ToF works by embedding analytes in a crystalline matrix. A laser is utilized to vaporize the analytes, which are then ionized and detected. MALDI-ToF/MS measures the mass-to-charge ratio of a given sample and compares it to a library of reference spectra to establish identification. Results can be obtained either from subculture or directly from blood cultures, and a protein extraction step may be utilized depending on the platform, especially for fungal organisms. This technology is useful for the identification of cultures containing a single species but is currently insufficient to detect mixtures of organisms [74]. The sample is placed with the matrix on a conductive metal plate that can then be inserted directly into the mass spectrometer. Obtained spectra results are compared to reference spectra within the database, thereby leading to identification of the organism. Currently, two commercially available MALDI-ToF/MS platforms have received the Conformité Européenne (CE) mark for *in vitro* diagnostic use in Europe. The Bruker Biotyper system, utilizing the Microflex LT mass spectrometer and Biotyper database (Figure 13.14; Bruker Daltonics), and the bioMérieux Vitek MS system (Figure 13.15), utilizing the MS-ID database (bioMérieux, previously developed by AnagnosTec and Shimadzu Corporation), are currently available for the identification of bacterial and fungal pathogens [307]. An additional Andromas database (Andromas SAS, Paris, France) can be utilized in conjunction with the Bruker system. Interpretation of

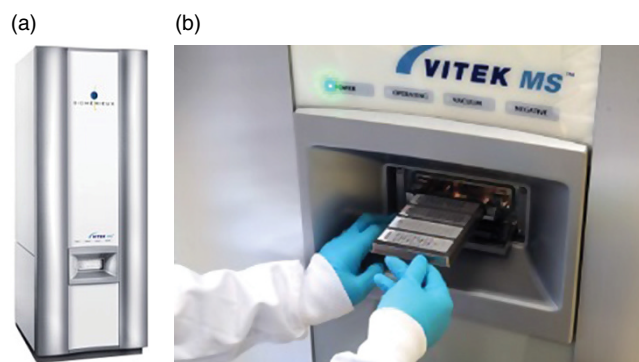


Figure 13.15 (a) Vitek MS MALDI-ToF instrument. (b) Loading of specimen slide for analysis. (Courtesy of bioMérieux.)

MS data is conducted by algorithms such as the Bruker Main Spectrum analysis or the bioMérieux Advanced Spectra Classifier for the profiling of the presence or absence of peaks. At the time of this writing, the majority of published studies relating to fungal identification utilize the Bruker MALDI-ToF platform coupled to the Biotyper database. The Sepsityper system (Bruker Daltonics) can be utilized to identify organisms directly from blood culture [148]. For fungal organisms, a protein extraction step substantially improves identification for the Bruker system in combination with the Biotyper database; however a protein extraction step is not required for the Vitek MS system [144]. At the time of this writing, the bioMérieux Vitek MS system has received FDA approval for the diagnosis of 193 clinical bacterial and yeast organisms, while the Bruker platform has FDA approval for an aerobic Gram-negative organism database including 40 species and species complex (100 total identifications). Advantages of MALDI-ToF/MS include low cost of consumables, fast analysis turnaround times, and easy interpretation of identifications. The reader should consult current publications and vendor web sites for up-to-date information since this is an extremely rapidly changing field of study.

13.3.4.2 MALDI biotyper

A 2009 study compared MALDI-ToF/MS (Bruker Daltonics, Bremen, Germany) versus conventional identification methods including the API ID 32C (bioMérieux) for identification of 267 clinical yeast isolates composed of *Candida*, *Cryptococcus*, *Saccharomyces*, *Trichosporon*, *Geotrichum*, *Pichia*, and *Blastoschizomyces* species subcultured on Sabouraud agar [187]. Utilizing the provided Biotyper software, MALDI-ToF identified 247 isolates (92.5%) on the first round compared to 244 isolates in the first round for the API ID 32C. Following the addition of complementary reference spectra to

the database for the remaining isolates, MALDI-ToF was able to identify all 267/267 isolates while the API ID 32 C identified 256/267 isolates in the second round. Another study in 2009 found that MALDI-ToF/MS showed promise in identifying fluconazole resistance in *C. albicans* isolates by assaying the minimum concentration of fluconazole that induces a detectable mass spectrum profile change. Results correlated with the Clinical Laboratory Standards Institute (CLSI) methodology of minimum inhibitory concentration (MIC) by broth microdilution [185]. Further studies need to be conducted to better define the efficacy and usefulness of MALDI-ToF in this context.

A 2010 study [308] compared MALDI-ToF/MS (Bruker Daltonics) to conventional techniques (Vitek2, API, and biochemical) in the identification of 80 subcultured yeast isolates (19 retrospective and 61 prospective) covering seven genera and 12 species. In the prospective study, MALDI-ToF achieved 85.2% species identification and 96.7% correct genus identification compared to 77.0% species and 98.4% genus identification for routine biochemical methods. Misidentifications for MALDI-ToF were largely the result of an incomplete database that could be resolved with the addition of appropriate reference spectra. Stevenson et al. [288] conducted a study in 2010 where 192/194 (99%) of subcultured clinical yeast isolates were correctly identified using a Bruker MALDI-ToF with BioTyper software. One *C. rugosa* isolate (1/7) and one *Cryptococcus neoformans* isolate (1/3) could not be identified in this study. Low MALDI-ToF scores requiring repeat testing were observed commonly for isolates of *C. guilliermondii*, *C. krusei*, *C. lusitanae*, and *C. neoformans*. Another 2010 study established a proof of concept study where various *Candida* species could be identified directly from blood cultures with appropriate sample preparation, thus allowing identification 1–2 days more quickly than if the isolates were subcultured [186]. Ferroni et al. [102] conducted a 2010 study in which the Bruker MALDI-ToF/MS system combined with the Andromas database (Andromas SAS) successfully identified 20/20 (100%) spiked blood cultures composed of 10 *C. albicans* isolates and 10 non-*albicans Candida* species in addition to 11/11 patient blood cultures containing *C. albicans*. Also in 2011, Bader et al. [13] analyzed 1192 clinical yeast and yeast-like isolates and compared two MALDI-ToF/MS platforms, the Bruker system/Biotyper database versus the AXIMA Assurance system/Saramis database (AnagnosTec/Shimadzu) along with traditional biochemical techniques (API 20 C AUX and ID 32 C systems from bio-Mérieux). With the Biotyper database, 97.6% of the 1192 isolates were correctly identified compared to 96.1% with the Saramis database and 96.9% for classic methods. The two MALDI platforms performed comparably and had

lower rates of misclassification (Biotyper: 8 misclassifications, 21 unknown; Shimadzu: 2 misclassifications, 44 unknown) than classical techniques (30 misclassifications, 7 unknown). With supplementation of the MALDI databases, the rate of misidentifications and unknown assignments could be decreased. The MALDI-ToF/MS was much better at resolving *C. orthopsilosis/metapsilosis/parapsilosis* and *C. glabrata/bracarensis* than traditional biochemical approaches. Analysis with the Biotyper database required a protein extraction step while analysis using the Saramis database did not. Alanio et al. [2] constructed a MALDI database including the reference spectra of 28 clinically relevant species from seven *Aspergillus* sections comprised of five common and 23 unusual species. They then utilized the database to identify 140 previously characterized isolates (124 clinical and 16 environmental) following processing on a Microflex MALDI-ToF/MS spectrometer (Bruker Daltonics) with flex control software along with comparative data analysis using the Andromas SAS software. Out of the 140 samples, 127 were identified with one run, 11 were identified with two runs, and two samples could not be identified. Identified species included *Aspergillus* species from the *Fumigati*, *Flavi*, *Terrei*, *Nigri*, *Nidulantes*, *Usti*, and *Circumdati* sections. The two unidentified samples were ultimately identified as atypical *Aspergillus fumigatus* isolates (absence of conidiogenesis or atypical sporulation) by multilocus sequencing. With no misidentifications, MALDI was able to correctly identify 98.6% of tested isolates with 100% sensitivity utilizing the constructed database.

A 2011 study [284] compared the MALDI Biotyper MALDI-ToF MS system (Bruker Daltonics) to conventional techniques directly from blood cultures with the utilization of a protein extraction step. MALDI-ToF correctly identified 95.9% of *C. albicans* and 86.5% of non-*albicans Candida* species. MALDI exhibited difficulty in the identification of *C. guilliermondii*, *C. krusei*, and *C. lusitanae* isolates as well as polyfungal bloodstream infections. Dhiman et al. [68] conducted a 2011 study comparing a Bruker MALDI-ToF to traditional phenotypic methods using 138 common and 103 archived yeast strains. MALDI-ToF correctly identified 96.3% of common and 84.5% of uncommon isolates using a score threshold of 1.8. Unreliable identifications were noted for *C. guilliermondii*, *Aureobasidium/Hormonema*, *C. neoformans*, *Blastoschizomyces capitatus*, *C. colliculosa*, *C. inconspicua*, *C. albidus*, and *Trichosporon asahii*. Low identification scores for *Cryptococcus neoformans* were thought to result at least partially from the organism's thick capsule, and it was noted that reference spectra for *C. gattii* were not in the database. McTaggart et al. [193] addressed the issue of identifying *Cryptococcus* species in a 2011 study that analyzed 137 *Cryptococcus* strains and 23 non-*Cryptococcus* strains subcultured on Inhibitory Mold Agar by

MALDI-ToF using intergenic spacer DNA sequencing as the reference method. Following supplementation of the Biotyper 2.0.1 database with *C. gattii* reference spectra, 27/27 *C. gattii* isolates were correctly identified. Overall, all 137 isolates were correctly identified to species level. A single *C. neoformans* var. *neoformans* (1/4) isolate was misidentified to subspecies level as *C. neoformans* var. *grubii*. Another 2011 study conducted by Pinto et al. [251] utilized the Bruker Biotyper MALDI-ToF/MS system to retrospectively analyze 30 reference and 167 clinical isolates as well as prospectively analyzing 67 clinical isolates subcultured to Sabouraud's dextrose agar in parallel with biochemical methods, where discrepancies were addressed by sequencing of the internal transcribed spacer region. MALDI-ToF/MS successfully identified 80% of the 30 isolates to species level. Out of 167 clinical isolates, 140/167 (84%) were identified to species level (threshold score > 2.0), and 160/167 (96%) were identified to genus level (threshold score > 1.70). Prospective analysis of 67 clinical isolates yielded successful species-level identification for 79% of isolates. Protein extraction of yeasts was found to be necessary for the generation of reliable mass spectra as compared to the direct colony preparation method. MALDI-ToF exhibited difficulty with identification of *C. ciferrii*, *C. zeylanoides*, *C. nivariensis*, *C. bracarensis*, *Pichia kluyveri*, *Arxiozyma telluris*, *Debaryomyces carsonii*, and *Cryptococcus neoformans*. Another study conducted in 2011 [324] investigated the Bruker MALDI Sepsityper kit (Bruker Daltonics) designed for the processing of blood cultures with a dedicated lysis step that disrupts red blood cells while leaving the bacterial and yeast cell walls intact. A total of 42 blood cultures containing yeast were isolated with correct identification of all samples. Serial dilutions of a *C. parapsilosis* culture revealed the limit of detection of this MALDI-ToF system is 5.9×10^5 colony-forming units (CFU).

In 2011 Bille et al. [24] investigated the Bruker MALDI-ToF/MS system along with the Andromas SAS database for the identification of yeast and *Aspergillus* species. MALDI-ToF successfully identified 96.3% of yeast and 92.2% of *Aspergillus* species following a single acquisition, with identification of 98.8% of yeasts and 98.4% of *Aspergillus* species from the second acquisition. Difficulty was found with identification of *C. lambica* (not in database). Utilization of MALDI-ToF with the Andromas SAS database could successfully classify 63/64 morphologic *Aspergillus* species into *Fumagati*, *Terrei*, and *Flavi* sections. Another study in 2011 [43] utilized the Bruker MALDI-ToF/MS system and a variety of extraction protocols for the identification of mold isolates. The most effective method was to grow molds on Sabouraud–chloramphenicol–gentamicin agar and conduct a formic-acid-based extraction step following mechanical scraping. Utilizing this process, this group identified 87% (154/177) of isolates. Identification failed in 12% (21/177)

of isolates due to the absence of reference spectra in the database. Appropriate reference spectra were lacking for *Alternaria*, *Penicillium*, *Scedosporium*, *Paecilomyces*, *A. clavatus*, *A. melleus*, *A. oryzae*, *A. sydowi*, *A. alliaceus*, *Geotrichum*, and *Fusarium* species. One *Beauveria bassiana* isolate was not identified, and one *Rhizopus oryzae* isolate was misidentified as *Mucor circinelloides*.

MALDI-ToF/MS is also a promising technology for the identification of dermatophytes. Theel et al. [296] utilized the MicroFlex LT MALDI-ToF/MS system (Bruker Daltonics) to identify 100 well-characterized, archived dermatophyte isolates and 71 fresh dermatophyte cultures with either the supplied MALDI Biotyper library (MBL version 3.0) or a supplemented library containing an additional 20 dermatophyte species. Dermatophyte specimens were submerged in 70% ethanol, pelleted, and dried prior to suspension in 70% formic acid and 50% acetonitrile with subsequent matrix (HCCA, α -cyano-4-hydroxycinnamic acid) application and analysis. Out of 171 isolates, the provided Biotyper library identified 37.4% of isolates to the genus level and 20.5% to the species level while the supplemented database identified 93% of isolates to the genus level and 59.6% of organisms to the species level. It was also found that lowering cutoff score values to at least 1.5 for genus and 1.7 for species from the manufacturer-recommended values of 1.7 for genus and 2.0 for species increased the number of obtained identifications without increasing misidentifications. With the manufacturer-provided database, two isolates of *Trichophyton mentagrophytes* were misidentified as *T. tonsurans* while passing the 10% criterion for species identification. With the supplemented database, seven isolates of *Trichophyton rubrum* were misidentified as *T. soudanense* while passing the 10% criterion, however all *T. rubrum* isolates were correctly identified following deletion of the single *T. soudanense* reference spectrum from the supplemented database. A 2012 study [5] utilized the Andromas SAS software to identify 381 isolates of clinical dermatophytes and *Neoscytalidium* following culture on Sabouraud's dextrose agar. Dermatophyte colonies were harvested in 70% formic acid and applied directly on a plate with subsequent matrix application. A reference database was constructed from a set of 50 reference strains from 12 clinically relevant species of dermatophytes and two species of *Neoscytalidium*. Utilizing MALDI-ToF/MS and the constructed database, 331/360 dermatophytes (91.9%) and 18/21 *Neoscytalidium* isolates (85.7%) were correctly identified. Two *T. mentagrophytes* var. *interdigitale* isolates were misidentified as either *T. rubrum* or *T. tonsurans*. In addition, spectral acquisition failed in 19% of *Microsporum langeronii*, 28% of *T. soudanense*, and 14% of *Neoscytalidium* isolates. Utilizing the Andromas SAS software, the authors were able to obtain higher levels of dermatophyte identification than observed results from a previous study utilizing the Bruker

Biotyper database [296], and no colony extraction step was needed with the Andromas SAS software. Thus, with appropriate database reference spectra, MALDI-ToF/MS is a powerful method for the identification of dermatophytes.

13.3.4.3 Vitek MS

A 2012 study [280] compared the predecessor to the Vitek MS (bioMérieux, SA, Marcy l'Etoile, France), the AXIMA Assurance MALDI-ToF/MS system (Shimadzu Biotech) with Saramis database (AnagnosTec) to the API ID 32C system (bioMérieux) in identifying yeasts from dermatological patients. Sequencing of the internal transcribed spacer (ITS) regions of the ribosomal DNA was used for confirmation, and no extra protein extraction step was utilized. From a total of 83 isolates, MALDI-ToF results concurred with ITS sequencing in 94% of cases (78 isolates), while API ID 32C results concurred in 84.3% of cases. MALDI-ToF misidentified 1/35 isolates of *C. albicans* as *C. glabrata* and misidentified 1/10 *C. glabrata* isolates as *C. tropicalis*. One *C. colliculosa* isolate (1/1) was not identified by MALDI-ToF as there was no reference spectrum in the Saramis database. One *Rhodotorula mucilaginosa* isolate (1/1) and one *Trichosporon mucoides* isolate (1/1) were insufficiently identified to genus only. The API ID 32C was especially susceptible to the repeated misidentification of *C. krusei* isolates (5/7 isolates misidentified). In this study, MALDI-ToF/MS compared favorably to the API ID 32C system for the identification of dermatological yeasts. Iriart et al. [144] utilized the Vitek MS coupled with the "advanced spectra classifier" algorithm and version 1.0 of the MS-ID database for the identification of yeast-like and *Aspergillus* isolates compared to either the Vitek 2 (bioMérieux, St. Louis, MO) or routine laboratory identification techniques. Yeast-like isolates were applied directly from a Sabouraud agar plate to the Vitek MS disposable target with no protein extraction step. Following lysis with 25% formic acid and drying, α -cyano-4-hydroxycinnamic acid (CHCA) matrix was applied to the spot prior to analysis. For *Aspergillus* isolates, CHCA matrix was applied directly to the fungal spot under laminar flow with no protein extraction or formic acid lysis step. Out of 192 yeasts and yeast-like isolates, Vitek MS correctly identified 184 isolates (95.8%) to species compared to 183 isolates (95.3%) by routine laboratory tests and 169 (88.0%) by Vitek 2. For yeast organisms present in the database, Vitek MS failed to identify 1/60 (1.7%) of *C. albicans* isolates, 1/45 (2.2%) of *C. parapsilosis* isolates, and 2/2 (100%) of *Geotrichum candidum* isolates for an overall identification rate of 98.2%. In addition, Vitek MS failed to identify *C. norvegensis* and *C. orthopsilosis* isolates, as these reference spectra were not in the database. A single misidentification (major error) was made for an isolate of *C. palmioleophila* (not in database) being identified as *C. haemulonii*. Out of 44 *Aspergillus* isolates, Vitek MS

correctly identified 36 (81.8%) and 39 (88.6%) were correctly identified by routine morphological identification. For *Aspergillus* species in the database, all *A. fumigatus* (33/33), *A. flavus* (2/2), and *A. niger* (1/1) isolates were properly identified. Isolates of *A. sydowi* (0/2), *A. terreus* (0/1), *A. tubingensis* (0/1), *A. calidoustus* (0/1), *A. nidulans* (0/2), and *A. puniceus* (0/1) could not be identified as these organisms were not in the Vitek MS database. The Vitek MS system allows for rapid high-quality identification of yeasts and *Aspergillus* species with only a single sample deposit and no protein extraction step. At the time of this writing, the Vitek MS system has received FDA approval for the identification of 193 clinical bacterial and yeast isolates. Continued supplementation of the provided MS-ID database and validation for additional organisms will be required to improve performance in the identification of clinically relevant fungal isolates.

Overall, MALDI-ToF/MS represents a new and promising technology for the identification of clinically important fungal organisms. Results can be obtained within approximately 1 h of culture, and the assay requires minimal consumable resources and technical manipulation. It is important to obtain more comprehensive databases to fully utilize the potential of this technology. MALDI-ToF/MS will likely replace many of the current frontline biochemical techniques, however, conventional methods will be required for antibiotic susceptibility profiling for the foreseeable future. The MALDI-ToF/MS technology exhibits difficulty in mixed specimens thereby requiring subculturing of specimens containing multiple organisms.

13.3.4.4 Rapid yeast identification panel

The Microscan Rapid Yeast Identification panel (Siemens Healthcare, West Sacramento, CA) is composed of chromogenic and modified conventional tests for identification of yeasts and yeast-like organisms, such as *Prototheca* spp., from clinical isolates. The 96-well plate format contains 27 dehydrated chromogenic substrates, which are designed to identify yeasts without the need for ancillary tests. A heavy suspension of a fresh yeast isolate, prepared in water and standardized to a known turbidity standard, is subsequently used to hydrate the plates. The plates are incubated for 4 h at 35–37°C before the reactions can be interpreted. The chromogenic readings can be made manually, read by eye, or with the Autoscan instrument. Interpretation of the biochemical profile occurs as the results are transformed into a nine-digit biotype number, which is compared with the Microscan cumulative database via the Autoscan instrument or manually, using the Microscan Rapid Yeast Biotype Codebook. The database identifies at least 42 yeasts and yeast-like organisms. In each case, profile identifications are assigned a cumulative relative probability of identification. When the probability of identification is not sufficient to confirm

identification, supplemental testing of morphological and phenotypic characteristics is recommended.

When the Rapid Yeast Identification Panel was compared to the API 20 C AUX system, early studies reported 94.1% correlation in identifying species of *Candida*, *Hansenula*, *Pichia*, *Rhodotorula*, and *Saccharomyces* when results were determined after 4 h. A lower correlation (65%) was observed for slower-growing species, such as *Blastoschizomyces* spp., *Cryptococcus* spp., *Trichosporon* spp., and yeast-like organisms, such as *Prototheca* spp. The overall correlation, using the first limited database was approximately 85% [171, 286].

The same system was also tested with results obtained after 72 h. The results were again compared to those with the Microscan Rapid Yeast Identification Panel at 4 h and the API 20 C AUX system with cornmeal-Tween 80 agar at 72 h. Both visual and Autoscan readings of the plates correctly identified 78% of the 357 yeast isolates tested with no supplementary tests required. With supplementary tests, Autoscan identified 96.6% (99.5% of common strains; 92.1% of less common strains, like *C. glabrata*, *C. fumata*, *C. lusitaniae*, *C. lambica*, *C. rugosa*, *C. albidus*, *C. laurentii*, and *C. uniguttulatus*), and the API 20 C AUX system identified 98.9%. Autoscan was shown to be reliable for the identification of common yeasts when supplementary tests were performed [286].

More recently, when the results were compared to those obtained with the API 20 C AUX system and from morphology on Tween-80 agar, the Microscan Walk-Away-96 correctly identified 67–82% of the 150 yeasts tested. The most commonly misidentified yeasts were *C. tropicalis*, *C. glabrata*, and *C. parapsilosis* [264].

13.3.4.5 Vitek YBC/Vitek 2 YST

The Vitek YBC system (bioMérieux) is an automated system for yeast identification. The system uses the Vitek test card, which is composed of microwells containing various identification substrates. The YBC is used for the yeasts that are most commonly encountered in hospitals. Yeast suspensions are prepared in sterile water. Tubes are placed in the Vitek filler-sealer, which uses a vacuum to load the liquid into the Vitek test card through a sterile straw. The sealer module removes the straw and seals the test card. The card contains dehydrated medium for a variety of biochemical tests. The reader-incubator unit maintains a controlled environment while the cards incubate; it rotates the cards so that readings can be measured by the laser unit and photodetector. The yeast database contains at least 36 species of commonly encountered yeasts. Results are maintained so that cumulative reports can be generated and can be formatted according to the user's needs. Add-on software is available for purchase to maintain workflow and quality control data. The maximum capacity for the

Vitek Junior model is 30 cards per run. The Vitek Senior comes in models that can analyze 60, 120, and 240 cards simultaneously.

In an early study [76], the Vitek yeast database was compared to the API 20 C AUX system. The Vitek system compared favorably for the identification of common yeasts but less favorably for identifying others. For all yeasts identified, most of the identifications were made within 24 h of incubation.

In another report [70], the YBC was compared to the API 20 C AUX system with additional biochemicals for the identification of 222 germ-tube-negative yeast isolates. The YBC identifications of commonly isolated yeasts compared favorably; however, the 48-h identifications of less common yeasts were not as successful: 55% were correctly identified, and the remaining isolates were either misidentified or not identified. In a separate study [126] the system was not able to identify an isolate of *S. cerevisiae* or *C. tropicalis* but provided correct identification for 98% of the common yeasts ($n = 100$).

Similarly, the Vitek YBC, with 24–48-h results, was compared with the API 20 C AUX system and morphology on Tween-80 agar [263]. Of 150 yeast isolates, 85–95% were correctly identified with the Vitek YBC. The most common misidentified yeasts were *C. tropicalis* and *C. glabrata*. In another study [99], 409 germ-tube-negative yeasts and *Geotrichum* spp. were also tested. The results were compared with identification using the API 20 C AUX system and yeast morphology agars. Both systems identified most yeast isolates within 24 h. The YBC correctly identified 89.7% of 409 germ-tube-negative yeasts. The API 20 C AUX system correctly identified 99.3%. Of the remaining isolates, some profiles provided no identification of the organism, some profiles converted to misidentifications (the YBC incorrectly identified 7.3% of the isolates tested; the API 20 C AUX system misidentified only 0.7%). Results were comparable for *C. glabrata*, *C. parapsilosis*, and *C. neoformans*. The Vitek YBC had difficulty with *C. tropicalis*, *C. krusei*, *Trichosporon*, and some *Cryptococcus* spp. [97, 98, 100, 101].

A current instrument, the Vitek 2 system, allows identification of medically important yeasts and yeast-like organisms in 15 h by using a compendium of 47 biochemical reactions that are monitored by a sensitive fluorescence-based chemistry. The new database with 51 taxa was evaluated using the Vitek ID-YST card. Compared to the ID32C strip (bioMérieux) for 241 strains from 21 species, the Vitek 2 system correctly identified 92.1% of the isolates [120].

A second-generation yeast identification card (YST), the currently available card – converted from fluorescence-based to colorimetric chemistry, was introduced with the Vitek 2. The card has 64 wells and an expanded database of 53 species and 14 genera. There are several comparisons of the ID-YST and the new YST cards

[9, 133, 177, 273, 304]. Loiez et al. [177] tested 172 strains representing 19 Taxa by the ID-YST, YST, and the API 32C system, which was considered the reference. The recorded concordance between the Vitek 2 cards and results with different identifications that could not be resolved by simple additional phenotypic testing were submitted for sequencing of the 18S–28S rDNA. Correct identifications to the species level were 93.6% for ID-YST and 83.7% for the YST card.

A different outcome was reported in another 2006 publication [9]. Ninety-seven isolates from CHROMagar *Candida* were compared with the ID-YST, YST, and API 20C as the reference identification. Their results show a superiority of the YST over the ID-YST, with correct identifications of 94.8% and 83.5% respectively. A 2007 study [273] evaluated the YST card against the RapID Yeast plus (Remel) system. A total of 750 clinical isolates were tested by both systems and rRNA ITS region sequencing as the reference standard identification. The Vitek 2 YST card and the RapID correctly identified 98.2% and 95.5% isolates, respectively.

13.3.4.6 BD Phoenix Yeast ID Panel

The BD Phoenix Yeast ID Panel (Becton Dickinson, Sparks, MD) is a recently introduced panel for their Phoenix Microbiology System. It utilizes modified, conventional, fluorogenic, and chromogenic substrates for the identification within 4–18 h. Early studies demonstrate high accuracy compared to other systems. The reader should refer to recent publications for this relatively new system.

13.3.4.7 Biolog YT Microplate

In the YT Microplate test (Biolog, Hayward, CA), yeast cells are suspended in sterile water. The YT Microplate is configured with both of its carbon source oxidation tests using tetrazolium violet as the colorimetric indicator. Carbon-based assimilation tests are scored turbidimetrically and can only be read with the automated Biolog MicroStation. A few wells contain two carbon sources and a test for colorization of various carbon sources with D-xylose. Software support exists for the Biolog Yeast Database. In addition, the SF-N and SF-P Microplates can be used for metabolic testing of sporulating and filamentous microorganisms, such as the actinomycetes and fungi. Special culture medium is used to enhance sporulation, and a gel-forming colloid, which keeps the organisms more evenly suspended, is used to prepare the organism suspension. The SF Microplates are identical to the GN and GP microplates used for aerobic bacteria except they do not contain the tetrazolium redox dye, which is toxic to many of these species. The plates are incubated at 25–27°C for several days and read with the automated MicroStation.

Sand et al. [272] compared the Biolog YT to the YBC and API 20 C AUX system with 171 germ-tube-negative yeasts; the Biolog system identified 48% of the isolates. The YBC and the API 20 C AUX system correctly identified 97% of the 171 strains tested. Both systems required repeat testing less than 10% of the time and required supplemental testing with 28% of the strains. With the Biolog system, there is a need for considerable expertise and a battery of supplemental reagents for correct identification of germ-tube-negative yeasts.

13.3.4.8 Microbial Identification System

The Microbial Identification System (MIS; Microbial ID, Inc., Newark, DE) relies on fatty acid analysis to identify yeast isolates. Cellular fatty acid chromatograms are analyzed and compared to the fatty acid profiles in the system's database. Prior to analysis, yeast isolates must be subcultured, with subsequent saponification, methylation, and extraction of fatty acids. In one study using MIS (with the yeast clinical library version 3.8), 477 isolates and 23 different species of yeast were tested. The MIS generated results with a performance of 75% correct identifications compared to a combination of identification methods, including the Vitek YBC, morphology on cornmeal agar, and the API 20 C AUX system. Predictive values for different species ranged from 47 to 100%, depending on the species [155].

In another similar study [60], the MIS was compared to the API 20 C AUX system and conventional methods. The results indicated that 374 (68%; $n = 550$) yeasts were correctly identified to the species level. After repeat testing, 18 more were identified, bringing the overall correct identification to 71.3%. Of all yeasts tested, 15.8% were incorrectly identified and 16.2% were given no identification. The isolates tested represented a variety of species: *C. albicans* (294), *C. glabrata* (145), *C. tropicalis* (58), *C. parapsilosis* (33), and other yeasts (20). *Candida glabrata* was the most commonly misidentified yeast, and it was misidentified as *S. cerevisiae* 32.4% of the time. Another study performed with the MIS provided evidence that the results can be dependent on the supplier of the agar on which the yeast isolates are grown [156].

13.4 Molecular testing

13.4.1 Accuprobe

Because of the slow growth of dimorphic fungi and the potential difficulty and safety risks involved in their confirmatory identification, molecular identification techniques are extremely useful for their detection. Nucleic acid hybridization tests are based on the complementary alignment and binding of nucleic acid strands to form stable double-stranded complexes. The AccuProbe System

(Gen-Probe, San Diego, CA) uses a single-stranded DNA probe with a chemiluminescent acridinium label as the complement of the rRNA of the target organism. Lysates of an organism are prepared by sonication with glass beads and heat treatment. The rRNA is released from the organism, and the labeled DNA subsequently binds with the target organism rRNA to form a stable labeled DNA-RNA hybrid, the presence of which can be detected in the Gen-Probe luminometer. The selection reagent, provided in the kit, provides for differentiation of hybridized and nonhybridized probes during readings taken by the luminometer. Positive reactions are based on cutoff values of relative light units derived from the chemiluminescence. FDA-approved kits for *in vitro* diagnostic use are available for the identification of *Coccidioides immitis*, *Histoplasma capsulatum*, and *Blastomyces dermatitis*. Molds can be tested from growth in both liquid and solid media; however, the assay is not reliable for formalin-killed cultures of *C. immitis* [123].

The AccuProbe DNA probe assay was originally compared to the traditional exoantigen test. For 105 isolates of various subspecies of *H. capsulatum*, all were identified with the AccuProbe assay with a more rapid result. A total of 103 isolates were identified within 2 h, while the remaining two cultures required additional purification due to contamination with bacteria [226]. In another 1992 study, the AccuProbe correctly identified 53 of 54 isolates as *H. capsulatum*, but one isolate of *A. niger* was incorrectly identified as *H. capsulatum*. In this study, the age of the culture, the isolation medium, and the morphological state of the organisms did not adversely affect the results [127]. In 1993, the Gen-Probe DNA probes for the identification of *H. capsulatum* var. *capsulatum* and *C. neoformans* were tested. A total of 95 fungal isolates were tested with the *Histoplasma* kit, 41 of which were *H. capsulatum* var. *capsulatum*. A total of 98 yeasts, 42 of which were *C. neoformans* isolates, were tested with the *Cryptococcus* probes. The probes exhibited 100% sensitivity and 100% specificity and significantly reduced the time necessary for identification [142, 290]. The *Cryptococcus neoformans* AccuProbe assay has also been found to detect eucalyptus-originated *C. gattii*, previously known as *C. neoformans* var. *gattii*, but was negative for eucalyptus-originated *C. laurentii* [169].

In a 1994 study [225], the AccuProbe assays were evaluated for detection of *B. dermatitidis* and *C. immitis*. Seventy-four mycelial cultures of *B. dermatitidis* and 72 cultures of *C. immitis* were tested. Seventeen *Paracoccidioides brasiliensis* isolates were included to test for cross-reactivity in addition to the inclusion of heterologous *Chrysosporium* species, *Malbranchea* species, *Geotrichum* species, and *Onychocola canadensis* isolates. All 72 *C. immitis* isolates were identified correctly with the AccuProbe assay for *C. immitis*, and no cross-reactive fungal isolates were

identified. For *B. dermatitidis*, all 74 isolates were correctly identified by the AccuProbe assay within 2 h. However, the *B. dermatitidis* AccuProbe assay was found to cross-react with 10 of the 17 *P. brasiliensis* isolates, leading to misidentification as *B. dermatitidis*. No other cross-reactive fungal isolates were identified. Conventional exoantigen tests were able to identify the 74 *B. dermatitidis*, 72 *C. immitis*, and 17 *P. brasiliensis* isolates correctly within 48–72 h with no cross-reactivity. Therefore, results from the *B. dermatitidis* AccuProbe assay should be interpreted carefully in conjunction with other microscopic and morphological laboratory data if *P. brasiliensis* is suspected.

Due to the safety risks involved with the manipulation of *C. immitis*, it is impractical to maintain viable cultures for positive control. In 1997, a method was published to prepare heat-killed *C. immitis* mycelial cultures frozen in 10% aqueous glycerol [306]. This preparation has been found to be stable when frozen at –70°C for at least 13 years [305]. Alternatively, the genetically modified, nonvirulent strain of *Coccidioides posadasii* has been found to cross-react with the *Coccidioides* AccuProbe test [189].

More recently, a 2001 study found that the *Histoplasma* AccuProbe could identify *H. capsulatum* directly from an excised aortic valve following sonication [46]. In addition, the *Blastomyces* AccuProbe was utilized to detect *B. dermatitidis* from knee joint fluid following environmental exposure in a nonendemic area [309]. Additional instances of cross-reactivity have been described. One case report documented an isolate of *Gymnascella hyalinospora* causing pneumonia in a leukemia patient that was found to exhibit a positive *Blastomyces* AccuProbe result [145]. A 2005 study found that the *Histoplasma* AccuProbe cross-reacted with a white mold morphologically inconsistent with *H. capsulatum* that displayed the closest GenBank match (94%) to *Nannizziopsis vriesii* (sequence AJ131687) based on sequencing of the ribosomal internal transcribed spacer (ITS) region [35]. While useful for the detection of dimorphic fungal organisms, AccuProbe assays should be interpreted carefully in conjunction with other microscopic and morphological laboratory findings.

13.4.2 *Candida* peptide nucleic acid fluorescence *in situ* hybridization

An important issue in treating *Candida* infections results from varying antibiotic susceptibility profiles of individual species. Physicians may initiate treatment with a relatively expensive echinocandin before switching to the less costly fluconazole for susceptible organisms. *Candida albicans*, *C. parapsilosis*, and *C. tropicalis* exhibit widespread susceptibility to fluconazole; while *C. krusei* exhibits intrinsic resistance and *C. glabrata* exhibits a reduced proportion of isolates susceptible to fluconazole. As culture identification can take between 2 and 5 days, AdvanDx

(Woburn, MA) developed a system using peptide nucleic acid (PNA) probes for fluorescence in situ hybridization (FISH) to establish species identification in 90 min from a positive blood culture. Early studies found that PNA FISH could accurately distinguish *C. dubliniensis* from the closely related *C. albicans* [223]. Peptide nucleic acids are synthesized by attaching nucleobases to a polyamide backbone. The resulting probe can hybridize complementary nucleic acid targets and displays favorable diffusion and stability characteristics. The AdvanDx system uses a mixture of fluorescein and rhodamine-labeled PNA probes designed specific to the target *Candida* species. Hybridization is performed at 55°C for 30 min and is followed by a 55°C 30 min wash in the proprietary Wash Solution. The smear is mounted with provided Mounting Medium and is examined by fluorescence microscopy using a dual fluorescein isothiocyanate (FITC)/Texas Red filter. Currently, two FDA approved AdvanDx PNA FISH products are available for diagnostic *Candida* species identification (*C. albicans*/*C. glabrata* PNA FISH assay and Yeast Traffic Light PNA FISH assay).

Initial studies utilized only a fluorescein-conjugated PNA probe specific for *C. albicans*. Positive results consisting of the presence of green fluorescent yeast forms led to an identification of *C. albicans*, while *C. glabrata* served as a negative control. The original AdvanDx PNA FISH *C. albicans* assay, cleared by the FDA in 2004, required a 90-min hybridization step and took approximately 2.5 h for identification. A 2005 study analyzed 244 clinical blood cultures using *C. albicans* PNA FISH and found a sensitivity of 99%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 99.3% [320]. There was one *C. albicans* isolate that exhibited an initial negative PNA FISH result, but was positive following repeat testing. The *C. albicans* PNA FISH probe did not cross-react with the closely related *C. dubliniensis* that may sometimes be misidentified as *C. albicans* by traditional testing, including a positive germ tube test. Additional studies in 2006 found significant cost savings with the early identification of *C. albicans* leading to a significant decrease in echinocandin usage [3, 104]. However, the generalization of results should be treated with caution as prescription patterns and antibiotic susceptibilities at individual institutions are major factors in potential cost savings.

The second-generation FDA-approved AdvanDx *C. albicans*/*C. glabrata* PNA FISH assay was developed for the rapid identification of these organisms directly from blood culture. PNA fluorescent probes specific for either *C. albicans* or *C. glabrata* were designed, where *C. albicans* exhibits green fluorescence, and *C. glabrata* exhibits red fluorescence. The assay utilizes a 90-min hybridization step and a 30-min wash. A 2008 study [281] found that the

sensitivity for *C. albicans* was 98.7% (78/79 isolates), and the sensitivity for *C. glabrata* was 100% (37/37 isolates) in 197 blood cultures. The specificity was 100% for both *C. albicans* and *C. glabrata* components (82/82 isolates). Analysis of reference strains found that *C. bracarensis* (2/2 isolates), *C. nivariensis* (3/3 isolates), and *Nakaseomyces delphensis* (1/1 isolate) cross-reacted with the *C. glabrata* PNA probe resulting in a false-positive red fluorescence, while *C. orthopsilosis* (1/1 isolate) cross-reacted with the *C. albicans* PNA probe resulting in false-positive green fluorescence. The *C. albicans*/*C. glabrata* PNA FISH assay is currently available and serves as a rapid and objective method to accurately identify *Candida* species.

The third-generation FDA approved AdvanDx Yeast Traffic Light PNA FISH assay (Figure 13.16a) utilizes a three probe system which can detect *C. albicans*/*C. parapsilosis*, *C. glabrata*/*C. krusei*, and *C. tropicalis* from positive blood cultures. Cultures positive for *C. albicans* or *C. parapsilosis* exhibit green fluorescence, while cultures positive for *C. glabrata* or *C. krusei* exhibit red fluorescence. *Candida tropicalis* positive cultures exhibit yellow fluorescence. The assay utilizes a 30-min hybridization step and a 30-min wash. Early studies focusing on PNA FISH detection of multiple *Candida* species from 238 clinical specimens found that the technology exhibited 99% sensitivity and 99% specificity [261]. There was one false-negative *C. albicans* result and one false-positive *C. albicans* result on a *C. parapsilosis* isolate. A 2012 study [130] utilizing the commercial AdvanDx Yeast Traffic Light assay tested a 30-min versus a 90-min hybridization and found that 39/39 *C. albicans* isolates were correctly identified with both hybridization times. The study found that 31/33 *C. parapsilosis* isolates exhibited green fluorescence (1/33 isolates yellow) following a 90-min hybridization, and 31/33 *C. parapsilosis* isolates were green after a 30-min hybridization (1/33 yellow, 1/33 no color) with a potential for rare misidentification as *C. tropicalis*. All *C. glabrata* isolates (34/34 red) and *C. krusei* isolates (24/24 red) were correctly identified following both 90- and 30-min hybridizations. Out of 33 *C. tropicalis* isolates, 31/33 (2/33 no color) exhibited expected yellow fluorescence after 30 min, and 32/33 (1/33 no color) isolates exhibited yellow fluorescence after 90 min. *Candida bracarensis*, *C. nivariensis*, and *Nakaseomyces delphensis* isolates cross-reacted with the *C. glabrata*/*C. krusei* probe resulting in red fluorescence, and *C. orthopsilosis* isolates cross-reacted with the *C. albicans*/*C. parapsilosis* probe resulting in green fluorescence. *Candida colliculosa*, *C. lambica*, and *Rhodotorula mucilaginosa* isolates exhibited some nonspecific cross-reactivity.

Review of the literature revealed an initial experiment for a prototype AdvanDx PNA FISH^{Flow} kit for rapid and accurate identification of *C. albicans* [301]. The kit

is designed to be analyzed either by flow cytometry or fluorescence microscopy and takes approximately 1 h for identification. A total of 36 reference isolates and 150 clinical isolates were tested, and 58/58 *C. albicans* isolates were correctly identified by both flow cytometry and fluorescence microscopy. Three non-*C. albicans* isolates (1/33 *C. glabrata* isolates, 1/6 *C. dubliniensis* isolates, and 1/6 *Saccharomyces cerevisiae* isolates) exhibited inconclusive results by flow cytometry, while all non-*C. albicans* isolates were read as negative by fluorescence microscopy. Additional *Candida* species probes will likely be added to this system, and further studies will be required to demonstrate efficacy.

The most recent development in the AdvanDx product line is the QuickFISH Technology (Figure 13.16b) for the identification of *C. albicans*, *C. parapsilosis*, and *C. glabrata*. This system utilizes unique self-reporting probe/quencher

complexes that allow for a 15-min hybridization step while eliminating the wash step. This system also includes positive and negative control samples on the slide, further decreasing handling time. The identification process utilizes a small amount of blood culture transferred to a slide followed by two short fixation steps. Hybridization is accomplished by mixing PNA Blue and Yellow on the slide and incubating for 15 min at 55°C. Samples are then read by fluorescence microscopy utilizing an oil objective. At the time of this writing, no published studies are available for the performance of QuickFISH in the identification of *Candida* species. However, QuickFISH was found to demonstrate sensitivities and specificities similar to previous PNA FISH assays for the detection of *Staphylococcus aureus* and coagulase-negative *Staphylococcus* with a turnaround time of less than 30 min and hands-on-time of less than 5 min [66].

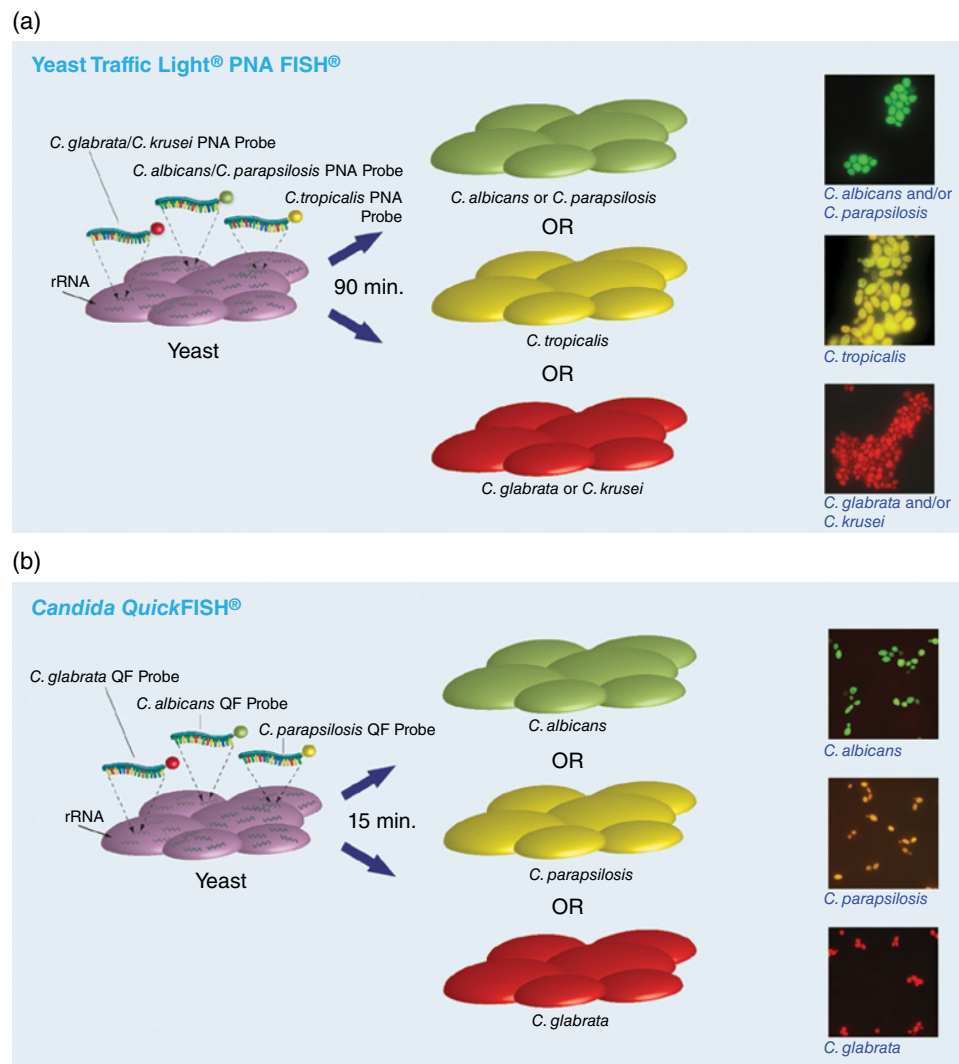


Figure 13.16 (a) Yeast Traffic Light PNA FISH. (b) *Candida* QuickFISH. (Courtesy of AdvanDX.) (See insert for color representation of the figure.)

13.4.3 PCR-electrospray ionization/MS

The Abbott PLEX-ID (Abbott, Abbott Park, IL) is a novel molecular diagnostic system that utilizes MS to measure the mass/charge ratio of PCR amplicons derived from conserved regions in the genomes of various pathogens. The PLEX-ID, developed from the previous Ibis T5000 system (Abbott), is the only currently available technology that allows for detection of bacteria, viruses, fungi, and certain parasites on a single platform. The PLEX-ID system has received the CE mark for distribution in Europe but as of this writing has not received FDA clearance for use in the United States. Currently, the PLEX-ID Viral IC Spectrum assay, the PLEX-ID BAC Spectrum BC bacterial assay, and the PLEX-ID Flu influenza assay are commercially available in Europe. The PLEX-ID technology can also be utilized for the detection of fungal organisms. PCR-electrospray ionization (ESI)/MS utilizes multiple primer sets to amplify specific genomic regions from microorganisms displaying the appropriate proportion of conservation and variability to successfully establish accurate identification. Following amplification, the specimen is desalted and subjected to ESI for subsequent analysis of the PCR products by MS. The sample is dissolved in an organic, aqueous solvent and subjected to a high voltage, thereby resulting in the emission of aerosolized charged droplets that are subsequently ionized [74, 274]. Following ionization, MS measures the mass to charge ratio of produced ions to determine the nucleotide composition of each amplicon within a sample. The obtained genomic data is compared to a database of known microbial genomic signatures to establish identification [78]. Quantification of the genomic data is made possible through the use of an internal calibration standard [75].

Advantages of this technology compared to the more widely used MALDI-ToF/MS systems include an increased ability to identify new pathogens, novel mutations, and antibiotic resistance genes. In addition, PCR-ESI/MS can allow detection of pathogens directly from patient samples and can resolve mixed specimens without the need for subculture [74]. This technology is extremely rapid with the ability to obtain results approximately 4–6 h following sampling. Disadvantages include the need for batching and relatively high cost (US\$50–100/sample) derived from the need for specialized equipment along with DNA extraction and PCR kits [149].

Review of the literature revealed a study that focused on the identification of various *Candida* species utilizing PCR-ESI/MS compared to MALDI-ToF/MS [149]. The Ibis T5000 was able to identify 9/11 *Candida* isolates. There were false-negatives for 1/4 *C. albicans* isolates and 1/1 *C. lusitaniae* isolates. These results were similar to those found with MALDI-ToF/MS (MALDI Biotyper, Bruker) that correctly identified 10/11 *Candida* isolates with one misidentification of *C. albicans* as *Staphylococcus aureus*

[149]. A 2012 study [206] showed that PCR-ESI/MS on the PLEX-ID platform (Abbott) was capable of identifying *Aspergillus terreus* from bronchoalveolar lavage (BAL) and brain abscess fluid in an acute myelogenous leukemia (AML) patient with fatal left lingular pneumonia and bilateral brain abscesses. The patient exhibited a negative CSF *Aspergillus* galactomannan antigen EIA and GMS stain but exhibited rare septate hyphae on GMS stain of BAL fluid. Culture of the brain abscesses exhibited numerous septate hyphae eventually identified as *A. terreus*; however, the patient was empirically treated with intrathecal amphotericin-B prior to identification and eventually succumbed to infection. In immunocompromised patients, molecular methods can be useful in the rapid identification of invasive *Aspergillus*. Identification from the brain abscess fluid using PCR-ESI/MS took 6–7 h compared to several days for identification using traditional methods. Future studies will be necessary to determine the overall utility of PCR-ESI/MS in the identification of fungal isolates in the clinical microbiology laboratory. It is also likely that improvement of the amplicon database will lead to increased ability for identification.

13.4.4 LightCycler SeptiFast Test MGRADE

Although not available in the United States at the time of this writing, the LightCycler SeptiFast Test MGRADE (Roche Diagnostics, Basel, Switzerland) has received the CE mark for European distribution, and the FDA approval process is ongoing. The SeptiFast assay combines Roche Diagnostics PCR-based analytical platforms with Spacer Probe Technology that was developed by Innogenetics and licensed to Roche in 2001 for the development of diagnostic bacterial and fungal identification assays. The SeptiFast platform utilizes multiplex PCR to target the 18S and 5.8S rDNA sequence of fungal organisms as well as the internal transcribed spacer between the 16S and 23S rRNA genes in bacteria [302]. This technology was designed for the rapid diagnosis of infections in septic patients and should be able to accurately detect polymicrobial infections. The SeptiFast platform is currently configured to directly detect the 25 most common bacterial and fungal pathogens known to cause blood-stream infections, including *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, and *A. fumigatus*, from a 1.5 mL sample of whole blood. While costs are relatively expensive, this assay should lead to improved patient care and better targeted antibiotic therapy deriving from a rapid turnaround time of between 6 and 12 h. Potential problems with the SeptiFast platform include the presence of microbial contaminants in addition to the detection of nonviable or nonpathogenic genetic material. This has been at least partially addressed by software design.

A number of studies have compared the SeptiFast platform to conventional blood cultures in the detection of infectious organisms. Interestingly, studies found a generally lower contamination rate for SeptiFast when compared to conventional blood culture. The SeptiFast platform detected more fungal isolates than conventional blood culture with a higher specificity and positive predictive value, especially in patients already treated with antibiotics [10, 180, 228, 271]. A 2012 study in a Czech Republic ICU evaluated results of patients with solid malignancies to assay SeptiFast workflow [72]. All of the submitted samples (100%) had a turnaround time of less than 10 h, 97% of samples were reported within 8 h, and 59% of results were reported within 6 h. Comparison of manual DNA extraction to an automated platform (MagNA Pure Compact System, Roche) found that the average turnaround time with manual DNA extraction was 6 h 11 min while the automated platform shortened this to 5 h 17 min. The SeptiFast platform appears to be especially useful in the early identification of invasive aspergillosis in repeat blood-culture-negative patients. Lodes et al. [174] conducted a 2011 study where SeptiFast detected four *C. albicans* and one *C. glabrata* isolate. SeptiFast also detected four *A. fumigatus* isolates in blood-culture-negative patients, and in three cases led to a change in antibiotic therapy. A 2008 study [183] found that SeptiFast identified *A. fumigatus* in two neutropenic patients with persistently negative blood cultures subsequently confirmed by positive culture of bronchoalveolar lavage fluid. Another 2009 study [310] evaluated SeptiFast compared to blood culture on 784 serum samples from 119 febrile neutropenic patients following chemotherapy. One *C. albicans* isolate was SeptiFast positive/blood-culture negative, one *C. glabrata* isolate was SeptiFast negative/blood-culture positive, and two *A. fumigatus* isolates were SeptiFast positive/blood-culture negative. Rath et al. [258] evaluated the SeptiFast test compared to blood culture in detecting pathogens from 225 blood samples in 170 ICU patients with suspected sepsis after liver transplantation or other major abdominal surgery. With respect to isolated fungal organisms, both SeptiFast and blood culture detected six *Candida* spp. isolates, four *Candida* isolates were SeptiFast positive/blood-culture negative, and three isolates were SeptiFast negative/blood-culture positive. SeptiFast also detected seven *A. fumigatus* isolates (four in liver transplant patients and three in nontransplant patients) in patients who exhibited negative blood cultures. By EORTC/MSG consensus group criteria, five of these seven patients had probable invasive aspergillosis and two had proven invasive aspergillosis. It can be concluded that the SeptiFast assay is more sensitive in the early identification of *Aspergillus* organisms than traditional blood culture. Overall, results using the SeptiFast platform are comparable to conventional blood cultures for the identification of clinical infections, and SeptiFast is

likely more efficacious in certain situations, such as with prior antibiotic administration and in the early detection of invasive aspergillosis.

13.4.5 Dermatophyte PCR kit

The Dermatophyte PCR kit (Statens Serum Institut Diagnostica, Copenhagen, Denmark) is a multiplex PCR system that combines a pan-dermatophyte PCR with a *Trichophyton rubrum* specific PCR for identification within 5 h compared to up to 4 weeks for traditional culture. Fungal DNA is extracted directly from nail specimens utilizing the provided buffers in the kit in a two-step 15-min procedure. Amplification primers are directed towards genes encoding for chitin synthase 1 for the pan-dermatophyte reaction and towards the internal transcribed spacer 2 (ITS2) region for specific detection of *T. rubrum*. All reactions contain an internal amplification control. A positive pan-dermatophyte reaction displays a 366 bp fragment on agarose gel electrophoresis while a positive *T. rubrum* reaction exhibits a 203 bp fragment. The Dermatophyte PCR kit is CE marked for distribution in the European Union but as of this writing is not yet approved for diagnostic use in the United States.

Brillowska-Dabrowska et al. [36] utilized the Dermatophyte PCR kit to extract DNA and analyzed a total of 118 nail samples. By PCR analysis, 50/118 (42.4%) of samples were positive for dermatophytes by PCR while 45/118 (38.1%) were positive by traditional diagnostics including microscopy and culture. Conventional diagnostics identified 27/118 (22.9%) isolates to species level while PCR identified 49/118 (41.5%) of isolates. One isolate was identified as *T. tonsurans* by culture and *T. rubrum* by PCR, and further examination could not be performed to resolve this discrepancy. Another isolate exhibited a mixed infection with *T. rubrum* and *T. mentagrophytes*; however, the PCR was pan-dermatophyte positive and *T. rubrum* negative. It was not definitively determined that *T. rubrum* was present in the test sample; however it is also possible that the *T. rubrum* PCR was not sensitive enough to identify the mixed infection. In 2010, Kondori et al. [164] utilized the Dermatophyte PCR kit to test 177 nail samples with suspected onychomycosis. PCR detected the presence of dermatophytes in 78/177 (44%) of samples compared to 59/177 (34%) in culture. Approximately 95% (56/59) of culture-positive specimens were positive for *T. rubrum*, and PCR confirmed 55/56 of these isolates as *T. rubrum*. Overall, PCR analysis was found to increase dermatophyte and specific *T. rubrum* detection in nail samples with a rapid turnaround time relative to conventional culture.

13.4.6 MycAssay

The MycAssay PCR kits developed by Myconostica Ltd (Cambridge, UK) are real-time PCR kits developed for use on the Cepheid SmartCycler, Roche LightCycler

2.0, Stratagene Mx3000, or AB 7500 platforms. Two MycAssay kits are currently available, MycAssay *Aspergillus* and MycAssay *Pneumocystis*. These kits are CE marked for distribution in the European Union but as of this writing are not approved for diagnostic use in the United States. For MycAssay *Aspergillus*, the molecular target is the 18S ribosomal RNA, and the reactions include an internal amplification control. Time to identification is approximately 2 h following DNA extraction, and the limit of detection is less than 50 target copies. For MycAssay *Pneumocystis*, the molecular target is the mitochondrial ribosomal large subunit (rLSU), and the limit of detection is less than 35 target copies. DNA extraction can be accomplished using either the companion MycXtra DNA extraction kit or another suitable method. The MycXtra DNA extraction kit is designed for the purification of fungal DNA from clinical bronchoalveolar lavage and other respiratory samples. This kit utilizes a bead-beating method to disrupt fungal cell walls and is designed to remove many real-time PCR inhibitors. With the MycXtra kit it takes approximately 1 h to perform ten extractions.

White et al. [318] utilized the MycAssay *Aspergillus* (Myconostica) assay compared to a validated in-house PCR assay to test for the presence of *Aspergillus* in 170 clinical serum specimens. DNA was extracted using the Roche High Pure Template DNA extraction kit. Both MycAssay and in-house PCR assays exhibited a limit of detection of five genomes and a linear dynamic range of five to $> 5 \times 10^4$ genomes. Sensitivities and specificities for MycAssay were 70.0 and 90.5% for a single positive sample compared to 60 and 100% for two or more positive specimens per patient. The in-house PCR exhibited sensitivities of 80.0% for a single positive sample and 50.0% for two or more positive samples, with specificities of 100% for single and multiple positive samples. Both MycAssay and the in-house PCR assay were capable of detecting *A. fumigatus*, *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans*, *A. versicolor*, *A. sclerotiorum*, and *A. glaucus* at an input level of 5000 genomes per reaction. Torelli et al. [298] utilized MycAssay *Aspergillus* to test 158 bronchoalveolar lavage (BAL) fluid specimens from hematology and ICU patients. DNA was extracted utilizing the MycXtra fungal DNA extraction kit (Myconostica), and extracts were frozen until batch PCR was performed. The MycAssay PCR exhibited a sensitivity of 94.1% (negative predictive value 99.3%) compared to 88.2% (NPV of 98.6%) for an in-house PCR and 94.1% (NPV of 99.3%) for a galactomannan ELISA with an index cutoff of at least 1.0. The MycAssay exhibited a specificity of 98.6% and a positive predictive value of 88.9% compared to a specificity of 97.9% and positive predictive value of 84.2% for the galactomannan ELISA. The MycAssay *Aspergillus* is a sensitive and specific assay for the detection of invasive aspergillosis with performance comparable to that of the

galactomannan assay. Both assays exhibit clinical utility in diagnosing and ruling out the presence of *Aspergillus*.

Hauser et al. [134] utilized the MycAssay *Pneumocystis* assay (Myconostica) to diagnose *Pneumocystis jirovecii* pneumonia (PJP, commonly referred to as PCP) in 110 respiratory samples. Alternative methods for the diagnosis of PCP include immunofluorescence, staining with Calcofluor white, silver staining, and Diff-Quik staining. This study compared the efficacy of MycAssay following DNA extraction by the MycXtra kit to the Merifluor-*Pneumocystis* direct fluorescent antigen test (MP-DFA). The MycAssay was positive in 13/14 subjects with a clinical diagnosis of PCP and in 9/96 subjects without a clinical diagnosis of PCP thus exhibiting a sensitivity of 93%, specificity of 90%, positive predictive value of 65%, and negative predictive value of 98% relative to clinical diagnosis. This compares to the MP-DFA values as follows: sensitivity of 93%, specificity of 100%, PPV of 100%, and NPV of 98%. Two samples tested in the study were reported as "Negative/Fail", suggesting the presence of a PCR inhibitor. One patient exhibited a positive PCR reaction prior to ultimate clinical diagnosis of PCP. Sequential MycAssay reactions conducted on patients during the course of therapy show that samples become negative during the course of treatment. MycAssay PCR was positive in one patient with a clinical diagnosis of PCP in which immunofluorescence was negative, potentially suggesting an increased trophozoite-to-cyst ratio where microscopy and immunofluorescence exhibit difficulty in detection [262]. Another study conducted by McTaggart et al. [194] compared the MycAssay *Pneumocystis* PCR assay to the laboratory standard of direct immunofluorescence microscopy (DFA) (Monofluo *Pneumocystis jirovecii* IFA kit, Bio-Rad Laboratories), real-time PCR, or conventional PCR and sequencing to determine if the presumed false-positives previously noted for the MycAssay were truly false-positives or if they represent a true analytical positive reaction with a low fungal burden. One hundred five bronchoalveolar lavage specimens were analyzed by the MycAssay following MycXtra DNA extraction and compared to the laboratory gold standard that considered a specimen *Pneumocystis* positive if it was positive by at least one of three alternative assays: IFA, *cdc2* real-time PCR, or mtLSU conventional PCR and sequencing. MycAssay specimens were considered positive if the cycle threshold value was less than 39.0, and the reaction exhibited exponential amplification. Out of 105 isolates, the MycAssay detected 27 positive samples compared to 9/105 positive samples for IFA, 16/105 positive samples for *cdc2* real-time PCR, and 26/105 positive samples for mtLSU conventional PCR/sequencing. Utilizing the presence of another positive laboratory test rather than clinical criteria, the MycAssay exhibited 100% sensitivity, 100% specificity, 100% PPV, and 100% NPV. Molecular methods

were found to be significantly more sensitive than IFA for *Pneumocystis* detection, and the most sensitive methods were MycAssay (27/105) real-time PCR, and the in-house conventional PCR/sequencing protocol, both targeting the mtLSU. Therefore, putative false-positive MycAssay reactions determined using clinical, microscopic, or IFA criteria may in fact be confirmed as true positives if alternative molecular methods are utilized to detect the presence of low fungal burdens.

13.4.7 Luminex xTAG technology

A novel multiplex PCR technology has been developed by Luminex Molecular Diagnostics (Austin, TX) that incorporates a proprietary universal tag system coupled with reagent coated microspheres. For fungal identification, Luminex has developed a set of analyte-specific reagent primers to detect the most common clinically relevant fungal pathogens. Luminex xTAG technology works by conducting multiplex PCR with provided ASR fungal primers following DNA extraction. The PCR reaction is then treated to remove excess nucleotides and primers and subjected to a primer extension step specific for the analyzed isolate that is attached to an xTAG universal tag sequence. The universal tag sequence coupled amplicons are then hybridized to the complementary anti-tag sequence coupled to a particular xMAP bead set. Hybridized beads or microspheres are color-coded and coated with specific reagents thereby allowing for detection of specific analytes. Inside the Luminex xMAP analyzer, a light source excites combinations of dyes specific to one of 500 microsphere sets, each with a unique spectral signature, as well as captured reporter dyes; results are analyzed by the accompanying software to achieve fungal identification. While the Luminex xMAP fungal assay has not yet received FDA approval as of this writing, the same technology has already been FDA approved for the detection of respiratory viruses (xTAG RVP FAST, Luminex).

Babady et al. [12] utilized a *Candida* 7-plex panel (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. lusitanae*, *C. guilliermondii*, and *C. krusei*) as well as a mold 11-plex panel (*A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *Scedosporium prolificans*, *Scedosporium apiospermum*, *Fusarium oxysporum*/*Fusarium solani*, *Rhizopus arrhizus*, *Rhizopus microspores*, *Mucor indicus*, and *Cunninghamella bertholletiae*) incorporating Luminex analyte specific reagents. DNA extraction was accomplished utilizing beads in a yeast lysis buffer with mechanical disruption. A total of 43 *Candida* culture isolates and 16 bacterial strains were tested on the *Candida* 7-plex assay with correct identification of all samples and no cross-reaction. The *Candida* 7-plex assay was also tested directly on blood-culture bottles (29 positive and 5 negative). The 7-plex assay correctly identified all *C. albicans* (9/9), *C. parapsilosis* (10/10), and *C. glabrata* (3/3) isolates, and

tested negative for *Rhodotorula glutinis* (0/2), *Fusarium* (0/5), and negative bottles. For the mold 11-plex assay, a total of 51 mold culture isolates were tested. The panel correctly identified all *A. fumigatus* (8/8), *A. terreus* (4/4), *A. flavus* (5/5), *S. apiospermum* (7/7), *S. prolificans* (2/2), *F. oxysporum*/*F. solani* (3/3), and *C. bertholletiae* (2/2) isolates. However, the assay failed to identify any *A. niger* (0/8) or *Mucor* (0/6) isolates and only identified 1/6 *Rhizopus* isolate. The 11-plex assay was also used to test 43 respiratory specimens and one appendix tissue specimen (23 positive, 21 negative) for the presence of molds. The assay correctly identified 1/1 (100%) *S. apiospermum*, 11/19 (58%) *A. fumigatus*, and 2/3 (67%) *A. flavus* isolates while failing to detect *Rhizopus* (0/1) and *A. niger* (0/1) isolates. The 11-plex assay did show promise in the detection of mixed infections, with simultaneous detection of *A. fumigatus* and *A. flavus* from two bronchial wash specimens. Detection of *Aspergillus* isolates from clinical specimens was suboptimal, possibly due to low organism burden or inadequate extraction. The inability of the assay to detect *A. niger* could be partially explained by the presence of phenotypically indistinguishable *A. tubingenensis* that can only be differentiated by molecular methods. The *Candida* 7-plex assay performed well, however, the mold 11-plex assay could use further development for certain organisms. A 2012 study [15] utilized the multiplex-PCR Luminex xTAG fungal ASR assay for the detection of clinically significant *Candida* species as well as *C. neoformans*, *H. capsulatum*, and *B. dermatitidis* organisms from blood cultures. A total of 132 blood cultures were tested (97 fungal positive, 25 bacterial positive, and 10 negative) for the presence of yeast species. The Luminex assay successfully identified all *Candida* positive blood cultures with the exception of three samples. One mixed infection of *C. glabrata* and *C. dubliniensis* was only identified as *C. glabrata*, one *C. dubliniensis* was not identified, and one *C. cijferrii* was misidentified as *Cryptococcus neoformans*. The assay successfully identified 6/7 mixed *Candida* infections. In addition, the assay was negative for all bacterial and negative blood cultures. Overall, the Luminex xTAG technology is a promising method for the identification of *Candida* and other fungal organisms with a rapid turnaround time and ability for simultaneous detection of mixed infections.

13.4.8 SepsisTest and Universal Microbe Detection systems

Molzym (Bremen, Germany) offers a series of universal PCR or real-time PCR-based systems for the culture-independent diagnosis of pathogens in a variety of clinical specimens. Primers are included for amplification and sequencing of the 16S and 18S rRNA regions with resulting identification of culture-negative infections,

including those caused by fungi, anaerobes, and intracellular bacteria. The SepsiT_{est} is designed for extraction and identification from blood. The Universal Microbe Detection (UMD) kit is designed for use with a variety of clinical specimens including blood, primary body fluids, exudates, tissues, and prostheses. UMD Liquid, UMD Swab, and UMD Tissue kits are available for the denoted specimens, and all have received the CE mark for *in vitro* diagnostic use in Europe. These kits are not currently available in the United States. Molzym also offers the CE-marked SepsiT_{est} SelectNA automated DNA extraction platform combined with PCR/real-time PCR pathogen analysis. The time to pathogen detection takes approximately 240 min with 75 min of hands on time. Sequencing analysis of positive samples takes around 260 min with 70 min of hands on time. The Molzym kits utilize pre-analytic removal of human DNA from clinical specimens utilizing MoLYsis technology to enrich the pathogen DNA, thereby allowing for increased sensitivity in detection. Twenty fungal species can be detected utilizing Molzym kits, including *A. fumigatus*, *A. niger*, *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. neoformans*, *Saccharomyces cerevisiae*, and *Malassezia* species.

A 2008 study [116] found that *C. albicans* DNA prepared using the MoLYsis DNA Complete kit (Molzym) could be detected at a level of 260–330 CFU/mL of spiked blood sample with *Candida* spp. PCR. The SepsiT_{est} has been found to be useful in the detection of mixed infections as well, with detection of multiple bacterial or mixed bacterial and *Candida* infections. A 2011 study [166] tested 30 patients with suspected infectious endocarditis with PCR testing utilizing the UMD-Tissue and SepsiT_{est} kits from both the affected heart valve and blood in comparison to culture. The SepsiT_{est} detected a mixed infection of *Streptococcus gallolyticus* and *C. albicans* from heart valve PCR but only detected *S. gallolyticus* from blood. Valve cultures were positive for only *C. albicans* and blood culture was negative. Previous blood cultures were positive for *S. hominis* (5 days previous) and *C. albicans* (30 days previous). Another patient displayed a valve PCR positive for *S. epidermidis* and *C. glabrata* with a negative blood PCR. Both valve and blood cultures taken at the time were negative although previous cultures were positive for coagulase-negative staphylococci (venous catheter swab, 19 days prior) and *C. glabrata* (blood culture, 10 days prior). These findings suggest that the SepsiT_{est} is more sensitive than culture for the detection of certain organisms in culture negative endocarditis. Additional studies are needed to determine the efficacy of SeptiFast.

13.4.9 Pyrosequencing

Pyrosequencing technology (PyroMark, Qiagen GmbH, Kungsgatan, Germany) is based on the principle of sequencing by synthesis and is a relatively rapid and

inexpensive method to obtain high-quality sequencing data. The pyrosequencing reaction occurs in five steps (reader can refer to the Qiagen website). First, a sequencing primer is hybridized to a single-stranded PCR amplicon in the presence of DNA polymerase, ATP sulfurylase, luciferase, and apyrase enzymes with adenosine 5' phosphosulfate (APS) and luciferin serving as substrate. Next, the first deoxyribonucleotide triphosphate (dNTP) is added. DNA polymerase incorporates the added dNTP into the DNA strand if it is complementary to the template. An incorporation event is accompanied by release of pyrophosphate proportional to the amount of incorporated dNTP. ATP sulfurylase then converts pyrophosphate into ATP in the presence of APS. The produced ATP fuels the luciferase-mediated conversion of luciferin to oxyluciferin with generation of visible light proportional to the amount of ATP. The produced light is detected by a charge-coupled device and seen as a peak in the data output with the height of the signal being proportional to the number of incorporated nucleotides. Following degradation of ATP and unincorporated nucleotides by apyrase, the next nucleotide is added. Nucleotides are added sequentially, and the signal peaks produced subsequently determine the sequence. Deoxyadenosine α -LFA-thio triphosphate (dATP-S) is used in the reaction rather than natural dATP as it can be incorporated by DNA polymerase but cannot be utilized by luciferase. A commercially available pyrosequencing platform (PyroMark Fungal ASR) developed by Biotage Inc. (Charlotte, NC) for the identification of fungal organisms has specific primers targeting a hypervariable region of ITS2. The technology is currently available as the PyroMark Q96 ID along with improved Pyro Gold reagents through Qiagen. Proprietary primers for fungal identification are also available through Qiagen as analyte-specific reagents.

For pyrosequencing identification, fungal organisms are subcultured on Sabouraud agar and subjected to DNA extraction. Commercially available DNA extraction kits for molecular grade isolation suitable for pyrosequencing include the UltraClean Microbial DNA Isolation kit (MoBio Laboratories) and Whatman FTA cards (GE Healthcare). Extracted DNA is then added to PCR reactions utilizing either laboratory designed or manufacturer-provided primers and reagents (Qiagen). Primers target conserved sequences flanking the ITS2 with subsequent amplification of the ITS2 hypervariable region. Following PCR amplification, amplicons can be analyzed by gel electrophoresis and purified prior to pyrosequencing. PCR products are denatured by alkali treatment, and appropriate sequencing primers are added. The template and primer are then loaded into a PSQ 96 reagent cartridge for pyrosequencing and analyzed using the PyroMark Q96 ID instrument and accompanying software (Qiagen).

A 2008 study [34] utilized the PyroMark Fungal ASR kit (Biotage Inc, Charlotte, NC), for pyrosequencing of the ITS2 hypervariable region and identification of various pathogenic *Candida* species according to manufacturer specifications. Pyrosequencing data were analyzed using the PSQ 96MA software v2.1 automated interpretation with a user-defined fungal sequence database containing ITS2 sequences constructed using IdentiFire software v1.0.5.0 (Biotage). The PSQ software analyzes sequence data and labels each base as “good,” “check,” or “bad” quality. Data were also reviewed manually. The mean read length of good and check quality bases in this study was 39 bases with a range of 19–50 bases. A minimum of 19 bases of good or check quality was required to make species calls. Pyrosequencing results yielded species-level identification for all 60 *Candida* isolates (51 clinical isolates and 9 reference isolates) following subculture and DNA extraction using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories Inc, Carlsbad, CA). Identified species include *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis*. Some DNA sequence heterogeneity was noted in homopolymeric tracts that can be difficult to interpret based on the reliance of pyrosequencing on signal heights. The average time following culture for species identification was 6 h for pyrosequencing compared to 20 h for biochemical and 77 h for morphologic identification. Borman et al. [28] described a procedure where fungal DNA is prepared through the application of an aqueous suspension of yeast cells or hyphal fragments and conidia to Whatman FTA cards (GE Healthcare) followed by brief microwave treatment. Pyrosequencing was conducted on the isolated DNA from 153 isolates encompassing 47 species using the commercially available Fungal ID kit (Biotage). Pyrosequencing correctly identified all test organisms including common *Candida* species in addition to the more rare *C. norvegensis*, *C. inconspicua*, *C. rugosa*, *C. fabianii*, *C. lipolytica*, *C. pelliculosa*, and *C. nivariensis* organisms. A subsequent study [29] showed that the ITS2 pyrosequencing protocol could discriminate among the closely related *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis* species. A 2010 study [30] analyzed 477 yeast isolates (88 reference and 389 clinical isolates) encompassing 43 species. Pyrosequencing of ITS2 successfully identified 469/477 isolates representing 40/43 species. No pyrosequence was generated from two *C. lambica* isolates, two *C. lipolytica* isolates, and four *C. pararugosa* isolates even after successful PCR amplification, suggesting variability in the sequencing primer binding site of these isolates. Molecular identification could be accomplished approximately 6 h after starting from pure culture.

Montero et al. [208] utilized pyrosequencing of the ITS2 region (PyroMark Fungi ASR, Biotage) compared to cycle sequencing of the ITS1 region to identify 133 isolates

of clinically relevant nondematiaceous yeasts belonging to the following genera: *Candida* (18 sp.), *Trichosporon* (10 sp.), *Cryptococcus* (7 sp.), *Malassezia* (3 sp.), *Rhodotorula* (2 sp.), *Geotrichum* (1 sp.), *Blastoschizomyces* (1 sp.), and *Kodamaea* (1 sp.). Cycle sequencing identified 78.9% of the isolates to the species level while pyrosequencing identified 69.1%. Out of 133 clinical isolates (43 sp.), pyrosequencing generated interpretable sequence data in 119 isolates encompassing 38 species. No signal was generated for 14 strains corresponding to isolates of *C. lambica*, *C. lipolytica*, *C. zeylanoides*, *C. pararugosa*, *Geotrichum candidum*, and *Kodamaea ohmeri*. With the exception of *Geotrichum candidum*, the other isolates exhibited successful PCR amplification. In addition, the pyrosequencing assay could not differentiate among species within the *C. neoformans* complex and *Trichosporon* species complex. A 2012 study [227] utilized pyrosequencing that targeted a 40 nucleotide ITS2 sequence with amplification primers designed based on alignment of available yeast rRNA gene sequences. DNA was extracted by freeze-thawing cultured isolates, and pyrosequencing was conducted using a PyroMark ID instrument, Pyro Gold reagents, and a PSQ 96 reagent cartridge (Biotage AB). Pyrosequencing of the 40 nucleotide ITS2 region was able to identify all 940 clinical yeast isolates to the species level encompassing 11 *Candida* species (931 isolates) as well as *Saccharomyces cerevisiae* (four isolates), *Malassezia pachydermatis* (three isolates), and *Rhodotorula mucilaginosa* (two isolates).

Pyrosequencing is a relatively inexpensive, rapid, and flexible method for the identification of fungal organisms. With continuing product development by Qiagen and others, this technology will likely become an important diagnostic method in the clinical microbiology laboratory.

13.4.10 Fast MicroSeq D2 LSU rDNA Fungal Identification Kit

The Fast MicroSeq D2 (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) large subunit (LSU) ribosomal DNA fungal identification kit combines the Fast MicroSeq D2 LSU rDNA Fungal PCR Kit and the MicroSeq D2 LSU rDNA Fungal Sequencing Kit (Applied Biosystems). This technology utilizes PCR to amplify the D2 large subunit of the ribosomal DNA from fungal isolates. The Fast MicroSeq kit is available for research use only and has not received FDA approval for diagnostic use in humans. DNA is extracted from fungal cells grown on Sabouraud's dextrose or inhibitory mold agar utilizing the recommended PrepMan Ultra Sample Preparation Reagent (Applied Biosystems) or another suitable protocol. Lysates can either be stored at –20°C or analyzed immediately. PCR is set up and run according to manufacturer instructions with provided reagents. A sample of produced amplicons is loaded onto a gel to check for DNA, and the remaining PCR

product is purified by ExoSAP-IT (USB) or the Montage PCR Filter Unit (Millipore) prior to cycle sequencing utilizing a GeneAmp PCR System 9700. Extension products are purified using a suitable DyeEx kit (Qiagen) to remove excess dye terminators and primers prior to electrophoresis (ABI Prism, ABI PRISM3100/3100-Avant, or Applied Biosystems 3730/3730xt instruments). Sequence data are then analyzed utilizing the Data Collection 2.0 Software and compared to those in the MicroSeq D2 database to establish identification.

A 2003 study investigated the ability of the MicroSeq D2 LSU rDNA identification kit to identify commonly encountered, clinically important yeast species [128]: approximately 93.9% (123/131) yeast isolates were correctly identified to the appropriate genus and species. Distance scores were obtained by comparing sequence data to MicroSeqD2 database sequences. The closest match was considered to be the correct identification as long as the distance score did not exceed 1.00%. One isolate of *C. dubliniensis* was identified as *C. albicans* (distance score of 3.42%). An isolate of *Rhodotorula glutinis* was identified as *Sporidiobolus johnsonii* (distance score of 8.72%), as the MicroSeq library did not contain a *R. glutinis* sequence. The MicroSeq system identified three *T. mucoides* isolates as *Trichosporon jirovecii* (*T. mucoides* not in database) and identified two isolates of *Saccharomyces cerevisiae* as *Zygosaccharomyces fermentati* and *Debaryomyces hansenii*, respectively. Sequencing of the D1/D2 large subunit region would be expected to increase the resolution of fungal species compared to sequencing D2 alone. The use of the 1.00% distance score cutoff also needs to be further analyzed.

A 2004 study investigated the ability of the MicroSeq D2 LSU rDNA identification kit to identify filamentous fungi in the clinical laboratory [129]. Of 234 tested isolates, 158 were correctly identified to the genus or species identification. Approximately 65% of 80 tested dematiaceous isolates and 51.2% of hyaline fungi isolates yielded sequence results concordant with conventional identification. The MicroSeq library lacked sequences for 70/234 isolates (29.9%) representing 27 genera.

Overall, sequencing the D2 region gives accurate results for clinically important filamentous fungi. However, the accompanying MicroSeq library lacks numerous commonly encountered isolates including *B. dermatitidis*, *C. immitis*, *O. canadensis*, *P. marneffei*, and several *Aspergillus* species. Although the library is somewhat limited, individual institutions can add data from clinical isolates to improve performance.

13.4.11 VYOO system

The VYOO system (SIRS-Lab, Jena, Germany) is a multiplex PCR-based method for the identification of 34 bacterial organisms, seven fungal organisms, and five resistance genes in blood samples. Fungal organisms detected are

A. fumigatus, *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*. Up to 5 mL blood collected using EDTA as the anticoagulant is subjected to a glass-bead-based lysis step. Following extraction, the blood contains a very low pathogen/human DNA ratio. The VYOO system utilizes the proprietary LOOKSTER preparation process that selectively enriches pathogen genetic material using an immobilized zinc-finger containing protein that targets different methylation patterns between human and bacterial/fungal DNA [74, 266]. Multiplex PCR then amplifies the target DNA, and microarray analysis reads the PCR results to identify the pathogens present in the clinical specimen. The time to identification is approximately 7–8 h. The VYOO system has received the CE mark for *in vitro* diagnostic use in Europe but as of this writing is not currently available in the United States. The majority of published studies using the VYOO system have focused on bacterial detection in sepsis. Fitting et al. [103] tested 72 sepsis patients for DNAemia using the VYOO system. Microorganisms were detected in the blood of 37/72 patients (51.4%) including 70% of patients with a positive blood culture. Nine patients exhibited a positive VYOO result without a positive blood culture. For fungal organisms, the VYOO system failed to detect *C. albicans* in one patient with a positive blood culture. Additional studies will be required to determine the efficacy of the VYOO system in detection of fungal organisms in septic patients.

13.4.12 DNA extraction/PCR amplification

With the arrival of additional sequencing methods and the increasing ability of individual institutions to develop so-called “home-brew” PCR and sequencing assays, the ability to efficiently obtain fungal DNA from a variety of specimens is of critical importance. There are numerous commercial DNA extraction kits available, but there are no currently accepted standards to determine if a given extraction protocol yields DNA of sufficient quantity and purity for analysis. DNA extraction on fungal specimens is especially difficult due to the need for cell-wall disruption and the presence of environmental fungal contaminants in numerous PCR assay components [175].

To improve standardization in the PCR testing of invasive aspergillosis using whole blood, the EORTC/MSG has conducted studies to determine the best and most reproducible methods for fungal DNA extraction [317]. Whole-blood panels were distributed to a total of 24 centers on two separate occasions in 2007 and 2008. Centers were determined to be compliant or noncompliant based on whether they followed certain recommendations on DNA extraction and PCR analysis. Individual DNA extraction protocols were found to have a large impact on the overall detection thresholds. Results from the centers found that compliant centers exhibited

improved sensitivity and specificity of fungal detection compared to noncompliant centers. The use of white-cell lysis buffer, bead beating for lysis, and an internal control PCR were associated with improved PCR performance. The European *Aspergillus* PCR Initiative (EAPCRI) has issued a number of recommendations for extraction and PCR targeting *Aspergillus*. The recommended protocol for fungal DNA extraction includes use of at least 3 mL blood, with ethylenediaminetetraacetic acid (EDTA) serving as the anticoagulant because heparin and sodium citrate have been found to inhibit PCR to an extent [113, 317]. Red and white blood cells should be treated with lysis buffer prior to disruption by bead beating rather than lyticase digestion [316]. Following fungal lysis, commercial DNA extraction kits can be utilized to obtain pure DNA. Suitable DNA purification kits are available from a variety of manufacturers including Qiagen, Roche, Zymo Research, Cepheid, bioMérieux, and others. Following extraction and purification, a suitable real-time or conventional PCR amplification system can be used to amplify DNA. Suitable PCR platforms include, but are not limited to, the Corbett Rotorgene apparatus, the Roche LightCycler product line, the Applied Biosystems TaqMan product line, the Cepheid SmartCycler apparatus, and the Stratagene Mx 300P platform. It should be noted that assay performance is more related to the DNA extraction protocol than the given PCR amplification platform. According to the EAPCRI, the PCR testing should be performed in duplicate, and reagents should be tested for potential contamination. An internal control target should be utilized to prevent false-negative reactions. Following these recommendations should improve the efficacy and cross-institutional reproducibility of aspergillosis detection.

Another 2010 study [217] compared the utility of five commercial kits and seven individual protocols in the diagnosis of *A. fumigatus* spores. Water samples and blood cultures were spiked with between 10^1 through 10^7 spores/mL. DNA was extracted from spiked samples using a previously described blood-cell lysis procedure [176]. The first method (method A) utilized the QIAamp DNA Mini kit (Qiagen) in combination with lyticase in a protocol described previously. Method B utilized the QIAamp DNA Mini kit with bead beating rather than lyticase [122, 176]. Method C utilized the QIAampDNA Micro kit (Qiagen) with lyticase treatment while method D utilized the QIAampDNA Micro kit with bead beating. Method E included the use of the Dynabeads DNA Direct Blood kit (Life Technologies, Grand Island, NY) and bead beating. Method F used the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA), and Method G utilized the YeastStar Genomic DNA Kit (Zymo Research). Quantitative PCR was conducted on fungal DNA isolated from the various extraction methods at a range of spore concentrations.

Method B (mechanical lysis with QIAamp DNA minikit) exhibited the highest sensitivity in water samples with 100% detection of 10^1 *A. fumigatus* spores/mL followed by 75% detection with method F, 50% detection with methods A and G, and 25% detection with methods C, D, and E. In spiked blood samples, methods F and G exhibited 75% sensitivity in the detection of 10^1 *A. fumigatus* spores followed by 50% sensitivity for methods A and B. Both methods B and F satisfied all requirements of the EAPCRI consisting of a suitable detection limit with bead beating. Due to improved sensitivity in blood and decreased cost relative to method B, the authors concluded that method F (ZR Fungal Bacterial DNA Kit) was the most suitable for *A. fumigatus* extraction.

It is particularly difficult to efficiently extract high-quality DNA from formalin-fixed paraffin-embedded (FFPE) tissue as formalin can cause protein–DNA cross-links thereby leading to fragmentation and decreased levels of intact DNA. A 2010 study [212] investigated the ability of five commercially available DNA extraction kits to obtain sufficient material from FFPE tissues to amplify a panfungal PCR product utilizing internal transcribed spacer 3 (ITS3) and ITS4 primers. The five DNA extraction kits tested include the QIAamp DNA FFPE Tissue kit (Qiagen), the TaKaRa Dexpat kit (Clontech Laboratories, Inc., Mountain View, CA), the PureLink Genomic DNA Mini Kit (Life Technologies), the WaxFree DNA kit (TrimGen Genetic Diagnostics, Sparks Glencoe, MD), and the QuickExtract FFPE DNA Extraction Kit (Epicenter Biotechnologies, Madison, WI). Following extraction, PCR was conducted to obtain appropriate amplicons. Reactions using the ITS3 and ITS4 primers generated a 300–400 bp product, while ITS1/ITS5 and ITS4 primers generated fragments between 400 and 600 bp. All PCR products were cleaned with ExoSap-It (USB Corporation, Cleveland, OH) before undergoing cycle DNA sequencing. Sequences were analyzed, and identification was accomplished using BLASTn to obtain closest homology. The QIAamp DNA FFPE Tissue Kit was utilized according to the manufacturer's instructions with slight modifications, including a 1 h room temperature incubation following the xylene and ethanol wash, digestion with ATL buffer (Qiagen) and proteinase K, and incubation with recombinant lyticase. The TaKaRa Dexpat kit was utilized according to Paterson et al. [230] using recombinant lyticase with omission of the B-mercaptoethanol step. The PureLink Genomic DNA Mini Kit was utilized according to manufacturer's instructions with modifications including the addition of a xylene and ethanol wash step, proteinase K digestion, and incubation with recombinant lyticase. The WaxFree DNA kit was utilized according to manufacturer's instructions with modifications, including incubation with recombinant lyticase and addition of an ethanol precipitation step following centrifugation in the

kit's WR filter. The QuickExtract FFPE DNA Extraction kit was utilized according to the manufacturer's instructions with modifications, including the use of 150 µL QuickExtract FFPE DNA Extraction Solution, incubation with recombinant lyticase, and the addition of an ethanol precipitation step following centrifugation. Modifications to manufacturer protocols were added because the commercial kits have not been thoroughly evaluated in the extraction of fungal DNA. The tested specimens consisted of 51 human cases, 24 human mock tissues, and six animal cases (81 total) infected with a combination of yeasts (18 isolates) and molds (63 isolates). A housekeeping gene served as an internal control. Results found that the shorter ITS3-ITS4 panfungal products were more likely to be positive than the longer ITS1-ITS4 or ITS5-ITS4 products for all tested extraction protocols. Additional assayed parameters include DNA quality, which was calculated as the number of the housekeeping gene PCR positives per total samples, and PCR efficiency, which was calculated as the number of panfungal PCR positive samples per number of housekeeping gene PCR positives. The QIAamp kit took 6 h and exhibited a DNA quality of 89% (72/81 samples) and a PCR efficiency of 90.3% (65/72 samples), while the TaKaRa kit took 2.5 h and exhibited a DNA quality of 91.4% (74/81 samples) and efficiency of 93.2% (69/74 samples). The PureLink kit took 6 h with a quality of 83% (67/81 samples) and an efficiency of 83.6% (56/67 samples), the WaxFree kit took 5 h with a quality of 79% (64/81 samples) and an efficiency of 57.8% (37/64 samples), while the QuickExtract kit took 3.8 h with a quality of 60.5% (49/81 samples) and an efficiency of 59.2% (29/49 samples). This study found that the QIAamp and TaKaRa kits performed similarly, however, the TaKaRa kit was more rapid with a simpler protocol and lower costs. Detected specimens included numerous fungi including *H. capsulatum*, *B. dermatitidis*, various *Aspergillus* species, and *Zygomycoses*. The strategy of DNA extraction from formalin-fixed paraffin-embedded tissues coupled to PCR and sequencing is a promising method for fungal identification in clinical specimens.

DNA extraction is a critical part of any PCR-based assay for fungal detection. As there are few standards currently in place, it is important for each institution to follow certain guidelines when preparing DNA for analysis. Numerous commercial fungal DNA extraction kits are available; however, it is important to test reagents for potential environmental fungal contamination. Based on guidelines from the EAPCRI, mechanical bead beating is preferable to lyticase digestion for fungal cell disruption due to a decreased chance of contamination and cost. PCR reactions should be conducted at least in duplicate, with appropriate negative and internal positive controls.

13.5 Susceptibility testing

Following publication of the first edition of this text, considerable progress has been made in the standardization and implementation of antifungal susceptibility testing in the clinical microbiology laboratory. The Clinical and Laboratory Standards Institute (CLSI) [formerly the National Committee for Clinical Laboratory Standards, NCCLS] has published updated standards for broth microdilution (BMD) testing of yeasts [50] and for filamentous fungi [49]. Disk diffusion testing for selected antifungal agents against *Candida* spp. has also been established [48, 51]. The CLSI BMD methodology has been accepted as the standard against which commercially available testing systems are evaluated.

Using the CLSI BMD assay, clinical interpretive break point (CBP) values for selected antifungal agents have been determined for *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. guilliermondii* [239]. In addition to CBPs, the concept of an epidemiological cutoff value (ECV) has been proposed. An ECV is a MIC threshold value that allows the discrimination of wild-type (WT) strains, which lack mutational or acquired resistance mechanisms, from non-WT strains [239]. ECVs are considered a sensitive method for detecting emergence of strains with decreased susceptibility.

There has been a recent revision of the CBPs for some *Candida* spp. for triazoles and echinocandins, and there may be further adjustments in the future. It is beyond the scope of this section to provide a detailed analysis of the interpretive criteria for antifungal testing, and the reader is referred to approved editions of the CLSI standards for current information [48–53].

13.5.1 Sensititre YeastOne

The Sensititre YeastOne system (Trek Diagnostics) combines a dried microdilution panel and a colorimetric method with an oxidation-reduction indicator to signal the growth of yeasts for susceptibility testing. This system has been compared with the CLSI M27-A document for microdilution susceptibility. When 600 isolates of *Candida* spp. and *C. neoformans* were tested against amphotericin B, fluconazole, and flucytosine, equivalent results were observed (>95% agreement) for all three drugs with the reference and the colorimetric indicator methods at 24 h, and > 98% agreement was observed at 48 h, except for fluconazole, which dropped to 84% agreement [250].

When the microdilution test, read after 24 h at 35°C, was compared with the M27-P method for fluconazole susceptibility testing, excellent agreement (97.7%) was demonstrated for 45 susceptible and resistant isolates of *C. albicans*. There was also *in vivo* correlation with fluconazole resistance. Preliminary data indicate that the microdilution methods may serve as less subjective alternatives

to the M27-P method for the determination of fluconazole MIC endpoints [84].

The Sensititre system was compared to the current standard microdilution method to compare 100 isolates of *C. albicans* and susceptibility to 5-fluorocytosine (5-FC), fluconazole, and itraconazole. The MICs were determined after 48 h of incubation. Discrepancies of no more than two dilutions were used to calculate the percent agreement. With this relatively wide agreement range, the agreement rate was 83% for itraconazole and 93% for 5-FC. Both were determined to be acceptable rates for those drugs [197].

In 1998, the Alamar Blue indicator was specifically tested for its utility in combination with the broth microdilution MIC method. It was shown that when used with RPMI media, Alamar Blue did not improve the ability of RPMI to detect strains of *C. albicans* resistant to amphotericin B [179].

In 1999, 1176 isolates of yeasts and yeast-like organisms were tested with both the CLSI reference method for broth microdilution and the Sensititre YeastOne Panel [94]. This study is noteworthy for the large number and the variety of isolates tested. Included were isolates of *Blastoschizomyces capitatus*, a *Cryptococcus* sp., common and emerging *Candida* spp., a *Hansenula* sp., a *Rhodotorula* sp., *S. cerevisiae*, *Sporobolomyces salmicolor*, and *T. beigellii*. The results for the YeastOne Panel were most comparable with those for the 24 h tests of the azoles and flucytosine, with 92–100% agreement for most species tested, except for *C. albicans* with 87–90% agreement. The best agreement for amphotericin B was at 48 h for *C. albicans* and most other species tested (92–99%), with the exception of *C. neoformans* at only 76% agreement. In addition, the results were comparable when the methods were compared at 48 h for amphotericin B and at 24 h for ketoconazole and itraconazole. The colorimetric test tended to influence the results by only one dilution and was not deemed to represent a substantial difference in overall results [270]. Good correlation was also observed between the Alamar Blue and the microtiter tray (MTT) tests for *Candida* susceptibilities, and the MTT method also generated interpretable data for *Aspergillus* spp. [146].

Overall, the YeastOne system has much potential value for clinical mycology laboratories. The test is generally easy to read and is likely to gain more practical utility as further *in vivo* comparisons are performed. Limitations to the method exist in that discordances with MIC occur for fluconazole and 5FC. One recent report documented resistance by the MIC reference method but sensitive results for the YeastOne method [178].

In 2004, Pfaller et al. [245] evaluated the Sensititre YeastOne colorimetric panel for susceptibility testing of *Candida* species for voriconazole, posaconazole, and ravuconazole relative to the CLSI M27-A2 BMD reference

method. Reference MIC endpoints were determined after 48 h, and YeastOne endpoints were determined after 24 h. Overall agreement between the two methods was 95.4% with 92.3% agreement for posaconazole, 95.3% agreement for voriconazole, 96.0% agreement for ravuconazole, and 98.0% agreement for fluconazole. Similarly, another 2004 study [93] conducted a multicenter comparison of the YeastOne panel for susceptibility testing of *Candida* species for posaconazole, ravuconazole, voriconazole, caspofungin, amphotericin B, and fluconazole relative to the CLSI M27-A2 BMD method. YeastOne and reference results were read at both 24 and 48 h. The best agreement between YeastOne and CLSI was when 24 h MIC values were used for each, resulting in overall agreement values of 95–99% for all organisms and antifungal agents. At 48 h (agreement 80–97%), YeastOne MICs were consistently greater than two dilutions higher than the reference BMD method. Interlaboratory agreement ranged from 90 to 99% for the CLSI method and from 83 to 99% for the YeastOne method for all organisms and antifungal agents. In 2005, Canton et al. [41] compared the YeastOne panel to the CLSI M27-A2 BMD method for antifungal susceptibility testing of 36 *Candida* clinical isolates, including six known caspofungin resistant strains with *FKS1* mutations. Overall MIC agreement (within 2 log₂ dilutions) was 87.16% between the two methods. Interlaboratory agreement ranged from 71.1% (amphotericin B) to 96.6% (flucytosine) for BMD and from 86.7% (fluconazole) to 97.2% (amphotericin B) for YeastOne.

Pfaller et al. [238] evaluated the YeastOne system for antifungal susceptibility testing of 404 *Candida* spp. isolates against anidulafungin, caspofungin, and micafungin, relative to the CLSI BMD method in three laboratories. Both YeastOne and CLSI panels were read at 24 h, and YeastOne endpoints were the lowest concentration at which the color changed from red (positive growth) to blue (negative for growth). Essential agreement between the two methods (MIC within two dilutions) was 100% for each *Candida* species and echinocandin tested. Categorical agreement was 99.3% for anidulafungin and 100% for micafungin and caspofungin. In 2009, Avolio et al. [11] tested the YeastOne panel for direct antifungal susceptibility testing of positive *Candida* blood cultures for amphotericin B, fluconazole, itraconazole, ketoconazole, 5-FC, and voriconazole. The YeastOne panel on a standard subcultured specimen was used as the reference method in this study. Out of 40 tested isolates, there was a 98% agreement rate between the direct and standard YeastOne panels for 240 tested *Candida*/antifungal combinations with only four minor errors. The Sensititre YeastOne colorimetric antifungal panel is an effective method for determining susceptibility patterns in yeasts.

The Sensititre YeastOne technology can also be applied to filamentous fungi and dermatophytes. Carrillo-Munoz et al. [42] tested the YeastOne panel for antifungal susceptibility testing of 100 opportunistic filamentous fungi (*Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp.) for amphotericin B, fluconazole, itraconazole, ketoconazole, and flucytosine. Minimum inhibitory concentration sub-90 values were obtained for various organism–antifungal combinations, however, potential discrepancies *in vitro* versus *in vivo* drug activities should be considered. Patel et al. [229] evaluated the YeastOne panel for testing susceptibility of filamentous fungi to posaconazole compared to the reference CLSI M38-A BMD method. Two hundred and forty-nine filamentous fungi were tested, including *A. fumigatus* (96), *A. flavus* (32), *A. niger* (20), *A. terreus* (19), *A. nidulans* (3), *A. ustus* (3), *A. versicolor* (3), *A. oryzae* (3), *F. solani* (14), *F. oxysporum* (7), *F. moniliforme* (6), *F. proliferatum* (six), *Fusarium* spp. (8), *Mucor* spp. (3), *Absidia* spp. (3), *Rhizopus* spp. (17), *Rhizopus arrhizus* (4), and *Rhizopus* microspores (2). Overall agreement between the YeastOne and BMD for these organisms was 97% (within 2 log₂ dilutions). In addition, YeastOne was able to detect laboratory generated *A. fumigatus* mutants with reduced susceptibility to posaconazole.

In 2008, Castro et al. [44] tested the YeastOne panel for antifungal susceptibility testing of dermatophytes for itraconazole, fluconazole, and voriconazole. The CLSI M38-A BMD guidelines were used as the reference method. Forty-six dermatophyte strains (30 *Trichophyton mentagrophytes*, 8 *Trichophyton rubrum*, and 8 *Microsporum gypseum*) were tested for azole susceptibility. Agreement between the two methods was low for the dermatophytes for the tested antifungals (0–37.5% for itraconazole, 12.5–53.3% for fluconazole, and 66.6–83.3% for voriconazole). In contrast, agreement was good (between 96.6 and 99%) for the reference *C. parapsilosis*, *C. krusei*, *A. flavus*, and *A. fumigatus* strains tested. Thus, the YeastOne system is promising for susceptibility testing in yeasts and filamentous fungi although its use for dermatophytes requires additional optimization.

13.5.2 Etest

The Etest (bioMérieux) has been used to determine MIC for bacteria and has also recently been tested for use with yeast isolates. The Etest is an agar-based method that uses strips impregnated with antifungal agents that are placed on the surfaces of the agar plates containing the inoculum. As the organism grows, the antifungal will inhibit the growth of susceptible organisms around the position of the Etest strip. The amount of inhibition correlates with the MIC for the organism (Figure 13.17).

Reproducibility was determined as results were compared in an interlaboratory study with broth macrodilution as the



Figure 13.17 MIC determination of fluconazole (FL), voriconazole (VO), and amphotericin B (AP) for *Candida* spp. by Etest. (Courtesy of bioMérieux.)

reference standard. In this report, the Etest quality control limits were one dilution broader (overall a four dilutions range) than those of the CLSI broth tube macrodilution method for the following combinations: ketoconazole with *C. krusei* and amphotericin B and ketoconazole with *C. parapsilosis* [248]. In another study, MICs of fluconazole, itraconazole, and ketoconazole were tested for 100 clinical isolates of *C. albicans* (25), *C. neoformans* (25), *C. glabrata* (20), *C. tropicalis* (15), and *C. parapsilosis* (15). Agreement was observed to be 71% for ketoconazole, 80% for fluconazole, and 84% for itraconazole [55].

Both broth macrodilution and microdilution tests for fluconazole susceptibility were compared to the Etest for 238 isolates of *C. albicans* and *C. glabrata*. While a 93% correlation was observed within ± 2 doubling dilutions of the broth methods at 24 h, only 37% was observed by the Etest for *C. glabrata*, 56% for *C. tropicalis*, 93% for *C. albicans*, and 90% for other *Candida* spp. The results were slightly better at 48 h [279].

A variety of agars have been used to evaluate Etest results, and the type of agar has been found to play a role in the performance of the Etest method. When amphotericin B, fluconazole, and itraconazole were tested, the results indicated that Casitone agar may be preferable for use in testing [63, 97]. Good correlation was shown between the results with fluconazole and the clinical outcome of the disease. Clinical failure resulted when MICs were $> 48 \mu\text{g/mL}$ [63].

In 1996 [92], an interlaboratory evaluation of the Etest was performed with Casitone agar and solidified RPMI 1640 medium with 2% glucose. The MICs of amphotericin B, fluconazole, flucytosine, itraconazole, and ketoconazole were tested with 83 isolates of *Candida* spp., *C. neoformans*, and *C. glabrata*. Analysis of 3,420 MICs demonstrated a

higher agreement between laboratories when Casitone medium was used to test amphotericin B, itraconazole, and ketoconazole. In contrast, flucytosine and fluconazole results were more reproducible when Roswell Park Memorial Institute (RPMI) medium was used. Recently [249], the performance of the Etest was compared to the fluconazole results from the CLSI microdilution method for 402 yeast isolates. Mueller–Hinton (MH) agar, RPMI agar with 2% glucose, and Casitone agar were tested with isolates of various species: *C. albicans* (161 isolates), *C. glabrata* (41), *C. tropicalis* (35), *C. parapsilosis* (29), *C. krusei* (32), *C. lusitanae* (31), other *Candida* spp. (19), *C. neoformans* (40), and miscellaneous species (14). The overall agreement was 97% for Casitone medium and 94% for RPMI. These media may allow the Etest to become a viable alternative to broth microdilution for testing the fluconazole susceptibilities of yeasts.

Several comparisons with broth microdilution have also been reported. When 169 yeasts were tested, the Etest results generally correlated with those of the reference method. There was at least 80% agreement within two dilutions of the reference method except for *C. neoformans* isolates, in which only 8% of the results correlated [282]. Another study, in which a variety of agar supplements were used to increase the percent agreement, documented 77–100% agreement with broth microdilution; however, problems were noted for the determination of azole susceptibility using the Etest [97].

The Etest and the CLSI broth microdilution test were compared using 602 yeast isolates for amphotericin B testing [247]. The clinical species tested were *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. lusitanae*, *C. krusei*, *Candida* spp., *C. neoformans*, and *S. cerevisiae*. The overall agreement between the Etest with RPMI medium and 2% glucose and the reference method was 98.3%. All Etest methods identified two resistant strains for which the MICs were 4–16 µg/mL, while the reference method failed to distinguish them from 18 other isolates for which the MICs were 2 µg/mL. The significance of this report is clear: the Etest may identify subpopulations of yeast isolates for which the amphotericin B MICs are high.

A 2008 study [67] found that the Etest in either RPMI or AM3 media (Antibiotic Medium 3; BBL/BD) can successfully discriminate between 28 susceptible and resistant *C. albicans*, *C. tropicalis*, and *C. krusei* isolates characterized by mutations in the *FKS1* protein as previously determined by the AFST-EUCAST BMD method. In 2009, Kiraz et al. [158] found that the Etest exhibited good agreement (within $\pm 2 \log_2$ dilutions) with the BMD for the determination of MICs against *C. glabrata*, with 86% agreement for amphotericin B and 90% agreement for caspofungin. Another 2010 study [90] examined the ability of the Etest to determine essential and categorical MIC values relative

to the CLSI BMD method for various *Candida* species with respect to anidulafungin susceptibility. For *C. albicans* (33 isolates), there was 93.9% agreement using a breakpoint of > 0.5 and 97% agreement using the breakpoint of > 2 . For *C. tropicalis* (15 isolates) and *C. glabrata* (13 isolates) there was 100% agreement between the two methods using either breakpoint, and for *C. krusei* (12 isolates) there was 91.7% agreement using either breakpoint. For *C. parapsilosis* (57 isolates) there was 54.4% agreement using > 0.5 breakpoint and 73.8% agreement using the > 2 breakpoint, while categorical agreement for *C. guilliermondii* (9 isolates) was 66.7% for both breakpoint values. Thus, agreement between Etest and the CLSI broth microdilution method for anidulafungin susceptibility determination appears somewhat based on the individual *Candida* species.

Alexander et al. [4] evaluated the Etest and YeastOne commercial assays against the CLSI M27-A2 reference BMD method for testing of 212 *Candida* spp. isolates for susceptibility to fluconazole, itraconazole, voriconazole, posaconazole, flucytosine, caspofungin, and amphotericin B. For Etest, overall essential agreement relative to BMD was 96% for flucytosine, 92% for fluconazole, 95% for itraconazole, 92% for voriconazole, 99% for posaconazole, 99% for amphotericin B, and 92% for caspofungin. For YeastOne, overall essential agreement was 92% for flucytosine, 82% for fluconazole, 96% for itraconazole, 85% for voriconazole, 100% for amphotericin B, and 92% for caspofungin. Overall categorical agreement was 90% for Etest and 88% for YeastOne. For the individual organisms, categorical agreement was lowest for *C. glabrata* (76% Etest/72% YeastOne), *C. tropicalis* (89% Etest/81% YeastOne), and *C. krusei* (40% Etest/75% YeastOne) organisms. The authors suggest additional evaluation of the commercial methods for determining azole susceptibility in *C. glabrata* isolates.

In 2010, Pfaller et al. [237] tested Etest and the EUCAST BMD method against the CLSI BMD method for echinocandin susceptibility testing of 133 *Candida* isolates that were characterized for the presence or absence of *FKS1* or *FKS2* gene mutations. *Candida albicans* (32 isolates), *C. glabrata* (34), *C. parapsilosis* (25), *C. tropicalis* (12), *C. krusei* (11), and *C. guilliermondii* (19) were tested for susceptibility to anidulafungin, caspofungin, or micafungin using epidemiological cutoff values. Categorical agreement of Etest with the CLSI method was greater than 90% for all strain–drug combinations with the exception of *C. krusei* and caspofungin (81.8%). The Etest identified 6/7 *FKS* mutant strains using anidulafungin as the reagent and 7/7 mutants using caspofungin as the reagent, while CLSI identified 5/7 for anidulafungin and 7/7 for caspofungin. Another 2010 study [125] tested the ability of Etest performed directly on blood samples from patients with fungemia to determine susceptibility of 195 prospectively

collected and 133 laboratory prepared yeast isolates to fluconazole, voriconazole, posaconazole, isavuconazole, caspofungin, and amphotericin B as compared to the CLSI M27-A3 method. In this study, Etest exhibited high percentages of very major errors (Etest direct indicated susceptible and CLSI indicated resistant) or major errors (Etest direct indicated resistant and CLSI indicated susceptible) with determination of yeast amphotericin B susceptibility. The Etest direct method exhibited lower error rates with the determination of azole susceptibility, with 0.6% very major errors and 14% major errors. Error rates for Etest direct relative to CLSI were higher for posaconazole than the other azoles, especially for *C. glabrata*.

In 2012, Ranque et al. [257] examined the interlaboratory reproducibility of Etest susceptibility testing of yeasts for amphotericin B and caspofungin relative to the CLSI method across four university hospital laboratories in the southeast region of France. The MIC values were determined for 198 consecutive clinical yeast isolates, and interclass correlation coefficients as well as essential and categorical agreement values were determined for the two methods at either 24 or 48 h. For the Etest, the interlaboratory reproducibility was 0.80 and 0.81 for caspofungin at 24 and 48 h, respectively, and 0.51 and 0.69 for amphotericin B at 24 and 48 h, respectively. Categorical agreement ranged from 87.9 to 91.4% for caspofungin and from 97.5 to 99.5% for amphotericin B between the Etest and CLSI methods. One major factor in the use of antifungal susceptibility testing for clinical decision-making is the use of MIC breakpoint values.

Arendrup et al. [6] examined the impact of revised CLSI recommended breakpoints for Etest susceptibility testing. The motivation for altering the breakpoints was data suggesting that an overall *Candida* spp. echinocandin susceptibility value of ≤ 2 mg/L was insufficient to identify isolates encoding an *FKS* gene mutation. In this study, the breakpoints for anidulafungin were set as follows (S, susceptible; R, resistant): for *C. albicans*, $S \leq 0.03$, $R > 0.03$, for *C. glabrata*, *C. krusei*, and *C. tropicalis*, $S \leq 0.06$, $R > 0.06$. For caspofungin, the breakpoints for *C. albicans*, *C. krusei*, and *C. tropicalis* were $S \leq 0.25$, intermediate (I) = 0.5, $R > 0.5$, for *C. glabrata* $S \leq 0.125$, I = 0.25, $R > 0.25$, and for *C. parapsilosis* $S \leq 2$, I = 4, and $R > 4$. Using the revised CLSI breakpoints, 65/496 susceptible blood *Candida* isolates (13.1%) as determined by the EUCAST BMD method were misclassified as either intermediate or resistant by the Etest. Misclassifications were most common for *C. krusei* (73.1%) and *C. glabrata* (33.1%) isolates, and the authors concluded that the revised CLSI breakpoints cannot be adopted for these two species.

Susceptibility testing for mold isolates is more ambiguous than yeast susceptibility testing. When the Etest was compared to broth microdilution for mold isolates, 75%

agreement was observed [291]. Both amphotericin B and itraconazole were retested by both methods. Among the molds tested were *Absidia corymbifera*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Exophiala dermatitidis*, *F. solani*, *S. apiospermum*, *S. prolificans*, and *S. brevicaulis*. The best agreements were seen for *A. fumigatus* and *A. terreus* (100% of the results for other agents). In every instance, where lack of agreement was observed, it was because of low MICs for molds in broth microdilution and much higher MICs in the Etest. The Etest appears to be a reasonable approach to susceptibility testing of *Aspergillus* spp. and a few other molds. Correlative studies that compare *in vivo* response to treatment may shed some light on discrepant results observed in this study.

Fuller et al. 2010 [110] examined the efficacy of the caspofungin Etest on RPMI or supplemented MH (MGM) agar for determination of MIC values for 345 clinical *Aspergillus* isolates relative to the reference CLSI M38-A2 BMD method minimum effective concentration (MEC) values. Essential agreement between the Etest MIC and reference MEC values was 18% for MGM agar and 26% for RPMI agar. The Etest MIC values were reproducible, with 91% of paired MGM and RPMI results within 2 log₂ dilutions of each other. Additional studies will be required to improve concordance of these assays prior to widespread use for clinical decision-making. In 2012, Badiie et al. [14] tested antifungal susceptibility of 108 clinical *Aspergillus* isolates against amphotericin B, ketoconazole, itraconazole, and voriconazole by Etest on RPMI agar as compared to the reference CLSI BMD method. Etest plates were read at 24 h, and CLSI method plates were read at 48 h. For amphotericin B, the Etest found 61.1% sensitive isolates and 38.9% resistant isolates compared to 63.9% sensitive and 36.1% resistant for CLSI. For ketoconazole, the Etest found 88.9% sensitive isolates and 11.1% resistant isolates, while CLSI found all 100% of isolates sensitive. For voriconazole, both Etest and CLSI methods found that all isolates were sensitive. For itraconazole, the Etest found 86.1% susceptible and 13.9% resistant while the CLSI found 69.4% sensitive and 30.6% resistant. In this study, the Etest was a useful method for determining antifungal susceptibility profiles for *Aspergillus* isolates.

In 2006, Espinel-Ingroff [86] compared Etest and YeastOne to the CLSI BMD M27-A2 and M38-A BMD reference methods for susceptibility testing of yeasts and molds to posaconazole and amphotericin B. This study included 328 clinical *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp., and Zygomycetes isolates. Overall agreement for YeastOne (% MIC pairs within a three dilution range) was 98–100% for Zygomycetes and yeasts and 99% for *Aspergillus* spp. for posaconazole. For the Etest, agreement was 95.7% for Zygomycetes and 97% for *Aspergillus* for posaconazole at 24 h. Etest agreement for posaconazole was 83% for yeasts at 24 h and 94% at 48 h.

For amphotericin B, YeastOne agreement with BMD was 99.3% for Zygomycetes and 95% for *Aspergillus* spp. while for the Etest agreement was 96.5% for Zygomycetes and 96% for *Aspergillus* spp. This study shows that both Etest and YeastOne commercial methods are effective methods for susceptibility determination.

13.5.3 Agar disk diffusion

The time tested methodology of agar disk diffusion testing of susceptibility of bacteria to antibiotics has also found application in mycology. There are CLSI-approved guidelines which incorporate methods and zone-size interpretations for fluconazole, voriconazole, posaconazole, and caspofungin when tested against *Candida* spp. [48, 51].

In 1996, Barry et al. [19] proposed a disk diffusion procedure for fungal susceptibility testing that was performed on RPMI 1640 broth supplemented with 1.5% agar and 2.0% glucose. Fluconazole disk diffusion testing was compared to the standard macrotube dilution reference method as well as the fluconazole Etest for determination of susceptibility for 250 *Candida* species selected to provide a broad range of MIC values. It was found that isolates exhibiting an inhibition zone diameter of ≥ 19 mm were all susceptible by the Etest, and 94% were susceptible by the macrotube dilution method. The predictive value for a susceptible result was 89% at 24 h and 94% at 48 h in comparison to the CLSI macrotube method. Isolates exhibiting smaller zones of inhibition were found to be resistant, intermediate, or susceptible by the Etest or macrotube methods. The disk diffusion method could not adequately determine intermediate or dose-dependent versus resistant isolates. Subsequent disk diffusion studies on Mueller–Hinton (MH) agar supplemented with 2% glucose showed significant growth in the zones of inhibition and poor definition of the zone edges, making accurate measurement difficult [20]. Addition of a low concentration of methylene blue (5 $\mu\text{g/mL}$) to MH 2% glucose agar was found to allow for more precise measurement of zones of inhibition. A 2001 study [172] utilized a 25 μg fluconazole disk diffusion test using RPMI 1640 broth MH agar plates supplemented with 2% glucose and 5 $\mu\text{g/mL}$ methylene blue (MH-GMB; Sigma) for susceptibility testing of *Candida* species. Agar plates were incubated in air at 35°C and results were read at 24 and 48 h of incubation. Minimum inhibitory concentrations were determined after 24 and 48 h using the CLSI macrodilution method M27 with RPMI 1640 broth. Inhibitory zones on agar plates were measured at the point where there was a sharp decline in growth corresponding to approximately 80% inhibition. Two hundred and ten *Candida* isolates were assayed including *C. albicans* (150), *C. glabrata* (18), *C. tropicalis* (16), *C. guilliermondii* (14), *C. parapsilosis* (9), *C. intermedia* (1), *C. sake* (1), and *C. utilis* (1) isolates. *Candida albicans*

ATCC 90028, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258 were used as quality control strains. Isolates were subcultured onto Sabouraud dextrose agar plates and suspended in 5 mL sterile saline. Following mixing and addition of saline, a suspension containing 2×10^4 to 4×10^4 CFU/mL was obtained, and MH-GMB agar plates were inoculated with a swab moistened in the suspension. Zones of inhibition were measured at 24 and 48 h, and isolates showing a ≥ 19 mm zone were considered susceptible, isolates showing a zone of 15–18 mm were considered intermediate, and those showing a zone of ≤ 14 mm were considered resistant. Predictive values of disk diffusion susceptibility for all tested *Candida* isolates compared to the macrodilution reference method was 97.6% for susceptible isolates, 56.3% for intermediate isolates, and 76.5% for resistant isolates at 24 h and 98.6% for susceptible isolates, 5.9% for intermediate isolates, and 31.4% for resistant isolates at 48 h. For *C. albicans* isolates, predictive values were 97.8, 50, and 85.7% at 24 h respectively and 99.3, 100, and 58.3% at 48 h. This study suggested that disk diffusion testing can be used as a routine screening procedure for susceptibility testing of *Candida* isolates in clinical laboratories.

In 2003, Barry et al. [18] conducted an international collaborative study in eight laboratories to propose quality control limits for fluconazole disk diffusion susceptibility testing on *Candida* isolates using MH-GMB agar plates. Plates were prepared with 2% glucose–0.5 $\mu\text{g/mL}$ methylene blue in advance and stored until needed or flooded with 200 μL of a GMB solution the day before testing. Quality control strains included *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, and *C. krusei* ATCC 6258. Disk diffusion tests were performed by preparing a saline suspension of freshly isolated colonies to match the turbidity of a McFarland 0.5 standard. A sterile applicator swab was utilized to inoculate 150 mm MH-GMB agar plates that were then incubated for 20–24 h. On 10 separate days, each laboratory tested each reference strain on both advance prepared and flooded MH-GMB plates. The two preparation methods were found to provide essentially identical results. It was found that the *C. krusei* ATCC 6258 strain was not useful for fluconazole susceptibility testing because 55% of tests had no zone of inhibition, and the remainder had zones of ≤ 18 mm. Utilizing data from the eight participating institutions, zone-size limits were generated for *C. parapsilosis* ATCC 22019 (22–33 mm), *C. tropicalis* ATCC 750 (26–37 mm), and *C. albicans* ATCC 90028 (28–39 mm). One participant reported unusually large zones for *C. tropicalis* ATCC 750 and *C. albicans* ATCC 90028, and another participant reported exceptionally large zones for *C. parapsilosis* ATCC 22019. This suggests the potential for variability among different laboratories. The CLSI Subcommittee for Antifungal Susceptibility Testing subsequently approved the quality control limits.

The CLSI document M44-A recommended zone interpretive criteria for the evaluation of susceptibility of *Candida* isolates to fluconazole: S $\geq$ 19 mm, susceptible dose-dependent (S-DD) 15–18 mm, and R $\leq$ 14 mm. In 2004, Testore et al. [295] determined the *in vitro* fluconazole susceptibility of 1565 clinical isolates of *Candida* species using the CLSI M44-A guidelines. Isolates were cultured on Sabouraud dextrose agar, and 0.5 McFarland standard suspensions were prepared in sterile saline. The suspension was streaked to MH-GMB agar plates with a 25 μ g fluconazole disk (Pfizer Inc.). After 24 h incubation, zones of inhibition were evaluated using the Biomic image analysis plate reader system (version 5.9, Giles Scientific, Inc., 1999) that determined 80% growth inhibition and zone edges. *Candida albicans* comprised 68% of the isolates, and 92.6% of isolates were susceptible to fluconazole, 2.7% were susceptible dose-dependent, and 4.7% were resistant. *Candida tropicalis* exhibited 91% susceptible, 4% susceptible dose-dependent, and 5% resistant isolates, while *C. parapsilosis* exhibited 97% susceptible, 3% susceptible dose-dependent, and 0% resistant isolates. Approximately 24% of *C. krusei* isolates were susceptible by disk diffusion, while 3% were susceptible dose-dependent, and 72% were resistant. Of course, *C. krusei* should be considered inherently resistant to fluconazole even though repeated testing of susceptible isolates by disk diffusion confirmed initial results. Disk diffusion was found to be a reliable method for determination of *Candida* susceptibility to fluconazole.

In 2006, Sims et al. [283] compared the M27-A2 BMD method, the Etest, and the M44-A disk diffusion method for susceptibility testing of 270 bloodstream *Candida* isolates to posaconazole. Isolates were subcultured at least twice on Sabouraud dextrose agar, and inoculum suspensions were adjusted to a 0.5 McFarland standard prior to spreading on MH-GMB agar plates and incubating for 24–48 h. Paper disks containing 5 μ g posaconazole were obtained from Oxoid Ltd. (Basingstoke, UK) and BD Diagnostics (Sparks, MD). The M44-A disk diffusion method at 24 h showed 4% major errors and 0.7% very major errors compared to BMD at 48 h. The Etest showed 11.7% major errors and 0.3% very major errors at 24 h compared to BMD at 24 h and 3.6% major errors and 2.5% very major errors for at 48 h compared to BMD at 48 h. No consistent differences were observed between the two manufacturers of posaconazole disks. A 2007 study [69] compared disk diffusion, Etest, and BMD methods for susceptibility testing of 2171 clinical *Candida* isolates against posaconazole. Cell suspensions of isolates were adjusted to the turbidity of a McFarland 0.5 standard and inoculated to MH-GMB plates containing 5 μ g posaconazole disks (Oxoid, Lenexa, KS). Zone diameters were read at 24 h using a Biomic image analysis plate reader system (version 5.9; Giles Scientific,

Santa Barbara, CA). As no interpretive breakpoints for posaconazole exist, accepted breakpoints for voriconazole were utilized: S $\leq$ 1 μ g/mL, $\geq$ 17 mm, S-DD 2 μ g/mL, 14–16 mm, and R $\geq$ 4 μ g/mL, $\leq$ 13 mm). For disk diffusion, there was 98% agreement with the reference BMD method with 1% minor errors, < 1% (8/2,171) major errors, and < 1% (2/2,171) very major errors. For the Etest, there was 93% agreement with BMD, with 4% minor errors, 3% major errors, and 0% very major errors. However, disk diffusion was 89% sensitive for the detection of posaconazole resistant isolates while Etest was 100% sensitive for the detection of resistant isolates. Also in 2007, a multilaboratory collaborative study [37] was performed to propose quality control limits for posaconazole disk diffusion susceptibility testing of *Candida* isolates on MH-GMB plates. Replicate testing on three lots of media using 5 μ g posaconazole disks in eight laboratories was conducted on ATCC *Candida* reference strains. For *Candida albicans* ATCC 90028, a quality control inhibition zone of 24–34 mm was proposed, for *C. parapsilosis* ATCC 22019 a zone of 25–36 mm was proposed, for *C. tropicalis* ATCC 750 a zone of 23–33 mm was proposed, and for *C. krusei* ATCC 6258 a zone of 23–31 mm was proposed.

In 2007, Milici et al. [201] compared the M44-A disk diffusion assay with the reference M27-A2 BMD method for susceptibility testing of 106 yeast isolates (50 *Candida albicans*, 10 *C. parapsilosis*, 10 *C. glabrata*, 10 *C. tropicalis*, 6 *C. guilliermondii*, 5 *C. rugosa*, 5 *C. krusei*, 2 *C. kefyr*, 2 *C. pelliculosa*, and 3 *Saccharomyces cerevisiae* isolates) to caspofungin at concentrations of 2, 10, and 25 μ g per disk following incubation on MH-GMB plates for 24–48 h. After 24 h incubation, there was 78.0% agreement with BMD for *C. albicans* isolates, with 100% of isolates with a MIC $\geq$ 2 μ g/mL showing an inhibition zone of $\leq$ 11 mm. For non-*C. albicans* isolates there was 76% agreement, and 100% of isolates with a MIC $\geq$ 2 μ g/mL showed an inhibition zone of $\leq$ 11 mm. Thus, the disk diffusion method is a promising alternative to BMD testing although additional testing is needed to determine utility and reproducibility.

13.5.3.1 Surveillance studies

The ARTEMIS global antifungal surveillance program [240–242, 246] is a comprehensive and long-running study that utilizes standardized disk diffusion according to CLSI M44-A guidelines and broth microdilution (BMD) to establish internal quality control, external quality assurance measures, and validation of test results. Results are analyzed electronically using the Biomic image analysis plate reader (Giles Scientific, Santa Barbara, CA) and are stored in a central database. For fluconazole, susceptible isolates have a zone diameter of $\geq$ 19 mm, susceptible dose dependent isolates have a zone diameter of 15–18 mm, and resistant

isolates have a zone diameter of ≤ 14 mm. For voriconazole, susceptible isolates have a zone diameter of ≥ 17 mm, susceptible dose dependent isolates have a zone diameter of 14–16 mm, and resistant isolates have a zone diameter of ≤ 13 mm. Between June 1997 and December 2007, fluconazole *in vitro* susceptibility results for 256,882 *Candida* spp. isolates were collected from 142 sites in 41 countries. From 2001 to 2007, voriconazole susceptibility data were collected from 197,619 isolates. Data are utilized to analyze trends of resistance to fluconazole and voriconazole and to monitor potential resistance patterns in emerging *Candida* species. Approximately 90.2% of *Candida* isolates were susceptible to fluconazole, and 95% were susceptible to voriconazole. Increasing fluconazole resistance was observed in *C. parapsilosis*, *C. guilliermondii*, *C. lusitanae*, *C. sake*, and *C. pelliculosa* isolates. Emerging fluconazole resistant species were *C. guilliermondii* (11.4% resistant), *C. inconspicua* (53.2% resistant), *C. rugosa* (41.8% resistant), and *C. norvegensis* (40.7% resistant). Thus, the M44-A disk diffusion method is a vital assay in the determination of yeast antifungal susceptibility patterns.

13.5.3.2 Dermatophytes

The CLSI M44-A document focused on disk diffusion testing for yeasts, however, no agar-based susceptibility testing method has been standardized for testing of dermatophytes. In 2004, Karaca et al. [152] tested a total of 56 strains from infected skin, nail, and hair belonging to four species of dermatophytes that were tested against 10 antifungal drugs using a disk diffusion method in comparison to the broth microdilution method according to the CLSI M38-P guidelines for obtaining MIC values. Antifungals tested for disk diffusion were itraconazole (8 $\mu\text{g}/\text{disk}$), fluconazole (15 $\mu\text{g}/\text{disk}$), ketoconazole (15 $\mu\text{g}/\text{disk}$), miconazole (10 $\mu\text{g}/\text{disk}$), suconazole (25 $\mu\text{g}/\text{disk}$), oxiconazole (25 $\mu\text{g}/\text{disk}$), bifonazole (25 $\mu\text{g}/\text{disk}$), griseofulvin (25 $\mu\text{g}/\text{disk}$), ciclopiroxolamine (50 $\mu\text{g}/\text{disk}$), and terbinafine (5 $\mu\text{g}/\text{disk}$). Isolates included 50 strains of *Trichophyton rubrum*, three strains of *T. mentagrophytes*, two strains of *T. tonsurans*, and one strain of *T. verrucosum*. Agar plates for disk diffusion consisted of RPMI 1640 broth supplemented with 1.5% agar and 2% glucose. All dermatophyte isolates produced detectable growth at time points ranging from 7 to 15 days. Favorable correlation between disk diffusion and broth microdilution were observed for fluconazole, ketoconazole, itraconazole, oxiconazole, miconazole, terbinafine, and griseofulvin. No correlation between the two methods was observed for bifonazole, sulconazole, and ciclopiroxolamine. Terbinafine was found to demonstrate the highest antifungal activity against tested dermatophytes. Thus, disk diffusion susceptibility testing of dermatophytes is highly dependent on the antifungal being tested, and further standardization is needed. A 2010 study [219] utilized

MH (Remel, KS) agar plates for disk diffusion susceptibility testing of 47 dermatophyte isolates against eight antifungals. Isolates were subcultured on potato dextrose agar or oatmeal agar (for *T. rubrum*) for 4–15 days, and a conidial suspension was adjusted to 1×10^6 conidia/mL. Plates were streaked with a swab and incubated at 30°C for 4–7 days. Commercially available disks (9 mm diameter) preloaded with ciclopirox (50 $\mu\text{g}/\text{disk}$), fluconazole (25 $\mu\text{g}/\text{disk}$), itraconazole (8 $\mu\text{g}/\text{disk}$), ketoconazole (15 $\mu\text{g}/\text{disk}$), miconazole (10 $\mu\text{g}/\text{disk}$), and voriconazole (1 $\mu\text{g}/\text{disk}$) were utilized (Rosco NeoSensitabs, Key Scientific, TX). Disks containing 10 μg of griseofulvin (Acros Organics, NJ) or 1 μg terbinafine (Norvartis, NJ) were not commercially available and were prepared in the laboratory. Strains tested included *Trichophyton tonsurans* (12), *T. rubrum* (10), *T. mentagrophytes* (9), *Microsporum canis* (8), and *Epidermophyton floccosum* (8) isolates as well as *T. rubrum* ATCC MYA 4438, *T. mentagrophytes* ATCC MYA 4439, *Candida albicans* ATCC 9028, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258 quality control strains. This study found no benefit to supplementation of MH agar plates with 2% glucose or 0.5 $\mu\text{g}/\text{mL}$ methylene blue for dermatophyte testing. Optimal incubation time for reproducible inhibition zones ranged from 3 days for *T. mentagrophytes* to 7 days for *T. rubrum*, *T. tonsurans*, *E. floccosum*, and *M. canis* isolates. All tested isolates were found to yield clear inhibition zones in nonsupplemented MH plates. Five *T. rubrum* isolates known to be resistant to terbinafine showed a 0 mm zone of inhibition, as expected. Disk diffusion data were found to correlate with MIC data determined by the CLSI M38-A2 microdilution method for terbinafine susceptibility patterns of *T. mentagrophytes* and *T. rubrum* isolates. Further testing is needed to determine the role of disk diffusion antifungal susceptibility testing for the dermatophytes.

13.5.3.3 Filamentous fungi

While the utility of agar disk diffusion susceptibility testing of yeasts has been demonstrated, no reference guidelines are available for disk diffusion testing of filamentous fungi. In 2007, Messer et al. [196] compared the Etest, CLSI M38-A BMD, and agar disk diffusion methods for the determination of posaconazole susceptibility in 146 clinical isolates of filamentous fungi, including *Aspergillus fumigatus* (68), *A. flavus* (45), *A. niger* (15), *A. terreus* (9), *A. niveus* (3), *A. nidulans* (2), *Mucor* spp. (2), and *Rhizopus* spp. (4). Isolates were subcultured to potato dextrose agar (Remel, Inc.) and incubated at 35°C for 7 days. Stock suspensions of conidia or sporangia were made in saline supplemented with 0.2% Tween and adjusted according to CLSI M28-A guidelines. Two different agar media were tested: MH agar (Difco) supplemented with 2% glucose and 0.5 $\mu\text{g}/\text{mL}$ methylene blue and RPMI agar buffered with

morpholinepropanesulfonic acid and supplemented with 2% glucose (Remel, Inc.). Five microgram posaconazole (Schering Plough) disks (Becton Dickinson, Sparks, MD) were prepared in the laboratory. Zone diameters were measured using digital calipers, and plates were incubated at 35°C for 48 h. Disk diffusion and MIC interpretive criteria for posaconazole were set as the accepted CLSI criteria for voriconazole: S $\geq$ 17 mm and $\leq$ 1 $\mu\text{g/mL}$, S-DD 14–16 mm and 2 $\mu\text{g/mL}$, and R $\leq$ 13 mm and $\geq$ 4 $\mu\text{g/mL}$. Categorical agreement between disk diffusion and BMD was 97.9% with 0% very major errors, 0% major errors, and 2.1% minor errors. For *Aspergillus* spp. isolates, categorical agreement between BMD and disk diffusion was 100%. The categorical agreement between Etest and BMD was 96.6% with 0% very major errors, 0% major errors, and 2.1% minor errors. Thus disk diffusion testing is highly promising as a simple and reliable alternative to BMD for susceptibility testing of filamentous fungi.

Another 2007 study [95] evaluated disk diffusion testing parameters for 555 isolates including *Absidia corymbifera* (15), *Alternaria* sp. (23), *Bipolaris spicifera* (18), *A. fumigatus* (56), *A. flavus* (38), *A. niger* (38), *A. terreus* (40), *A. nidulans* (31), *F. solani* (29), *F. moniliforme* (28), *F. oxysporum* (20), *M. circinelloides* (17), *Mucor ramosissimus* (5), *R. arrhizus* (37), *R. microspores* var. *rhizopodiformis* (10), *Paecilomyces lilacinus* (24), *S. apiospermum* (28), and *S. prolificans* (26). A set of 483 isolates was evaluated at three laboratories while a separate set of 72 isolates was evaluated at two laboratories. Disk diffusion zone diameters were compared with CLSI M38-A BMD MIC or MEC values to determine optimal testing conditions for mold disk diffusion testing. Both plain MH agar plates and MH plates supplemented with 2% dextrose and 0.5 $\mu\text{g/mL}$ methylene blue were tested. Reproducibility of inhibition zone diameters was tested at 24, 48, and 72 h. Antifungals tested were posaconazole, voriconazole, itraconazole, caspofungin, and amphotericin B. Results from the study found that reproducibility of zone diameters was best on plain MH agar (91–100%) with incubation times of 16–24 h for *Zygomycetes*, 24 h for *A. fumigatus*, *A. flavus*, and *A. niger*, and 48 h for the remaining species. Agreement of inhibition zone diameters with BMD MIC or MEC values was 92% for the posaconazole 5 μg disk, 83% for the 10 μg itraconazole disk, 86% for the 1 μg voriconazole disk, 96% for the 5 μg BBL caspofungin disk, and 73% for the amphotericin B disk. Among the three laboratories testing the 483 isolate set, rates of major errors ranged from 0.2 to 6.4%, and rates of very major errors ranged from 0 to 2.7%. Only two major errors were observed for the 203 *Aspergillus* isolates using the itraconazole disk, and none were observed using the posaconazole disk. The amphotericin B disk provided the lowest correlation with MIC values, with 1.7% very major errors, 2.7% major errors,

and 23.1% minor errors. Thus, while disk diffusion testing correlates well with BMD testing overall, disk diffusion could not differentiate intermediate from susceptible or resistant for some species–antifungal combinations.

In 2010, CLSI developed reference guidelines (M51-A) for testing the susceptibility of nondermatophyte filamentous fungi using disk diffusion. In 2011, a multicenter study [88] documented reproducibility of disk diffusion susceptibility testing for one isolate each of five molds (*Paecilomyces variotii* ATCC MYA-3630, *A. fumigatus* ATCC MYA-3626, *A. flavus* ATCC MYA-3631, *A. terreus* ATCC MYA-3633, and *Fusarium verticillioides* [moniliforme] ATCC MYA-3629 as well as *C. krusei* ATCC 6258). Inoculation suspensions of molds were prepared from 7-day-old isolates grown on potato dextrose agar and spread on nonsupplemented MH agar plates. Susceptibility to amphotericin B (10 μg ; Abtek Biologicals, Liverpool, UK), itraconazole (10 μg ; Abtek Biologicals), voriconazole (1 μg ; BD Diagnostics), and posaconazole (5 μg ; BD Diagnostics, Sparks, MD) disks were tested. Zone diameters were determined by visual examination after incubation for 24–48 h, and slight trailing growth around zone edges was ignored for the triazoles but not for amphotericin B. No significant differences were observed among the different lots of MH agar, and there were no consistent discrepancies among the participant laboratories. Zone diameter ranges were proposed for the quality control isolates as follows: *P. variotii* ATCC MYA-3630, amphotericin B (15–24 mm), itraconazole (20–31 mm), and posaconazole (33–43 mm); *A. fumigatus* ATCC MYA-3626, amphotericin B (18–25 mm), itraconazole (11–21 mm), posaconazole (28–35 mm), and voriconazole (25–33 mm). Zone diameter ranges were not proposed for *A. flavus*, *A. terreus*, or *F. verticillioides* quality control isolates due to low testing reproducibility. Zone diameters for *A. flavus* ATCC MYA-3631 were 27–37 mm for posaconazole and 25–36 mm for voriconazole, while zones for *A. terreus* ATCC MYA-3633 were 33–41 mm for posaconazole and 23–33 mm for voriconazole. Data for *F. verticillioides* ATCC MYA-3629 showed zone diameters of 21–32 mm for posaconazole with 100% interlaboratory agreement.

In 2012, Martos et al. [188] tested susceptibility of 65 clinical *Aspergillus* spp. isolates (28 *A. fumigatus*, 18 *A. flavus*, 16 *A. terreus*, 2 *A. niger*, 1 *A. glaucus*, and 4 *Fusarium* spp.) to the echinocandins caspofungin, micafungin, and anidulafungin in comparison to BMD MEC values. Isolates were subcultured on potato dextrose agar for 7 days, and a stock suspension was prepared equivalent to a 0.5 McFarland turbidity standard. Disks (Becton Dickinson) were prepared in the laboratory to obtain a final concentration of 2 $\mu\text{g/disk}$ for anidulafungin and caspofungin and 1 $\mu\text{g/disk}$ for micafungin. After application of the isolate suspension to nonsupplemented MH

agar plates, zones of inhibition were measured after 24–48 h. The caspofungin disk provided zones of inhibition ranging from 14 to 29 mm, while the micafungin disk provided zones ranging from 14 to 40 mm, and the anidulafungin disk zones ranged from 22 to 45 mm. No inhibition zones were detected for the four *Fusarium* isolates, as expected. For caspofungin, MEC values for the *Aspergillus* isolates were ≤ 0.25 $\mu\text{g/mL}$, and inhibition zones were ≥ 15 mm except for *A. terreus* isolates (11–22 mm). For micafungin and anidulafungin, MEC values were ≤ 0.015 $\mu\text{g/mL}$, however, micafungin inhibition zones were ≥ 14 mm while anidulafungin inhibition zones were ≥ 22 mm. Further studies will be needed to determine the reproducibility and utility of disk diffusion for echinocandin susceptibility testing.

13.5.4 Neo-Sensitabs tablet diffusion assay

The Neo-Sensitabs assay (A/S Rosco Diagnostica, Taastrup, Denmark) utilizes a tablet containing 10 μg amphotericin B, 5 μg caspofungin, 8 μg itraconazole, 5 μg posaconazole, or 1 μg voriconazole for antifungal susceptibility testing of yeasts and molds. A 2006 study [86] compared susceptibility of 92 *Zygomycetes*, 126 *Aspergillus*, 110 *Candida*, and 15 *C. neoformans* isolates to posaconazole and amphotericin B using reference M27-A2 and M38-A BMD MIC values, M44-A disk diffusion zones, and the Neo-Sensitabs tablet disk diffusion zones. Both nonsupplemented MH agar plates and MH plates supplemented with 2% glucose and 0.5 $\mu\text{g/mL}$ methylene blue (MGM) were tested. The Neo-Sensitabs tablet performed similarly to posaconazole and amphotericin B disks for determination of inhibition zones. For molds and posaconazole, the Neo-Sensitab assay yielded a correlation coefficient with reference method MIC values of 0.799 after 24 h incubation and 0.799 after 48 h incubation on nonsupplemented MH plates compared to the M44-A disk diffusion values of 0.804 and 0.826 at 24 and 48 h, respectively. For molds and amphotericin B, the Neo-Sensitab assay exhibited correlation coefficients of 0.765 and 0.749 at 24 and 48 h, respectively compared to M44-A disk diffusion values of 0.736 and 0.8118 at 24 and 48 h, respectively. Likewise for yeasts and posaconazole, correlation coefficients were 0.769 and 0.717 at 24 and 48 h compared to values of 0.810 and 0.685 for the M44-A disk diffusion method on MGM plates. However, for yeast and amphotericin B, correlation coefficients were 0.478 and 0.503 at 24 and 48 h for Neo-Sensitabs compared to 0.681 and 0.681 at 24 and 48 h for the M44-A method.

A 2007 study [89] compared the Neo-Sensitabs tablet assay to reference M27-A2 broth microdilution and M44-A disk diffusion methods for susceptibility testing of 110 isolates of *Candida* spp. and *C. neoformans* to amphotericin B, caspofungin, fluconazole, itraconazole, and voriconazole on MGM plates, RPMI 1640 plates supplemented with 2% dextrose, and Shadomy agar plates.

Plates were incubated for 24–48 h for *Candida* isolates and 48–72 h for *C. neoformans* isolates. The Neo-Sensitabs assay performed well for susceptibility testing against fluconazole (95.5% agreement with BMD on MGM with 4.5% minor errors, 96.4% on RPMI, and 89.1% on Shadomy), voriconazole (95.5% agreement on MGM with 3.6% minor errors, 97.3% on RPMI, and 95.5% on Shadomy), itraconazole (92.7% agreement on MGM with 7.3% minor errors, 87.3% on RPMI, and 84.5% on Shadomy), and caspofungin (94.5% agreement on MGM with six discrepancies, 60.9% on RPMI, and 93.6% on Shadomy). However, for amphotericin B, the Neo-Sensitabs assay showed two discrepancies of resistant isolates being called susceptible on both MGM and RPMI agar and five discrepancies on Shadomy agar. The M44-A disk diffusion method correctly classified all isolates for amphotericin B testing. Optimal conditions for the Neo-Sensitabs assay include the use of MGM agar and 24 h of incubation for *Candida* and 72 h of incubation for *C. neoformans* isolates. While the Neo-Sensitabs assay is a viable alternative for susceptibility testing against the triazoles or caspofungin, additional improvement is required to optimize utility for amphotericin B testing.

A 2008 study by Espinel-Ingroff et al. [87] compared the Neo-Sensitabs tablet assay to the M38-A BMD assay and disk diffusion methods for susceptibility testing of 183 filamentous isolates (6 *A. corymbifera*, 7 *Alternaria* spp., 10 *B. spicifera*, 27 *A. fumigatus*, 17 *A. flavus*, 14 *A. niger*, 17 *A. terreus*, 12 *A. nidulans*, 9 *F. solani*, 6 *F. moniliforme*, 7 *F. oxysporum*, 19 *R. arrhizus*, 2 *M. circinelloides*, 2 *M. ramosissimus*, 3 *Rhizopus microsporus*, 9 *P. lilacinus*, 10 *S. apiospermum*, and 6 *S. prolificans*) to amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole. Isolates were tested on nonsupplemented MH plates and incubated from 16 to 48 h. Correlation coefficients with M38-A MIC values were 0.686 for Neo-Sensitabs and 0.76 for disk diffusion for posaconazole, 0.788 for Neo-Sensitabs and 0.84 for disk diffusion for itraconazole, 0.806 for Neo-Sensitabs and 0.891 for disk diffusion for voriconazole, and 0.72 for Neo-Sensitabs and 0.813 for disk diffusion for amphotericin B. Correlation with M38-A MEC values was 0.92 for Neo-Sensitabs and 0.91 for disk diffusion for caspofungin. With posaconazole, there was 84% agreement with BMD for Neo-Sensitabs (2% major and 0.5% very major errors) and 96% for disk diffusion (0% major and 0% very major errors). For itraconazole there was 85% agreement with BMD for Neo-Sensitabs (1.0% major and 0.5% very major errors) and 87% agreement for disk diffusion (0% major and 0% very major errors). For voriconazole there was 90% agreement with BMD for Neo-Sensitabs (0% major and 0% very major errors) and 93% agreement for disk diffusion (1.0% major and 0% very major errors). For caspofungin there was 95% agreement with BMD for Neo-Sensitabs (0.5% major and 0.5% very major errors)

and 96% agreement for disk diffusion (1.0% major and 0% very major errors). For amphotericin B there was 79% agreement with BMD for Neo-Sensitabs (2% major and 0.5% very major errors) and 83% agreement for disk diffusion (0.5% major and 0% very major). The highest numbers of errors with amphotericin B were among *Aspergillus* (mainly *A. terreus*) and *Fusarium* isolates. The authors concluded that the Neo-Sensitabs assay was suitable for testing all molds for caspofungin, voriconazole, and posaconazole susceptibility, all molds except *Zygomycetes* for itraconazole susceptibility, and only *Zygomycetes* for amphotericin B susceptibility.

13.5.5 Screening with CHROMagar

When CHROMagar *Candida* containing 8 or 16 µg of fluconazole/mL was used as a screen for fluconazole resistance, the results compared favorably with those of the standard broth macrodilution methods when the following criteria were used for interpretation. Susceptible *C. albicans* colonies are smaller on fluconazole medium than on fluconazole-free medium. The correlation with this observation was 96% for MICs with a 16 µg/mL breakpoint and 95% for MICs with an 8 µg/mL breakpoint. The results indicate that this method may be useful as a screen for fluconazole resistance [231]; however, a substantial amount of technical expertise will be required for proper interpretation of the results.

A 2005 study [292] tested 95 blood cultures positive for *Candida* species to determine direct susceptibility testing of fluconazole (25-µg disk) on CHROMagar *Candida* versus disk diffusion using a standardized inoculum plated onto CHROMagar *Candida* as well as MH-GMB, relative to reference YeastOne microdilution MIC determination. At 24 h there was categorical agreement among the methods for all *C. albicans*, *C. tropicalis*, *C. lusitaniae*, and *C. krusei* isolates. For *C. parapsilosis*, there was one very major error for direct CHROMagar *Candida* and one major error for standardized MH-GMB at 24 h. For *C. glabrata*, there were 10 minor errors and one very major error for direct CHROMagar *Candida*, 12 minor and two very major errors for MH-GMB, and 13 minor and two very major errors for standardized CHROMagar *Candida* at 24 h. There were no major errors for *C. glabrata* at 48 h relative to the reference YeastOne MIC determination. These data suggest that the subculturing of *Candida* isolates onto CHROMagar with a fluconazole disk can be a useful method for identification and prediction of fluconazole susceptibility at 24 h, with the exception of *C. glabrata*.

In 2010, Kirkpatrick et al. [161] examined the utility of supplementation of CHROMagar *Candida* with echinocandins and azoles for the determination of yeast antifungal susceptibility. Clinical *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *C. lusitaniae*, *C. rugosa*, and *C. dubliniensis* isolates were tested. Antifungal susceptibility

against anidulafungin, caspofungin, micafungin, posaconazole, and voriconazole utilizing CHROMagar agar dilution were shown to be 96, 80, 94, 90, and 97% accurate, respectively, relative to the CLSI BMD reference method (within one tube dilution of CLSI MICs). Categorical determination of resistance versus susceptibility was more problematic, especially for *C. glabrata* and *C. parapsilosis* isolates. For *C. glabrata*, there were 12% major errors for posaconazole, 17% very major errors for anidulafungin, 33% very major errors for micafungin, 10% very major errors for caspofungin, and 20% very major errors for voriconazole. Thus, determination of resistance or susceptibility utilizing agar dilution screening may be problematic and species dependent.

13.5.6 Vitek 2

The first fully automated approach to yeast susceptibility testing was developed using the Vitek 2 (bioMérieux, Inc.). FDA approval for clinical use was granted in 2006 and initially limited to fluconazole and *Candida* spp. Endpoint reading is by spectrophotometric measurements on the instrument, resulting in less subjective and more reproducible readings. Initial studies compared the Vitek 2 AST-YST (VT2) card to the reference CLSI broth microdilution method for fluconazole [243]. A total of 426 *Candida* spp., including the most common and less frequent clinically significant isolates were tested by the BMD and VT2. A challenge set of 80 isolates within range MICs were selected and tested at one site. Three hundred forty-six clinical isolates were also tested, and interlaboratory reproducibility was determined using a panel of 10 isolates. The essential agreement between the methods for isolates for fluconazole was 97.9% (24 h BMD) and 93.7% (48 h BMD) with a range of 95.3 to 100% (24 h) and 68.6 to 100% (48 h). The low 48 h reading was for *C. krusei* and excluding that value the range for the BMD 48 h reading was 96.4 to 100%. Categorical agreement between VT2 and BMD was excellent with only 0.2% very major errors (VME), all associated with *C. glabrata*. Interlaboratory reproducibility was excellent.

The same set of *Candida* spp. used by Pfaller et al. [244] to compare fluconazole BMD to the VT2 was used in a separate publication extending the test drugs to amphotericin B, flucytosine, and voriconazole. Results for these drugs were very similar to those for fluconazole, with an overall essential agreement of 97% for amphotericin B, 99.1% for flucytosine, and 96.7% for voriconazole (BMD 24 h). Categorical agreement between VT2 MICs and BMD for flucytosine and voriconazole (no interpretative criteria for amphotericin B) was 100% for most species, with an overall agreement of 98.2 and 98.6% respectively for flucytosine and voriconazole (24 h BMD readings).

A 2009 study [252] tested the VT2 test for detection of *in vitro* resistance to fluconazole and voriconazole in *C. albicans* and *C. glabrata* clinical isolates. Out of

122 isolates (36 *C. albicans* and 86 *C. glabrata*), the VT2 method exhibited excellent categorical agreement (97.5%) for both azoles relative to the reference CLSI BMD method. The mean time for obtaining results for the VT2 system was 14.88 h (range of 13–19 h), while the BMD wells were read at either 24 or 48 h with the 48 h result serving as the reference, according to the CLSI procedure. Essential agreement between VT2 and BMD was 91.6% for *C. albicans* isolates against fluconazole, 94.4% against voriconazole, and 100% for *C. glabrata* isolates against both azoles.

Borghetti et al. [27] tested the VT2 system (AST-YS01 card) against the CLSI BMD reference method for determining susceptibilities of 614 *Candida* spp. isolates, 10 *C. neoformans* isolates, one *Geotrichum capitatum* isolate, and two reference strains to amphotericin B, fluconazole, and voriconazole. Overall categorical agreement between the two methods was 99.5% for amphotericin B, 92% for fluconazole, and 98.2% for voriconazole. The VT2 system exhibited difficulty with determining susceptibility of *C. glabrata* and *C. krusei* isolates to fluconazole, with categorical agreement values of 51.4% and 53.3%, respectively, relative to the 48 h BMD value as recommended by CLSI. In addition, the VT2 system could not determine susceptibility of *C. neoformans* to voriconazole due to the absence of an established MIC breakpoint.

In 2010, Cuenca-Estrella et al. [61] compared the VT2 system (AST-YS01 card) to the CLSI and EUCAST BMD reference methods in addition to the Sensititre YeastOne and Etest techniques for the determination of susceptibility of 154 clinical yeast isolates to amphotericin B, flucytosine, fluconazole, and voriconazole. Overall essential agreement of the VT2 system to the reference BMD methods was comparable to the Etest and YeastOne methods (>95%). VT2 results were available after an average time of 15.5 h (range 14–18 h) for *Candida* species while *C. neoformans*, *R. mucilaginosa*, and *Dipodascus capitatus* required 34 h. The VT2 exhibited 2.6% very major errors relative to EUCAST BMD and 1.6% relative to CLSI BMD.

A 2011 study [96] evaluated interlaboratory agreement for either the VT2 or Sensititre YeastOne method for determining susceptibility of 70 *Candida* isolates to amphotericin B, fluconazole, flucytosine, and voriconazole. The VT2 results were obtained at 18 h while YeastOne results were read at 24 h. Intralaboratory agreement for VT2 was 100% for amphotericin B, 90.3–96.1% for fluconazole, 93.7–97.9% for 5-FC, and 96.5–99.7% for voriconazole. For YeastOne, agreement was 93.5–98.1% for amphotericin B, 83.6–91.4% for fluconazole, 71.4–81.4% for 5-FC, and 85.7–91.9% for voriconazole. Thus, overall VT2 exhibited better interlaboratory agreement than YeastOne in this study.

A recent study [33] compared the Vitek 2 YS01 card to both the CLSI M27-A3 BMD and the Etest methods using 208 strains of *Candida*. All strains of *Candida* were

isolated from patients with clinical disease. Of these, 81 were from blood cultures, 62 from other sterile sites, 51 from pulmonary samples, and 14 from different sites. Species identification was determined from chromogenic medium, YST Vitek 2 system cards or ID32C strips. Etests were performed using RPMI agar plates. Endpoints for the Etest and BMD were read after 24 and 48 h incubation at 37°C. Azole MICs were read as lowest concentration resulting in an 80% reduction in growth and amphotericin B at the point of no growth. The VT2 cards were read at a time that the instrument detected adequate growth in the control well, with average times of 15 h and a range of 12 h to 26 h. For amphotericin B there was an excellent correlation between MICs of VT2 and the BMD. However, MICs obtained by the Etest method had a poor essential agreement with a range of 29.8 to 100% when compared to BMD. Azoles tested by VT2 were more related to 24 h BMD and Etest results than with the 48 h Etest readings. It should be noted that the VT2 gave fluconazole MICs of *C. krusei* below the level of resistance that were flagged by the software and corrected as resistant. This is an important point to be observed when using manual methods to determine fluconazole susceptibility of *C. krusei*, which may be performed before identification has been determined.

A multicenter study comparing the VT2 antifungal susceptibility of *Candida* spp. to caspofungin, micafungin, and posaconazole with the CLSI BMD reference method was reported in 2011 [235]. The study included 250 clinical isolates tested at each of three participating sites, to include *C. albicans* (150) and *Candida* spp. other than *C. albicans* (100). The echinocandins, caspofungin, and micafungin, were tested against all species, and posaconazole was tested only against *C. albicans*. Each site also tested 112 well-characterized challenge strains and an additional panel of 10 isolates tested in triplicate to determine intra- and interlaboratory reproducibility. Categorical agreement between the VT2 and BMD was assessed by combining data obtained with clinical and challenge isolates from all three sites (total 867). Categorical agreement for all drug–organism combinations was greater than 98% with the exception of micafungin and *C. parapsilosis* (84.1%). Considering VME, the VT2 (none) was superior to BMD (15.9%) for *C. parapsilosis* against micafungin.

The automated performance and reading of antifungal susceptibility testing for *Candida* spp. is an equivalent method to the CLSI BMD for many relevant antifungal agents and has a shorter turnaround time for many species.

A 2013 study by Astvad et al. [8] tested the newly available VT2 AST-YS06 card to determine caspofungin susceptibility of 98 *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. dubliniensis* isolates, including those

exhibiting *FKS* mutations. The CLSI and EUCAST BMD methods were also tested. With the *FKS* genotype used as the gold standard, VT2 exhibited a categorical agreement of 93.9% relative to 88.4% categorical agreement for CLSI and 98.7% for EUCAST. VT2 misclassified 50% of *C. tropicalis*, 20% of *C. albicans*, and 9.1% of *C. glabrata* *FKS* mutants as susceptible. If CLSI had been used as the gold standard, VT2 essential agreement would have been 92.6%, and categorical agreement would have been 96.5%. The VT2 could not determine whether *C. glabrata* isolates were susceptible or intermediate for caspofungin due the fact that the detection range did not span the MIC breakpoint value for this organism. Additional studies testing the efficacy of VT2 for determining susceptibility to echinocandins will be beneficial.

13.5.7 ATB Fungus 2

In 2007, Torres-Rodriguez et al. [299] evaluated the microbroth ATB Fungus 2 (ATBF2) system (bioMérieux, La Balme-les Grottes, France) compared to the Sensititre YeastOne system for antifungal susceptibility testing using a panel of 100 yeasts from clinical samples. CLSI M27-A2 BMD was used as the reference method. The ATBF2 system is a modification of the previous ATB Fungus system that consists of ready-to-use strips including a growth control, amphotericin B, 5-FC, fluconazole, and itraconazole for MIC determination. The ATBF2 strip is composed of 32 wells including 5-FC at concentrations from 0.5 to 64 mg/L, amphotericin B from 0.5 to 16 mg/L, fluconazole from 0.25 to 128 mg/L, and itraconazole from 0.125 to 4 mg/L. Following incubation at 35°C, MIC values were determined either visually or automatically with the mini-API instrument (bioMérieux). No, or slight, reduction in turbidity (scores 4 and 3) were considered as no inhibition while prominent decrease to no growth (scores 2–0) was considered inhibition for the azoles and 5-FC. For amphotericin B, only optically clear samples were determined as inhibition. For *Candida* species, incubation was 24–48 h, and for *C. neoformans*, incubation was 48 h. For visually read ATBF2 strips there was 100% agreement with M27-A2 for amphotericin B, 100% agreement for flucytosine, 97% agreement for fluconazole, and 98% agreement for itraconazole. For automatically read ATBF2 strips there was 97% agreement for amphotericin B, 97% agreement for flucytosine, 93% agreement for fluconazole, and 89% agreement for itraconazole. This compares to Sensititre YeastOne values of 85% agreement for amphotericin B, 90% agreement for flucytosine, 95% agreement for fluconazole, and 95% agreement for itraconazole, relative to the reference M27-A2 method. The authors concluded that the ATBF2 strip is a simple, objective, and accurate method for determining MICs for the applicable antifungals.

A 2008 study [81] compared ATBF2 to the Sensititre YeastOne system for determining antifungal susceptibility in a panel of 133 *Candida* strains (60 *C. albicans*, 18 *C. dubliniensis*, 29 *C. glabrata*, and 26 *C. krusei*). The panel consisted of eight reference strains and 125 clinical isolates. Susceptible, susceptible dose dependent, intermediate, and resistant isolates were included in the panel. Categorical agreement between the ATBF2 and Sensititre YeastOne systems was 93.2–98.5% for 5-FC and amphotericin B but only 72.9–75.9% for fluconazole and itraconazole. The ATBF2 system failed to identify three of the 12 itraconazole and four of the 21 fluconazole resistant isolates, relative to the Sensititre YeastOne system.

At the time of this writing, the updated commercially available ATB Fungus 3 assay has not received US FDA approval but has received the CE mark for European distribution. According to the manufacturer (bioMérieux website), this assay provides concordant results with those obtained using the CLSI (97.5%) and EUCAST (94.3%) reference techniques. Additional testing of ATBF3 relative to the CLSI or EUCAST reference methods would be beneficial to further evaluate this system.

13.5.8 MALDI-ToF/MS

Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-ToF/MS) is an emerging technology that has demonstrated proficiency in the identification of a wide variety of bacteria and fungi by generating species or strain-specific protein profiles and comparing them to reference spectra in a database. Currently, there are two commercially available instruments, the Bruker Biotyper (Bruker Daltonics, Bremen, Germany) and the Vitek MS (bioMérieux, Marcy-l'Etoile, France) for the identification of clinical isolates [39]. Under certain conditions, MALDI-ToF/MS can be utilized to test drug susceptibility of pathogens. In 2009, Marinach et al. [185] conducted a study based on the hypothesis that the protein composition of *C. albicans* would change in a reproducible manner in response to exposure to varying concentrations of antifungal agents. As a proof of concept, mass spectra were obtained using a P3S platform for 16 previously characterized *C. albicans* strains and a reference strain subjected to fluconazole concentrations ranging from 0.125 to 128 µg/mL. Eight strains were known to have low MICs using BMD ranging from 0.125 to 8 µg/mL, while the remaining strains exhibited high MICs ranging from 16 to 128 µg/mL. Yeast pellets were obtained from cultures grown for 15 h, subjected to acid extraction, and supernatants were analyzed by MALDI-ToF/MS. The endpoint to determine susceptibility for an isolate was defined as the lowest drug concentration where a mass spectrum profile change was detected, resulting in determination of a minimal profile change concentration (MPCC) value. Statistical analysis

is required to determine where a given reference spectra corresponding to a particular antifungal concentration would be plotted on a range of observed spectra for a single isolate at fluconazole concentrations ranging from 0 to 128 µg/mL. Comparison of MPCC values of the 17 *C. albicans* strains with the BMD MIC values showed full agreement for between 94 and 100% of the strains, depending on whether concordance was scored within ± 1 or ± 2 drug dilution values. The single discrepancy concerned a strain classified as susceptible by MALDI-ToF/MS and susceptible dose-dependent by BMD. Results were independent of drug resistance mechanism (ERG11 mutations, TAC mutation, CDR1/2, or MDR hyperexpression) or particular clade of the isolates.

A 2012 study [64] utilized MALDI-ToF/MS for caspofungin susceptibility testing of *Candida* and *Aspergillus* species using a Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany) and with the MALDI Biotyper 3.0 software. A panel of 44 fungal isolates was tested including *C. albicans* (14), *C. glabrata* (12), *C. parapsilosis* (4), *C. krusei* (4), *A. fumigatus* (6), and *A. flavus* (4) isolates in addition to the *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 reference strains. Spectra were collected from three biological replicates of each isolate at 14 drug concentrations ranging from 0.008 to 64 µg/mL caspofungin as well as in the absence of drug. Raw spectra were divided into intervals, automatically viewed in a correlation matrix view, and translated into a heat map. This allowed determination of MPCC values for isolates exposed to caspofungin. The MPCC values were compared to reference BMD MIC values using CLSI species-specific clinical breakpoints for *Candida* isolates for caspofungin susceptibility to determine categorical agreement using the two methods. There was 100% essential agreement (within two drug dilutions) between the MPCC and MIC values for the *Candida* and *Aspergillus* isolates. In addition, there was 94.1% categorical agreement for the *Candida* isolates with misclassification (minor errors) of two *C. krusei* isolates as intermediate. Although there are no available clinical breakpoints for *Aspergillus* species susceptibility, good correlation was observed between MPCC and MIC values. Thus, MALDI-ToF/MS is a promising technology for the determination of antifungal susceptibility based on analysis of isolate-specific protein reference spectra. Further studies are required to determine the utility, practicality, and reliability of MALDI-ToF/MS in antifungal susceptibility testing.

13.5.9 Miscellaneous susceptibility methods

In a 1993 study, the Mycototal, Mycostandard, Candifast, ATB Fungus, and Difftest systems were compared for reproducibility and repeatability, as well as for the concordance of

results with MICs. A total of 630 yeast–antifungal agent results for Mycototal and Mycostandard, 540 for Candifast and 450 for ATB Fungus and Difftest, were obtained with a repeatability of > 95% for all kits. Reproducibility was > 95% for all tests except Candifast, for which it was 80.1%. The highest concordance with MICs was observed with the Mycototal, Mycostandard, and Difftest systems at 90.3, 90.2, and 80.9%, respectively. Concordance was more limited for the ATB Fungus and Candifast systems at 75.3–91.7 and 51.6%, respectively [71, 83, 91, 255].

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Mycobacteria

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14.1 Introduction

The practice of clinical mycobacteriology has changed significantly over the past several decades. In the United States, with the steady declining number of patients with tuberculosis, the workload on mycobacteria in most hospitals is declining. Larger better-equipped reference laboratories perform more mycobacteriology work. On the other hand, nontuberculous mycobacteria are assuming more significance due to better recognition and more opportunistic infections in the increasing immunocompromised population. Worldwide, however, tuberculosis remains one of the most common causes of fatal infections, exacerbated by the human immunodeficiency virus pandemic and emergence of drug-resistant *Mycobacterium tuberculosis* strains. Public and private enterprises are joining hands to tackle the global tuberculosis problems.

Better commercial supplies of various reagents, culturing systems, and molecular detection and identification methods have substantially improved the quality and timeliness of mycobacterial detection and identification. This chapter, built on its first edition, will summarize our current practice and methods. The methods or systems are described first, and when applicable, clinical evaluation studies are summarized.

14.2 Specimen processing

Most clinical specimens are nonsterile and may contain non-acid-fast bacteria that would out-grow any *Mycobacterium* spp. soon upon culture. Specimen processing is essential to remove or kill those contaminants and to concentrate mycobacteria so that the culture can be kept for several weeks for best recovery and detection of mycobacteria. Treatment with *N*-acetyl-L-cysteine (NALC), a mucolytic

agent, and with sodium hydroxide (NaOH), a lytic base, serves these purposes. Upon this chemical treatment, the specimen pellet is then resuspended in phosphate-buffered saline for culture inoculation. These reagents can be prepared readily, and many hospital laboratories do so. Commercially available reagents are convenient and also provide more consistent results within and between institutions. Included in these commercial manufacturers (but not limited to) are:

- 1 AlphaTec Systems (Vancouver, WA, www.alphatec.com). The reagents for digestion and decontamination include NAC-PAC, NAC-PAC Red, and NAC-PAC- EA₃. The buffers include neutralization buffer (XPR-plus) to neutralize 3% NaOH used in heavy processing and pellet resuspension buffer (NPC-67).
- 2 BD MycoPrep (BD Diagnostic Systems, Sparks, MD, www.bd.com). This specimen digestion/decontamination kit consists of ten 75 mL bottles of NALC-NaOH solution and five packages of phosphate buffer (pH 6.8).
- 3 Remel Microbiology Products (Lenexa, KS, www.remel.com). The digestants include TB Base Digestant (sodium hydroxide/sodium citrate) and TB Digestant (4% sodium hydroxide).

14.3 Acid-fast staining reagents

Direct microscopic examination to identify mycobacteria in a specimen after proper staining is common and useful in mycobacteriology. This is particularly important in resource-poor countries where culture may be difficult to carry out. Acid-fast staining may use carbol fuchsin-based methods, such as Kinyoun and Ziehl-Neelsen, and fluorochrome-based methods, such as auramine alone or auramine and rhodamine (Truant stain). These reagents can be

prepared in-house; a number of commercial sources are also available (but not limited to), such as:

- 1 AlphaTec Systems. Common TB staining reagents are available along with TB processing reagents. Positive and negative staining control slides are also available.
- 2 BD Diagnostic Systems. These are Difco/BBL brand TB staining reagents. They are available in individual reagents and in kits. The kits include TB Stain Kit K (carbol fuchsin KF, decolorizer, and brilliant green), TB Stain Kit ZN (carbol fuchsin ZN, decolorizer, and methylene blue), TB Fluorescent Stain Kit M (auramine M, decolorizer TM, and potassium permanganate), TB Fluorescent Stain Kit T (auramine-rhodamine T, decolorizer TM, and potassium permanganate).
- 3 Remel Microbiology Products. All individual TB staining reagents are available. Two kits are TB Auramine O Stain Kit and TB Kinyoun AFB Stain Kit.
- 4 Aerospray TB Stainer (Wescor Biomedical Systems, Logan, UT, www.wescor.com; also PREVI Color TB in association with bioMérieux). This is an automated nondip-type stainer. The primary stain can be carbol fuchsin or fluorochrome. Excess primary stain is then spun off and the slide is rinsed with water to avoid precipitate formation. Decolorizer and counterstain are then applied stepwise. Finally, the slide is air dried. This automated stainer provides speed, economy, consistent performance, and no cross-contamination. Figure 14.1 shows an Aerospray TB Stainer.

Other methods, reagents, and instruments may also be available (such as the RAL Stainer, RAL Diagnostics, Montesquieu, Martillac, France; and QS-AFB, GG&B Co., Wichita Falls, TX). The user should investigate current commercially available products in his/her locale and consult company web sites and current literature.

14.4 Direct detection of mycobacteria from clinical specimens

14.4.1 GeneXpert MTB/RIF Test

The Xpert MTB/RIF system (Cepheid, Sunnyvale, CA, www.cephid.com) was developed several years ago. It combines detection of *M. tuberculosis* (MTB) and resistance to rifampin (RIF) in a single assay using hemi-nested real-time polymerase chain reactions (PCR) to amplify an MTB-specific sequence of the *rpoB* gene, which is probed with molecular beacons for mutations within the RIF-resistance determining region. The system integrates sample processing and PCR in a disposable plastic cartridge containing all reagents required for bacterial lysis, nucleic acid extraction, amplification, and amplicon detection. The only manual step is the addition of a bactericidal buffer to the sputum specimen before transferring to the cartridge. The MTB/RIF cartridge is then inserted into the GeneXpert device; the run completes within 2 h.



Figure 14.1 Aerospray TB Stainer. (Courtesy of ELITech Group, Inc.).

The cartridge is a closed system to avoid amplicon contamination. The system requires minimal technical experience and training and suits developing countries where the tuberculosis burden is particularly heavy.

The World Health Organization has adopted the Xpert MTB/RIF for implementation in resource-poor countries in campaigns against tuberculosis since 2010 [65]. As of March 31, 2013, a total of 1123 GeneXpert instruments (comprising of 6181 modules) and 2.3 million testing cartridges had been procured in 83 countries [65]. Xpert MTB/RIF received FDA clearance recently.

14.4.2 AMPLIFIED MTD2 Test

The AMPLIFIED MTD2 (*M. tuberculosis* Direct) Test (Hologic Gen-Probe Inc., San Diego, CA, www.gen-probe.com) detects MTB rRNA directly and rapidly from acid-fast smear-positive and negative specimens. The MTD test is an isothermal transcription-mediated amplification (TMA) method based on specific mycobacterial rRNA targets using DNA intermediates. The RNA amplicons produced are identified by hybridization protection assay using an acridinium ester-labeled MTB complex-specific DNA probe. The test is specific for the MTB complex. It is FDA-approved. The MTD test is intended for use with specimens from patients that are showing signs and symptoms consistent with active pulmonary tuberculosis. The test is used in conjunction with routine acid-fast bacillus smear and culture. The test has a turn-around time of 2.5–3.5 h.

14.4.3 COBAS TaqMan MTB Test

The COBAS TaqMan MTB Test (Roche Diagnostic Corp, Indianapolis, IN, www.roche.com) uses real-time PCR nucleic acid amplification and hydrolysis probes for the detection of MTB complex DNA in liquefied, decontaminated and concentrated human respiratory specimens, including expectorated and induced sputum and bronchial alveolar lavages (BAL). The test utilizes the AMPLICOR Respiratory Specimen Preparation Kit for

Table 14.1 Clinical evaluation of GeneXpert for detection of *M. tuberculosis* (MTB) and rifampin (RIF) resistance or susceptibility in comparison with culture methods

Study location/type and year	Number of culture samples and type*	Xpert detection of MTB		
		Sensitivity†	Specificity†	RIF‡
Peru, Azerbaijan, South Africa, India (2010) [7]	561 smear (+) sputa 171 smear (–) sputa	551 (98.2) 124 (72.5)	604 of 609	200 of 205 (97.6) RIF-resistant 504 of 514 (98.1) RIF-susceptible
Peru, Azerbaijan, South Africa, India, Philippines, Uganda (2011) [8]	648 smear (+) sputa 385 smear (–) sputa	637 (98.3) 296 (76.9)	2846 of 2876	236 of 250 (94.4) RIF-resistant 796 of 810 (98.3) RIF-susceptible
Spain (2011) [38]	85, most pulmonary	64 (75)	40 of 40	6 of 6
Germany (2011) [27]	45 extrapulmonary	35 (77.8)	425 of 433	No data
Turkey (2011) [66]	27 smear (+) pulmonary 35 smear (–) pulmonary 48 extrapulmonary	27 (100) 24 (69) 25 (52.1)	319 of 319	1 of 1 RIF-resistant 88 of 88 RIF-susceptible
Meta-analysis of 18 studies (2012) [12]	10,224 suspected TB specimens	90.4%	98.4%	94.1% RIF-resistant, 97.0% RIF-susceptible

+, positive; –, negative.

* Extrapulmonary cases did not have smear data.

† Specificity in a study was for all culture-negative specimens.

‡ Percent in parentheses.

manual specimen preparation and the COBAS TaqMan 48 Analyzer for automated amplification and detection. Each run can test 48 samples. This test is currently not available in the United States at the time of this writing.

14.4.4 LCx *Mycobacterium tuberculosis* assay

The LCx *Mycobacterium tuberculosis* assay (Abbott Diagnostics Division, Abbott Park, Ill., www.Abbott.com) uses the ligase chain reaction for the amplification of a segment of a gene in the MTB complex. The LCx assay was designed for use with processed respiratory specimens.

14.4.5 Clinical evaluation of direct tuberculosis tests

Many studies have been carried out to assess the performance of these direct test systems, particularly the Xpert MTB/RIF in recent years (Table 14.1). Large-scale multinational studies have shown that the Xpert MTB/RIF system provides sensitive and specific detection of tuberculosis and RIF resistance directly from untreated sputum in less than 2 h with minimal hands-on time. Overall, when compared with cultures (liquid and solid media), it performs best in smear-positive pulmonary TB (>90% sensitivity) and modest in smear-negative pulmonary TB and extrapulmonary TB (>70% sensitivity). It also shortens detection time to 1 day instead of weeks. In particular, it shortens initiation of treatment for RIF-resistant MTB from nearly 2 months to a single day, which will likely help to contain further dissemination of the resistant MTB strains.

Selected large-scale evaluations of the MTD2, LCx, and AMPLICOR systems are listed in Table 14.2. These studies show that these rapid tests perform well overall,

particularly for the smear-positive pulmonary specimens. One study recommends that, for smear-negative specimens, three consecutive specimens be tested to improve the test yield with AMPLICOR [17].

A novel rapid test, available in some markets, to detect the LAM antigen (lipoarabinomannan) in urine samples is the Alere Determine TB LAM Ag test (Alere, Waltham, MA). Please consult the vendor for additional information and performance characteristics.

14.5 Blood-culture recovery of mycobacteria

Regular blood cultures may also recover mycobacteria in the blood circulation [16], particularly rapidly growing mycobacteria (RGM), which can be cultivated after an incubation period of 3–5 days, similar to other fastidious organisms [24]. A Gram stain shows beaded Gram-positive or variable rods, and a positive acid-fast stain should be confirmative. The Isolator 10 system (Wampole Laboratories, Princeton, NJ) is particularly useful for recovering RGM because the intracellular mycobacteria are released from the lysed leukocytes and concentrated in the centrifuged sediment, and the culture plates support better growth of mycobacteria. Colony counts are also available from the plates. Other commercially available automated continuously monitoring culture systems (CMCS), such as BACTEC 9240, BacT/Alert 3D, and VersaTREK, can be used to recover rapid growers.

Blood-culture recovery of slowly growing mycobacteria commonly takes an incubation period of 2–3 weeks using the CMCS with appropriate media [11,26,39]. Some strains, however, may take up to 4–6 weeks. *Mycobacterium avium*

Table 14.2 Clinical evaluations of MTD2 and AMPLICOR tests in comparison to culture methods

Study location and year	Number of specimens (isolates)	Test	Sensitivity (%)	Specificity (%)	Note
Italy (2000) [48]	486 (167), pulmonary and extrapulmonary	MTD2	85.7 for pulmonary 82.9 for extrapulmonary	100 for both	Both tests comparable, culture to confirm
		AMPLICOR	94.2 for pulmonary 85 for extra-pulmonary	100 for both	
Singapore (1999) [60]	230 (72), all pulmonary	MTD2	98.6	99.4	Three tests comparable
		AMPLICOR	96.1	100	
		LCx	100	99.3	
Spain (1998) [19]	682 (163)	MTD2	94.7 for pulmonary 86.8 for extrapulmonary	100 for both	MTD2 useful
Denmark, Switzerland, Germany, UK (1996) [5]	7194 (654)	AMPLICOR	91.4 for smear (+) 60.9 for smear (–)	96.1	Enabling rapid diagnosis of MTB
France (six centers) (1995) [11]	2073 (184)	AMPLICOR	94.5 for smear (+) 74 for smear (–) 83 for extrapulmonary	98	Rapid and easy to perform
Germany (1998) [17]	1681 (65)	AMPLICOR	96.4 for smear (+) 45.5 for smear (–)	99.7	Smear (+) samples preferred
Australia (1999) [32]	2347 (152)	LCx	98.5 for smear (+), 41.5 for smear (–)	99.9	Smear (+) samples preferred
Italy (1999) [57]	697 (128)	AMPLICOR	78	Not stated	Comparable two tests
		LCx	88		

and *M. tuberculosis* are the two most commonly recovered mycobacteria from patients with AIDS/HIV infection [2,28]. *Mycobacterium avium* bacteremia in AIDS/HIV used to be common in the United States [28], but is considerably less common now with better anti-HIV treatment. *Mycobacterium tuberculosis* bacteremia in AIDS/HIV is common in developing countries and carries a high and quick mortality [2,3,23,39].

14.6 Mycobacteria-culturing methods and systems

Traditional culture methods for mycobacteria mainly use Lowenstein–Jensen agar slants or bottles and Middlebrook 7H9, 7H10, and 7H11 agar plates or liquid. Many manufacturers supply them commercially. These culture media are simple, cheap, low technology, and easy to set up. They are particularly useful in resource-poor settings. The disadvantages are that they are labor-intensive and relatively less sensitive to recover *M. tuberculosis*. Many studies have consistently demonstrated that Lowenstein–Jensen agar has a sensitivity of 70–80%. Therefore, three specimens on different days are recommended to be cultured to reach a positive probability near 99%, the reciprocal probability of the product of three negative probabilities $[1 - (1 - 0.75) \times (1 - 0.75 \times (1 - 0.75))]$.

14.6.1 BD Septi-Chek AFB System

The manual BD Septi-Chek AFB System (www.bd.com) system has been available since the early 1990s; it consists of a bottle with 20 mL culture media (7H9 broth) under an atmosphere of CO₂. The interior has a paddle containing three different media within a plastic tube, including

Lowenstein–Jensen, Middlebrook 7H11, and chocolate agars. In general, nonacid-fast bacterial contaminants will grow on the chocolate agar whereas the mycobacteria grow on the remaining agars. The Septi-Chek AFB System performs reasonably well in comparison to other manual and automated systems (please check the availability of this product in your region or country, since, according to the manufacturer, this product line has been discontinued).

In the past two decades, the culture methods for mycobacteria have evolved to combine the traditional tube methods and newer liquid medium automated system in the United States. Several liquid automated systems are available; they are briefly described first, followed by comparative studies on their clinical performance.

14.6.2 BACTEC 460TB System

The BACTEC 460TB System (BD Microbiology Systems, www.bd.com) is the first automated system for mycobacteria culturing and testing. The system uses radiometric technology to sensitively detect the growth of mycobacteria in media. The system can be used with either 12B medium supplemented with PANTA (an antimicrobial agent solution) or 13A medium for whole blood. Due to the use of radioactive isotopes and the issue of its disposal, and the advent of comparable nonradiometric systems, this system is infrequently used now in most healthcare settings.

14.6.3 BACTEC MGIT 960 System

The Mycobacterial Growth Indicator Tube (MGIT) 960 (Figure 14.2; BD Microbiology Systems, www.bd.com) is a fully automated system for high-volume mycobacteria growth, detection, and susceptibility testing. It offers a



Figure 14.2 The Mycobacterial Growth Indicator Tube (MGIT) 960 System. (Courtesy and © Becton, Dickinson and Company.)

capacity of 960 tubes for culture, safe operation, no radioactivity, and fast results. The system allows processing up to 8000 specimens per year, which meets the need of a medium to large hospital in the United States. The detection sensor uses fluorometric technology to detect oxygen consumption by the proliferating mycobacteria every 60 min to signal the positives as they occur. The EpiCenter Data Management System (an optional component) performs statistical analyses and accommodates different customer preferences. It also provides simple specimen tracking along with a flexible and dynamic reporting system for more in-depth data management needs. Depending on the hospital size, a smaller capacity MGIT 320 System is also available.

14.6.4 BacT/Alert 3D System

The BacT/Alert 3D System (bioMérieux, Durham, NC, www.biomerieux-usa.com), also known as the MB/BacT System, can be used for regular blood cultures and mycobacteria cultures, depending on selection of culture media. The system uses a colorimetric method to detect CO₂ production by bacterial growth periodically (10 min for blood cultures and 60 min for mycobacteria). The mycobacteria culture medium uses an MP bottle with 10 mL supplemented Middlebrook 7H9 broth and an intended inoculum of 0.5 mL processed sample. A supplemental solution kit (0.5 mL) is also added, consisting of nutrients (oleic acid, glycerol, bovine serum albumin, and amaranth in water) to boost recovery of mycobacteria and antibiotics (amphotericin



Figure 14.3 BacT/Alert 3D MB/BacT System. (Courtesy of bioMérieux.)



Figure 14.4 Thermo Scientific™ VersaTREK™ Automated Microbial Detection System. (Photo courtesy of ThermoFisher Scientific, copying is prohibited.)

B, azlocillin, nalidixic acid, polymyxin B, trimethoprim, and vancomycin) to inhibit nonacid-fast contaminants. Figure 14.3 illustrates a BacT/Alert 3D System.

14.6.5 VersaTREK System

The VersaTREK System (Trek Diagnostic System, ThermoFisher, Cleveland, OH, www.trekds.com) system (Figure 14.4), previously known as the ESP System, performs regular blood cultures as well as mycobacteria culture and susceptibility test, depending on the culture media used. The mycobacteria culture combines a liquid culture medium (VersaTREK Myco Broth), a growth supplement (Myco GS), and, for potential contaminants, an antibiotic supplement (Myco AS or Myco PVNA).

Table 14.3 Selected large-scale clinical studies of mycobacteria culture methods and systems

Study location and year	Number of specimens (isolates)	System	Recovery		Contamination (%)
			Rate (%)	Mean days	
Spain (2000) [1]	1068 (120)	BACTEC 460	80	11.8	Not reported
		MB/BacT	83.3	12.6	
		MGIT	82.5	15.9	
		LJ agar	70	22.2	
USA (multicenter) (1999) [25]	3330 (362)	BACTEC 460	75	15.2	4.9
		MGIT	80	14.4	8.1
		LJ-7H10 agar	69	24.1	Not reported
Spain (1998) [33]	600 (106)	BACTEC 460	90.2	11.7	1.8
		MB/BacT	94.3	14.2	5.0
		LJ + Coletsos	92.5	26.1	1.5
Switzerland (1997) [45]	1078 (73)	BACTEC 460	91.8	14.3	2.9
		MB/BacT	86.3	17.5	4.3
		Two agars	79.5	24.2	Not reported
Italy (1999) [9]	1830 (173)	BACTEC 460	99.4	11.2	Not reported
		MB/BacT	96.5	11.7	
		LJ agar	95.9	26.8	
Italy (multicenter) (1998) [53]	2673 (219)	BACTEC 460	89	18.0	4.0
		VersaTREK	79	18.0	7.9
		LJ agar	64	28	8.9
USA (1997) [64]	2,283 (149)	BACTEC 460	81	14.4	4.0
		VersaTREK	87	13.1	8.6
		7H11 agar	65	17.8	0.8
USA (2000) [61]*	3,151 (231)	MGIT	63.9	13.1	17.1, overall
		VersaTREK	71.2	16.9	18.9, overall
		7H11 agar	61.8	19.2	11
		MGIT + 7H11	81.5		
		VersaTREK+7H11	84.1		
Total or summary	16,013 (1433)	BACTEC 460	75–99.4 (89)*	11.2–18 (14)*	1.8–4.9 (3)*
		MGIT	63.9–82.5 (80)	13.1–15.9 (14)	8.1–17.1 (11)
		MB/BacT	83.3–96.5 (85)	11.7–17.5 (14)	4.3–5 (5)
		VersaTREK	71.2–87 (79)	13.1–18 (16)	7.9–18.9 (11)
		Solid media	61.8–92.5 (69)	17.8–28 (23)	0.8–11 (5)

*Numbers in parenthesis represent median or mean values. Coletsos is a solid medium used in Europe.

*Please refer to original manuscript for complete description of contamination rates in this study.

The bottle also contains cellulose sponge material to provide greater surface area for optimal mycobacterial growth. The system detects mycobacterial growth by automatically monitoring (every 24 min) the rate of oxygen consumption within the headspace of the culture bottle. A positive growth is reported via a visible and audible signal.

14.6.6 Clinical evaluation of mycobacterial recovery systems

Many clinical studies, performed about 15 years ago when these mycobacterial recovery systems were rolled out, have assessed and compared their clinical performance. Table 14.3 summarizes results of several relatively large studies. Most studies used BACTEC 460TB as the standard system; the newer nonradiometric systems all performed at similar, acceptable levels. As a result, it is recommended now that an automated liquid culture system should be used together with a traditional solid medium to achieve best recovery of mycobacteria in a

clinical specimen [13]. In general, a Lowenstein–Jensen tube and a liquid system together can reach 90% culture sensitivity. Therefore, if a patient has two quality specimens on different days, the combined culture sensitivity should reach 99% [$1 - (1 - 0.90) \times (1 - 0.90)$].

14.7 Identification of mycobacteria

Identification of clinical isolates of mycobacteria has traditionally included acid-fast stains, growth rates, macroscopic examination of colony morphologies (e.g., flat or raised, irregular or entire, dry or smooth, chromogenic or buff, light exposure), utilization of substrates, and metabolic byproducts. This method requires considerable technical experience and is time-consuming. Several biochemical tests take a week or more to complete and may have to be repeated if results are equivocal. Therefore, traditional methods are labor-intensive and slow. Fortunately, the past two decades have seen the advent of many quick identification methods, which meet today's

need for evidence-based medical decisions, rising health-care cost, tight budget, and fast pace. These methods can offer identification upon culture positivity to obviate the need of subculture or colonies. Selected products will be mentioned in this chapter. However the reader is advised to consult other vendors who may offer similar products. Please refer to the Appendix of this book and company web sites and published information for additional product availability.

14.7.1 Biochemicals, reagents, and media

Several biochemical reagents are available from BD Diagnostic Systems (Sparks, MD, www.bd.com): Lowenstein–Jensen medium with 5% NaCl for salt tolerance; Lowenstein–Jensen medium deeps for semiquantitative catalase; Taxo TB niacin test strips for the detection of niacin production; Taxo Urea for rapid detection of urease production; Taxo X factor strips for optimal growth and isolation of *Mycobacterium haemophilum*; arylsulfatase broth.

Available media and reagents from Remel Microbiology Products (Lenexa, KS, www.remel.com) are: Middlebrook 7H9 Broth with polysorbate 80 (tellurite reduction medium), for differentiation of *Mycobacterium* spp. based on tellurite reduction (requiring potassium tellurite reagent); Lowenstein–Jensen Medium w/5% NaCl, for detection of sodium chloride tolerance; Niacin Reagent Strip, for detection of niacin; Nitrate Reagent A and B, for differentiation and identification of mycobacteria, used in conjunction with Nitrate Substrate Broth; Pyrazinamidase Agar, to determine pyrazinamidase activity; TCH (thiophen-2-carboxylic acid hydrazide) agar for 1 mcg and 5 mcg respectively, for TCH susceptibility for identification of *Mycobacterium bovis*, in biochemical differentiation with *M. tuberculosis*; Polysorbate 80 Hydrolysis Substrate Concentrate, to determine lipase production and the hydrolysis of Polysorbate 80; Urea Broth, to determine urea hydrolysis.

14.7.2 Nucleic acid probe from Gen-Probe

The AccuProbe Culture Identification Reagents system (Hologic Gen-Probe Inc., San Diego, CA, www.gen-probe.com) uses a single-stranded DNA probe with a chemiluminescent acridinium label to hybridize with the ribosomal RNA released from the target *Mycobacterium*. The DNA:RNA forms a stable double strand hybrid. A selection reagent differentiates nonhybridized from hybridized probe that is detected by a luminometer. The probe sequence defines the specificity of hybridization and individual kits identify each of the following: MTB complex, *M. avium-intracellulare* complex, *M. gordonae*, and *M. kansasii*. Using a species-specific probe for the *M. avium-intracellulare* complex, each species can be further probed.

A major advantage of the AccuProbe is its speed: it probes a positive culture directly, from liquid media or solid agar, and provides identification within the same day of culture positivity. Algorithms have been developed to use the probes in a cost-effective fashion [21,35,40]. Studies have evaluated the performance of these AccuProbes; in general, both the sensitivity and specificity are above 95% [21,31,56].

14.7.3 16S rRNA gene sequencing by MicroSEQ 500

This kit is the sequencing component of the MicroSEQ 500 16S rDNA Bacterial Identification System (Life Technologies, CA, www.invitrogen.com), which provides an easy-to-use DNA sequence-based method to identify most bacteria. This kit can be used to sequence PCR products that have been generated using the MicroSEQ 500 16S rDNA PCR Kit. The two kits together permit the quick and convenient amplification and sequencing of 500 base pairs of the 16S rRNA gene. Sequences can then be compared to the MicroSEQ 16S rDNA 500 Library to generate an identity match.

In the past two decades, sequencing the bacterial 16S rRNA gene has emerged as an excellent method to identify bacteria rapidly. Its application to mycobacterial identification is particularly useful, for speed (within a day or two), accuracy, and recognition of many novel *Mycobacterium* species in the past decade. The 16S rRNA gene of almost all (approximately 150) mycobacterial species has been sequenced and analyzed by various laboratories. This wealth of knowledge and the availability of these sequences through public and private databases, such as GenBank, RIDOM, and others, provide a solid basis for potentially wide clinical application. In addition to the commercial MicroSEQ 500, many in-house developed sequencing methods are in use in various laboratories.

Ideally, sequencing the entire 1.5 kbp (kilobase pairs) of the 16S rRNA gene provides maximum possible identification accuracy; however, its associated cost and requirement of high proficiency limits its use to large reference laboratories and/or academic purposes. Most clinical laboratories need efficient and cost-effective identification without significant compromise of accuracy. Thus, the MicroSEQ 500 system meets this purpose. Several studies have found that MicroSEQ 500 performed well [15,43,63].

14.7.4 Other identification methods

1 High performance liquid chromatography (HPLC): HPLC was developed and used by the Centers for Disease Control and Prevention decades ago before the advent of DNA sequencing method, and also used by many state health laboratories. This method identifies mycobacteria by profiling mycolic acids. This method is rapid from a positive culture.

2 Line Probe Assay (LiPA) (InnoGenetics Inc., Ghent, Belgium, www.Innogenetics.com): Inno-LiPA Mycobacteria v2 assay is a DNA probe test, based on reverse hybridization, for the detection and identification of 16 different mycobacterial species from culture. The species differentiation is based on nucleotide differences in the 16S–23S rRNA spacer region. This test is available in Europe, but not in the United States at the time of this writing. A few studies have evaluated and noted its acceptable performance in identifying commonly encountered *Mycobacterium* species [37,41,51,55].

14.8 Susceptibility tests for mycobacteria

Susceptibility tests for various mycobacteria are assuming more importance in view of the emergence and spread of drug-resistant *M. tuberculosis* strains and varying susceptibility of other slowly growing and rapidly growing mycobacteria. To this end, the US Clinical and Laboratory Standards Institute (CLSI) has published and updated standard procedures for antimycobacterial drug testing methods [14]. Thanks to recent technologic advancements [62], these tests, particularly those for the *M. tuberculosis* complex, can be performed with various formats, including culture-based methods and gene mutation analysis-based methods. CLSI recommends that all MTB complex strains should be tested when first isolated.

14.8.1 Traditional assays

Traditional antimycobacterial susceptibility tests generally refer to the agar proportion method established since the 1960s. The basis is that at least 1% of the population of cells of a susceptible isolate of *M. tuberculosis* will exhibit innate resistance to the primary antimycobacterial drugs used, including isoniazid, ethambutol, streptomycin, rifampin, and pyrazinamide. The breakpoint of $\geq 1\%$ is used to define resistance of an isolate cultured on Middlebrook 7H10 or 7H11 agar. The proportion method is considered the standard method for anti-MTB complex testing, to which all other testing methods are measured. However, this method is not rapid.

14.8.2 BBL Sensi-Disk

The BBL Sensi-Disk (www.bd.com) serves as a convenient method for addition of antimicrobial agents to culture media, especially for qualitative studies of mycobacteria and related organisms. The discs are added to measured amounts of liquefied agar medium, e.g., Middlebrook and Cohn 7H10 agar or 7H11 agar, to prepare recommended concentrations of the antimicrobial agent in solidified agar. A number of drugs at a variety of concentrations are available: ethambutol at 25 and 50 mcg/mL, ethionamide

Table 14.4 Susceptibility tests of primary anti-MTB complex drugs in FDA-cleared commercial broth systems

Drug	System and concentration (mcg/mL)		
	BACTEC 460TB	BACTEC MGIT 960	VersaTREK
Isoniazid	0.1	0.1	0.1
Rifampin	2.0	1.0	1.0
Ethambutol	2.5	5.0	5.0
Pyrazinamide	100	100	300

Source: Adapted from CLSI 2011 (reference 14).

at 25 mcg/mL, isoniazid 1 and 5 mcg/mL, *p*-aminosalicylic acid at 10 and 50 mcg/mL, RIF at 25 mcg/mL, and streptomycin at 50 mcg/mL. Disks may also be available from other vendors. Please refer to other chapters and to the Appendix of this book for additional vendors and their web sites and products.

14.8.3 Automated broth culturing and testing systems

Three systems, i.e., BACTEC 460TB, BACTEC MGIT 960, and VersaTREK, perform automated culturing as well as anti-tuberculosis susceptibility tests (AST). These systems are FDA-cleared for AST. The main advantages of these systems are: shortening turnaround time substantially by a few weeks, i.e., at the time of culture positivity if AST is set up at the same time of culture (usually smear-positive specimens) or 7 to 14 days from an isolate (indirect method), less labor-intensive, and less subjective in result interpretation. Table 14.4 shows usual drugs and concentrations in these ASTs.

The BACTEC MGIT 960 SIRE (streptomycin, isoniazid, RIF, and ethambutol) and PZA (pyrazinamide) susceptibility test for the MTB complex features automatic interpretation for susceptibility or resistance to eliminate subjectivity. This instrument automatically directs the placement of the AST Set within the instrument, and AST results are available, on average, in 7–8 days, which is comparable to the BACTEC 460TB System. Tubes incubate and are read without the need of any further action by the technologist. Susceptible or resistant results are produced when the instrument compares the drug-containing tube to the growth control tube.

The VersaTREK system was the first automated system cleared by the FDA for the MTB AST, for use with continuous monitoring systems. The testing kits include RIF, isoniazid, ethambutol, PZA, and streptomycin.

Several studies from settings of different countries have evaluated the performance of the AST of BACTEC MGIT 960 in comparison with either BACTEC 460TB or the method of proportions; they all found comparable results [6,20,42,47,52,59]. Similarly, studies have also found good AST results from the VersaTREK system [18,30,46].

Currently, the BACTEC MGIT 960 and VersaTREK systems are commonly used in industrialized countries for MTB AST.

14.8.4 Susceptibility tests for rapidly growing mycobacteria

Anti-rapidly growing mycobacteria (RGM) susceptibility tests use a broth microdilution method as the standard. CLSI has established interpretation breakpoints for 12 drugs [14]. There are no particular commercial systems yet for these RGM tests.

14.8.5 Susceptibility by Etest

The Etest (bioMérieux Inc., Durham, NC, www.biomerieux-diagnostics.com) is a predefined, stable gradient of approximately 15 antibiotic concentrations on a plastic strip. The Etest is used to determine the on-scale minimum inhibitory concentration of antimicrobials against non-acid-fast bacteria, fungi, and mycobacteria. Several studies (cited in the first edition) have evaluated its utility against mycobacteria.

14.8.6 Gene-sequence-based drug resistance tests

In addition to the GeneXpert MTB/RIF test, as discussed above, other gene-sequence-based methods are also available. These methods offer speedy detection of multidrug resistant MTB from a few weeks to 1 or 2 days.

14.8.6.1 GenoType MTBDR_{plus}

The GenoType MTBDR_{plus} (Hain Lifescience GmbH, Nehren, Germany, www.hain-lifescienc.de) method analyzes three genes responsible for the multidrug resistance of MTB: mutations of the *rpoB* gene (coding for the β -subunit of the RNA polymerase) for RIF resistance, the *katG* gene (coding for the catalase peroxidase) for the high level isoniazid resistance, and the promoter region of the *inhA* gene (coding for the NADH enoyl ACP reductase) for low-level isoniazid resistance. Two studies, one from South Africa and another from Russia, have shown usefulness of this assay [4,36].

14.8.6.2 TB-Biochip

The TB-Biochip (Engelhardt Institute of Molecular Biology, Russian Academy of Science, Moscow, Russia, www.biochip.ru) detects resistance to both RIF and isoniazid. The method is based on PCR amplifications of gene targets in the MTB strain and hybridization of the amplicons with fluorescence-labeled probes on a chip. The resistance or susceptibility is determined by fluorescence patterns. The analysis requires less than 1 day, a speed of importance in countries with high prevalence of resistant MTB strains. In clinical evaluations [10,29], the TB-BIOCHIP detected over 95% of MTB strains resistant

to RIF and above 80% of those resistant to isoniazid. These results are comparable with the GenXpert direct test and BACTEC MGIT 960 broth AST.

14.8.6.3 Inno-LiPA Rif.TB

The Inno-LiPA Rif.TB (www.innogenetics.com) is a line probe assay for the detection of the MTB complex and its resistance to RIF directly from clinical specimens. Studies have found reasonable sensitivity (82–94%) in the detection of MTB, particularly from smear-positive specimens, and high sensitivity and specificity (both > 90%) in the detection of RIF resistance [36,49,54,58]. The method has been adapted in a few MTB prone countries [44,49,50].

14.9 Immunodiagnosis of tuberculosis

Nearly all cases of tuberculosis represent reactivation of latent primary *M. tuberculosis* infection. This raises the need of diagnosing latent MTB infection. Traditionally, this is achieved through a tuberculin skin test. But this test has innate issues, such as low sensitivity, anergy due to patient's underlying conditions, cross-reaction to BCG vaccine, and others. Two relatively recent commercial immunodiagnostic tests are available now: the QuantiFERON Gold In-Tube (QFNG-IT; Cellestis, Chadstone, Victoria, Australia, www.cellestis.com) and the T-SPOT.TB (Oxford Immunotec, Oxford, UK, www.tspot.com). These tests are based on interferon-gamma release from lymphocytes upon *in vitro* exposure to MTB-specific antigens (ESAT-6, CFP-10, and TB-7.7). The assay format is enzyme-linked immunosorbent assay. Many recent studies have reported evaluations of these tests in various target populations, mainly in industrialized countries with low rates of latent MTB infection. Overall, they offer relatively more specificity than the tuberculin skin test, but similar sensitivity. The Centers for Disease Control and Prevention has established guidelines for proper use of these tests [34]. A recent publication also provided updates [22].

14.10 Conclusion

Tuberculosis remains one of the major threats to human health. This requires global joint efforts from public, private, and industry sectors to tackle the problem. Adequate detection and treatment are the first steps towards long-term and sustainable control of this disease.

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Diagnostic Medical Parasitology

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With the increase in world travel and access to many different populations and geographic areas, it is very likely that we will see more tropical diseases and infections due to the rapidity with which people and organisms can be transmitted from one place to another. The transportation of infectious agents, as well as human travelers, has been clearly demonstrated during the past few years, particularly via air travel. Travel has become available and more affordable for many people throughout the world, including those who are in some way compromised in terms of their overall health status. With the increase in the number of patients whose immune systems are compromised, through underlying illness, chemotherapy, transplantation, AIDS, or age, we are much more likely to see increasing numbers of opportunistic infections, including those caused by parasites. Also, we continue to discover and document organisms that were thought to be nonpathogenic that, when found in the compromised host, can cause serious disease [8,20,21].

Control of parasitic infections depends on a number of different factors, including geographic location, public health infrastructure, political stability, available funding, social and behavioral customs and beliefs, trained laboratory personnel, healthcare support teams, environmental constraints, poor understanding of organism life cycles, and opportunities for control and overall commitment. Vectors and other carriers of infectious agents do not recognize political or control boundaries, both of which become meaningless. When newer infectious agents and/or diseases are recognized, there is often very little information available regarding the organism life cycle, potential reservoir hosts, and environmental requirements for survival. Priorities may change, and in areas of the world where disease epidemiology was considered important in the past, these important issues may have

been moved lower on the priority list. Unfortunately, funding often plays a major role in decisions that impact disease control measures.

As new etiologic agents are discovered and the need for new therapeutics increases, more sensitive and specific diagnostic methods will become mandatory in order to assess the efficacy of newer drugs and alternative therapies.

15.1 Diagnostic parasitology testing

15.1.1 General comments

Diagnostic procedures in the field of medical parasitology require a great deal of judgment and interpretation and are generally classified by the Clinical Laboratory Improvement Amendments of 1988 (CLIA '88) as high-complexity procedures.

Very few procedures can be automated, and organism identification relies on morphologic characteristics that can be very difficult to differentiate. Although morphology can be learned at the microscope, the amount of training to reach proficiency, and eventually mastery, is extensive. Knowledge about the life cycle, epidemiology, infectivity, geographic range, clinical symptoms, range of illness, disease presentation depending on immune status, and recommended therapy are critical to the operation of any laboratory providing diagnostic services in medical parasitology. As laboratories continue to downsize and reduce staff, cross-training will become more common and critical to financial success. Maintaining expertise in fields such as diagnostic parasitology will become more difficult, particularly when standard manual methods are used. Also, the lower the positive rate for parasitic infections (i.e., the prevalence of disease), the more likely we are to generate both false-positive and false-negative laboratory reports. It is difficult to maintain proficiency when

positive specimens are rarely encountered. It is important for members of the healthcare team to thoroughly recognize those areas of the clinical laboratory that require experienced personnel and why various procedures are recommended above others. Healthcare delivery settings where physicians provide parasitology diagnostic testing may occasionally provide simple test results (CLIA '88 waived tests) based on wet mount examinations. However, in spite of the CLIA classification of these diagnostic methods, wet mount examinations are often very difficult to perform and are not as thorough as a full examination, which includes a permanent stained smear. The key to performance of diagnostic medical parasitology procedures is formal training and experience. As the laboratory setting changes during the early part of the 21st century, it is important to recognize that these changes will continue to require a thorough understanding of the skills required to perform diagnostic parasitology procedures and the pros and cons of available diagnostic methods. Laboratories will continue to have a number of diagnostic options: whatever approach is selected by an individual laboratory, it is important that the clinical relevance of the approach be thoroughly understood and conveyed to the client user of the laboratory services.

The majority of diagnostic parasitology procedures can be performed either within the hospital setting or in an offsite location. There are very few procedures within this discipline that must be performed and reported on a short turnaround time (STAT) basis. Two procedures fall into the STAT category: request for examination of blood films for the diagnosis of malaria and examination of cerebrospinal fluid for the presence of free-living amebae, primarily *Naegleria fowleri*. Any laboratory providing diagnostic parasitology procedures must be prepared to examine these specimens on a STAT basis 7 days a week, 24 h a day [20,28,45]. Unfortunately, these two procedures can be very difficult to perform and interpret; cross-trained individuals with little microbiology training or experience will find this work difficult and subject to error. It has been well documented that automated hematology instrumentation lacks the sensitivity to detect malaria infections, particularly since most patients seen in an emergency room have a very low parasitemia [28]. However, even a low parasitemia can be life threatening in an infection with *Plasmodium falciparum* or *Plasmodium knowlesi*.

Diagnostic laboratories outside of the hospital setting are also appropriate settings for this type of diagnostic testing; the test requests, for the most part, are routine and are batch tested, rather than tested singly. With very few exceptions, STAT requests are not relevant and/or sent to such laboratory locations and do not require immediate testing and reporting. Point-of-care testing within the hospital (ward laboratories, intensive care

units, emergency rooms, and bedside) is usually not considered appropriate sites for diagnostic parasitology testing; one exception might be the emergency room, where patients with malaria may first present with fever and general malaise. Alternative testing sites (outpatient clinics, shopping malls, senior citizen groups, and others) are generally not considered appropriate settings for diagnostic parasitology testing, although relevance might be dictated by geographic location, particularly outside of the United States, and by the development of newer, less subjective methods. The majority of physician office laboratories are not involved in diagnostic parasitology testing; however, as more molecular (nonmicroscopic) methods are developed, they may become more widely used in this setting.

15.1.2 Specific options

15.1.2.1 Diagnostic tests

The selection and use of diagnostic procedures depend on a number of factors, including geographic area, population served, overall positivity rate, client preference, number of test orders, staffing, personnel experience, turnaround time requirements, epidemiology considerations, clinical relevance of test results, and cost. Diagnostic tests generally have a wide range in sensitivity and specificity. As an example, the ova and parasite (O&P) examination (direct wet mount, concentration, and permanent stained smear) could be considered a screening method for the detection of a number of different intestinal protozoa and helminth infections; this procedure is moderately sensitive and highly specific when performed by expert microscopists. Molecular test methods tend to be very specific (generally for a single organism such as *Giardia lamblia*) and often more sensitive than the routine O&P examination. However, the test results are limited in scope; either the organism is present or it is not, and none of the other possible etiologic agents can be ruled in or out! In certain situations a laboratory may offer tests on request; an example would be testing for the presence of *Cryptosporidium* spp. If a potential waterborne outbreak were suspected, the laboratory might change its approach and begin testing all stool specimens submitted for an O&P examination rather than testing only those specimens accompanied by a specific test request. These decisions require close communication with other laboratories, water companies, public health personnel, pharmacies (e.g., communication of an increase in the purchase of antidiarrheal over-the-counter medications), and physicians.

It is critical that laboratory clients be educated regarding appropriate test ordering protocols; tests are ordered based on patient history and symptoms (Table 15.1). These ordering guidelines can be extremely helpful for the physicians and the laboratory.

Table 15.1 Stool testing order recommendations (for physicians)

Patient and/or situation	Test ordered*	Follow-up test ordered
Patient with diarrhea and AIDS or other cause of immune deficiency	<i>Cryptosporidium</i> or <i>Giardia</i> / <i>Cryptosporidium</i> immunoassay	If immunoassays are negative and symptoms continue, special tests for microsporidia (modified trichrome stain) and other coccidia (modified acid-fast stain) and O&P exam should be performed
Potential waterborne outbreak (municipal/city water supply)		
Patient with diarrhea (nursery school, day-care center, camper, backpacker)	<i>Giardia</i> or <i>Giardia</i> / <i>Cryptosporidium</i> immunoassay (perform testing on two stools before reporting as negative)	If immunoassays are negative and symptoms continue, special tests for microsporidia and other coccidia (see above) and O&P exam should be performed
Patient with diarrhea and potential waterborne outbreak (resort setting)	<u>Particularly relevant for areas of the United States where <i>Giardia</i> most common organism found</u>	
Patient with diarrhea from areas where <i>Giardia</i> is the most common parasite found		
Patient with diarrhea and relevant travel history	O&P exam, <i>E. histolytica</i> / <i>E. dispar</i> immunoassay; immunoassay for confirmation of <i>E. histolytica</i> ; various tests for <i>Strongyloides</i> may be relevant (even in the absence of eosinophilia)	If exams are negative and symptoms continue, special tests for coccidia and microsporidia should be performed
Patient with diarrhea who is a past or present resident of a developing country		
Patient in an area of the United States where parasites <u>other than</u> <i>Giardia</i> are found		
Patient with unexplained eosinophilia and possible diarrhea; if chronic, patient may also have history of respiratory problems (larval migration) and/or sepsis or meningitis (hyperinfection)	Although the O&P exam is a possibility, the agar plate culture for <i>Strongyloides stercoralis</i> (more sensitive than the O&P exam) is recommended	If tests are negative and symptoms continue, additional O&P exams and special tests for microsporidia and other coccidia should be performed
Patient with diarrhea (suspected food-borne outbreak)	Test for <i>Cyclospora cayentanensis</i> (modified acid-fast stain, autofluorescence)	If tests are negative and symptoms continue, special procedures for microsporidia and other coccidia and O&P exam should be performed

* Depending on the particular immunoassay kit used, various single or multiple organisms may be included. Selection of a particular kit depends on many variables: clinical relevance, cost, ease of performance, training, personnel availability, number of test orders, training of physician clients, sensitivity, specificity, equipment, time to result, etc. Very few laboratories will handle this type of testing exactly the same. Many options are clinically relevant and acceptable for good patient care. It is critical that the laboratory report indicate specifically which organisms could be identified using the kit; a negative report should list the organisms relevant to that particular kit.

15.1.2.2 Routine methods

Routine methods may also be screening methods; however, this is not always the case. "Routine" can imply a widely used, well-understood laboratory test; it can also imply a low or moderately complex method, rather than a high complexity procedure. Routine diagnostic parasitology procedures could include the O&P exam, preparation and examination of blood films and pinworm tapes and/or paddles, occult blood tests, and examination of specimens from other body sites (urine, sputum, duodenal aspirates, urogenital sites, etc.).

Diagnostic parasitology includes laboratory procedures that are designed to detect organisms within clinical specimens using morphologic criteria and visual identification. Some clinical specimens, such as those from the intestinal tract, contain numerous artifacts that complicate the differentiation of parasites from surrounding debris. Final identification is usually based on light microscopic examination of stained preparations, often using high magnification techniques such as oil immersion (1000×) [20,38,39,42].

Specimen preparation often requires some type of concentration, all of which are designed to increase the chances of finding the organism(s). Microscopic examination requires review of the prepared clinical specimen

using multiple magnifications and different examination times; organism identification also depends to a great degree on the skill of the microscopist.

Protozoa range from 1.5 µm (microsporidia) to approximately 80 µm (*Balantidium coli* [ciliate]) in size. Some parasites are intracellular, and multiple isolation and staining methods may be required for identification. Helminth infections are usually diagnosed by finding eggs, larvae, and/or adult worms in various clinical specimens, primarily those from the intestinal tract. Identification to the species level for many human parasites may require microscopic examination of the specimen. The recovery and identification of blood parasites can require concentration, culture, and microscopy. Confirmation of suspected parasitic infections depends on the proper collection, processing, and examination of clinical specimens, and multiple specimens may be required in order to confirm the presence of organisms [12,20,22,24,27,29,35,37,41,44,56,60].

15.1.2.3 Special testing

Special procedures such as parasite culture are performed in a limited number of laboratories. These procedures require the maintenance of positive control

cultures used for quality control checks on all patient specimens and special expertise and time often not available in many clinical laboratories [59]. Although some standardized reagents are now commercially available, many clinical laboratories choose to send their request for serologic testing for parasitic disease to other laboratories. Often, the Centers for Disease Control and Prevention (CDC) perform serologic testing on specimens submitted to each state's Department of Public Health. Generally, specimens for parasitic serologies are not submitted directly to the CDC, but are forwarded to CDC by the state public health laboratories. In an emergency situation, consultation with your city, county, or state public health laboratory may allow shipment of a specimen directly to the CDC.

15.1.3 Diagnostic medical parasitology

15.1.3.1 Equipment

Equipment required for diagnostic parasitology work is very minimal; however, the one expense that is totally justified would be for one or more microscopes with good optics. Each microscope should be equipped with high quality (flat-field) objectives (10×, 40×, 50× or 60× oil immersion, and 100× oil immersion objectives). The oculars should be a minimum of 10×. Depending on the range of immunoassay testing available, a fluorescence microscope or enzyme immunoassay (EIA) reader might be desirable. The availability of this equipment will vary from one laboratory to another and may be shared with other groups within the laboratory. Another option would be a fume hood, in which the staining could be performed; this is not required but is recommended, particularly if the laboratory is still using xylene for dehydration of permanent stained fecal smears. The rest of the equipment is quite common and can be shared with other areas within the laboratory. Equipment would include refrigerators, freezers, pipette systems, and other common items normally found within a clinical laboratory.

15.2 Solicitation of product information

Commercial companies that supply collection systems, stool concentration devices, reagents, test kits, and other relevant products used in the practice of diagnostic medical parasitology were contacted for inclusion in this book on laboratory medicine diagnostic products. Those companies that replied have been included and are presented below. Not all companies provided the same information, and you will see differences as you review the available products used in this field. In some cases, peer reviewed manuscripts on the products have not been published. Based on company responses, the data are current at the time of this writing.

15.3 Specimen collection systems

Various collection systems are available for clinical specimens suspected of containing parasites or parasitic elements (Table 15.2). The selection of collection options should be based on a thorough understanding of the value and limitations of each. The final laboratory results are based on parasite recovery and identification and will depend on the initial handling of the organisms. Unless the appropriate specimens are properly collected and processed, the examination may lead to false-negative results. Specimen rejection criteria have become much more important for all diagnostic parasitology procedures. Laboratory results based on improperly collected specimens may require inappropriate expenditures of time and supplies and may also mislead the physician.

15.4 Fresh stool specimen collection

Fecal specimens should be collected in clean, wide mouth containers; often a waxed cardboard or plastic container with a tight fitting lid is selected for this purpose. These collection containers are available from most of the microbiology product distributors and are not difficult to locate; there are very minor differences, none of which are critical. Stool specimen containers should be placed in plastic biohazard bags when transported to the laboratory for testing. Specimens should be identified with the following information: patient's name and identification number, physician's name, the date and time the specimen was collected (if the laboratory is computerized, the date and time may reflect arrival in the laboratory, not the actual collection time), and the laboratory procedures requested. Although it may be helpful to have information concerning the presumptive diagnosis or relevant travel history, this information is rarely available.

Fresh specimens are required for the recovery of motile trophozoites (amebae, flagellates, or ciliates). The protozoan trophozoite stage is normally found in cases of diarrhea; the gastrointestinal tract contents are moving through the system too rapidly for cyst formation to occur. Once the stool specimen is passed from the body, trophozoites do not encyst but may disintegrate if not examined or preserved within a short time after passage. However, most helminth eggs and larvae, coccidian oocysts, and microsporidian spores will survive for extended periods. Liquid specimens should be examined within 30 min of passage, not 30 min from the time they reach the laboratory. If this general time recommendation of 30 min is not possible, then the specimen should be placed in one of the available fixatives. Soft (semiformed) specimens may have a mixture of protozoan trophozoites and cysts and should be examined within 1 h of passage; if this time frame is not possible, then preservatives should be used. Immediate examination of formed specimens is not as critical; if the specimen is examined any time within 24 h after passage, the protozoan cysts should still be intact (Table 15.3) [20–22,41,44].

Table 15.2 Body sites and specimen collection*

Site(s)	Specimen option	Collection method recommendations
Blood	Smears of whole blood Anticoagulated blood	Thick and thin films: fresh or with anticoagulant EDTA (first choice); heparin (second choice)
Bone marrow	Aspirate	Sterile
Central nervous system	Spinal fluid Brain biopsy specimen	Sterile Sterile
Cutaneous ulcer	Aspirates from below surface Biopsy specimen	Sterile, plus air-dried smears Sterile, nonsterile to histopathology (formalin acceptable)
Eye	Biopsy specimen Scrapings Contact lens Lens solution	Sterile (in saline), nonsterile to histopathology Sterile (in saline) Sterile (in saline) Sterile; unopened commercial solutions not acceptable
Intestinal tract	Fresh stool Preserved stool Sigmoidoscopy material Duodenal contents Anal impression smear Adult worm or worm segments	Half-pint waxed or plastic container 5 or 10% formalin, MIF, SAF, Schaudinn's fluid (mercury, copper, or zinc base) (with PVA), single vial systems/Universal Fixative (no mercury, no formalin, no PVA) Fresh, preserved, or smears submitted in Schaudinn's fluid Entero-Test or aspirates Cellulose tape (pinworm exam) or other collection device. Saline, 70% alcohol
Liver and/or spleen	Aspirates Biopsy specimen	Sterile, collected in four separate aliquots (liver) Sterile, nonsterile to histopathology
Lung	Sputum Induced sputum Bronchoalveolar lavage Tracheobronchial aspirate Brush biopsy Open lung biopsy Aspirate	True sputum (not saliva) Sterile, air-dried smears Sterile, air-dried smears Sterile, air-dried smears Sterile, air-dried smears Sterile Sterile, air-dried smears
Muscle	Biopsy specimen	Fresh, squash preparation, nonsterile to histopathology (formalin acceptable)
Skin	Scrapings Skin snip BiopsysSpecimen	Aseptic, smear or vial No preservative Sterile (in saline), nonsterile to histopathology
Urogenital system	Vaginal discharge Urethral discharge Prostatic secretions Urine	Saline swab, transport swab (no charcoal), culture medium, plastic envelope culture; air-dried smear for fluorescent antibody Saline swab, transport swab (no charcoal), culture medium, plastic envelope culture; air-dried smear for fluorescent antibody Saline swab, transport swab (no charcoal), culture medium, plastic envelope culture; air-dried smear for fluorescent antibody Single unpreserved specimen (early morning), 24-h unpreserved specimen

Source: adapted from references 19 and 20.

* Most products used for specimen collection are available from any major medical supply house.

Table 15.3 Stool collection: pros and cons of fresh and preserved specimens*

Stool type	Pros	Cons
Fresh	No requirements for stool fixatives. Ability to see motile trophozoites. Lower cost. Can perform direct wet exam, concentration and permanent stained smear (O&P), fecal immunoassays, special stains for coccidia and microsporidia. Relevant <u>only</u> if time from stool passage to laboratory is acceptable; in symptomatic patients, the trophozoite form of the intestinal protozoa is present and will not encyst when outside of the body: liquid or watery stool – 30 min semiformed – 1 h formed – 24 h	May have excessive lag time between stool passage and fixation or processing; trophozoites may disintegrate, thus giving a false negative result. Ova and parasite examination (direct wet exam, concentration, and permanent stained smear) may be negative due to lack of organism preservation and morphology integrity. Poor results may also result for special stains for coccidia and microsporidia.
Preserved	Organism morphology is preserved when lag time between stool passage and fixation is short, which can be accomplished by allowing the patients to collect and fix stool specimens at home. Once the specimen is mixed with the preservative, the delivery time to the laboratory is not critical. Can perform concentration and permanent stain (O&P), fecal immunoassays, special stains for coccidia and microsporidia. Fixatives now available that contain no mercury, no formalin, and no PVA (universal fixatives)	Cost of collection vials may represent a cost increase; however, may be overall less expensive because of much more accurate result (patient outcome). Disposal of vials may be a problem if the laboratory is using preservatives containing mercuric chloride

MIF, merthiolate–iodine–formalin; SAF, sodium acetate–acetic acid–formalin; PVA, polyvinyl alcohol; FA, fluorescent antibody; O&P, ova and parasite examination.

* Most products used for specimen collection are available from any major medical supply house.

15.5 Preservation of stool specimens

If there are likely to be delays in specimen delivery to the laboratory, fecal preservatives should be used (Table 15.4). To preserve protozoan morphology and to prevent the continued development of some helminth eggs and larvae, the stool specimens can be placed in preservative, either immediately after passage (by the patient using a collection kit) or once the specimen is received by the laboratory (Figure 15.1). There are several fixatives available, and these include formalin, sodium acetate–acetic acid–formalin (SAF), and Schaudinn's fluid; some of these fixatives will contain polyvinyl alcohol (PVA), a plastic powder that is used as a glue to adhere the stool to the glass slide (Table 15.4). Regardless of the fixative selected, adequate mixing of the specimen and preservative is mandatory (Figures 15.1 to 15.9) [24,27,29,37,56,60]. When selecting one or more stool fixatives, remember that a permanent stained smear is mandatory for a complete examination for parasites. You may also want to perform methods such

as fluorescence, EIA, or the rapid cartridge immunoassay devices; however, you will need to confirm that the fixative you are using is compatible with the diagnostic procedures you have selected [1–7,10,11,13,14,16,18,19,23,25,26,30–34,42,48,50,52–55,61]. It is also important to remember that disposal regulations for compounds containing mercury are becoming more restrictive; each laboratory will have to check applicable state and federal regulations to help determine fixative disposal options. Another responsibility may be the need to validate specific protocols and/or kits for uses that are not clearly defined in the package insert.

15.5.1 Formalin

Formalin is used as an all-purpose fixative that is appropriate for helminth eggs and larvae and for protozoan cysts. Two concentrations are commonly used: 5%, which is recommended for preservation of protozoan cysts, and 10%, recommended for helminth eggs and larvae.

Table 15.4 Preservatives used in diagnostic parasitology (stool specimens)

Preservative	Concentration	Permanent stained smear	Immunoassays* (<i>Giardia lamblia</i> , <i>Cryptosporidium</i> spp.)	Comments
5% or 10% formalin	Yes	No	Yes	EIA, FA, cartridge
5% or 10% buffered formalin	Yes	No	Yes	EIA, FA, cartridge
MIF	Yes	Polychrome IV stain	ND	ND
SAF	Yes	Iron hematoxylin (best)	Yes	EIA, FA, cartridge
Schaudinn's fixative [†] (mercury base) with or without PVA	Yes, but rarely used	Trichrome or iron hematoxylin	No	PVA interferes with immunoassays
Modified Schaudinn's [‡] (copper base) with PVA	Yes, but rarely used	Trichrome or iron hematoxylin	No	PVA interferes with immunoassays
Modified Schaudinn's [§] (zinc base) with PVA	Yes, but rarely used	Trichrome or iron hematoxylin	No	PVA interferes with immunoassays
Single-vial systems [¶] (with or without PVA)	Yes	Trichrome or iron hematoxylin	Some, but not all	Check with the manufacturer
Universal fixative ^{**} (single-vial system)	Yes	Trichrome or iron hematoxylin	Yes	EIA, FA, cartridge; contain no mercury, no formalin, no PVA; does not require albumin for adhesion (no PVA, no albumin); has been validated for some molecular/PCR testing

EIA, enzyme immunoassay; FA, fluorescent antibody; MIF, merthiolate–iodine–formalin; ND, no data; PCR, polymerase chain reaction; PVA, polyvinyl alcohol; SAF, sodium acetate–acetic acid–formalin.

* Fecal immunoassays for the *E. histolytica*/*E. dispar* group or the true pathogen, *E. histolytica* require fresh or frozen specimens; in some cases Cary–Blair may be acceptable (check with the manufacturer).

[†] These two fixatives use the mercuric chloride base in the Schaudinn's fluid; this formulation is still considered to be the “gold standard”, against which all other fixatives are evaluated (organism morphology after permanent staining). Additional fixatives prepared with nonmercuric chloride-based compounds are used; however, the overall organism morphology is not as good.

[‡] This modification uses a copper sulfate base rather than mercuric chloride; organism morphology is marginal.

[§] This modification uses a zinc base rather than mercuric chloride and works well with both trichrome and iron-hematoxylin stains.

[¶] These modifications use a combination of ingredients (including zinc), but are prepared from proprietary formulas. The aim is to provide a fixative that can be used for the fecal concentration, permanent stained smear, and available immunoassays for *Giardia lamblia*, *Cryptosporidium* spp.; *Entamoeba histolytica* (or the *E. histolytica*/*E. dispar* group).

^{**} Universal fixatives contain no mercury, no formalin, and no PVA. The complete O&P (concentration, permanent stained smear), fecal immunoassays for *G. lamblia* and *Cryptosporidium* spp., and special stains for the coccidia and microsporidia can be performed using these options. Fecal immunoassays for the *E. histolytica*/*E. dispar* group or the true pathogen, *E. histolytica* require fresh or frozen specimens; in some cases Cary–Blair may be acceptable (check with the manufacturer). An example would be TOTAL-FIX (Medical-Chemical Corporation, Torrance, CA), a universal fixative that has also been found to be compatible with a number of molecular test options, including PCR: it may be necessary to validate reagents for test options other than those appearing in the package inserts.



Figure 15.1 Sample of Para-Pak parasitology products. (Courtesy of Meridian Bioscience, Inc.)



Figure 15.2 Representative sample of stool collection vials. From left to right they are modified PYA (prepared with a copper base), SAF fixative, 5% buffered formalin, 10% buffered formalin, ZPVA (prepared with a zinc base), Unifix (single-vial collection system), and PVA (prepared with a mercury base). (Courtesy of Medical Chemical Corporation.)



Figure 15.3 Fecal collection and transport vials. (Courtesy of Evergreen Scientific.)



Figure 15.4 PVA preservative (prepared with the zinc base). (Courtesy of Evergreen Scientific.)

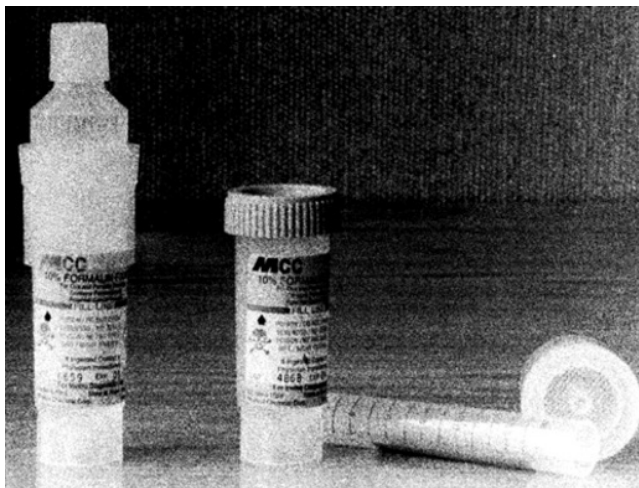


Figure 15.5 Example of a 10% formalin fecal concentration system, the PARA-SED system. (Courtesy of Medical Chemical Corporation.)

Although 5% is often recommended for all-purpose use, most commercial manufacturers provide 10%, which is more likely to kill all helminth eggs. To help maintain organism morphology, the formalin can be buffered with sodium phosphate buffers, i.e., neutral formalin. Selection



Figure 15.6 Example of a 10% formalin vial, the Para-Pak Ultra. (Courtesy of Meridian Bioscience, Inc.)



Figure 15.7 Example of a single-vial fecal collection system, Para-Pak ECOFIX. (Courtesy of Meridian Bioscience, Inc.)

of specific formalin formulations is at the user's discretion. Aqueous formalin will permit the examination of the specimen as a wet mount only, a technique much less accurate than a permanent stained smear for the identification of intestinal protozoa. Most companies offer both 5 and



Figure 15.8 Example of a single-vial fecal collection system, PROTO-FIX. (Courtesy of Alpha-Tec. Systems, Inc.)



Figure 15.9 Example of a single-vial fecal collection system, universal fixative, TOTAL-FIX (no mercury, no formalin, no PVA). (Courtesy of Medical Chemical Corporation.)

10% formalin (most are buffered); most laboratories use 10% formalin [20,21,41].

Protozoan cysts (not trophozoites), coccidian oocysts, helminth eggs, and larvae are well-preserved for long periods of time in 10% aqueous formalin. Hot (60°C) formalin can be used for specimens containing helminth eggs, since in cold formalin some thick-shelled eggs such as *Ascaris*, *Toxocara*, or *Baylisascaris* may continue to

develop, become infective, and remain viable for long periods. Several grams of fecal material should be thoroughly mixed in 5 or 10% formalin.

15.5.2 Merthiolate-iodine-formalin

Merthiolate-iodine-formalin (MIF) is a good stain preservative for most kinds and stages of parasites found in feces; it is especially useful for field surveys, but is less commonly used in the routine clinical laboratory. It is used with all common types of stools and aspirates; protozoal eggs, and larvae can be diagnosed without further staining in temporary wet mounts, either made immediately after fixation or prepared several weeks later. Although some laboratories maintain that a permanent stained smear can be prepared from specimens preserved in MIF, most laboratories using such a fixative examine the material only as a wet preparation (direct smear and/or concentration sediment). The MIF preservative is prepared in two stock solutions, stored separately and mixed immediately before use [20].

15.5.3 Sodium acetate-acetic acid-formalin

Both a concentration and a permanent stained smear can be performed from specimens preserved in the liquid fixative SAF; this fixative does not contain mercuric chloride, as is found in Schaudinn's fluid (some of which contain PVA). The sediment is used to prepare the permanent smear, and the stool material is generally placed on an albumin-coated slide to improve adherence to the glass [20,25]. SAF is considered to be a "softer" fixative than mercuric chloride. The organism morphology will not be quite as sharp after staining as will that of organisms originally fixed in solutions containing mercuric chloride. The pairing of SAF-fixed material with iron hematoxylin staining provides better organism morphology than does staining SAF-fixed material with trichrome (personal observation). Although SAF has a long shelf life and is easy to prepare, the smear preparation technique may be a bit more difficult for less experienced personnel who are not familiar with fecal specimen techniques. Laboratories that have considered using only a single preservative have selected this option. Helminth eggs and larvae, protozoan trophozoites and cysts, and coccidian oocysts and microsporidian spores are preserved using this method; a number of distributors provide prepared SAF, ready to use.

15.5.4 Schaudinn's fluid

Schaudinn's fluid is used with fresh stool specimens or samples from the intestinal mucosal surface and is prepared using mercuric chloride. Many institutions that have problems meeting recommended specimen delivery times select this approach. Permanent stained smears are then prepared from fixed material. When using this

approach, a second vial is required for the concentration and/or various screening methods [20,21,41,44].

15.5.5 Polyvinyl alcohol

PVA is a plastic resin that is incorporated into Schaudinn's fixative (mercury base) or other fixatives (copper, zinc bases). The PVA powder serves as an adhesive for the stool material; i.e., when the stool PVA mixture is spread onto the glass slide, it adheres because of the PVA component. Fixation is still accomplished by the Schaudinn's fluid. Perhaps the greatest advantage in the use of PVA is the fact that a permanent stained smear can be prepared. Fixatives containing PVA are highly recommended as a means of preserving cysts and trophozoites for examination at a later time. The use of PVA also permits specimens to be shipped (by regular mail service) from any location in the world to a laboratory for subsequent examination. PVA is particularly useful for liquid specimens and should be used in the ratio of three parts PVA to one part fecal specimen [12,20,21,41,44].

15.5.6 Modified PVA (copper, zinc base – nonmercury)

Although preservatives have been developed that do not contain mercury compounds, substitute compounds have not quite provided the quality of preservation necessary for good protozoan morphology on the permanent stained smear. Copper sulfate has been tried but does not provide results equal to those seen with mercuric chloride. Zinc sulfate has recently proven to be an acceptable mercury substitute and is used with trichrome stain. Although zinc substitutes have become widely available, each manufacturer has a proprietary formula for the fixative. There are different opinions; however, many feel that the zinc sulfate substitute provides better fixation and organism morphology than that prepared with copper sulfate. Most manufacturers who supply stool collection vials offer both of these options [8,29,37].

15.5.7 Single-vial collection systems

Several manufacturers now have available single-vial stool collection systems, similar to SAF or modified PVA methods. From the single vial, the concentration and permanent stained smear can be prepared. It is also possible to perform immunoassay procedures from some of these vials, as well as specialized stains for coccidia and the microsporidia. Make sure to ask the manufacturer about all capabilities (concentrate, permanent stained smear, immunoassay procedures, special stains for coccidia and microsporidia) and for specific information indicating there are no formula components that would interfere with any of the three methods. Like the zinc substitutes, these formulas are proprietary. Several examples are Unifix, ECOFIX, and ProtoFix [20,21,24].

15.5.8 Single-vial universal fixative collection system

The purpose of the universal fixative is to provide a single vial collection system that contains no mercury (disposal problems), no formalin (exposure problems, may prevent performance of a permanent stained smear), and no PVA (interferes with fecal immunoassays). These modifications use a combination of ingredients (including zinc), but are prepared from proprietary formulas. The aim is to provide a fixative that can be used for the fecal concentration, permanent stained smear (routine plus special stains), and available immunoassays for *G. lamblia*, *Cryptosporidium* spp. and *Entamoeba histolytica* (or the *E. histolytica*/*Entamoeba dispar* group). An example would be Total-Fix (Figure 15.9). However, even with the universal fixatives, fecal immunoassays for the *E. histolytica*/*E. dispar* group or the true pathogen, *E. histolytica* require fresh or frozen specimens; in some cases Cary–Blair may be acceptable. Also, it is important to remember that appropriate validation studies must be performed and documented if reagents and/or kits are used for any purposes other than those specified in the package insert. In some cases, these fixatives are also being validated for use with molecular methods. These fixatives have been termed “universal” fixatives, since they can be used for many parasitology diagnostic options.

15.5.9 Stool preservatives: user notes and suggestions

- 1 Most of the commercially available kits have a “fill-to” line on the vial label to indicate how much fecal material to add to ensure adequate preservation of the fecal material. However, patients often overfill the vials; remember to open the vials with the vials turned away from your face.
- 2 Although the two-vial system (one vial of 5 or 10% buffered formalin [concentration] and one vial of fixative containing PVA [permanent stained smear]) has always been the “gold standard,” laboratories are beginning to use other options, including the universal fixative. Changes in the selection of fixatives are based on the following considerations.
 - (a) Problems with disposal of mercury-based fixatives and lack of multilaboratory contracts for disposal of such products, as well as the elimination of formalin and PVA in the systems.
 - (b) The cost of a two-vial system compared with the cost of a single collection vial.
 - (c) Selection of specific stains (e.g., trichrome or iron hematoxylin) to use with specific fixatives.
 - (d) Whether the newer immunoassay kits can be used with stool specimens preserved with that particular fixative.
 - (e) Polymerase chain reaction (PCR) and other molecular methods.

15.5.10 Stool preservatives: procedure limitations

- 1 Adequate fixation still depends on the following parameters.
- (a) Meeting recommended time limits for lag time between passage of the specimen and fixation.
 - (b) Use of the correct ratio of fixative to specimen (3:1).
 - (c) Thorough mixing of the fixative and specimen.
- Once the specimen is received in the laboratory, any additional mixing at that time will not counteract the lack of fixative/specimen mixing and contact prior to that time.
- 2 Unless the appropriate stain is used with each fixative, the final permanent stained smear may be difficult to examine (organisms hard to see and/or identify). Examples of appropriate combinations are:
- (a) Schaudinn's fixative (with or without PVA) with trichrome or iron hematoxylin stains;
 - (b) SAF fixative with iron hematoxylin stain (trichrome is second choice);
 - (c) single-vial mercuric chloride substitute systems with trichrome, iron hematoxylin, or company-developed

proprietary stains matched to their specific fixatives (an example would be ECOFIX coupled with ECO-STAIN [24]).

- 3 If you want to investigate one of the products for potential use in your laboratory, ask to see the package inserts and ask for actual laboratory user contacts with whom you can discuss product performance. Make sure to check the references to see if clinical studies have been published in peer-reviewed journals. If not, ask why not.

15.6 Intestinal tract specimens (stool)
15.6.1 Immunoassay methods

The use of immunoassay methods for fecal specimens has become incorporated into many laboratories, particularly during the past couple of years (Tables 15.5 and 15.6). The selection and ordering of various procedures depend on a number of variables, including patient history and symptoms, geographic area, population served, positivity rate, client test preference, number of test requests, laboratory staffing, personnel training and experience, equipment availability, turnaround time requirements, epidemiology considerations, clinical relevance of test results, and cost.

Table 15.5 Ova and Parasite Examination vs. Rapid Tests (stool)

Fecal specimen procedure	Traditional procedure(s)	Alternative options, rapid tests	Comments
Concentration	Specimen sampling (pipettes), use of slides and coverslips	Specimen sampling (DiaSys semiautomated system), DiaSys glass viewing chambers (Now Apacor)	The traditional sampling approach using pipettes and the preparation of wet smears using glass slides and coverslips can be replaced with a semiautomated sampling and viewing system from DiaSys Corporation. The specimen is drawn through tubing from the mixed concentrated stool sediment into two viewing chambers that fit onto the microscope stage. The quality of the glass is excellent and organism morphology can be easily seen within the viewing chambers. However, this system is most appropriate for laboratories with a large workload (O&P testing).
Permanent stained smear	Microscopic examination of stained fecal smear using oil immersion objectives	Several tests provide a solid phase qualitative immunochromatographic procedure that can be used for the detection of <i>Cryptosporidium</i> spp. and <i>Giardia lamblia</i> antigen. The test can be performed on fresh, frozen, unfixed, formalin-fixed (5%, 10%, SAF), or those fecal specimens in universal fixative. Another available fecal immunoassay is a qualitative EIA procedure for the rapid detection of <i>G. lamblia</i> , the <i>E. histolytica</i> and <i>E. dispar</i> group, and <i>Cryptosporidium</i> antigens. The test can be performed on fresh or fresh frozen, unfixed human fecal specimens. Direct fluorescent antigen detection is also available for <i>Giardia</i> and <i>Cryptosporidium</i> , although this method does require a fluorescence microscope. Fecal immunoassays are also under development for <i>Blastocystis hominis</i> and <i>Dientamoeba fragilis</i> .	Although these tests are limited to <i>Cryptosporidium</i> and <i>Giardia</i> , they can be very beneficial in the absence of trained microscopists. However, in patients who remain symptomatic after a negative result, the O&P examination should always remain an option. If <i>Giardia</i> is suspected, if the first stool is negative, a second stool should be tested prior to reporting a negative; this is not required when testing for <i>Cryptosporidium</i> antigen. Although this test is limited to the use of fresh or fresh frozen fecal specimens, it can be helpful in the absence of trained microscopists. It is important to remember that the test does not differentiate between <i>E. histolytica</i> (pathogen) and <i>E. dispar</i> (nonpathogen). In patients who remain symptomatic after a negative result, the O&P examination or special stains should always remain options. For laboratories that are equipped with a fluorescence microscope, this test provides an excellent method for few or many specimens. It is recommended that the test be performed on centrifuged material to enhance the sensitivity (one sees the actual <i>Giardia</i> cysts and <i>Cryptosporidium</i> oocysts, rather than just antigen detection).

Table 15.6 Sources of available reagents for immunodetection of parasitic organisms or antigens*

Company	Cryptosporidium EIA or IFA	Cryptosporidium- <i>Giardia</i> combination (various methods)	Entamoeba <i>histolytica</i> EIA	<i>E. histolytica</i> / <i>E. dispar</i> group EIA	<i>Giardia</i> EIA or IFA	Crypto- <i>Giardia</i> Cartridge and/ or separate cartridges	Crypto- <i>Giardia</i> - <i>E.</i> <i>histolytica</i> / <i>E. dispar</i> group cartridge	<i>Trichomonas</i> <i>vaginalis</i> (various methods)	Other parasites (methodology varies, often EIA)	Water- testing reagents
Alere (Wampole, Inverness Medical)									Binax NOW Malaria, Clearview Malaria Combo, Rapid Toxoplasma Ab	
Becton-Dickinson								X (DNA Probe) (Affirm VP III) (<i>Trichomonas</i> , <i>Gardnerella</i> , <i>Candida</i>)		
Alere							X (Triage Parasite Panel)			
BioFire		FilmArray Gastrointestinal Panel (also includes <i>Cyclospora</i>) X (DFA)	FilmArray Gastrointestinal Panel							
Cellabs	X (IFA)				X (IFA, EIA)				Filariasis Ab <i>Leishmania</i> Ag, Ab <i>Plasmodium</i> Ag, Ab <i>T. cruzi</i> Ab Toxocara Ab	
Hologic GenProbe								Aptima <i>Trichomonas</i> amplification assay		
Luminex, Inc.										
Medical Chemical Corp.	X	xTAG [†] X (DFA)			X					
Meridian	X	X (DFA)			X	X				
Diagnostics, Inc. Remel	X	X (EIA)		X	X	X				
(ThermoFisher Scientific)										
Sekisui Diagnostics (Genzyme Diagnostics)								OSOM <i>Trichomonas</i> rapid test		
TechLab, Inc/Alere. Waterborne, Inc.	X	X (DFA, EIA, Rapid)	X		X					X

* Tests for the *E. histolytica*/*E. dispar* group or *E. histolytica* require the use of fresh or frozen fresh stools, or those submitted in Cary-Blair. All other tests (with the exception of DFA for *Cryptosporidium*/*Giardia* combination) can be used with fresh or preserved stools (formalized base, not PVA – some single-vial systems may be acceptable (universal fixative); consult manufacturer). Since the combination *Cryptosporidium*-*Giardia* tests (DFA) are based on visual recognition of the fluorescent oocysts and/or cysts, the specimens must not be frozen if fresh stools are used for testing; it is also recommended that these DFA tests be performed on centrifuged stool sediment to enhance sensitivity.

[†] xTAG, multiplexed nucleic acid test that simultaneously detects common viral, bacterial, and parasitic causes of infectious gastroenteritis from a single patient sample.



Figure 15.10 MERIFLUOR DFA kit for the detection of *Cryptosporidium* oocysts and *Giardia* cysts. (Courtesy of Meridian Bioscience, Inc.)

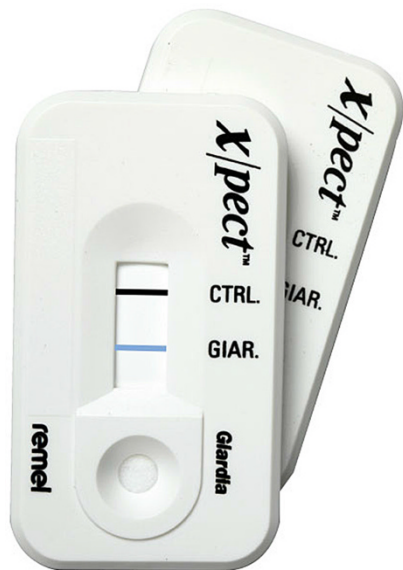


Figure 15.11 Thermo Scientific™ Xpect™ *Giardia* Test. (Photo Courtesy of ThermoFisher Scientific, Copying is Prohibited.)

Like any type of diagnostic test option, there are pros and cons. Certainly the specificity of the fecal immunoassay methods is very high; however, the tests are limited to specific organisms. A negative test does not rule out many other possible etiologic agents. See Table 15.1 for specific ordering options and recommendations (O&P, fecal immunoassays, special stains for coccidia, microsporidia).

Test formats include fluorescent antibody assay, EIA, and the newer immunochromatographic cartridge immunoassays. The reagents have been used for direct fluorescent antibody (DFA) microscopy, microplate immunoassays, and the newer cartridge test systems. The sensitivity and specificity of all of these reagents have been comparable; selection of a particular format will vary from laboratory to laboratory. Specific format examples can



Figure 15.12 *E. HISTOLYTICA* II Immunoassay for the true pathogen, *Entamoeba histolytica*. (Courtesy of TECHLAB, Inc.) (See insert for color representation of the figure.)

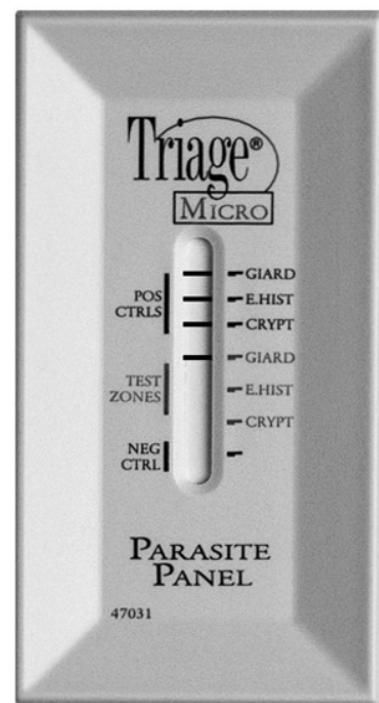


Figure 15.13 Triage parasite panel. The specimen shown is positive for *Giardia* spp. (GIARD). (Courtesy of BIOSITE Diagnostics/Alere, Inc.)

be seen in Figures 15.10 to 15.13. Organisms for which these commercial products have been developed include *G. lamblia*, *Cryptosporidium* spp., *E. histolytica*/*E. dispar* group, *E. histolytica*, and *Trichomonas vaginalis* (urogenital system). These procedures are more sensitive than the routine O&P examination. However, as mentioned above, the test results are limited in scope. Either the organism

is present or it is not, and no other possible etiologic agents can be ruled in or out. Also, if low antigen levels or very few organisms are present in the specimen, a false-negative result may occur; this is a possibility for any of the fecal immunoassay test kits available.

15.7 Ova and parasite examination

The specimen most commonly submitted to the diagnostic parasitology laboratory is the stool specimen, and the most commonly performed procedure in parasitology is the O&P examination, which comprises three separate protocols: the direct wet mount, the concentration, and the permanent stained smear. The direct wet mount requires fresh stool. It is designed to allow detection of motile protozoan trophozoites, and is examined microscopically at low and high dry magnifications (100 $\times$, entire 22-by-22 mm coverslip; 400 $\times$, one-third to one-half of the 22-by-22 mm coverslip). Iodine can also be used to examine the wet mount (Figure 15.14). However, due to potential problems with lag time between the time of specimen passage and receipt in the laboratory, the direct wet examination has been eliminated from the routine O&P examination in favor of receipt of specimens collected in stool preservatives; if specimens are received in the laboratory in stool collection preservatives, the direct wet preparation is not performed. A list of products and suppliers can be seen in Table 15.7.

The second part of the O&P examination is the concentration that is designed to facilitate recovery

of protozoan cysts, coccidian oocysts, microsporidial spores, and helminth eggs and larvae. Both flotation and sedimentation methods are available, the most common procedure being the formalin-ethyl acetate sedimentation method (formerly used was the formalin-ether method). The concentrated specimen is examined as a wet preparation, with or without iodine, using low and high dry magnifications (100 $\times$ and 400 $\times$) as indicated for the direct wet-smear examination. Various commercial concentration devices are available and provide results that are comparable with the gold standard gauze-and-funnel method (Table 15.6; Figures 15.15 to 15.24). A semiautomated system for sampling and examination of concentration sediments is also available (FE-5; Apacor Ltd USA, Brooklyn and New York) (Figures 15.23 and 15.24; Table 15.6).

The third part of the O&P examination is the permanent-stained smear that is designed to facilitate identification of intestinal protozoa. Several staining methods are available, the two most common being the Wheatley modification of the Gomori tissue trichrome and the iron hematoxylin stains. This part of the O&P examination is critical for the confirmation of suspicious objects seen in the wet examination and identification of protozoa that might not have been seen in the wet preparation. The permanent stained smear is the most important procedure performed for the identification of intestinal protozoan infection; permanent stained smears are examined using oil immersion objectives (60 $\times$ for screening and 100 $\times$ for final review of a minimum of 300 oil immersion fields). Specific modified trichrome stains for the microsporidia can be seen in Figure 15.25. Those working with SAF-preserved specimens will require the use of albumin for the preparation of permanent stained slides (Figures 15.25 to 15.29). Commercially available quality control slides are also available (Table 15.6).



Figure 15.14 D'Antoni's iodine (used during the examination of fecal wet preparations). (Courtesy of Hardy Diagnostics.)

15.8 Molecular methods

There are several FDA-approved molecular diagnostic tests for parasites. They include the APTIMA *Trichomonas vaginalis* Assay (GenProbe, San Diego, CA). This assay utilizes the same amplification technology that has been used for years to detect *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. It also uses the same specimen types (i.e., clinician-collected vaginal and endocervical swabs, female urine and PreservCyt solution). It has reported high sensitivity and specificity, and does not require organism viability or motility. It, as well as other antigen-based assays, represents advances in the detection of this parasite.

The Affirm VPIII DNA probe technology (Becton Dickinson, Franklin Lakes, NJ) offers a dependable, rapid means for the early identification of three organisms causing

Table 15.7 Sources of commercial reagents and supplies⁵

Reagent or supply	AJP Scientific	ALPHA-TEC	Avantor (J.T. Baker)	bioMérieux (PML Microbiologicals)	City Chemical Corporation	Evergreen Scientific	Fisher Scientific (ThermoFisher Scientific)
Specimen collection kit(s)							
Formalin/PVA		X		X		X	
PVA, modified (copper base)		X					
PVA, modified (zinc base)		X		X		X	
MIF	X	X					
SAF		X		X		X	
Universal fixative*							
Pinworm Paddles							
Preservatives (bulk)							
Schaudinn's fixative solution (mercury)		X + –					
PVA fixative solution	X	X					
PVA powder [†]			X				X
MIF solution	X	X					
SAF solution		X		X			
Concentration systems							
Formalin–ethyl acetate		X		X		X	
Zinc sulfate (sp gr, 1.18/1.20)		X	X				
Stains							
Trichrome – solution	X(W)	X(W)		X			
Trichrome – modified [†]	X	X		X			
Trichrome – dye powders							
Chromotrope 2R			X				
Fast green FCF			X				X
Light green SF yellowish			X				X
Hematoxylin – solution				X			
Hematoxylin – powder			X				X
Chlorazol-black E-powder					X		
Giemsa – solution	X	X	X				X
Giemsa – powder			X				X
Rapid blood stains							
Carbol fuchsin–Kinyoun's	X	X		X			X
Modified acid-fast	X	X					
Modified acid-fast with DMSO		X		X			
Calcofluor white	X						X
Auramine–rhodamine				X			X
Acridine orange				X			
Toluidine blue O							
Miscellaneous							
Lugol's iodine dilute 1:5	X	X		X			
Dobell and O'Connor's iodine		X					X
Triton X–100		X	X				
Eosin/saline solution (1%)							X
Mayer's albumin		X					
Control slides or suspensions		X		X			
Alcohols, xylene							
Methanol (thin blood film)	X			X			X
Ethanol 70%	X	X					X
Ethanol 90%		X					X
Ethanol 95%	X	X					X
Ethanol absolute							X
							(200 proof)
Xylene	X						X
Carbol-xylene	X						X
Xylene substitute		X					X

Reagent or supply	Hardy Diagnostics	Harleco	Medical Chemical Corp	Meridian Bioscience, Inc	Rowley Biochem	Scientific Device Laboratory	ThermoFisher Scientific (Remel)
Specimen collection kit(s)							
Formalin/PVA	X		X	X		X	X
PVA, modified (copper base)	X		X	X			X
PVA, modified (zinc base)	X		X	X			X
MIF	X		X	X			X
SAF	X		X	X		X	X
Universal fixative*			X				
Pinworm paddles	X					X	

Reagent or supply	Hardy Diagnostics	Harleco	Medical Chemical Corp	Meridian Bioscience, Inc	Rowley Biochem	Scientific Device Laboratory	ThermoFisher Scientific (Remel)
Preservatives (bulk)							
Schaudinn's fixative solution	X +		X +	X +	X –		X –
PVA fixative solution	X		X	X		X	X
PVA powder [†]							
MIF solution	X		X	X			
SAF solution	X						
Concentration systems						X	
Formalin–ethyl acetate	X		X	X			
Zinc sulfate (sp gr, 1.18/1.20)	X		X			X	
Stains							
Trichrome – solution	X(W)		X(W)	X(W)	X(W, G)	X(W)	X(W)
Trichrome – modified [†]	X		X	X		X	X
Trichrome – dye powders							
Chromotrope 2R		X			X		
Fast green FCF		X			X		
Light green SF yellowish		X			X		
Hematoxylin – solution	X		X		X		
Hematoxylin – powder		X			X		
Chlorazol – black E-powder					X		
Giemsa – solution	X	X	X		X		X
Giemsa – powder		X			X		
Rapid blood stains			X				X
Carbol fuchsin–Kinyoun's	X	X	X		X		X
Modified acid-fast	X	X					
Modified acid-fast with DMSO							X
Calcofluor white	X						X
Auramine–rhodamine			X				X
Acridine orange							X
Toluidine blue O		X	X				
Miscellaneous							
Lugol's iodine dilute 1:5	X	X	X	X	X		X
D'Antoni's iodine	X		X			X	X
Triton X–100	X		X	X		X	X
Eosin/saline solution (1%)			X				
Mayer's albumin	X						X
Control slides or suspensions	X			X		X	X
Alcohols, xylene	X		X				
Methanol (thin blood film)					X		X
Ethanol 70%	X		X		X		X
Ethanol 90%							X
Ethanol 95%	X				X		
Ethanol absolute	X		X				
Xylene	X		X		X		
Carbol-xylene	X						
Xylene substitutes	X		X				X

W, Wheatley; G, Gomori; +, with acetic acid; –, without acetic acid.

* Contains no mercury, formalin, or polyvinyl alcohol (PVA) (TOTAL-FIX).

[†] Use grade with high hydrolysis and low viscosity for parasite studies.

[‡] Used for the identification of microsporidia spores in stool or other specimens.

[§] Since products can frequently vary, please consult the vendor's web site and representatives for current product availability and pricing.

vaginitis: *Candida* species, *Gardnerella vaginalis*, and *T. vaginalis*. During the test protocol, the assay aligns complementary nucleic acid strands to form specific, double-stranded complexes called hybrids. For each organism, this hybrid is composed of capture and color development single-stranded nucleic acid probes, complementary to the released target

nucleic acid analyte. Enzyme conjugate then binds to the captured analyte.

In January 2013, the FDA approved the first test that can simultaneously detect common viral, bacterial, and parasitic (*Cryptosporidium*, *Giardia*) causes of infectious gastroenteritis from a single patient sample (xTAG

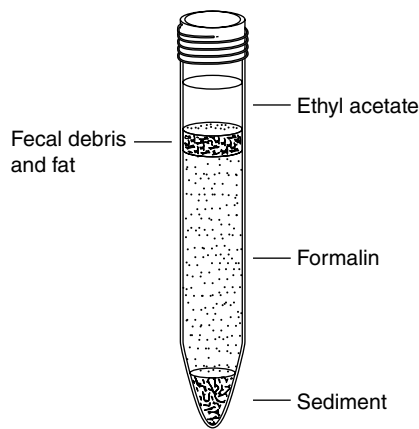


Figure 15.15 Traditional fecal concentration system (sedimentation method diagram). (Courtesy of Hardy Diagnostics and Garcia, LG 2016, *Diagnostic Medical Parasitology*, 6th edn, ASM Press, Washington, DC.)



Figure 15.17 FPC JUMBO fecal concentration system. (Courtesy of Evergreen Scientific.)

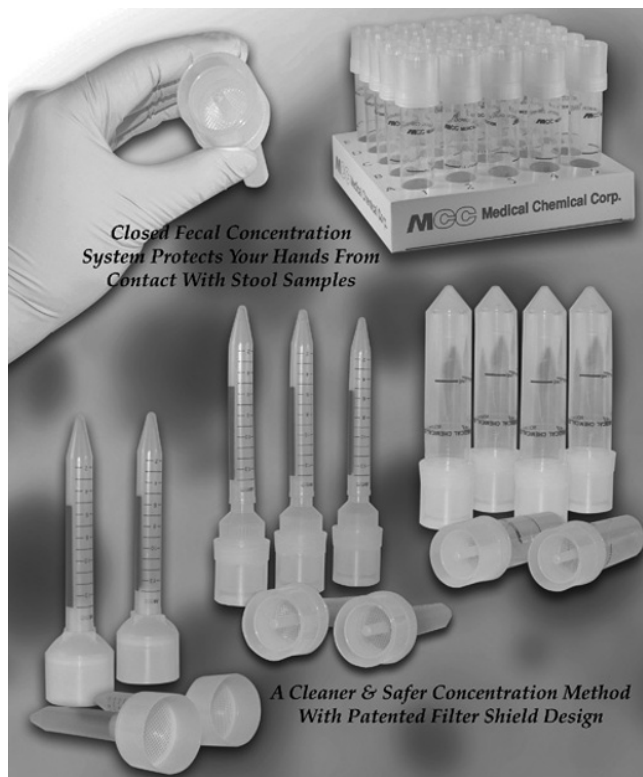


Figure 15.16 Closed fecal concentration system. (Courtesy of Medical Chemical Corporation.)



Figure 15.18 FPC Hybrid fecal concentration system. (Courtesy of Evergreen Scientific.)



Figure 15.19 Para-Pak Plus fecal concentration system. (Courtesy of Meridian Bioscience, Inc.)

Gastrointestinal Pathogen Panel, Luminex, Inc., Austin, TX). The xTAG GPP is a multiplexed nucleic acid test that detects various causes of gastroenteritis, inflammation of the stomach and intestines.

The BioFire FilmArray Gastrointestinal Panel is another FDA-approved multiplex PCR test that can simultaneously detect gastrointestinal pathogens in about an hour (BioFire FilmArray Gastrointestinal Panel, Salt



Figure 15.20 Para Kit, fecal concentrator kit. (Courtesy of Hardy Diagnostics.)



Figure 15.21 Para Kit, fecal concentration funnel. (Courtesy of Hardy Diagnostics.)



Figure 15.22 CONSED CLR and concentration tube. (Courtesy of Alpha-Tec Systems Inc.)



Figure 15.23 FE-5 semiautomated sampling and examination system for fecal concentration sediments. (Courtesy of Apacor Ltd USA, Brooklyn and New York.) (See insert for color representation of the figure.)



Figure 15.24 Detailed view (from Figure 15.23) of the glass viewing chamber that holds a portion of the concentration sediment and rests on the microscope stage. The fecal material is viewed through the microscope focused on the contents in the chamber. (Courtesy of Apacor Ltd USA, Brooklyn and New York.) (See insert for color representation of the figure.)



Figure 15.25 Example of parasitology stain: Trichrome-Blue stain for the microsporidia. (Courtesy of Medical Chemical Corporation.)

Lake City, UT). The panel includes *Cryptosporidium*, *Cyclospora cayatanensis*, *E. histolytica*, and *G. lamblia*, along with a number of bacteria, and viruses. The BD Max Enteric Panel (Becton, Dickinson and Co., Sparks, MD)



Figure 15.26 Example of parasitology stain: Trichrome. (Courtesy of Scientific Device Laboratory)

is another multiplex test that can simultaneously detect *Giardia*, *Cryptosporidium* and *E. histolytica* in both unpreserved and preserved (10% formalin) stool specimens, in addition to other bacteria and toxins.

There are also a number of other molecular tests that are in clinical trials for the detection of select gastrointestinal parasites. These tests are molecular gastrointestinal panels and target the most commonly occurring bacterial, viral, and parasitic stool pathogens. Although there are laboratory-developed tests for most parasites, these are not commercially available or available only in specialized testing centers. When such tests are used, there should be attention given to the use of internal amplification controls to detect inhibition, since common specimens, such as blood and



Figure 15.27 Various stains used in diagnostic parasitology. (Courtesy of Medical Chemical Corporation.)



Figure 15.28 Various reagents and stains used in the ECO-Stain system. (Courtesy of Meridian Bioscience, Inc.)



Figure 15.29 Mayer's albumin used as an adhesive for preparation of permanent stains from SAF fixative. (Courtesy of Hardy Diagnostics.)

stool, may contain PCR inhibitors. Thorough validation is required before these are implemented for clinical testing.

15.9 Other diagnostic methods

Several other diagnostic techniques are available for the recovery and identification of parasitic organisms. Most laboratories do not routinely offer all of these techniques, but many are relatively simple and inexpensive to perform. The clinician should be aware of the possibilities and the clinical relevance of information obtained from using such techniques. Occasionally it is necessary to examine stool specimens for the presence of scolices and cestode proglottids and adult nematodes and trematodes to confirm the diagnosis and/or for identification to the species level.

15.10 Collection of specimens from other body sites

Although clinical specimens for examination can be obtained from many other body sites, these specimens and appropriate diagnostic methods are not as commonly performed as those used for the routine stool specimen.

The majority of specimens from other body sites (Table 15.2) would be submitted as fresh specimens for further testing. Another example would be examination of paddles or devices designed to sample perianal skin for the presence of pinworm eggs (Figures 15.30 and 15.31). Another simplified plastic pouch culture option has been developed for the isolation and identification of *T. vaginalis*. This approach has proven to be much more sensitive than the examination of wet preparations alone and has been incorporated into use in many

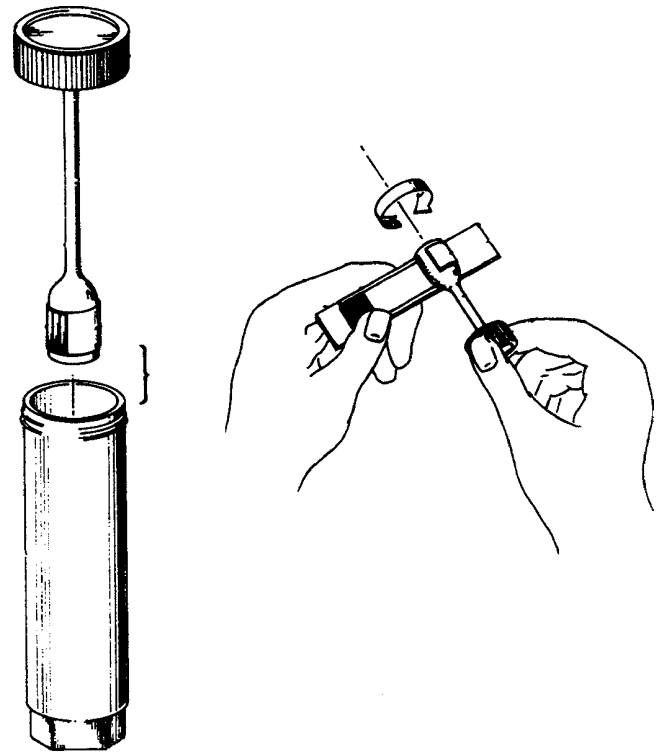


Figure 15.30 PCA pinworm egg collection-transport apparatus. The PCA combines the utility of the cellulose tape with the ready-to-use convenience of the adhesive paddle. (Courtesy of Evergreen Scientific.)



Figure 15.31 BD Falcon SWUBE pinworm paddle. (Courtesy and © Becton, Dickinson and Company.)

institutions with dramatic increases in the number of positive specimens identified (Figures 15.32 and 15.33) (Table 15.6) [11,15,31,36,40,51,58].

15.11 Blood collection

There are a number of parasites that may be recovered in a blood specimen, either whole blood, buffy coat preparations, or various types of concentrations



Figure 15.32 InPouch TV transport/culture medium for the growth and identification of *Trichomonas vaginalis*. (InPouch™ TV™ Image Courtesy of BIOMED Diagnostics, Inc.). (Garcia, LS. 2016. *Diagnostic Medical Parasitology*, 6th edn, ASM Press, Washington, DC.)

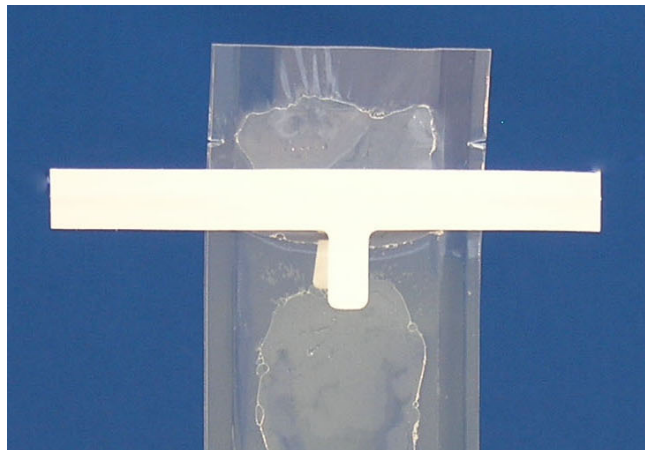


Figure 15.33 Inoculation of the InPouch™ TV™ culture pouch for the growth and identification of *T. vaginalis*. (InPouch™ TV™ Image Courtesy of BIOMED Diagnostics, Inc.)

[9,17,33,47,49,57]. These parasites include *Plasmodium*, *Babesia*, and *Trypanosoma* species; *Leishmania donovani*; *Toxoplasma gondii*, and microfilariae. Although some organisms may be motile in fresh, whole blood, usually organism identification is accomplished from the examination of permanent stained blood films, both thick and thin films. Blood films can be prepared from

fresh, whole blood collected with no anticoagulants, anticoagulated blood, or sediment from the various concentration procedures [20,21,38,39,45].

Many laboratories receive blood for these procedures in a tube rather than relying on finger-stick blood. Ethylenediaminetetraacetic acid (EDTA) anticoagulant is preferred, and the tube should be filled with blood to provide the proper blood/anticoagulant ratio. For detection of stippling, the smears should be prepared within 1 h after the specimen is drawn. After that time, stippling may not be visible on stained films; however, the overall organism morphology will still be excellent. Most laboratories routinely use commercially available blood collection tubes that are readily available through most supply houses.

The time the specimen was drawn should be clearly indicated on the tube of blood and also on the result report. The physician will then be able to correlate the results with any fever pattern or other symptoms of the patient. There should also be some comments on the test result report that is sent back to the physician that one negative specimen does not rule out the possibility of a parasitic infection.

Although microscopy of Giemsa-stained thick and thin blood films has been used to diagnose malaria for decades, alternative approaches have been developed



Figure 15.34 BinaxNOW rapid Malaria test. (Courtesy of Alere, Inc.)



Figure 15.35 (Top) BinaxNOW Malaria rapid test card interpretation. (Bottom) Examples of positive results for the BinaxNOW Malaria rapid test card. (Courtesy of Alere Inc.)

that are sensitive, specific, and rapid and might not require laboratory equipment (Figures 15.34 and 15.35). Various methods include fluorescence microscopy (acridine orange), fluorescent microscopy after centrifugation (QBC, glass capillary tube and closely fitting plastic insert; CBC malaria blood tubes [Becton Dickinson, Tropical Disease Diagnostics, Sparks, Md.] has been used for the detection of blood parasites), and flow cytometry [46]. A number of other stains are also available, including the rapid blood stains.

15.12 Malaria rapid diagnostic tests

Malaria is a serious parasitic disease, particularly in the non-immune traveler, and prognosis depends on timely diagnosis. Microscopy remains the gold standard of diagnosis; however, malaria rapid diagnostic tests (RDTs) are increasingly used in nonendemic settings [43]. Malaria RDTs, sometimes called “dipsticks” or malaria rapid diagnostic devices (MRDDs), are used in the diagnosis of malaria by providing evidence of the presence of malaria parasites in human blood. RDTs are an alternative to diagnosis based on clinical grounds or microscopy, particularly where good quality microscopy services cannot be readily provided; currently, there are more than 200 RDTs available commercially. RDTs are lateral flow immunochromatographic antigen-detection tests, which rely on the capture of dye-labeled antibodies to produce a visible band on a strip of nitrocellulose. Some examples of available products can be seen in Table 15.8; more extensive lists can be found on the web site http://www2.wpro.who.int/NR/rdonlyres/3B0EEC88-A85A-4C66-BED8-46F13F47D087/0/RDT_selection_criteria2012.pdf (last accessed May 22, 2014). Reported sensitivities vary between different RDT products but are generally good for *Plasmodium falciparum*, with RDTs detecting the *P. falciparum* antigen histidine-rich protein-2 (PfHRP2) scoring slightly better than *P. falciparum*-lactate dehydrogenase (Pf-pLDH)-detecting RDTs. Some detect one or more of the other three species of malaria that infect humans by detecting various other antigens. RDTs for the fifth human malaria, *Plasmodium knowlesi*, are currently under development.

However, these tests do have limitations, including persistence of the PfHRP2 antigen, cross-reactions of *P. falciparum* with the non-falciparum test line and vice versa and (rare) false-positive reactions due to other infectious agents or immunological factors. False-negative results occur in the case of low parasite densities, which may be a problem with immunologically naïve patients who tend to become symptomatic very early with parasitemias as low as $< 0.002\%$ or lower ($100/\mu\text{L}$). As an example, the BinaxNOW (the only FDA-approved RDT in the United States at the time of this writing), has a maximum sensitivity at 0.1% ($5000/\mu\text{L}$). The same test may achieve a high sensitivity in a population in which all infected people have high parasite density (e.g., above $10000/\mu\text{L}$), but achieve low sensitivity in areas where parasite densities are frequently below $200/\mu\text{L}$. RDTs are a valuable adjunct to (but not a replacement for) microscopy for the diagnosis of malaria in the returned traveler. In malaria endemic settings, many of which may be in remote areas, potential problems may include limited temperature control, poor resupply options, and limited training for health workers.

RDTs are available in three different formats. The simplest form is a dipstick, which is placed in wells containing blood and/or buffer. The nitrocellulose strip may be

Table 15.8 The following is a list of rapid diagnostic test kits (RDTs) for the detection of antigen of *Plasmodium* species that are currently available for sale (as of the time of writing). This is not a complete listing of available products, but reflects information that was readily available. Products for the detection of antibody are not included

Manufacturer	Product name	Catalog number	Malaria species*	Target antigen	Test format
Alere (FDA approved)	BinaxNOW Malaria	Not stated	Pf/Pan	Pf HRP2 LDH	Lateral flow
Alere	Clearview Malaria Pf test	Not stated	Pf	Pf HRP2 Aldolase	Lateral flow
Alere	Clearview Malaria Combo	Not stated	Pf/Pan	Pf HRP2 Aldolase	Lateral flow
Core Diagnostics	Core Malaria Pf	Not stated	Pf	Pf HRP2	Lateral flow
Core Diagnostics	Core Malaria Pan-Pf	Not stated	Pf/Pan	LDH Pf HRP2	Lateral flow
Core Diagnostics	Core Malaria Pv-Pf	Not stated	Pf/Pv	LDH	Lateral flow
Core Diagnostics	Core Malaria Pan-Pv-Pf	Not stated	Pf/Pv/Pan	LDH Pf HRP2	Lateral flow
Diagnostic Automation	Malaria (Pf/Vivax)	172110-25	Pf/ Pv	Pf HRP2 Pv LDH	Lateral flow
Cortez Diagnostics	Malaria (Pan-LDH)	172120-25	Pan	LDH	Lateral flow
Diagnostic Automation					
Human GmbH	Hexagon Malaria Combi	58054	Pf/Pan	Pf HRP2 Aldolase	Lateral flow
Immunospec	Malaria Pf	R14-537	Pf/Pan	LDH	Lateral flow WB
Immunospec	Malaria Pf	R14-530	Pf	LDH	Lateral flow WB
Immunospec	Malaria Pf/vivax Cassette	R14-538	Pf/Pv	LDH	Lateral flow WB
Immunospec	PF Malaria Rapid test	R6-537	Pf	LDH	Lateral flow WB
Omega Diagnostics	Visitect Malaria Pf	OD026	Pf	Pf HRP2	Lateral flow
Omega Diagnostics	Visitect Malaria Combo	OD206	Pf/Pan	Pf HRP2 LDH	Lateral flow
Omega Diagnostics	Paracheck Pf Dipstick	Not stated	Pf	Pf HRP2	Solid phase dipstick
Premier Medical Corp	First Response Malaria Pf, Pv Ag	106FRS48	Pf/Pan	LDH	Lateral flow strip
Premier Medical Corp	First Response Malaria Ag	112FRC30	Pan	LDH	Lateral flow card
Premier Medical Corp	First Response Malaria Ag	113FRC30	Pf	Pf HRP2	Lateral flow card
Premier Medical Corp	First Response Malaria Ag	115FRS30	Pf/Pan	LDH Pf HRP2	Lateral flow strip
Premier Medical Corp	First Response Malaria Ag Combo Rapid	116FRC30	Pf/Pan	LDH Pf HRP2	Lateral flow card
Span Diagnostics	ParaHIT-f	25977	Pf	Pf HRP2	Solid phase dipstick
Span Diagnostics	ParaHIT-f	25978	Pf	Pf HRP2	Lateral flow device
Span Diagnostics	ParaHIT Total	25988	Pf/Pan	Pf HRP2 Aldolase	Solid phase dipstick
Span Diagnostics	ParaHIT Total	25989	Pf/Pan	Pf HRP2 Aldolase	Lateral flow
Standard Diagnostics	SD BIOLINE Malaria Ag	05FK40	Pf/Pan	Pf HRP2 LDH	Lateral flow
Standard Diagnostics	SD BIOLINE Malaria Ag Pf	05FK50	Pf	Pf HRP2	Lateral flow
Standard Diagnostics	SD BIOLINE Malaria Ag Pf/Pan	05FK60	Pt/Pan	Pf HRP2 LDH	Lateral flow
Zephyr Biomedicals (Tulip Group)	PARABANK	50301010 50301025	Pan	LDH	Lateral flow device
Zephyr Biomedicals (Tulip Group)	PARABANK	50302010 50302025	Pan	LDH	Lateral flow dipstick
Zephyr Biomedicals (Tulip Group)	PARASCREEN	50310010 50310025	Pan	Pf HRP2 LDH	Lateral flow device
Zephyr Biomedicals (Tulip Group)	FALCIVAX	50300010 50300025	Pf, Pv	Pf HRP2 Pv LDH	Lateral flow device
Zephyr Biomedicals (Tulip Group)	PARAMAX-3	50320010 50320025	Pf/Pv/Pan	Pf HRP2 Pv LDH Pan LDH	Lateral flow device
Zephyr Biomedicals (Tulip Group)	MALASCAN	50402001 50402010 50402025	Pf/Pan	Pf HRP2 Pan LDH	Lateral flow device

WB, Western blot.

* Although these rapid malaria tests were not developed for detection of the fifth human malaria, *Plasmodium knowlesi*, a few cases have been confirmed using these rapid tests. Specific rapid tests that include *P. knowlesi* are under development.

placed in a cassette or the third option is to place the strip on a card. Although the cassettes and cards are more expensive, they are considered the easiest to use. As with any diagnostic test system, it is critical that directions be followed and interpretive criteria be thoroughly understood by the user. Also, when diagnosing malaria, patient signs and symptoms should always be considered, in addition to the RDT result.

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Molecular Microbiology

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16.1 Introduction

The advent of polymerase chain reaction (PCR) molecular techniques has revolutionized our approach and practice of clinical microbiology. Advances in technologies, reduction in operating cost and availability of complete automation from sample preparation to real-time amplification technology with true walk-away freedom capabilities have enabled the routine use of molecular assays in numerous clinical laboratories. Since publication of the first edition of this manual, in the past decade, with improvements and new approaches in technology the first-generation monoparametric nucleic acid assays are being slowly replaced by multiparameter platforms incorporating multiplex nucleic acid amplification techniques, microarrays, mass spectrometry, or sequencing. While first-generation molecular assays were predominantly directed towards detection and identification of single microbial pathogens, new technologies enable parallel determination of multiple microbial pathogens, virulence factors/resistant mechanisms, microbial typing, and surveillance on a genetic level.

While molecular technologies offer sensitive and specific results with a rapid turnaround time that has never before been possible, the necessity to adopt a consistent approach to assay validation, verification, standardization, and quality assurance/quality control issues have become of paramount importance. Any molecular diagnostic procedure can be divided into three components: (i) specimen processing and nucleic acid extraction, (ii) nucleic acid amplification, and (iii) amplification products (amplicons) detection and identification [45,65]. This chapter provides a brief overview of principles of various technologies used in the three components for multiparametric molecular diagnostics tests.

16.2 Specimen processing and nucleic acid extraction platforms

Paramount for any molecular diagnostic assay is the quality of sample. The best specimen for laboratory examination depends on the clinical setting of the patient as well as the site of infection. Nucleic acid extraction is one of the most basic requirements in a molecular biology laboratory, which has a major impact on the reliability of results in routine molecular diagnostics. The process of nucleic acid extraction consists of multiple steps beginning with disruption and lysis of the pathogen and/or host cells, inactivation of cellular nucleases, capture of nucleic acids, purification (removing proteins and cell debris), and elution of the purified nucleic acids. Also, different pre-PCR processing strategies such as optimizing the sample preparation and elimination of trace levels of extraction reagents in the purified sample can enhance the sensitivity of downstream assays.

Traditional manual nucleic acid extraction is labor intensive, time-consuming, and is susceptible to contamination, handling variations, and errors. Several commercial extraction methods are available that can completely automate the process, thus minimizing the above-mentioned variables at high throughput from a broad range of clinical specimens in a short time. Initial optimization of the extraction process is crucial and should include true clinical samples to evaluate matrix effects on the extraction method and assay performance. Coextraction of nucleic acids from patient samples, with internal or external controls (DNA or RNA, as appropriate), aid in monitoring assay performance and the presence of amplification inhibitors that could lead to false-negative results [5,50]. Choice of inappropriate methods for nucleic acid extraction may introduce bias to clinical diagnosis or epidemiological investigation, as only organisms susceptible to the method selected would be detectable at lower levels.

16.2.1 Isolation/extraction techniques

Several nucleic acid extraction methods have been used in clinical microbiology laboratories. The classic alkaline lysis and the traditional manual phenol–chloroform extraction/ethanol precipitation methods, although least expensive, are not the method of choice in diagnostic clinical laboratories as they are associated with serious practical limitations, including using toxic chemicals and the time required for processing a large number of samples [17]. Several nucleic acid extraction kits such as Isoquick (ORCA Research Inc., Bothell, WA) and RNazol (Molecular Research Center, Inc., Cincinnati, OH) are still used in the research and molecular genetic laboratories to handle small sample volumes [59].

Most commercially available extraction kits and automated extraction platforms are based on reversible binding of nucleic acid molecules on the silica in the presence of high salt concentration and optimal pH conditions. Silica-based technologies are founded on the principle that nucleic acids are adsorbed to the silica gel membrane in the presence of high concentrations of chaotropic salts. The ready-to-use nucleic acids eluted in small volumes of low-salt buffer are free of unwanted cellular debris, polysaccharides, and proteins. Silica can be coated on to membrane filters or magnetic particles. Plastic columns containing silica-impregnated filters are one commonly adopted design. Commercial kits, including those from Qiagen, Promega, Invitrogen and Ambion, are silica membrane-based methods [17,35,42].

Magnetic bead [4] based extraction processes use ion-seeded silica with switchable charges to bind with the target DNA/RNA sample in the specimen, followed by applying a magnetic field to collect the magnetic beads bound with the target nucleic sample from the crude clinical samples. Studies have shown that magnetic bead technology typically results in more efficient elimination of inhibitory substances from heavily contaminated specimens such as stool [52,66] and urine samples [64]. Advantages of magnetic beads compared to other technologies for isolating DNA/RNA include efficient binding of nucleic acids to beads, resulting in better quality, and higher and more consistent DNA/RNA yields. It has become the dominant nucleic acid extraction method in clinical microbiology laboratories.

The introduction and gradual mainstreaming of automated instruments for the extraction of nucleic acids from biological samples has had a significant impact on molecular diagnostics. Manual extraction procedures, owing to their limited throughput, turnaround time, expenses related to labor, variability in technique among analysts, and the potential for sample contamination, are rapidly becoming obsolete in clinical laboratories [17,35,50]. Automated nucleic acid extractors have the

potential to improve workflow and decrease variability in the clinical laboratory. In contrast to low-throughput manual kits, automated DNA and RNA purification systems can process 12–96 samples in less than 30 min or 1 h, respectively, and allow various handling volumes ranging from 1 uL to 1000 uL. Touch-screen and user-friendly interfaces provide nucleic acid purification simplicity, and robotic arms decrease contamination. The automated nucleic acid extraction platforms are easy to perform and result in a strong reduction of hands-on time compared with manual protocols [17,35,38]. Commercial extraction instruments are available in different formats including low-volume random access, and batched, and high-volume high throughput.

Automated high-throughput extraction systems purify nucleic acids that are free of proteins, nucleases, and other impurities. Automated processing, including barcode reading, load check, sample tracking with complete process documentation, improve workflow and decrease variability in the clinical laboratory. Comparative studies of automated commercial nucleic acid extraction systems have shown differences in nucleic acid recovery, reproducibility, and linearity in a pathogen-specific manner [68]. Criteria for selection of an automated instrument in a clinical laboratory should be based on practical considerations such as volume, throughput, flexibility, cost, size, ease of use, and performance [35]. Other important considerations for choice of systems are: number of samples processed daily and the available laboratory space, the ability to effectively extract nucleic acids from different organisms and from various sample substrates, and optimal isolation of nucleic acids and removal of inhibitors [54]. Determination of analytical and clinical sensitivity and specificity, as well as positive and negative predictive value are other important factors to thoroughly evaluate for any specific molecular diagnostic assay [5,54]. Furthermore, the results must be reproducible, and the process economical [42].

16.3 Amplification methods and platforms

The past decade has witnessed advances in the rapid detection assays, and clinical microbiology laboratories are increasingly relying on the sensitive and rapid enzyme-mediated *in vitro* nucleic acid amplification techniques. Advanced techniques in molecular assays have opened new avenues in diagnostic microbiology for the detection, identification, and characterization of pathogenic organisms. To that end, they offer a promising alternative to routine culture-based methods by reducing the time to identification and enhancing patient care. Conceptually, the amplification techniques can be divided into three

Table 16.1 Comparison of different nucleic acid methods

Amplification method	Reaction condition	Technology	Enzyme(s)	Specificity	Sensitivity	Multiplexing
Target	Thermal cycler	Polymerase chain reaction (PCR)	<i>Taq</i> DNA polymerase	++++	++++	++
Target	Isothermal	Transcription mediated amplification (TMA)	Reverse transcriptase, RNA polymerase, RNase H	++	+++	+
Target	Isothermal	Nucleic acid sequence-based amplification (NASBA)	Reverse transcriptase, RNA polymerase, RNase H	++	+++	+
Target	Isothermal	Strand displacement amplification (SDA)	Restrictive endonuclease, DNA polymerase	++	++++	+
Target	Isothermal	Loop-mediated isothermal amplification (LAMP)	DNA polymerase	++++	++++	+
Target	Isothermal	Helicase-dependent amplification (HDA)	Helicase, DNA polymerase	++	+++	+
Probe	Isothermal	Cycling probe technology (CPT)	Rnase H	++	+++	+
Probe	Thermal cycler	Ligase chain reaction (LCR)	DNA polymerase, DNA ligase	++++	++++	++
Signal	Isothermal	Invader technology	Cleavase	++	+++	+
Signal	Isothermal	Hybrid capture system	Alkaline phosphatase	+	+++	+
Signal	Isothermal	Branched DNA (bDNA)	Alkaline phosphatase	++	++++	+

broad categories: (i) target amplification systems, (ii) probe amplification systems, and (iii) signal amplification systems. Commercially available amplification methods based on these categories are summarized in Table 16.1. The common advantages are the ability to provide vastly improved sensitivity over conventional methods, particularly for the detection of fastidious, uncultivable, and/or highly contagious organisms.

16.3.1 Target amplification methods

A variety of technologies based on target amplification methods allow post-amplification analysis depending on the clinical needs for the assay. The basic principle relies on the *in vitro* production of multiple copies of the nucleic acid sequence of interest. The most common application of target amplification method is PCR. Other amplification methods, such as isothermal methods of nucleic acid sequence-based amplification (NASBA) [9] and loop-mediated isothermal amplification (LAMP) [43,46], unlike the PCR amplification chemistries, eliminate the requirement for different temperatures during amplification.

16.3.1.1 PCR amplification

PCR today has been adapted for various applications. Several modifications are used in the clinical laboratory. These methods are capable of detecting multiple targets in a single run (multiplex PCR), using RNA templates (reverse transcriptase PCR), or such amplified products as templates (nested PCR) and quantitating starting templates (quantitative PCR, or real-time PCR) [16]. Real-time PCR (RT-PCR) based on fluorogenic detection has become the technology of choice for accurate, reproducible quantification of DNA and RNA [51,65]. PCR-based typing methods such as repetitive-element PCR

(REP-PCR) requires shorter time to result and requires less technical skill and less hands-on time. REP-PCR typing is based on the presence of multiple copies of short repetitive sequences found in microbial genomes [30]. These sequences are interspersed throughout the genome and are usually located in noncoding regions of DNA. The number of repetitive elements and their respective genomic locations are used to genotype isolates and to differentiate highly related strains. The DiversiLab system [25] (bioMérieux, Inc., Durham, NC) is a commercial REP-PCR typing system that offers a complete package for typing and data analysis, with database libraries for organisms including methicillin-resistant *Staphylococcus aureus* and *Clostridium difficile*.

Today, several PCR assays are commercially available for specific organism detection. More recently, applications of qualitative and quantitative real-time PCR, nested PCR, and multiplex PCR are increasingly being adapted for commercial diagnosis of virus, bacterial, fungal, and parasitic infections [2,3,7,10–12,21,61]. Detection of amplification products real-time provides the advantage of simultaneous amplification and detection, thereby reducing the risk of contamination and subsequent false-positive results [24]. Multiplexing necessitates more rigorous assay design and provides a number of unique challenges to PCR chemistries and instrumentation. Robust quality control processes and informed data analyses are required to maintain assay reliability. Recent improvements in chemistries and instrumentation make it possible to create assays that bypass or mitigate previous limitations. Despite the success of currently available multiplex panels, further progress is required to bring their use to the forefront of molecular testing. Contamination potential, lack of standardization or validation for some assays, complex interpretation of results, and increased

cost are possible limitations of these tests, and must be carefully considered before implementing them in the clinical laboratory [5,54].

16.3.1.2 Loop mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a simple, rapid, and specific nucleic acid amplification method, which is characterized by the use of multiple primers specifically designed to recognize several distinct regions on the target gene. Two inner and two outer primers define the target sequence and an additional set of loop primers are added to increase the sensitivity of the reaction. Amplification and detection of target genes is generally completed in a single step, by incubating the mixture of samples, primers and DNA polymerase with strand displacement activity and substrates at a constant temperature. A significant advantage of the isothermal amplification process is the ability to detect a positive reaction by simple turbidity measurements or direct visualization by the naked eye without any expensive equipment. Another advantage of LAMP over PCR is the ability to detect the target of interest earlier, particularly at low gene copy number. However, primer design for LAMP is more complex than for PCR, with specialized training and software required for their design. The Illumigene molecular system (Meridian Bioscience, Inc, Cincinnati, OH), an FDA-cleared LAMP assay initially available for detection of *C. difficile* [36], now has been extended for both Group A and B *Streptococcus* detection [1]. Modification of LAMP has successfully been applied to the rapid and real-time detection of several emerging and reemerging human pathogens [44,48,49]. Several LAMP-based assays were reported for rapid detection of influenza viruses and severe acute respiratory syndrome coronavirus (SARS-CoV), with the advantages of rapid amplification, simple operation, and ease of detection. LAMP-based assays have also been used to detect other respiratory viral pathogens, such as mumps, measles, and adenoviruses. In comparison to conventional RT-PCR, RT-LAMP assays have demonstrated 10- to 100-fold-enhanced sensitivity, with a detection limit of 0.01 to 10 plaque-forming units of virus in most cases [31].

16.3.1.3 Transcription-mediated amplification

In contrast to PCR, transcription-mediated amplification (TMA) systems are based on isothermal amplification. There are a number of commercial variations of this process: TMA (Gen-Probe/Hologic, San Diego, CA) and nucleic acid sequence-based amplification (NASBA; bioMérieux, Durham, NC). TMA is similar to NASBA, except that it uses the RNaseH activity of reverse transcriptase, whereas NASBA uses a separate enzyme. Both TMA and NASBA have several distinct advantages over other RNA amplification techniques. The fact that the initial process

does not require prior denaturation before amplification, rendering the dsDNA sequences incapable of binding to primers in the reaction, obviates the problem of DNA contamination giving a falsely elevated determination of RNA. The TMA and NASBA systems-based products have been used successfully in a wide variety of applications, including viruses such as HIV, HCV, varicella zoster virus, cytomegalovirus (CMV), rhinovirus, enterovirus, measles, human papillomavirus, and bacteria such as *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma pneumoniae*, and *Campylobacter jejuni*. In addition, quantitative tests for HIV and HCV have been developed using this technology [8].

16.3.1.4 Strand-displacement amplification

Another example of the isothermal amplification process is strand-displacement amplification (SDA; Becton Dickinson, Sparks, MD). The principle is more similar to ligase chain reaction (LCR) in that the major amplification products are the probes/primers and not the product of *in vitro* synthesis of target DNA. The first stage of SDA is the denaturation of the double-stranded target and annealing of primers and probes tailed with sequences including a restriction enzyme site. The second stage of the reaction is the exponential probe/target amplification phase. The product of this amplification is millions of copies of the initial probe. SDA is used in the diagnosis of *M. tuberculosis*, *C. trachomatis*, and *N. gonorrhoeae*.

16.3.1.5 Helicase-dependent amplification

The helicase-dependent amplification (HDA) assay utilizes DNA helicase to unwind the double-stranded DNA. Polymerizing enzymes along with sequence-specific primers are then used to extend the primers annealed to the templates to produce a double-stranded DNA, resulting in exponential amplification of the selected target sequence. Some of the commercial uses of HDA technology available in the market are the direct, qualitative detection of the *C. difficile* Toxin A gene (*tcdA*) in unformed stool specimens (AmpliVue *C. difficile* Assay, Quidel, San Diego, CA) and direct, qualitative detection of herpes simplex virus (HSV-1 and HSV-2) DNA in male and female genital and oral lesions (IsoAMP HSV, BioHelix, Beverly, MA). Compared with the other amplification techniques it has the advantages of low cost for instrumentation, as isothermal DNA amplification system eliminates the need for a thermocycler, a versatile platform that can amplify both DNA and RNA (with a reverse transcriptase), and is compatible with multiple detection technologies. However, one of the major drawbacks is its inability to amplify DNA targets greater than 200 base pairs (bp).

16.3.2 Probe amplification methods

In probe amplification procedures, the number of target nucleic acid sequences in a sample is not changed, as it is in target amplification procedures like PCR. Rather, synthetic probes that are specific to the target sequences bind to the target where the probes themselves are amplified. Three major technologies that involve the amplification of probe sequences are: LCR, RNase H, and Q beta replicase. The LCR method of DNA amplification is similar to PCR but differs by amplifying the primers/probes complementary to target nucleic acid rather than producing amplicon through polymerization of nucleotides. LCR can have greater specificity than PCR since the entire target sequence must be known in order to prepare the oligonucleotide primers for LCR. Commercially, products based on probe amplification methods are currently unavailable. However, one technique, multiplex ligation-dependent probe amplification (MLPA), uses both ligation and PCR to greatly reduce probe concentrations and longer hybridization periods to generate conditions compatible with multiplex analysis [57].

16.3.3 Signal amplification methods

Signal amplification differs from target amplification procedures in that the large signal present in the sample is bound to the target sequences but the concentration of probe or target does not increase. As such the number of target molecules is not altered and, as a result, the signal is directly proportional to the amount of target sequence present in the clinical specimen. This reduces concerns about false-positives due to cross-contamination and simplifies the development of quantitative assays. Several signal amplification methods are available commercially. Branched DNA and hybrid capture are examples of signal amplification systems that have developed into diagnostic tests.

16.3.3.1 Branched DNA

Branched DNA (bDNA) is a signal amplification technology used in clinical and research laboratories to quantitatively detect nucleic acids. Unlike techniques that rely on in vitro amplification of the target sequence (i.e., PCR, transcription-mediated amplification, nucleic acid sequence-based amplification, and strand displacement amplification) the sensitivity of bDNA methods is based on signal amplification on the bDNA probe following direct binding of a large hybridization complex to the target sequence. Consequently, bDNA assays have lower susceptibility to contamination risks than those associated with PCR-based assays. Additional advantages compared to PCR assay include robust quantitation across genotypes, as minor sequence variation in probe-binding regions of target does not compromise assay performance, especially

at the low end of an assay's dynamic range. First-generation bDNA assays were developed for the detection of HIV-1 RNA and HCV RNA in human plasma (Quantiplex HIV-1 or HCV RNA 1.0 assay, Siemens, Tarrytown, NY). More recent efforts have focused on the development of target-specific oligonucleotides (label extenders and capture extenders), which hybridize with high stringency to the target nucleic acid. Third generation assays such as VERSANT HIV-1 RNA 3.0 Assay (bDNA) ("Versant Assay") with shorter target incubation drastically improve the turn around time and may be ideal for laboratories with especially stringent cost, contamination, or reliability requirements [69].

16.3.3.2 Hybrid-capture assays

Hybrid-capture technology provides an accurate, inexpensive, user-friendly signal amplification technology for RNA/DNA detection. Although considered less sensitive than other amplification methods, hybrid-capture assay is less prone to false-negatives due to target degradation by virtue of having multiple probes along an extended target. Additionally, this technology is well adapted to excellent high- or low-volume testing and reproducibility and low crossover contamination due to signal instead of target amplification. The Digene Hybrid Capture system (Qiagen, Valencia, CA) is a commercial assay for detection of human papillomavirus and combined qualitative detection of *C. trachomatis* and *N. gonorrhoeae* from cervical specimens [13,28,56].

16.3.3.3 Cleavase/Invader technology

The Invader chemistry is a probe amplification method based on two simultaneous isothermal reactions. The pathogen of interest is specifically and accurately detected in the primary reaction. This is followed by a second reaction for signal amplification and generic readout. Originally developed at Third Wave Technologies, now Hologic Gen-Probe Inc., San Diego, the technology is widely applied for diagnostic application such as the detection of high-risk genotypes of human papilloma viruses [27,58] in cervical samples and a wide variety of other applications such as inherited genetic disorders, including cystic fibrosis, drug metabolism, and agricultural plant and animal genetic testing.

16.4 Amplicon detection and identification platforms

The detection of the amplified product for the presence or absence of a target by a reliable method is quintessential for a molecular assay to be successful. The choice of detection method used in the diagnostic microbiology laboratory has significantly improved in recent years and can be classified as either qualitative or quantitative. The method

of choice can range in complexity from being able to detect one or two analytes to being able to identify multiple targets like those seen in a multiplex PCR.

16.4.1 Fluorescence *in situ* hybridization

Peptide nucleic acid (PNA) fluorescence *in situ* hybridization (PNA FISH) probes are highly sensitive novel diagnostic techniques combining to provide rapid and accurate diagnosis of infectious diseases [26]. PNA probes are DNA-like compounds with nucleotide bases attached to a neutral peptide backbone that allows improved hybridization characteristics with high degrees of specificity, strong affinities, and rapid kinetics resulting in enhanced hybridization to highly structured targets such as rRNA. Additionally, the relatively hydrophobic character of the PNA probes enables easy penetration of the hydrophobic cell wall after preparation of a standard smear. Commercially available PNA FISH (AdvanDx, Woburn, MA) probes for *S. aureus*, *Candida albicans* and *Enterococcus faecalis* offer rapid and accurate results for clinical diagnosis [29,62]. The PNA FISH probe is also a promising procedure for species-specific identification of culture-grown mycobacteria and for direct visualization of these organisms in tissue sections [37]. Recent improvements include development of the rapid 20-min QuickFish [14] and targeting *mecA* gene for determination of methicillin resistance.

16.4.2 Fluorescence resonance energy transfer

Numerous chemistries available allow the detection of amplified products in different RT-PCR platforms. Direct and indirect detection formats are available for diagnostic application including detection of bacteria, viruses, parasites, and fungi. Hydrolysis TaqMan probes are labeled with a fluorescent reporter molecule on the 5' end and a quencher molecule at the 3' end. If the target is present, the quenched probe hybridizes with the target, and DNA polymerase (having 5' exonuclease activity) then digests the probe. The reporter molecule is thereby freed from the quencher, and fluorescence is detected. Molecular beacon probes also contain a similar fluorescent reporter and a quencher molecule, but the constructed probe is folded in as a hairpin loop. Other probes based on the fluorescence resonance energy transfer (FRET) principle include linear oligoprobes commonly used in LightCycler assays, hybridization probes, and scorpions. For FRET product detection, a hybridization probe with a donor fluorophore, fluorescein, on the 3' end is excited by an external light source, which emits light that is absorbed by a second hybridization probe with an acceptor fluorophore at the 5' end. The acceptor fluorophore then emits light of a different wavelength that is measured with a signal that is proportional to the amount of specific PCR product. New and emerging technologies such as dual priming

oligonucleotides (DPO) and tagging oligonucleotide cleavage and extension (TOCE) could facilitate multiplex assays (Seegene Inc, Gaithersburg, MD) on existing real-time platforms that are currently found in most clinical laboratories. Automated sample-to-result multiplex RT-PCR assay formats for the detection and discrimination of multiple pathogens are continuously growing; however, on the downside laboratory validation of the multiplexed PCR panel can be laborious, expensive, and unreachable for many clinical laboratories.

16.4.3 Microarray

Array-based technologies offer high-level multiplexing capability providing a major advantage over conventional methods of detection. These have been used to differentiate numerous microbial pathogens within a group, as well as to identify a large variety of genetic determinants of drug resistance. FDA-approved microarray-based assays are currently available for the detection and differentiation of respiratory viruses, discussed in more detail in other chapters of this text. Briefly, the microarray system combines ligation-mediated amplification with detection of amplified products on a microarray to detect the pathogen and strain variation. Bacteria and viruses can be detected using a single array; other techniques, for example PCR, require separate tests for each suspected pathogen [41].

Multiplex PCR followed by hybridization of the amplified PCR products to specific capture probes on a solid or liquid microarray offers a major advantage over RT-PCR methods of detection. Suspension arrays are based on the coupling of oligonucleotide probes to microbeads that are color-coded using different ratios of two fluorescent dyes. A third dye is used for generating labeled target DNA, which is subsequently hybridized in suspension with a set of different beads, each carrying a different probe. The bead mixture is sorted by flow cytometry based on their internal colors and hybridized samples produce a fluorescent signal. The Luminex xMAP technique has been very active in developing and expanding the suspension array application. In addition to the respiratory panel, their gastrointestinal panel has recently received FDA clearance intended for the simultaneous detection and identification of multiple gastrointestinal pathogens including bacteria, viruses, and parasites [34,40].

Several multiplexing amplification and high-throughput detection systems are commercially available; examples include the FilmArray platform from BioFire Diagnostics, Inc. (Salt Lake City, UT); the Verigene Enteric Pathogen test from Nanosphere (Northbrook, IL); the Infiniti Respiratory Viral Panel from AutoGenomics, Inc. (Vista, CA); the BD Max Enteric Bacterial Panel and Enteric Parasite Panel from Becton Dickinson, (Sparks, MD); the Multi-Code-PLx respiratory virus panel from

EraGen Biosciences (Madison, WI; acquired by Luminex); the NGEN Respiratory Virus ASR from Nanogen (San Diego, CA); the proFLU1 and the proPARAFLU1, the Prodesse Progestro SSCS, and the Aptima System from Gen-Probe/Hologic (San Diego, CA); the ResPlex II assay from Qiagen (Valencia, CA); the Seeplex respiratory virus detection assay from Seegene, Inc (Gaithersburg, MD); and the xTAG Respiratory Viral Panel and xTAG Gastrointestinal Pathogen Panel from Luminex (Austin, TX).

16.4.4 Mass spectrometry

The availability of mass spectrometry (MS) as a routine diagnostic tool has revolutionized clinical microbiology. Matrix-assisted laser desorption/ionization with time-of-flight MS (MALDI-ToF/MS) has been used as a rapid tool for microbiological identification based on proteomic profiles.

MS has also been used to analyze PCR products in lieu of sequencing through a method called electrospray ionization (ESI) MS. The principle of PCR/ESI-MS is based on the ESI/MS ability to discriminate individual PCR products contained in complex amplicon mixtures according to the nucleotide base composition [6]. Deconvolution algorithms allow base composition of PCR products to be deduced from mass spectrometrically measured molecular weights and the complementary nature of DNA, leading to organism identification. Initially developed for identification of multiple pathogens and biodefense applications, PCR/ESI-MS is now adapted for detection and identification of a variety of microorganisms and drug resistance in cultured specimens or patient samples and molecular determination of TB drug resistance [18,20,23,67]. Integrated systems such as the Abbott Plex-ID (Des Plaines, IL) system couples broad-range nucleic acid amplification to high-performance ESI-MS; and base-composition analysis enables the identification and quantification of a broad set of pathogens, including all known bacteria along with the detection of virulence factors and antibiotic resistance markers, pathogenic fungi, and viruses that cause disease in humans and animals [19,32]. Commercial databases are available for a large spectrum of clinically relevant pathogens and one can anticipate that these databases will expand as the technology progresses. Nevertheless, the significant equipment costs present a challenge to current PCR-MS technologies.

MassTag PCR employs pathogen-specific primers that are tagged with molecules of known mass (mass codes) with ability to detect up to 20–30 target sequences from one reaction and a highly sensitive technique for the screening for pathogens [15]. Multiple microbes can be detected using primers labeled through a photocleavable link with tags that vary in molecular weight. After multiplex PCR, tags (mass codes) are liberated from the PCR products and read with a liquid chromatograph-mass

spectrometer. The identification of a microbe in a sample is determined by its cognate mass code.

16.4.5 Sequencing

Nucleic acid sequencing remains the gold standard for detection and identification of amplification products. The traditional method of post-amplification analysis consists of determining the nucleic acid sequence of the amplicon, usually through either Sanger or pyrosequencing. The commercially available ViroSeq HIV-1 genotyping system (Abbott Molecular, Des Plaines, IL) [22] and the Trugene HIV-1, HBV and HCV genotyping [39] kit for drug resistance (Siemens, Deerfield, IL), based on the conventional Sanger method, are widely used for sequence-based genotyping assays.

Pyrosequencing is based on enzymatic inorganic pyrophosphate release by DNA polymerase, which generates short sequence read lengths (less than 200 bp) [55]. Multiplexed pyrosequencing involves simultaneous extension of several primers hybridized to one or more target DNA templates. A unique feature of pyrosequencing is its theoretical adaptability to the analysis of any genetic marker, which allows for the detection of multiple known and unknown mutations in a single pyrosequencing reaction. Drawbacks of pyrosequencing include read length less than 100 bp, secondary structure formation, which affects quality of the results, particularly with guanine-cytosine (GC) rich targets and difficulty in quantifying the precise number of nucleotides in a homopolymeric region based on peak heights. Advantages of pyrosequencing over Sanger sequencing include decreased cost, less labor, and shorter time to results [47].

Next generation sequencing (NGS), has the ability to perform substantial parallel sequences of one million to several hundred million DNA fragments. Some of the commercial NGS products include the Roche 454 Life Science Genome Sequencer (Branford, CT), Illumina Genome Analyzer (San Diego, CA), and Applied Biosystems (Carlsbad, CA) Supported Oligonucleotide Ligation Detection System (SOLiD) [53]. The NGS technologies are also known as massively parallel sequencing technologies because of their ability to simultaneously sequence one million to several hundred million DNA fragments. Single-molecule DNA sequencing technologies are third-generation sequencing technologies (TGS) and differ from NGS in that they do not require whole-genome amplification steps such as emulsion PCR and bridge amplification. The TGS technologies include a single-molecule real-time (SMRT) sequencing technology by Pacific Biosciences (Menlo Park, CA). Important uses of sequencing in diagnostic microbiology include identification and characterization of organisms to the species, strain differentiation, and detection of resistance-associated mutations from bacteria, fungi, and viruses [33,60,63].

16.5 Future directions

Recent advances in nucleic acid diagnostic methods have revolutionized microbiology by facilitating rapid, sensitive microbial surveillance, and differential diagnosis of infectious diseases, including hospital infection control. Implementation of these methods may enable intervention when the prognosis is optimal for limiting replication, dissemination, transmission, morbidity, and mortality. Additionally, they may also reveal unappreciated links between infection and chronic diseases. Many clinical laboratories routinely implement multiplexed molecular methods because of the methods' sensitivity, speed (more rapid than culture), ability to detect new and emerging infections that are difficult or impossible to culture, and ability to detect multiple microbes simultaneously. However, it is imperative that molecular assays, regardless of intent or nature, demonstrate their utility by both providing a strong biological rationale for investigation and undergo a rigorous demonstration of the sensitive, selective, accurate, and precise assessment of the outcome for which they are proposed to measure.

The increasing use of molecular diagnostics for detection of microorganisms of medical importance raises many new questions for the laboratory and the clinical diagnostician, but also promises many benefits as these methods become available for routine use. Molecular techniques will continue to show improvements in speed and accuracy and promise to become part of the routine detection of respiratory viruses. Microarrays that will test for all types of infectious respiratory agents (viral, bacterial, fungal, and parasitic) on a single respiratory sample may emerge and find a place in the clinical laboratory. Customizable formats may allow institutions to design assays incorporating particular targets of interest in a given population. Until the molecular assays are more rapid and less expensive, however, some conventional methods will continue to be used by many laboratories. The application of molecular diagnostic tools opens a new era of clinical diagnostics for traditionally neglected pathogens in the clinical setting. The arrival of point-of-care molecular testing for emerging and reemerging infections is not far away and would eliminate the high complexity associated with molecular testing currently performed in large hospitals and reference and research laboratories. However, several hurdles still remain such as cost-effectiveness, commercial availability, automation, diagnostic platforms, availability of susceptibility information, and high-throughput technologies. The modern clinical laboratory places a substantial burden upon molecular diagnostics because issues regarding assay platform standardization and analytical performance are critical as new technologies are constantly evolving. For instance, in the United States availability of well-characterized, FDA-cleared (<http://www.accessdata.fda.gov/scripts/cdrh/devicesatfda/index.cfm>)

tests will reduce the burden on laboratories in developing or validating laboratory-developed tests, facilitate implementation of molecular testing in more laboratories, and allow standardization and comparability of tests, thus greatly improving healthcare outcomes.

16.6 Summary

PCR remains the molecular method of choice in many clinical settings due to its superb sensitivity and specificity. RT-PCR has replaced many conventional PCR methods, which allows more rapid detection and quantification of the PCR product, as well as detection of different strains of the pathogen by melting-curve analysis. Complex molecular diagnostics that use DNA sequencing and microarrays are available, but there are also significant efforts to make such assays easier to use and more reproducible so that they too may be implemented as diagnostic tools in more laboratories. The more widespread implementation of these and similar technologies will undoubtedly improve the laboratory-based diagnosis of infectious diseases. Utilization of high-throughput novel technologies, multiparametric testing, and bioinformatics will bring together new growth possibilities for clinical applications.

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Automated Immunoassay Analyzers

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Immunoassays designed to detect antibodies and/or antigens play an important role in the laboratory diagnosis and management of patients with infectious diseases. Over the past several decades, clinical laboratories have observed a significant evolution in not only the immunoassays that are available, but also the technology that is used and the potential to automate testing and increase sample throughput. Automated platforms were initially designed to accommodate enzyme immunoassays (EIA), which represented an important advance over traditional methods such as immunofluorescence (IFA) and manual agglutination assays. A number of semiautomated and fully automated immunoassay analyzers are now commercially available for the performance of a variety of bacterial, viral, fungal, and parasitic antibody and/or antigen detection tests (Table 17.1 and Figure 17.1). Many of these systems have found particular utility in the area of clinical virology, where extensive test menus exist for the automated performance of serologic assays for human immunodeficiency virus (HIV), hepatitis viruses, the so-called TORCH (*Toxoplasma gondii*, rubella, cytomegalovirus, herpes simplex, and HIV) agents implicated in congenital and peripartum infections, and other viruses of clinical significance. The basic technologies used in the instruments generally involve the capture of a particular analyte (e.g., virus-specific IgM, IgG, total antibodies, and/or antigen) using coated polystyrene beads, magnetic particles, microplate wells, solid phase receptacles, or fiber matrix filters. The captured analytes are then detected by colorimetric, chemiluminescence, fluorescence, phosphorescence, and electrochemiluminescence measurements. Recent advances have been made in the development of multiplex immunoassays, which allow for the simultaneous detection of multiple analytes in a single

reaction by combining immunoassay chemistries with flow cytometric analysis. In addition, new analyzers are available to automate the processing and interpretation of Western blot (WB) testing. The available automated platforms, whether designed for immunoassays, WB, or multiplex testing, are either compact benchtop units that take up a limited amount of space in the laboratory, or free-standing units with larger footprints which require more space. Many systems are formatted for batch testing only, but certain instruments provide continuous random-access and STAT-processing capabilities that allow the addition of tests and reagents without disruption of contemporaneous testing. The vast majority of the automated immunoassay analyzers provide walk-away simplicity to perform assays from sample processing through interpretation and reporting of results. The instruments can automatically generate work lists of specimens being tested, pipette specimens from primary sample tubes and then dilute the samples, dispense all reagents, time the incubations at a desired temperature, shake the assay vessels if needed, perform all washes, and read and electronically store the final results. Some analyzers have no sample-handling capabilities and perform only the post-sample analytical steps. The majority of manufacturers of automated instruments also provide software for the analysis and management of patient data and for monitoring the quality of the testing being performed. Most of the instruments can also interface with computer-based, hospital laboratory information systems for seamless and accurate reporting of results. The number and selection of automated instruments used depends primarily on the volume of specimens being tested and the number of individual tests to be performed. Some instruments will run only assay protocols developed and preset by the

Table 17.1 Automated immunoassay systems for bacterial, viral, fungal, and parasitic antibody and antigen detection

Manufacturer	Product name	Selected features	Test menu*		Selected reference(s)*
			Available in United States	In development	
Abbott Diagnostics†	Architect i1000SR	Closed system for Abbott Diagnostics products; chemiluminescence (i1000SR) or advanced chemiflex technology (i2000SR and i4000SR) with flexible protocols and magnetic particle or magnetic microparticle chemistries, respectively; free-standing units; modularity with seamless integration of multiple instruments to single workstations; supports batch and continuous random access testing; onboard capacity to run 25 (i1000SR and i2000SR) and 50 (i4000SR) different assays simultaneously and 100, 200, and 400 tests/h, respectively; primary tube sampling; STAT or standard processing; onboard dilution of samples; multiple reagent configurations; reagent and sample bar code reading; automatic detection of adequate reagent or specimen; clot detection; automatic sample rerun capability; onboard reagent refrigeration; onboard data management; onboard quality control monitoring and maintenance records; onboard instrument self diagnostics; LIS interface; autocalibration or autocalibration alert	Anti-HAV IgM, anti-HAV IgM, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBsAg, anti-HCV, HIV-1/2 Ag/Ab combination available on all three instruments	Anti-EBV panel on i1000SR and i2000SR	1, 3, 15, 22, 23, 28–31, 35, 44–46, 48, 50–55, 60, 61, 66
	i4000SR				
Alere, Inc.	DSX/ DS2	Open systems; EIA utilizing coated microplate wells and colorimetric measurement; benchtop units; full walk-away capability; modular design with flexible reagent configuration; fully automated microplate processor with precise liquid-handling capabilities; batch testing; can process up to two (DS2) or four (DSX) 96-well microplates; onboard capacity to run 12 different assays simultaneously; primary tube sampling with sample bar code reading capability; onboard dilution of samples; automatic detection of adequate reagent or specimen; clot detection; onboard data management; onboard instrument self diagnostics; LIS interface	Anti-HAV IgG, anti-HAV IgM, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBsAg, anti-HCV, HIV-1/2 Ag/Ab combination available on all three instruments	Anti-HAV IgG, anti-HAV IgM, HBsAg qualitative, HBsAg quantitative, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBsAg, anti-HBeAg, HBsAg, anti-HCV, HIV-1/2 Ag/Ab combo, anti-HTLV-I/HTLV-II, CMV IgG and IgM, CMV IgG avidity, rubella virus IgG and IgM, <i>Toxoplasma gondii</i> IgG and IgM, <i>T. gondii</i> IgG avidity, anti- <i>Trypanosoma cruzi</i> IgG (chagas), anti- <i>Treponema pallidum</i> (syphilis) IgG available on all three instruments; HCV Ag available on i2000SR and i4000SR	None identified
Awareness Technology Inc.	ChemWell 2910	Open system; EIA utilizing coated microplate wells and colorimetric measurement; fully automated modular benchtop design; handles up to 27 reagents and 96 samples and 200 tests/h with multiple reagent configurations; primary tube sampling; batch, random access or STAT capabilities; can make predilutions and automatically dilutes and retests samples when needed; automatic detection of adequate reagent and specimen; onboard data management; onboard quality control monitoring; onboard instrument self diagnostics		System can be programmed to perform all analytical steps for virtually any EIA. This includes sample dispensing, reagent addition, plate shaking, incubating and washing microplates, reading results, data reduction, and printing customized reports. DSX is best suited for medium to high throughput laboratories and the DS2 for smaller laboratories	None identified
				System can be programmed to perform all analytical steps for virtually any EIA. This includes sample dispensing, reagent addition, incubating and washing microplates, reading results, data reduction, and printing customized reports. Designed to complete 200 tests/h	None identified

Beckman Coulter, Inc.	Access/ Access 2	Closed system for Beckman Coulter products; Chemiluminescence detection with magnetic particle separation; free-standing unit with continuous random access capabilities; onboard capacity to run 24 different assays simultaneously and 100 tests/h; primary tube sampling; multiple reagent configurations; reagent and sample bar code reading; automatic detection of adequate reagent or specimen; clot detection; onboard reagent refrigeration; onboard data management; onboard quality control monitoring and maintenance records; onboard instrument self diagnostics; LIS interface	Rubella virus IgG, <i>T. gondii</i> IgG and IgM	None identified	Rubella virus IgG, <i>T. gondii</i> IgG and IgM, anti-HAV, anti-HAV IgM, anti-HBc total, anti-HBc IgM, anti-HBsAg, HBsAg, HBsAg confirmatory, CMV IgG and IgM, rubella virus IgM	12, 25, 29, 34, 36, 40, 43, 47
	UniCel Dxl 600 Dxl 800 DxC 600i DxC 660i DxC 680i DxC 860i DxC 880i	Closed system for Beckman Coulter products. Chemiluminescence detection with magnetic particle separation; free-standing units with continuous random access capabilities; onboard capacity to run 50 different assays simultaneously and 200 (Dxl600) and 400 (Dxl800) tests/h, respectively; primary tube sampling; multiple reagent configurations; reagent and sample bar code reading; automatic detection of adequate reagent or specimen; clot detection; onboard reagent refrigeration; onboard dilution of patient samples; autocalibration or autocalibration alert; onboard data management; onboard quality control monitoring and maintenance records; onboard instrument self diagnostics; LIS interface; Dxl 600 and Dxl 800 are immunoassay analyzers only; DxC 600 and 800 series perform parallel processing of immunoassay and chemistry tests on one system	Rubella virus IgG, <i>T. gondii</i> IgG and IgM	None identified	Rubella virus IgG, <i>T. gondii</i> IgG and IgM, anti-HAV, anti-HAV IgM, anti-HBc total, anti-HBc IgM, anti-HBsAg, HBsAg, HBsAg confirmatory, CMV IgG and IgM, rubella virus IgM	12, 29, 40, 41
bioMérieux, Inc.	VIDAS/ Mini-VIDAS	Closed system for bioMérieux products; enzyme-linked fluorescence assay; automated benchtop unit; random access and batch testing; reagent and sample bar code reading; autocalibration or autocalibration alert; onboard data management; onboard quality control monitoring and maintenance records; LIS interface; VIDAS contains five sections accepting 6 tests each and can handle 60 tests/h; Mini-VIDAS has 2 sections accepting 6 tests each and can handle 24 tests/h; number of different assays that can be run simultaneously is 30 for VIDAS and 12 for Mini-VIDAS	CMV IgG and IgM, measles virus IgG, mumps virus IgG, rubella virus IgG, VZV IgG, <i>H. pylori</i> IgG, <i>T. gondii</i> IgG and IgM, <i>T. gondii</i> IgG and IgM competition, <i>T. gondii</i> IgG avidity, anti-Borrelia burgdorferi (lyme) IgG and IgM, Chlamydia trachomatis Ag, C. trachomatis blocking assay, rotavirus Ag, RSV Ag, Clostridium difficile toxin A and B	None identified	CMV IgG and IgM, measles virus IgG, mumps virus IgG, rubella virus IgG, VZV IgG, <i>H. pylori</i> IgG, <i>T. gondii</i> IgG and IgM, <i>T. gondii</i> IgG avidity, <i>B. burgdorferi</i> (lyme) IgG and IgM, <i>C. trachomatis</i> Ag, <i>C. trachomatis</i> blocking assay, rotavirus Ag, RSV Ag, <i>C. difficile</i> toxin A and B, anti-HIV-1 and -2, HIV p24 Ag, HIV DUO (combined detection of p24 Ag and anti-HIV-1 and anti-HIV-2 Ab), EBV, anti-HAV total, anti-HAV IgM, HBsAg, anti-HBsAg, anti-HBc total, anti-HBc IgM, HBeAg, anti-HBeAg	2, 6, 10, 12, 22, 23, 26, 31, 37, 38, 40, 45-47, 49, 53, 54, 60, 65

(Continued)

Table 17.1 (Continued)

Manufacturer	Product name	Selected features	Test menu*			Selected reference(s)*
			Available in United States	In development	Available outside United States	
Bio-Rad Laboratories	PhD System/ PhD Ix System	Open systems; EIA or IFA utilizing coated microplate wells or slides; benchtop units with batch testing; multiple reagent configurations; capacity to do eight different EIAs or four different IFAs simultaneously; primary tube sampling with sample bar code reading; automatic detection of adequate reagent or specimen; onboard dilution of specimens; onboard data management; LIS interface	Systems can be programmed to automate a wide variety of enzyme or immunofluorescence assays. This includes the front end processing for sample dispensing, reagent addition, and the incubating and washing of microplates and slides. Reading results and data reduction are done outside the systems using additional equipment			None identified
	BioPlex 2200	Closed system for Bio-Rad products. Multiplex flow cytometric immunoassays with magnetic particle separation; free-standing unit with batch or continuous random access capabilities; onboard capacity to run 440 different assays simultaneously and 100 tests/h; primary tube sampling; reagent and sample bar code reading; automatic detection of adequate reagent or specimen; clot detection; onboard reagent refrigeration; onboard dilution of patient samples; autocalibration or autocalibration alert; onboard data management; onboard maintenance records; LIS interface	EBV VCA IgG and IgM, EBV EA diffuse IgG, EBV EBNA IgG, heterophile IgM, <i>T. pallidum</i> (syphilis) IgG, <i>T. gondii</i> IgG, measles virus IgG, mumps virus IgG, rubella virus IgG and IgM, CMV IgG and IgM, HSV-1 and HSV-2 IgG	<i>T. pallidum</i> (syphilis) IgM, <i>T. gondii</i> IgM, HIV, hepatitis tests, <i>B. burgdorferi</i> (lyme) IgG	EBV VCA IgG and IgM, EBV EA diffuse IgG, EBV EBNA IgG, heterophile IgM, <i>T. pallidum</i> (syphilis) IgG, <i>T. gondii</i> IgG and IgM, measles virus IgG, mumps virus IgG, rubella virus IgG and IgM, CMV IgG and IgM, HSV-1 and HSV-2 IgG, <i>T. pallidum</i> (syphilis) IgM	4-7, 9, 24, 33
	EVOLIS Premium/ EVOLIS Twin Plus	Open systems; EIA utilizing coated microplate wells and colorimetric measurement; benchtop units; full walk-away capability; modular design with flexible reagent configuration; fully automated microplate processor; batch testing; onboard capacity for 180 (EVOLIS Premium) or 144 (EVOLIS Twin Plus) samples; primary tube sampling; reagent and sample bar code reading capability; onboard dilution of samples; clot detection; onboard data management; onboard quality control monitoring and maintenance records; LIS interface	Systems can be programmed to perform all analytical steps for EIA, including Bio-Rad's immunoassay product lines. This includes sample dispensing, reagent addition, plate shaking, incubating and washing microplates, reading results, data reduction, and printing customized reports. EVOLIS Premium is best suited for medium to high throughput laboratories and the EVOLIS Twin Plus for smaller laboratories			13
Diamedix Corp.	MAGO 4S	Open systems; EIA or IFA utilizing coated microplate wells or slides; benchtop units with batch testing or continuous random access capabilities; multiple reagent configurations; capacity for 4 EIA plates, 16 IFA slides, and 120 samples; primary tube sampling; reagent and sample bar code reading capability; automatic detection of adequate reagent or specimen; onboard dilution of specimens; onboard quality control monitoring; autocalibration or autocalibration alert; onboard data management; LIS interface	System can be programmed to simultaneously process samples for EIA and IFA. This includes the front end processing for sample dispensing, reagent addition, the incubating and washing of microplates and slides, and the internal reading and data reduction of EIA results. The system supports Diamedix EIA and IFA kits as well as other manufacturers' assays			None identified

DiaSorin, Inc.	MAGO Plus	Open system; EIA utilizing coated microplate wells and colorimetric measurement; benchtop unit; fully automated microplate processor; batch or random access testing; can process up to four 96-well microtiter plates; onboard capacity to load 120 samples and run nine different assays simultaneously; multiple reagent configurations; primary tube sampling; reagent and sample bar code reading capability; automatic detection of adequate reagent or specimen; onboard dilution of specimens; onboard quality control monitoring; autocalibration or autocalibration alert; onboard data management; LIS interface	System can be programmed to perform all analytical steps for EIA. This includes sample dispensing, reagent addition, the incubating and washing of microplates, reading results, data reduction and printing customized reports. The system supports Diamedix EIA kits as well as other manufacturers' assays	None identified	None identified	None identified	None identified
	ETI-MAX 3000	Closed system for DiaSorin products; EIA utilizing coated microplate wells and colorimetric measurement; fully automated microplate processor; multiple reagent configurations; benchtop unit with batch or random access capabilities; onboard capacity to load 240 samples; primary tube sampling; reagent and sample bar code reading capability; automatic detection of adequate reagent or specimen; clot detection; onboard dilution of specimens; onboard quality control monitoring and maintenance records; onboard data management; LIS interface	EBV VCA IgG and IgM reverse capture, EBV EBNA IgG, measles virus IgG, mumps virus IgG, VZV IgG, HSV-1/2 IgG, CMV IgG and IgM capture, rubella virus IgG, <i>H. pylori</i> IgG, <i>T. pallidum</i> (syphilis) IgG, <i>T. gondii</i> IgG and IgM capture	None identified	None identified	14	
Dynex Technologies	LIAISON/ LIAISON XL	Closed systems for DiaSorin products; chemiluminescence detection with magnetic particle separation; benchtop (LIAISON) or free-standing (LIAISON XL) units with batch testing or continuous random access capabilities; onboard capacity to run 15 (LIAISON) and 25 (LIAISON XL) different assays simultaneously; primary tube sampling; reagent and sample bar code reading; automatic detection of adequate reagent or specimen; clot detection; onboard refrigeration; onboard dilution of patient samples; reagent data management; onboard quality control monitoring and maintenance records (LIAISON XL only); LIS interface; LIAISON XL has 50-90% more throughput capability than the LIAISON	LIAISON: EBV VCA IgG and IgM, EBV EBNA IgG, EBV EA IgG, CMV IgG and IgM, VZV IgG, HSV-1/2 type-specific IgG, rubella virus IgG, measles virus IgG, mumps virus IgG, <i>T. gondii</i> IgG and IgM, <i>T. pallidum</i> (syphilis) IgG and IgM, anti- <i>B. burgdorferi</i> (lyme) total, anti-HAV IgM, anti-HAV total LIAISON XL: <i>T. pallidum</i> (syphilis) IgG and IgM, mumps virus IgG, anti-HAV total	LIAISON: None identified LIAISON XL: anti-HCV, HIV, HBsAg	LIAISON: EBV VCA IgG and IgM, EBV EBNA IgG, EBV EA IgG, CMV IgG and IgM, VZV IgG, HSV-1/2 type-specific IgG, HSV-1/2 IgM, rubella virus IgG and IgM, measles virus IgG, mumps virus IgG, <i>T. gondii</i> IgG and IgM, <i>T. pallidum</i> (syphilis) IgG and IgM, anti- <i>B. burgdorferi</i> (lyme) total, anti-HAV IgM, anti-HAV total LIAISON XL: <i>T. pallidum</i> (syphilis) IgG and IgM, measles virus IgG, mumps virus IgG, anti-HAV total	4, 11, 17, 20, 23, 30, 32, 37-39, 49, 51, 53, 60	
	Agility	Open system; EIA utilizing coated microplate wells and colorimetric measurement; benchtop unit; full walk-away capability; flexible reagent configuration; fully automated microplate processor with precise liquid-handling capabilities; batch testing; onboard capacity to run 16 different assays simultaneously and 12 plates at once; primary tube sampling; reagent and sample bar code reading capability; onboard dilution of samples; automatic detection of adequate reagent or specimen; clot detection; onboard data management; onboard instrument self diagnostics; onboard maintenance records; LIS interface	Systems can be programmed to perform all analytical steps for virtually any EIA. This includes sample dispensing, reagent addition, plate shaking, incubating and washing microplates, reading results, data reduction, and printing customized reports. Reagents provided by various manufacturers are placed in self-contained, easy-to-use, direct-load reagent kits (SmartKits) prepackaged for each assay	None identified	None identified	None identified	

(Continued)

Table 17.1 (Continued)

Manufacturer	Product name	Selected features	Test menu*			Selected reference(s)†
			Available in United States	In development	Available outside United States	
GRIFOLS	DSX/DS2	Open systems; EIA utilizing coated microplate wells and colorimetric measurement; benchtop units; full walk-away capability; modular design with flexible reagent configuration; fully automated microplate processor with precise liquid-handling capabilities; batch testing; can process up to two (DS2) or four (DSX) 96-well microplates; onboard capacity to run 12 different assays simultaneously; primary tube sampling with sample bar code reading capability; onboard dilution of samples; automatic detection of adequate reagent or specimen; clot detection; onboard data management; onboard instrument self diagnostics; LIS interface	Systems can be programmed to perform all analytical steps for virtually any EIA. This includes sample dispensing, reagent addition, plate shaking, incubating and washing microplates, reading results, data reduction, and printing customized reports. DSX is best suited for medium to high throughput laboratories and the DS2 for smaller laboratories			None identified
	Triturus	Open system; EIA utilizing coated microplate wells and colorimetric measurement; benchtop unit; fully automated microplate processor; batch or random-access testing; can process up to four 96-well microtiter plates; onboard capacity to run eight different assays simultaneously; multiple reagent configurations; primary tube sampling with sample bar code reading capability; onboard dilution of samples; automatic detection of adequate reagent or specimen; clot detection; autocalibration or autocoloration alert; onboard data management; onboard quality control monitoring and maintenance records; LIS interface	Systems can be programmed to perform all analytical steps for virtually any EIA. This includes sample dispensing, reagent addition, incubating and washing microplates, reading results, data reduction, and printing customized reports			5, 24
Inova Diagnostics, Inc.	Quanta-Lyser 240/160/2	Open systems; EIA or IFA utilizing coated microplate wells or slides; benchtop units; batch testing; fully automated; multiple reagent configurations; primary tube sampling; reagent and sample bar code reading capability; onboard dilution of samples; clot detection; onboard data management; onboard instrument self diagnostics; LIS interface	Systems can be programmed to simultaneously process samples for EIA and IFA. This includes the front end processing for sample dispensing, reagent addition, the incubating and washing of microplates and slides, and the internal reading and data reduction of EIA results. The Quanta-Lyser 240 and 160 can process up to six 96-well microplates and 25 slides, while the Quanta-Lyser 2 can process up to 2 plates and 15 slides; sample capacity is 240, 160, and 96, respectively			None identified
	DSX/DS2	Open systems; EIA utilizing coated microplate wells and colorimetric measurement; benchtop units; full walk-away capability; modular design with flexible reagent configuration; fully automated microplate processor with precise liquid-handling capabilities; batch testing; can process up to two (DS2) or four (DSX) 96-well microplates; onboard capacity to run 12 different assays simultaneously; primary tube sampling with sample bar code reading capability; onboard dilution of samples; automatic detection of adequate reagent or specimen; clot detection; onboard data management; onboard instrument self diagnostics; LIS interface	Systems can be programmed to perform all analytical steps for virtually any EIA. This includes sample dispensing, reagent addition, plate shaking, incubating and washing microplates, reading results, data reduction, and printing customized reports. DSX is best suited for medium to high throughput laboratories and the DS2 for smaller laboratories			None identified

Ortho Clinical Diagnostics	VITROS ECI	Closed system for Ortho-Clinical Diagnostics products; coated microwells with enhanced chemiluminescence measurement; free-standing unit with continuous random access or STAT processing; up to 90 tests/h; onboard capacity to run 20 different assays simultaneously; capacity for 60 samples; primary tube sampling; reagent and sample bar code reading capabilities; onboard dilution of samples; clot detection; automatic detection of adequate reagent or specimen; onboard reagent refrigeration; automatic sample rerun capability; onboard data management; onboard quality control monitoring; LIS interface	HBsAg, HBsAg confirmatory, HBeAg, anti-HBeAg, anti-HBc total, anti-HBc IgM, anti-HBc IgG, anti-HAV total, anti-HAV IgM, anti-HIV-1/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG and IgM, <i>T. pallidum</i> IgG	HIV-1/2 Ag/Ab combo	HBsAg, HBsAg confirmatory, HBeAg, anti-HBeAg, anti-HBc total, anti-HBc IgM, anti-HBc IgG, anti-HAV total, anti-HAV IgM, anti-HIV-1/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG and IgM, <i>T. pallidum</i> IgG	1, 3, 27, 29, 41, 42, 44, 63
	VITROS 3600/5600	Closed system for Ortho-Clinical Diagnostics products; coated microwells with enhanced chemiluminescence measurement; free-standing unit with continuous random access or STAT processing; up to 189 tests/h; onboard capacity to run 31 different assays simultaneously; capacity for 80 samples; primary tube sampling; reagent and sample bar code reading capabilities; onboard dilution of samples; clot detection; automatic detection of adequate reagent or specimen; onboard reagent refrigeration; automatic sample rerun capability; onboard data management; onboard quality control monitoring and maintenance records; LIS interface; VITROS 5600 integrates chemistry and immunodiagnosics	HBsAg, HBsAg confirmatory, HBeAg, anti-HBeAg, anti-HBc total, anti-HBc IgM, anti-HBc IgG, anti-HAV total, anti-HAV IgM, anti-HIV-1/2, rubella virus IgG	HIV-1/2 Ag/Ab combo, HBeAg, anti-HBeAg	HBsAg, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBc IgG, anti-HAV total, anti-HAV IgM, anti-HIV-1/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG and IgM, CMV IgG and IgM, <i>T. pallidum</i> (syphilis) IgG	None identified
Roche Diagnostics	Cobas 8000 modular analyzer series with e602 module	Closed system for Roche Diagnostics products; electrochemiluminescence chemistry with magnetic particles; free-standing unit; fully automated; continuous random access; up to 176 tests/h; onboard capacity to run 25 different assays simultaneously; primary tube sampling; reagent and sample bar code reading capability; multiple reagent configuration; onboard reagent refrigeration; automatic detection of adequate reagent or specimen; clot detection; onboard dilution of samples; autocalibration or autocalibration alert; onboard data management; onboard quality control monitoring and maintenance records; LIS interface	Anti-HAV IgM, anti-HAV total, HBsAg, anti-HBsAg, anti-HCV, rubella virus IgG and IgM, <i>T. gondii</i> IgG	HBsAg confirmatory, HBsAg quantitative, anti-HBc total, anti-HBc IgM, <i>T. gondii</i> IgM, CMV IgG and IgG avidity, HSV-1/2 IgG, HIV combi PT, <i>T. pallidum</i> (syphilis) IgG	Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, HBeAg, anti-HBeAg, anti-HBc IgG, anti-HAV total, anti-HAV IgG, HIV Ag confirmatory, HIV Combi, <i>T. gondii</i> IgG and IgM, CMV IgG and IgM, rubella virus IgG and IgM	62

(Continued)

Table 17.1 (Continued)

Manufacturer	Product name	Selected features	Test menu*			Selected reference(s) [†]
			Available in United States	In development	Available outside United States	
Cobas 6000 analyzer series with e601 module		Closed system for Roche Diagnostics products; electrochemiluminescence chemistry with magnetic particles; free-standing unit; fully automated; continuous random access; up to 176 tests/h; onboard capacity to run 25 different assays simultaneously; primary tube sampling; reagent and sample bar code reading capability; multiple reagent configuration; onboard reagent refrigeration; automatic detection of adequate reagent or specimen; clot detection; onboard dilution of samples; autocalibration or autocalibration alert; onboard data management; onboard quality control monitoring and maintenance records; LIS interface	Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, anti-HBsAg, anti-HBc total, anti-HBc IgM, anti-HCV, rubella virus IgG and IgM, <i>T. gondii</i> IgG	HBsAg quantitative, anti-HBc total, <i>T. gondii</i> IgM, CMV IgG and IgM, HSV-1/2 IgG, CMV IgG avidity, HIV combi PT, <i>T. pallidum</i> (syphilis) IgG	Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, HBsAg quantitative, anti-HBc total, anti-HBc IgM, HBeAg, anti-HBeAg, anti-HBsAg, anti-HCV, HIV Ag, HIV Ag confirmatory, HIV Combi, <i>T. gondii</i> IgG and IgM, CMV IgG and IgM, rubella virus IgG and IgM	28, 40, 57
			Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, anti-HBsAg, anti-HBc total, anti-HBc IgM, anti-HCV, rubella virus IgG and IgM, <i>T. gondii</i> IgG	HBsAg quantitative, anti-HBc total, <i>T. gondii</i> IgM, CMV IgG and IgM, HSV-1/2 IgG, CMV IgG avidity, HIV combi PT, <i>T. pallidum</i> (syphilis) IgG	Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, HBsAg quantitative, anti-HBc total, anti-HBc IgM, HBeAg, anti-HBeAg, anti-HBsAg, anti-HCV, HIV Ag, HIV Ag confirmatory, HIV Combi, <i>T. gondii</i> IgG and IgM, CMV IgG and IgM, rubella virus IgG and IgM	29, 40
Cobas 4000 analyzer series with e411 module		Closed system for Roche Diagnostic products; electrochemiluminescence chemistry with magnetic particles; benchtop unit; fully automated; continuous random access; up to 86 tests/h; onboard capacity to run 18 different assays simultaneously; STAT processing; primary tube sampling; reagent and sample bar code reading capability; multiple reagent configuration; onboard reagent refrigeration; automatic detection of adequate reagent or specimen; clot detection; onboard dilution of samples; autocalibration or autocalibration alert; onboard quality control monitoring; LIS interface; system comes either as the stand-alone cobas e411 immunoassay analyzer or coupled with the cobas c311 chemistry analyzer as part of the combined 4000 analyzer series	HBsAg quantitative, anti-HBc total, <i>T. gondii</i> IgM, CMV IgG and IgM, HSV-1/2 IgG, CMV IgG avidity, HIV combi PT, <i>T. pallidum</i> (syphilis) IgG	Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, anti-HBsAg, anti-HBc total, anti-HBc IgM, anti-HCV, rubella virus IgG and IgM, <i>T. gondii</i> IgG	Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, HBsAg quantitative, anti-HBc total, anti-HBc IgM, HBeAg, anti-HBeAg, anti-HBsAg, anti-HCV, HIV Ag, HIV Ag confirmatory, HIV Combi, <i>T. gondii</i> IgG and IgM, CMV IgG and IgM, rubella virus IgG and IgM	1, 23, 35, 40, 42, 44, 45, 48, 53, 55, 58, 64, 66
			HBsAg, HBsAg confirmatory, anti-HBs	Anti-HAV total, anti-HAV IgM, anti-HBc total, anti-HBc IgM, anti-HBeAg, anti-HCV, HIV combi, CMV IgG and IgM, rubella virus IgG and IgM, <i>T. gondii</i> IgG and IgM	Anti-HAV IgM, HBsAg, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBeAg, anti-HBeAg, HIV antigen, HIV antigen confirmatory	
ELECSYS 2010		Closed system for Roche Diagnostic products; electrochemiluminescence chemistry with magnetic particles; benchtop unit; fully automated; continuous random access; up to 88 tests/h; onboard capacity to run 15 different assays simultaneously; STAT processing; primary tube sampling; reagent and sample bar code reading capability; multiple reagent configuration; onboard reagent refrigeration; automatic detection of adequate reagent or specimen; clot detection; onboard dilution of samples; autocalibration or autocalibration alert; onboard data management; onboard quality control monitoring; LIS interface	HBsAg, HBsAg confirmatory, anti-HBs	Anti-HAV total, anti-HAV IgM, anti-HBc total, anti-HBc IgM, anti-HBeAg, anti-HCV, HIV combi, CMV IgG and IgM, rubella virus IgG and IgM, <i>T. gondii</i> IgG and IgM	Anti-HAV IgM, HBsAg, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBeAg, anti-HBeAg, HIV antigen, HIV antigen confirmatory	1, 23, 35, 40, 42, 44, 45, 48, 53, 55, 58, 64, 66

Siemens Healthcare Diagnostics§	ADVIA Centaur CP	Closed system for Siemens Healthcare Diagnostics products; chemiluminescence detection with magnetic particle separation; benchtop unit with continuous random access; up to 180 tests/h; onboard capacity to run 15 different assays simultaneously; primary tube sampling; reagent and sample bar code reading capability; multiple reagent configuration; onboard reagent refrigeration; automatic detection of adequate reagent or specimen; clot detection; onboard dilution of samples; autocalibration or autocalibration alert; onboard data management; onboard quality control monitoring and maintenance records; LIS interface	Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, anti-HBc confirmatory, anti-HBsAg, anti-HBc total, anti-HBc IgM, anti-HCV, HIV-1/O/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG	HBsAg, anti-HBc total, anti-HBc IgM, anti-HBc IgG, HIV-1/O/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG	Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, anti-HBc confirmatory, anti-HBsAg, anti-HBc total, anti-HBc IgM, anti-HBc IgG, HIV-1/O/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG	1, 16, 19, 21, 35, 42, 44, 52, 56, 59, 63
	ADVIA Centaur XP	Closed system for Siemens Healthcare Diagnostics products; acridinium ester and chemiluminescence detection with magnetic particle separation; free-standing unit with continuous random access; up to 240 tests/h; onboard capacity to run 30 different assays simultaneously; primary tube sampling; reagent and sample bar code reading capability; multiple reagent configuration; onboard reagent refrigeration; automatic detection of adequate reagent or specimen; clot detection; onboard dilution of samples; autocalibration or autocalibration alert; onboard data management; onboard quality control monitoring and maintenance records; LIS interface	Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBc IgG, HIV-1/O/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG	HBsAg, anti-HBc total, anti-HBc IgM, anti-HBc IgG, HIV-1/O/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG	Anti-HAV IgM, anti-HAV total, HBsAg, HBsAg confirmatory, anti-HBc confirmatory, anti-HBsAg, anti-HBc total, anti-HBc IgM, anti-HBc IgG, HIV-1/O/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG	
	IMMULITE 2000/2000 XPI	Closed systems for Siemens Healthcare Diagnostics products; chemiluminescence detection with microsphere separation; free-standing unit with continuous random access; up to 200 tests/h; onboard capacity to run 24 different assays simultaneously; primary tube sampling; reagent and sample bar code reading capability; multiple reagent configuration; onboard reagent refrigeration; automatic detection of adequate reagent or specimen; clot detection; onboard dilution of samples; autocalibration or autocalibration alert; onboard data management; onboard quality control monitoring; LIS interface	HBsAg, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBsAg, anti-HBc IgG, HIV-1/O/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG, <i>H. pylori</i> IgG, <i>T. pallidum</i> (syphilis) IgG	None identified	HBsAg, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBsAg, anti-HBc IgG, HIV-1/O/2, rubella virus IgG and IgM, <i>T. gondii</i> IgG, <i>H. pylori</i> IgG, <i>T. pallidum</i> (syphilis) IgG	2, 4, 14, 16, 20, 47
	IMMULITE 1000	Closed system for Siemens Healthcare Diagnostics products; chemiluminescence detection with microsphere separation; benchtop unit with continuous random access; up to 120 tests/h; onboard capacity to run 12 different assays simultaneously; reagent and sample bar code reading capability; multiple reagent configuration; onboard reagent refrigeration; automatic detection of adequate reagent or specimen; onboard dilution of samples; autocalibration or autocalibration alert; onboard data management; LIS interface	HBsAg, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBsAg, CMV IgG and IgM, HSV-1/2 IgG, rubella virus IgG and IgM, <i>T. gondii</i> IgG, <i>H. pylori</i> IgG, <i>T. pallidum</i> (syphilis) IgG	None identified	HBsAg, HBsAg confirmatory, anti-HBc total, anti-HBc IgM, anti-HBsAg, CMV IgG and IgM, HSV-1/2 IgG, rubella virus IgG and IgM, <i>T. gondii</i> IgG, <i>H. pylori</i> IgG, <i>T. pallidum</i> (syphilis) IgG	40

(Continued)

Table 17.1 (Continued)

Manufacturer	Product name	Selected features	Test menu*			Selected reference(s) [†]
			Available in United States	In development	Available outside United States	
TOSOH Bioscience, Inc.	AIA-2000/ AIA-1800/ AIA-900	Closed systems for TOSOH Bioscience products; fluorescence detection with magnetic bead separation; free-standing units with continuous random access; up to 200, 180, and 90 results/h with onboard capacity to run 48, 45, and 31 different assays simultaneously for the AIA-2000, AIA-1800, and AIA-900, respectively; primary tube sampling; reagent and sample bar code reading capability; multiple reagent configuration; automatic detection of adequate reagent or specimen; clot detection; onboard dilution of samples; onboard data management; onboard quality control monitoring and maintenance records (except AIA-900); LIS interface	None identified	None identified	HBsAg, anti-HBsAg, HBcAg, anti-HBc total, HBeAg, anti-HCV	None identified
	AIA-600 II/ AIA-360	Closed systems for TOSOH Bioscience products; fluorescence detection with magnetic bead separation; benchtop units with continuous random access; up to 36 and 60 results/h with onboard capacity to run 25 and 26 different assays simultaneously for the AIA-360 and AIA-600 II, respectively; primary tube sampling; reagent and sample bar code reading capability; multiple reagent configuration; automatic detection of adequate reagent or specimen; clot detection; onboard dilution of samples (AIA600 II only); onboard data management; LIS interface	None identified	None identified	HBsAg, anti-HBsAg, HBcAg, anti-HBc total, HBeAg, anti-HCV	None identified
Viramed Biotech	BeeBlot 50/ ViraCam	Open system; fully automated Western blot and immunoblot processing on BeeBlot 50; automated scanning with densitometry-based detection and interpretation of results using ViraCam scanner and ViraScan software; up to 48 samples/run; raw data can be stored in image archives	Systems are designed for Virablot and ViraStripe assays from Viramed Biotech, but are adaptable to third party assays. A list of available immunoblot kits for use on the BeeBlot 50/ViraCam system includes <i>Borrelia</i> spp. IgG and IgM, <i>B. burgdorferi</i> B31 IgG and IgM (both assays are FDA cleared), <i>T. pallidum</i> IgG and IgM, <i>H. pylori</i> IgG and IgA, <i>Yersinia enterocolitica</i> IgG and IgA, <i>Bordetella pertussis</i> IgG and IgA, EBV IgG and IgM, and parvovirus B19 IgG and IgM			8

Ab, antibody; Ag, antigen; CMV, cytomegalovirus; EBV, Epstein-Barr virus; EBNA, Epstein-Barr nuclear antigen; EA, early antigen; EIA, enzyme immunoassay; HIV, human immunodeficiency virus; HIV-1, HIV type 1; HIV-2, HIV type 2; HAV, hepatitis A virus; HBc, hepatitis B core; HBeAg, hepatitis Be antigen; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HSV, herpes simplex virus; IFA, immunofluorescence; Ig, immunoglobulin; LIS, laboratory information system; RSV, respiratory syncytial virus; STAT, short turnaround time; VZV, varicella-zoster virus; VCA, viral capsid antigen.

* This information was updated in March 2013.

[†] An electronic search of citations from NCBI/PubMed was conducted to provide pertinent references on the use of automated immunoassay systems in the setting of a clinical microbiology laboratory. In some cases, no references could be found for the described use of certain instruments.

[†] The Abbott Diagnostics AxSYM automated analyzer is not included in this table because it was retired from the US market in early 2013 and will be removed from the international market at the end of 2014.

[‡] The references provided for the Siemens Healthcare Diagnostics ADVIA Centaur instruments did not specifically designate which model (CP versus XP) was used in the studies performed.

<p>Abbott ARCHITECT i1000SR</p> 	<p>Abbott ARCHITECT i2000SR</p> 	<p>Abbott ARCHITECT i4000SR</p> 	<p>Alere DSX</p> 
<p>Alere DS2</p> 	<p>Awareness Technology ChemWell 2910</p> 	<p>Beckman Coulter Access/Access 2</p> 	<p>Beckman Coulter UniCel Dxl 600</p> 
<p>Beckman Coulter UniCel Dxl 800</p> 	<p>bioMérieux VIDAS</p> 	<p>bioMérieux Mini-VIDAS</p> 	<p>Bio-Rad PhD System</p> 

Figure 17.1 Representative photographs of the various automated immunoassay analyzers.*

*Images were provided courtesy of: (a) For Abbott products, courtesy of Abbott, (b) For Alere products, courtesy of Alere, Inc., (c) For Awareness Technology Product, courtesy of Awareness Technology, Inc., (d) For Beckman Coulter products, © 2015 Beckman Coulter, Inc., all rights reserved, used with permission, (e) For bioMérieux products, courtesy of bioMérieux, (f) For Bio-Rad products, courtesy of Bio-Rad Laboratories, Inc. (g) For Diamedix products, courtesy of ERBA Diagnostics, (h) For Diasorin products, courtesy of Diasorin, Inc., (i) For Dynex products, courtesy of Dynex Technologies, (j) For Grifols product, courtesy of Grifols, (k) For Inova products, courtesy of Inova Diagnostics, Inc., (l) For Ortho products, courtesy of Ortho Clinical Diagnostics, (m) For Roche products, courtesy of Roche Diagnostics Corporation, (n) For Siemens products, © Siemens Healthcare Diagnostics, 2015, used with permission, (o) For Tosoh products, courtesy of Tosoh Bioscience, Inc., and (p) For Viramed product, courtesy of Viramed, Inc.

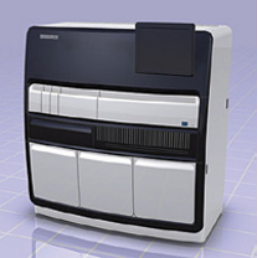
<p>Bio-Rad PhD Ix System</p> 	<p>Bio-Rad BioPlex 2200</p> 	<p>Bio-Rad EVOLIS Premium</p> 	<p>Bio-Rad EVOLIS Twin Plus</p> 
<p>DiaMedix MAGO 4S</p> 	<p>DiaMedix MAGO Plus</p> 	<p>DiaSorin ETI-MAX 3000</p> 	<p>DiaSorin LIAISON</p> 
<p>DiaSorin LIAISON XL</p> 	<p>Dynex Technologies Agility</p> 	<p>Grifols Triturus</p> 	<p>Inova Diagnostics Quanta-Lyser 240/160</p> 

Figure 17.1 (Continued)

<p>Inova Diagnostics Quanta-Lyser 2</p> 	<p>Ortho Clinical Diagnostics VITROS Eci</p> 	<p>Ortho Clinical Diagnostics VITROS 3600</p> 	<p>Ortho Clinical Diagnostics VITROS 5600</p> 
<p>Roche Diagnostics Cobas 8000 Series</p> 	<p>Roche Diagnostics Cobas 6000 Series</p> 	<p>Roche Diagnostics Cobas 4000 Series</p> 	<p>Roche Diagnostics Cobas e411</p> 
<p>Roche Diagnostics ELECSYS 2010</p> 	<p>Siemens Healthcare Diagnostics ADVIA Centaur CP</p> 	<p>Siemens Healthcare Diagnostics ADVIA Centaur XP</p> 	<p>Siemens Healthcare Diagnostics IMMULITE 2000</p> 

Figure 17.1 (Continued)

<p>Siemens Healthcare Diagnostics IMMULITE 2000 XPi</p> 	<p>Siemens Healthcare Diagnostics IMMULITE 1000</p> 	<p>TOSOH Bioscience AIA-2000</p> 	<p>TOSOH Bioscience AIA-1800</p> 
<p>TOSOH Bioscience AIA-900</p> 	<p>TOSOH Bioscience AIA-600 II</p> 	<p>TOSOH Bioscience AIA-360</p> 	<p>Viramed Biotech BeeBlot 50</p> 

Figure 17.1 (Continued)

manufacturer, while others will permit the incorporation of user-defined protocols or are completely open systems that can be programmed to perform all analytical steps of a wide variety of manufacturers' kits.

The incorporation and use of automated immunoassay analyzers can be highly advantageous to the laboratory that has a shortage of trained medical technologists or needs to reduce costs or improve the throughput and turnaround time for test results. These sophisticated instruments allow the users to consolidate methodologies and multiple workstations and improve productivity by offering speed, efficiency, standardization, less hands-on time, and ease of use. For laboratories that are consolidating departments, most analyzers also have extensive onboard menus to perform assays for cardiac biomarkers, thyroid function, reproductive endocrinology, anemia, drugs of abuse, diabetes, allergy, tumor markers, and autoimmune screening.

Although automated systems offer a number of advantages, there are limitations with these platforms that should be considered. First, there is often a higher cost associated with acquiring and operating automated analyzers. However, this cost may be offset by the significant reduction in time and labor that is required to perform the testing. Second, although automated instruments typically demonstrate a high level of reproducibility and precision, errors can still occur, including instrumentation failures and pipetting errors. Third, some analyzers have a relatively large footprint, and therefore, small laboratories may have to carefully consider the advantages of automated testing compared to the availability and configuration of the laboratory space.

Despite these concerns, automated immunoassay analyzers represent an important and growing area in clinical microbiology, allowing laboratories to meet increasing testing demands despite continued shortages in medical technologists. Future considerations for the widespread implementation of these systems include standardization among testing laboratories, which may require the consolidation of immunoassays onto a more limited number of platforms. In addition, there should be a continued emphasis on the development and implementation of multiplex immunoassays, which will allow for increased testing throughput with lower sample volume requirements.

An overview and representative photographs of the currently available automated immunoassay analyzers that have applicability in clinical microbiology are presented in Table 17.1 and Figure 17.1, respectively. The information was collated by reviewing individual manufacturer websites, published material [12,18], and/or direct conversations with company representatives. The data provided are not intended to represent an exhaustive overview of the described platforms, methods, and reagents; instead, we have attempted to include the most pertinent information

that will allow the reader to quickly compare and contrast the various instruments with respect to their basic features and the available menus of infectious disease tests that can be performed on each analyzer. Assay availability may vary by analyzer and between the United States and international markets, and some manufacturers provide different models of the same or similar instruments to accommodate the testing volume and capacity of different sized laboratories. The user should contact the manufacturer for a more comprehensive description and the most current assay menu and pricing for a particular immunoassay analyzer and corresponding reagents.

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Molecular Typing Instruments and Methods

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18.1 Introduction

Molecular typing allows for the identification of different strains of a particular bacterial species. While all of the strains tested are the same species (e.g. *Pseudomonas aeruginosa*), subtle differences in the bacterial genomes allow differentiation between strains that are unrelated and those that may have originated from a common source. Clonally related strains will create a cluster, a group of highly similar molecular fingerprinting patterns, when analyzed by molecular methods. A clonal relationship would suggest that they originated from the same bacterial cell and could indicate a lateral or point source of transmission.

Molecular epidemiological surveillance has become a routine tool for many infection control practitioners. The ability to track outbreaks in real time as well as ruling out potential new opportunities for nosocomial infections is greatly enabled by the in-house utilization of molecular typing techniques. Routine surveillance has also proven useful in determining whether changes in infection control guidelines are effective in preventing transmission of known strains [29]. With accurate clustering of a well-characterized group of isolates, further analysis can enable determination of risk factors for acquisition of a particular strain and outcome data, such as the potential association of greater morbidity and mortality with documented infection of a known strain.

18.2 Background

The discriminatory power of a molecular typing technique describes the technique's ability to differentiate unrelated isolates [50]. Techniques with reasonable discriminatory power for molecular typing include restriction fragment length polymorphism (RFLP), ribotyping, random

amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), arbitrarily-primed PCR (AP-PCR), repetitive element PCR (rep-PCR), multilocus sequence typing (MLST), and most recently, next-generation sequencing. Additional strategies for discriminating strains have been employed through the years, such as *spa* sequencing for methicillin-resistant *Staphylococcus aureus* typing [22,34], specific polymorphism-targeted approaches for *Mycobacterium tuberculosis* differentiation [33,63], and multilocus variable number tandem repeat analysis [30,37], but these techniques will not be discussed in detail in this chapter.

Various factors of each technique determine their acceptability for clinical use. Lack of reproducibility has hampered clinical application of AP-PCR and RAPD [54,62]. RFLP proved too labor-intensive for daily clinical use, and ribotyping provided only moderate discriminatory power compared to the other techniques [54]. PFGE was considered the gold standard for many years, but there are several factors that make this technology less than ideal for the clinical laboratory. PFGE requires a 2–4 days turnaround time, is costly to perform, is labor-intensive, and requires specialized expensive equipment [54,62]. In contrast, the major complaint of rep-PCR was that the technology lacked standardization [62], while others suggested the improved reproducibility of the technology would lend itself to standardization as a library typing system [54]. The issues with standardization would later be remedied by the introduction of the DiversiLab system (bioMérieux, Durham, NC) [23]. The DiversiLab system offered several improvements to the rep-PCR process, including optimized PCR chemistry provided in a reagent kit format, microfluidics-based DNA amplicon detection, and internet-based computerized analysis and data storage. While the DiversiLab

system was one of the first standardized molecular typing platforms, further advances in the areas of next-generation sequencing may ultimately prove to have greater discriminatory power [16,32].

When reviewing studies in molecular typing, the definitions of clones and strains become important. Clones are defined as genetically related isolates that are indistinguishable or so similar to each other that they are presumed to descend from a common parent, and a strain is an isolate or group of isolates that can be distinguished from other isolates of the same genus and species [57]. In regards to conventional typing strategies (nonsequencing-based), indistinguishable or closely related isolates should have no more than a two to three band difference (absence or presence of a band), isolates that are possibly related would have a four to six band difference, and those isolates that are clearly different, considered distinguishable, would have a seven or greater band difference [57].

Several current typing methods utilize bacterial DNA purified from a single microbial isolate. This generally requires routine culture in the clinical microbiology laboratory to isolate a single bacterial species (a pure culture) and provide enough cultured material for adequate extraction of bacterial DNA associated with that single isolate. Most laboratories then extract DNA using commercially available kits and platforms. The resulting nucleic acid is then assessed for both quantity and quality prior to use in downstream molecular typing applications. Currently, there is no FDA-approved assay or platform for molecular bacterial strain typing. This chapter will include selected methods, products, and platforms used for strain typing and data analysis. Other products and instruments may be available worldwide in this area. The reader is encouraged to review current scientific literature, web sites, and regional vendors and companies for additional information.

18.3 Current molecular typing methodologies

18.3.1 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is performed by digestion of chromosomal DNA with rare-cutting restriction enzymes and analysis of the resulting series of fragments by agarose gel electrophoresis [50]. The “pulsed-field” is obtained by constantly changing the direction of the electrical field during electrophoresis: the final molecular profiles can then be analyzed by various software programs including BioNumerics by Applied Maths (Austin, TX). A representative workflow of PFGE developed by the Centers for Disease Control (CDC) is shown in Figure 18.1.

Pure culture of the desired isolate is mixed with a cell-suspension buffer. The cell suspension is then added to

melted agarose and poured into a mold to form a gel plug. Cells are lysed within the agarose using a cell lysis buffer (usually containing 1% Sarcosyl), and the agarose plugs are washed with water and TE (Tris-EDTA) buffer. Restriction enzyme digestion is performed with a complement of enzymes specifically chosen for the bacteria to be typed. Digestion reactions are loaded into an agarose gel, and voltage conditions specific to the bacteria are applied. When electrophoresis is complete, a stain, such as ethidium bromide, is applied to visualize the DNA bands under UV light. The resulting profiles can then be captured by digital image and analyzed using the appropriate software program. Once PFGE profiles are obtained, comparisons can also be made with national data repositories such as PulseNet, a resource maintained by the CDC (<http://www.cdc.gov/pulsenet>). PulseNet was created to alert healthcare professionals about potential pathogenic outbreaks of food-borne illnesses and maintains national data for a limited number of key bacterial species [55].

PFGE has been used in clinical molecular typing studies in many institutions and has been instrumental in the discovery of outbreak situations. PFGE typing in a neonatal intensive care unit determined a clonal relationship between the strains of *Serratia marcescens* isolated from the patients and those found in the hand-washing disinfectant used by the nursing staff [59]. Isolating each of the patients infected with *S. marcescens* and more diligent hand washing and glove use by the staff contained the outbreak. PFGE was also shown to identify two endemic strains of methicillin-resistant *Staphylococcus aureus* [39] and a cluster of *Enterobacter cloacae* associated with a high mortality rate [27]. In another example of molecular typing of antibiotic resistant organisms, PFGE identified a strain of vancomycin-resistant *Enterococcus faecium* that was present in 85% of the samples typed [14]. The implementation of weekly surveillance cultures and the increased use of barrier precautions were effective in controlling the spread of the strain in the hematology unit of the hospital.

18.3.2 Repetitive element PCR

Repetitive element PCR (rep-PCR) capitalizes on the widespread distribution of repetitive DNA elements throughout the genomes of many microorganisms [58]. By positioning amplification primers in these repetitive regions, amplified fragments of various lengths and quantities are produced leading to a unique molecular fingerprint for each strain of a particular species. Rep-PCR has been significantly improved by the introduction of bioMérieux's DiversiLab system (Durham, NC). The complete DiversiLab system offers quality-controlled reagents in a kit format, microfluidics-based amplified fragment detection, and internet-based computer assisted analysis, reporting, and data

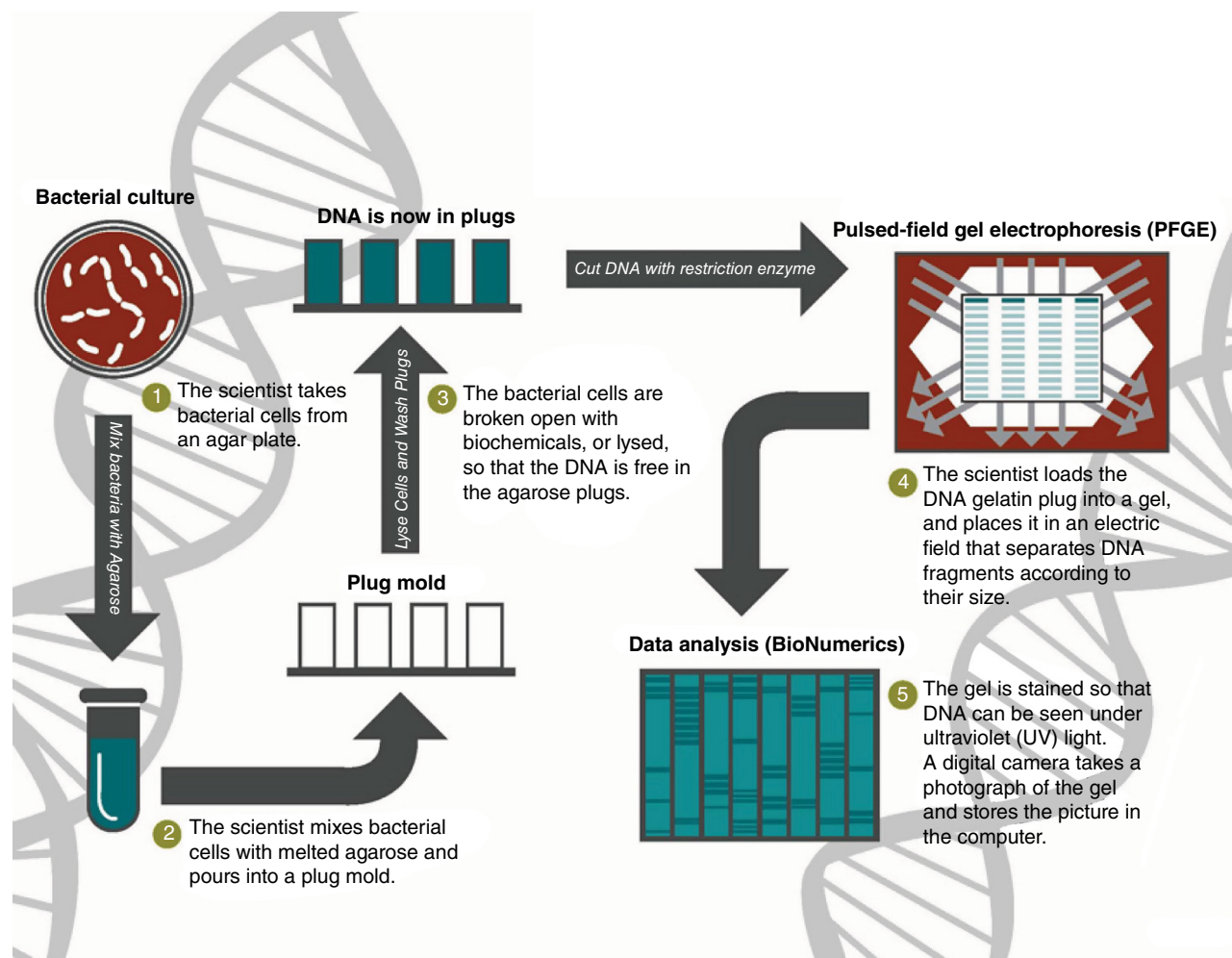


Figure 18.1 Simplified Process of Pulsed-Field Gel Electrophoresis. This schematic developed by the Centers for Disease Control explains the PFGE process from bacterial culture to data analysis. (Source: <http://www.cdc.gov/pulsenet/pathogens/protocol-images.html>). (See insert for color representation of the figure.)

storage [23]. The level of discrimination and reproducibility of the technology paired with the electronic storage of data created an ideal environment for the archiving of rep-PCR data for comparative longitudinal and epidemiological studies.

Rep-PCR oligonucleotide primers bind to conserved, interspersed repetitive sequences throughout the bacterial genome, and these primers direct amplification at multiple primer binding sites to produce multiple DNA fragments of various lengths, yielding strain-specific DNA profiles. Amplification kits are available for various bacterial genera and bacterial groups, with each kit containing an optimized set of primers to promote discriminatory power. The amplified products are loaded into the DNA LabChip, combined with a proprietary gel-dye matrix, and size-fractionated by microfluidics and electrophoresis in the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The Bioanalyzer uses a series of wells and microchannels to electrophoretically

separate DNA fragments, and each fragment is detected by laser-induced fluorescence (excitation wavelength of 635 nm, emission wavelength of 685 nm) of a proprietary dye that intercalates into DNA. The various sizes and relative intensities of the amplified fragments yield clone- or strain-specific chromosomal DNA profiles.

Automated DNA fingerprinting results are then transferred to the DiversiLab analysis software. The software uses the Pearson correlation coefficient and the unweighted pair group method with arithmetic mean (UPGMA) to compare the presence or absence of amplified fragments as well as the intensity of each fragment. This analysis leads to the generation of a dendrogram, similarity matrix, and scatterplot that conveys the relative similarities of isolates based on the DNA profiles generated by rep-PCR (Figure 18.2). The dendrogram is a hierarchical tree representation that uses group averages to display relative similarities or clusters of the isolates. The similarity matrix builds upon the information provided

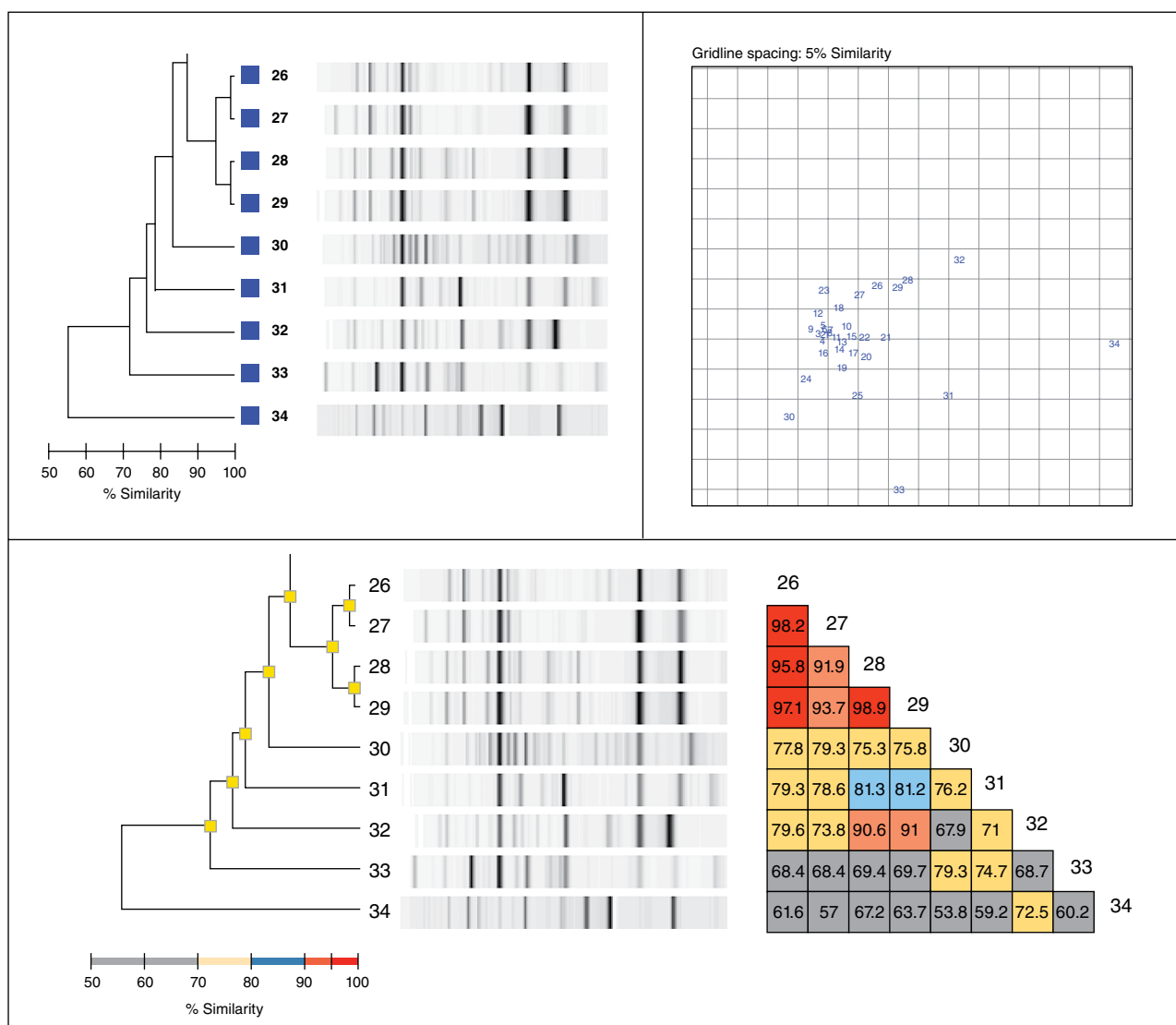


Figure 18.2 DiversiLab Report Components. This DiversiLab report is an example of an automated analysis and reporting system for molecular strain typing that provides several options for visualizing relatedness of clones including: (a) upper left, a dendrogram, (b) upper right, a scatterplot, and (c) lower, a similarity matrix. (Courtesy of bioMérieux.) (See insert for color representation of the figure.)

in the dendrogram by providing a percent similarity for each node of the tree. The scatterplot is a nonhierarchical data display that uses a two-dimensional spatial representation to display relative similarities of isolates by virtue of relative distances on a grid.

Rep-PCR is a proven technology for typing of various bacterial species. Published studies have used rep-PCR to type *Acinetobacter baumannii* [31] and *Vibrio vulnificus* [7], as well as several other bacteria isolated from the International Space Station [6]. One study that separated *Listeria monocytogenes* isolates into four separate clusters suggested that molecular fingerprints be considered different if there was a discrepancy (presence or absence) of at least two bands [25]. A larger study that evaluated the relatedness of several organisms (*Acinetobacter* spp., *Enterobacter* spp., and coagulase-negative *Staphylococcus*)

between an individual's hands and the immediate environment stated specific criteria for the identification of clusters [38]. Interestingly, greater than 25% of the bacterial isolates found on the hands and in the immediate environment of a single household were found to be the same strain. Different isolates were defined as those with less than 95% similarity and a two to three band difference. Similar isolates were described as those with less than 97% similarity but only one or two bands difference; and indistinguishable isolates were defined as those with greater than 95% similarity and no band differences, including intensities. Overall profile intensity differences were acceptable because that indicated a universal change throughout the whole rep-PCR profile most likely due to efficiency of the PCR rather than actual differences between the bacterial isolates.

In addition to bacterial typing, rep-PCR has been recommended as an effective tool for the typing of fungi [24] and dermatophytes [41]. Most importantly, rep-PCR has been proven for use in infection-control investigations. Rep-PCR of vancomycin-resistant *Enterococcus* was instrumental in the control of an outbreak in the neonatal intensive care unit (NICU) in one hospital [51]. In another institution, rep-PCR epidemiologically linked a NICU outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA) to a healthcare worker afflicted with chronic otitis externa and nasal colonization with a specific MRSA clone [4].

18.4 Comparison of typing techniques

Several studies have compared the discriminatory power of fragment-based molecular profiles for molecular typing. One study compared PFGE, rep-PCR, and AP-PCR for typing of *Bartonella henselae* and found that PFGE and rep-PCR had the highest discriminatory potential [46]. Another study compared rep-PCR and RFLP for the typing of *Pseudomonas* isolates from soil and concluded that rep-PCR and RFLP were comparable methods [9]. A comparison of rep-PCR, PFGE, and ribotyping in *Clostridium difficile* found a high correspondence between rep-PCR and PFGE results but lesser discrimination with the ribotyping results [52].

Multiple investigators chose to perform a direct comparison between PFGE and rep-PCR. *Candida albicans* was a specific pathogen of interest, and rep-PCR was found to be comparable to PFGE [8]. One study showed that the DiversiLab rep-PCR system was more rapid and conducive to ongoing epidemiologic studies in *Candida* species [61]. The DiversiLab system was also evaluated for typing of *S. aureus* strains, and the study found that rep-PCR provided more consistent results as compared with PFGE and was also less labor intensive [49]. Further studies confirmed the comparable discriminatory power and more rapid turnaround time of rep-PCR as compared to PFGE in reports of *Listeria monocytogenes* [10] and vancomycin-resistant *Enterococcus* [41].

18.4.1 Ribotyping

While ribotyping has been a mainstay in the molecular typing arena, the process is laborious and requires significant hands-on time in the laboratory. The introduction of the RiboPrinter (DuPont Qualicon, Wilmington, DE) has spurred the reincorporation of this technique in many laboratories. Traditional ribotyping targets the 16S and 23S rRNA genes. The panels developed by DuPont additionally target the 5S rRNA gene as well as spacer regions and flanking genes, allowing for greater discriminatory power than the originally developed methods.

The RiboPrinter entirely automates the ribotyping process. Once a cultured isolate is obtained, the bacterial cells are subjected to heat treatment, cells are lysed, and restriction enzyme digestion is performed using prepackaged enzymatic reagents. Customization of enzyme panels is also possible, but most kits utilize either EcoRI, PvuII, or PstI. Size fractionation is performed via on-board gel electrophoresis. Samples are transferred to a membrane, and DNA probes are hybridized. A chemiluminescent agent is applied, and a digital camera captures subsequent light emission. The captured images are translated into a RiboPrint pattern. DuPont maintains a large library of thousands of RiboPrint patterns that allows for accurate speciation and strain typing based on the obtained patterns.

A wealth of data exists on conventional ribotyping, but the RiboPrinter system offers standardization and robustness well beyond the original process [47]. While early studies found the traditional methods of ribotyping were more useful in the strain typing of *Vibrio cholerae* [12], improvements have been made in the system over the past several years. A similar study evaluated typing of *Salmonella enterica* and found consistent results between traditional and automated ribotyping [11]. While the RiboPrinter has successfully typed strains of vancomycin-resistant *Enterococcus* [18] and *Lactobacillus* and *Streptococcus* [36], ribotyping continues to fall short of the discriminatory power of PFGE. In the differentiation of strains of *Citrobacter* spp. [64], *Listeria monocytogenes* [20], and *Campylobacter* spp. [19], PFGE was found to be more effective than automated ribotyping.

18.4.2 Multilocus sequence typing

Relatively conserved housekeeping genes serve as the targets for multilocus sequencing typing (MLST). Sequence variations across several genes are utilized to group isolates based on overall relatedness. The ideal gene panel to provide sufficient discriminatory power varies depending on the bacterial species, and well-vetted gene lists of many bacterial species are publicly available on the Multi Locus Sequencing Typing website (<http://pubmlst.org/mlst>) from the University of Oxford [26]. In most cases, a total of seven to ten housekeeping genes are sufficient for strain typing. Selected genes are amplified by PCR with subsequent purification. Sequencing reactions are prepared, with many laboratories utilizing kits such as the ABI Big Dye Terminator Ready Reaction Mix, and sequencing is performed via capillary electrophoresis (i.e., ABI genetic analyzers; Life Technologies, Applied Biosystems, Grand Island, NY).

The resulting sequences are aligned, and sequence variations (generally single nucleotide variants) are compared. An example of a typical MLST pipeline is shown in Figure 18.3. Several software packages are available

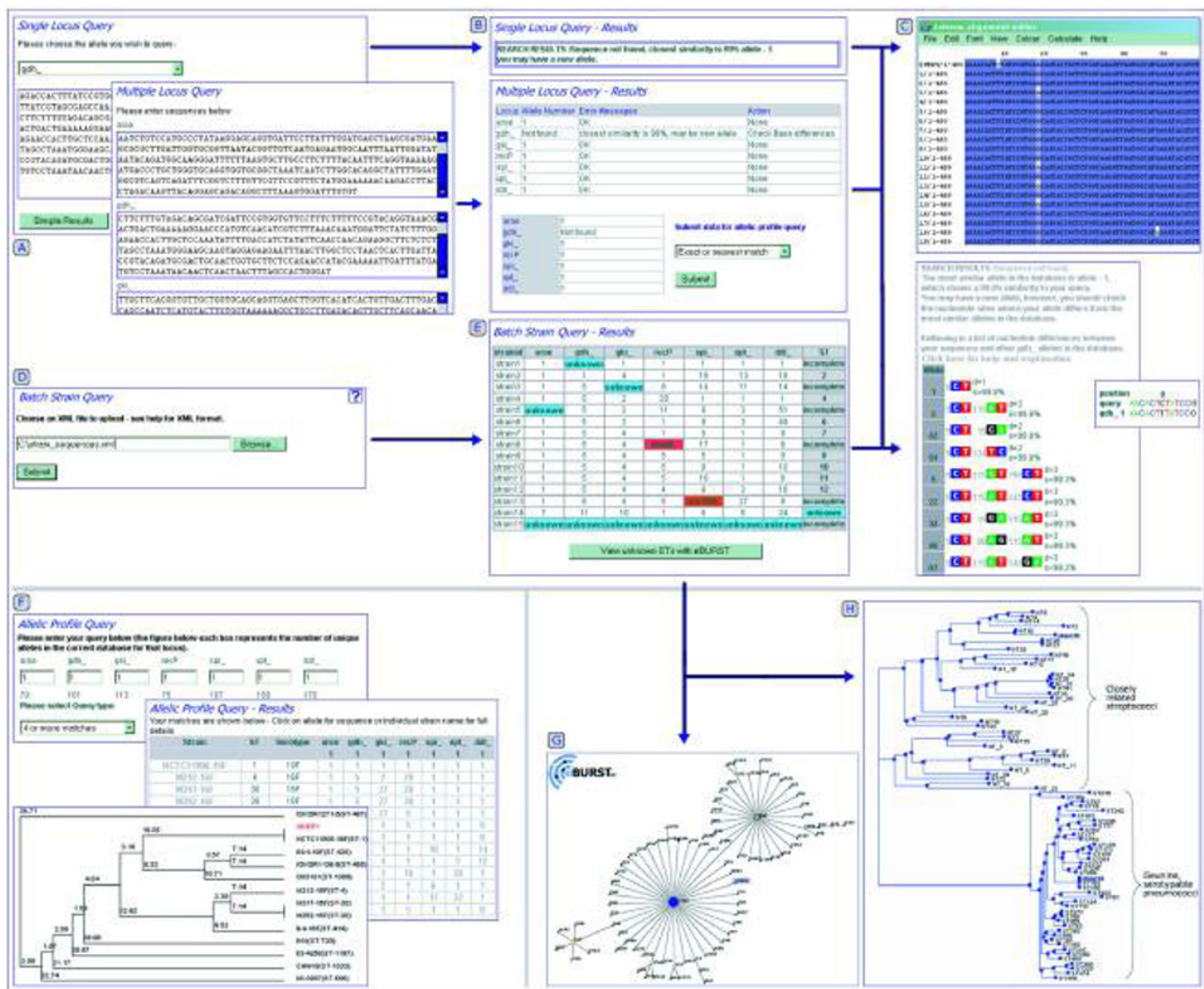


Figure 18.3 Overview of Multi Locus Sequencing Typing Workflow. Various details of the MLST process are presented in this workflow originally described in Aanensen et al. 2005 [1] (Source: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1160176/>). (See insert for color representation of the figure.) (See manuscript and website for expanded view and details.)

for data analysis, including the previously mentioned Bionumerics program, as well as various programs capable of sequence alignment. Publicly available databases such as www.mlst.net and <http://pubmlst.org/mlst> offer standardization and documented sequence variations by bacterial species. Additional analyses of sequence results may include programs like eBURST, which determines clonal complexes by predicting founding genotypes in a data set and computing potential descendant strains for each founder [53]. Commercial software, such as CLC bio's MLST Module (Qiagen, Valencia, CA), is also becoming more widely available.

MLST has been employed extensively in the typing of *Campylobacter* spp. as well as many other pathogens [56]. A recent study compares MLST to rep-PCR for typing *Propionibacterium acnes* and found the methods comparable, with rep-PCR deemed the best

option for rapid screening but MLST necessary for greater discrimination into clonal complexes [13]. MLST remains a current technology and was employed in the identification and tracking of an outbreak of ertapenem-resistant *Klebsiella pneumoniae* strain that persisted for 7 months [40].

While the discriminatory power of MLST is highly desirable, the total cost and time-consuming process of both the hands-on laboratory work and in-depth sequence analysis have driven laboratories to expand testing options. High-resolution melting (HRM) assays are being explored, such as in the typing of *Campylobacter jejuni* [28]. The approach utilizes typical real-time PCR reactions targeting genes associated with known MLST types. A post-PCR melting curve is generated to differentiate the strains based on melting points. Background work in MLST would be necessary to identify which specific MLST types to target,

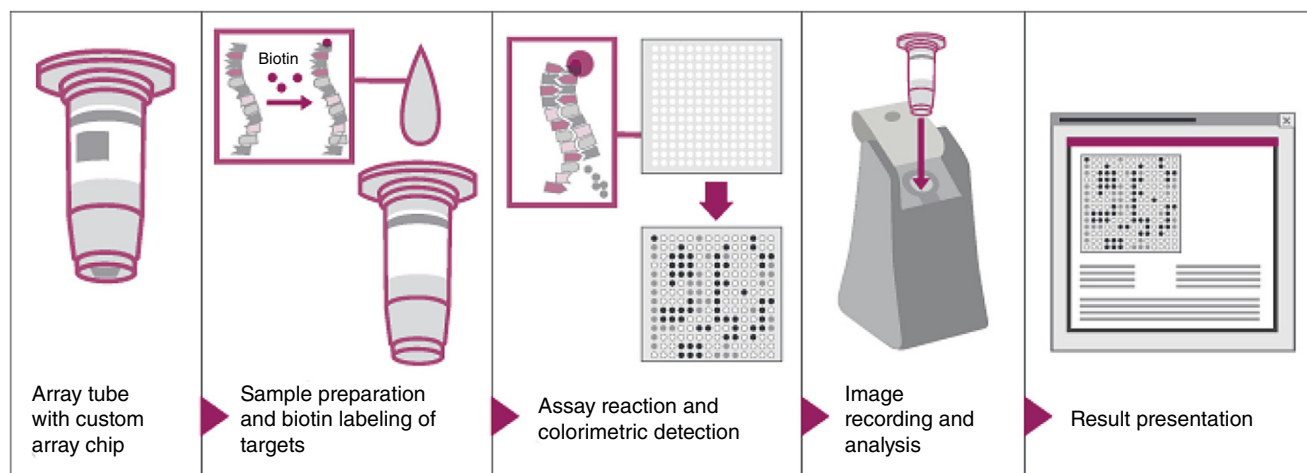


Figure 18.4 ArrayTube Technology. Custom arrays can also serve as effective methods for molecular strain typing as shown in this explanation of the ArrayTube system. (<http://alere-technologies.com/en/products/lab-solutions/platform-components/arraytube-at.html>). (Courtesy of Alere, Inc.) (See insert for color representation of the figure.)

and potential newly identified types would still need to reflex to MLST. The benefit of the HRM approach for rapid screening is that the results could be analyzed with the onboard real-time PCR software leading to a less costly and less time-consuming assay.

While the previous approach with HRM seeks to lower the complexity of the testing, a contrasting viewpoint seeks to increase the overall complexity by taking MLST into next-generation sequencing. Termed HiMLST (high-throughput MLST), initial studies have taken conventional MLST reactions and adapted them to the 454 sequencing amplicon-directed pipeline (454 Sequencing, Roche Diagnostics Corp, Branford, CT) [5]. Accurate profiling of hundreds of isolates including *Legionella pneumophila*, *S. aureus*, *P. aeruginosa*, and *Streptococcus pneumoniae* has been shown by HiMLST [15].

18.4.3 Microarray-based typing methods

Oligonucleotide microarrays with species-specific targets have also entered the molecular typing market. The ArrayTube (AT) Technology (Alere Technologies GmbH, Jena, Germany) employs small biochips embedded in a reaction vial. The microarray tubes can be customized for a variety of purposes including common sequences used in molecular typing (see Figure 18.4 for process workflow). The European Molecular Biology Laboratory maintains a large repository of microarray configurations, among other information, and this information can be utilized to design AT microarrays for strain differentiation (www.ebi.ac.uk/arrayexpress) [44].

Once the ArrayTube has been manufactured with the desired oligonucleotides, bacterial DNA from individual isolates can be amplified using random primers with simultaneous biotin-labeling of the amplicon.

Hybridization of the amplicons to the biochip is then performed in the ArrayTube followed by washing of the biochip. Hybridization with streptavidin-horseradish peroxidase (HRP) conjugate and a HRP substrate then enables colorimetric detection that is measured by the ArrayTube Reader. The recorded image is then used for analysis by the onboard IconoClust software.

The ArrayTube platform has been used in a variety of bacterial species, especially *Chlamydia* spp. and *P. aeruginosa*. The technology has proven useful in the typing of *P. aeruginosa* in eye infections [48] as well as *C. trachomatis* in males with urethritis [42]. With regard to discriminatory power, AT has shown to be concordant and in one case outperform RFLP-based typing strategies in *C. trachomatis* and *C. psittaci*, respectively [43,45]. In the case of *P. aeruginosa* strain typing, the data are conflicting. One study comparing PFGE, MLST, and AT testing modalities found that both MLST and PFGE had higher discriminatory power than AT [2]. In contrast, AT was found to more accurately type the Liverpool Epidemic Strain of *P. aeruginosa* when compared to RAPD and PFGE results [17]. In this case, it was believed that the genomic instability of the bacterial strains in patients with cystic fibrosis may lead to inconsistent PFGE results, a problem that appeared not to effect AT typing results in this group of bacteria.

18.4.4 Next-generation sequencing

The power of high-throughput next-generation sequencing is rapidly expanding into the world of molecular strain typing. As this is a new area, standardized protocols are still under development and quickly evolving. Even though this technology is still relatively new to the molecular diagnostic laboratory, recent publications have already shown the benefits of next-generation sequencing

for strain differentiation. Because of the massive amount of data obtained through sequencing, this approach easily has the greatest discriminatory power of all testing methodologies. However, the cost, labor, and extended turnaround times of next-generation sequencing in its current form make it a less than ideal solution for most laboratories. Most studies have taken a partnered approach, with conventional typing strategies helping to direct the use of sequencing.

Several studies utilizing whole genome sequencing (WGS) have focused on group A *Streptococcus*. With over 1 million reads obtained per sample, WGS on the Illumina HiSeq 2000 (Illumina, San Diego, CA) was shown to exceed the differentiation capabilities of PFGE [3]. A similar exploration of group A *Streptococcus* performed initial deep sequencing of epidemic strains using the Illumina HiSeq 2000 and the Roche 454 GS FLX Sequencer (454 Sequencing, Roche Diagnostics Corp., Branford, CT) followed by sequencing of the test samples on the smaller scale Illumina Genome Analyzer II instrument [16]. This approach accurately assigned clonality in 601 isolates.

A recent study by Martin et al. compared MLST, PFGE, and next-generation sequencing for the typing of *P. aeruginosa*, and found that the most consistent and discriminatory data were obtained with Illumina-based sequencing [32]. It is inevitable that as costs of next-generation sequencing continue to decrease and automation of the process continues to improve, this technology will become more widely used in routine molecular strain typing.

An outbreak of Enterohemorrhagic *Escherichia coli* (EHEC, 0104:H4) in Germany prompted strain typing via whole genome sequencing on the Ion Torrent Personal Genome Machine (Life Technologies, Grand Island, NY) (PGM) [35]. This approach allowed for the differentiation of the outbreak strain from a historical EHEC strain. Identification of genes that were specific to the outbreak strain also enabled the development of a rapid PCR-based assay.

Genome Sequence Scanning (GSS) (PathoGenetix, Woburn, MA) is a relatively new technology in the realm of molecular strain typing. Genomic DNA is digested into large fragments, tagged, and then linearized in a continuous-flow microfluidic funnel [21]. Fragment lengths and spatial patterns of the observed fluorescence are then combined to generate a genomic barcode. With the integration of automated extraction with subsequent Genome Sequence Scanning, the RESOLUTION Microbial Genotyping System provides a single platform that can provide strain-typing results in less than 5 h. Uniquely, this approach does not require a pure culture, thus reducing the amount of work to be done prior to extraction. Previous studies have shown that GSS is comparable to PFGE for

differentiation of strains, and in comparison to other methods, the complete RESOLUTION system requires less hands-on time and minimal training [60]. Complete data analysis is also incorporated into the benchtop instrument, eliminating the need for additional software or tools.

18.5 Summary

The power of molecular typing has increased exponentially over the past decade. While conventional methods like ribotyping and PFGE continue to be used in many laboratory settings, standardization of techniques has been enabled by the introduction of the DiversiLab system for rep-PCR, the RiboPrinter for ribotyping, the ArrayTube for microarray-based typing, and now the Genome Sequence Scanning technology. Even with these standardized platforms, the field will continue to migrate toward sequencing-based approaches with the highly utilized MLST methods and the introduction of next-generation sequencing in the clinical infectious disease laboratory.

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Commercial Methods in Clinical Veterinary Microbiology

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The field of clinical veterinary microbiology is becoming more standardized and adopting many of the tests and protocols that are commonly used in human clinical microbiology. Standard protocols for antimicrobial susceptibility testing of animal pathogens continue to be revised and advanced by the Clinical Laboratory Standards Institute and now include:

- VET01-A4 and VET01S-ED3: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard – Fourth Edition and Supplement
- VET01S-ED3: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Third Edition
- VET02-A3: Development of *In Vitro* Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents; Approved Guideline – Third Edition
- VET03-A: Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Approved Guideline
- VET04-A2: Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Approved Guideline – Second Edition
- VET03/VET04-S2: Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Second Informational Supplement
- VET05-R: Generation, Presentation, and Application of Antimicrobial Susceptibility Test Data for Bacteria of Animal Origin; A Report

These are available from <http://shop.clsi.org/veterinary-medicine-documents/>

The One Health Initiative is a collaborative effort designed to unite human and veterinary medicine, and recognizes that zoonotic infections are becoming more

common and have a greater impact on human health than ever before. Factors that compromise the immune system (advanced age, HIV and other chronic infections, chemotherapy, etc.) have resulted in animal pathogens or commensals being isolated from human specimens more commonly. Currently, 39% of US households own at least one dog and 33% of US households own at least one cat (http://www.humanesociety.org/issues/pet_overpopulation/facts/pet_ownership_statistics.html). Furthermore, 56% of American households own some type of pet (<https://www.avma.org/KB/Resources/Statistics/Pages/Market-research-statistics-US-pet-ownership.aspx>). Therefore, there is a greater demand for diagnostic services related to animal health care. While zoonotic agents are more commonly being isolated from human patients [37], anthrophilic infections are also more commonly being seen in pets (e.g. methicillin-resistant *Staphylococcus aureus* in dogs). Agents that are currently recognized as responsible for emerging infectious diseases are also often of zoonotic origin or use animals as intermediate hosts [87,101,108], and many of the select agents are zoonotic or animals are their primary host. Therefore, human and veterinary clinical and diagnostic laboratories need to be capable of identifying and determining the antibiotic susceptibility of microbial pathogens of animal origin.

Although the demand for the accurate identification of animal pathogens is increasing, the capability of commercial tests to identify these pathogens has not kept up with demand. The lack of commercial tests for these agents reflects the consumer cost of such tests, which is usually the full responsibility of the owner, as opposed to insurance. Such costs are most restricted in diagnostic and medical expenses for food animals because any disease intervention is based on the cost-benefit of the animal or animals (a disease that is likely to affect a large number of

animals in the herd warrants more attention to treatment than that of only an individual animal). Because many of the pathogens isolated from animals are identical or similar to those isolated from humans, veterinary diagnosticians will often use products made for human clinical microbiology. Most of the fungi responsible for animal infections are essentially identical to and cause the same diseases as those causing human infections. The enteric bacterial pathogens that cause infections in animals are also either identical or of the same genus. Many of the bacteria that colonize or infect host mucosal surfaces (e.g., *Bordetella*, *Haemophilus*, *Chlamydia* species, etc.) differ only at the species level. Parasites isolated from animals may either be similar or identical to those isolated from humans, or more specific to a host species. However, the viruses that commonly cause infections in animals are usually the most host-specific.

To address the need for identification of many animal-specific pathogens, many academic and state animal diagnostic laboratories offer “in-house” tests to identify these agents, although these tests are largely unregulated. Currently, most of these tests are based on polymerase chain reaction (PCR) and other molecular assays, or on serology. The American Association for Veterinary Laboratory Diagnosticians (AAVLD) voluntarily inspects and accredits laboratories offering veterinary diagnostic services. Currently, there are 45 public veterinary diagnostic laboratories that are AAVLD-accredited in the United States and Canada. AAVLD accreditation is designed to assist laboratories to meet or exceed the standards of the World Organization for Animal Health (OIE Quality Standard and Guidelines for Veterinary Laboratories: Infectious Diseases, 2008).

In this chapter, due to the scarcity of commercial diagnostic tests specific for many animal pathogens, reviews of how well tests designed for human pathogens perform to identify related species are reported, as well as diagnostic services that are provided by veterinary laboratories for specific agents. Tests that are primarily used in human clinical medicine and are highly relevant to use in veterinary medicine may be mentioned, but the reader will be referred to other chapters in the text that describe these tests in more detail. Recommendations on the collection and transport of veterinary clinical specimens are also described.

19.1 Collection and transportation of clinical samples

The successful detection of microorganisms by the laboratory depends critically on the proper collection and transportation of the clinical sample by the submitting veterinarian. In general, care should be taken to protect the agent in specimens from environmental damage and

maintain viability by using the proper transport system [70]. The materials intended for diagnosis should be collected as soon as possible after the onset of clinical disease, and in a manner to avoid contamination. The samples collected during the acute phase of infection usually contain adequate amounts of organisms, which are often readily detectable with available assays. Samples collected at later times, such as the convalescent phase of infection, may lead to false-negative results even though a microorganism was the cause of the disease. Certain viral infections can predispose the host to secondary viral or bacterial infections [115,165]. Therefore, samples collected late in the disease process may lead to a misdiagnosis when infection with secondary pathogens is involved.

The types of samples to be collected are also important, but will largely depend upon the diagnostic tests selected and the suspected agents or clinical diseases manifested. The knowledge of the pathogenesis of a particular disease by the submitting veterinarian is important for the selection of suitable clinical samples. In general, the appropriate samples for viral or bacterial detection are nasal swabs, body fluids, fecal, and blood samples from infected, live animals, and relevant tissues and organs from necropsied animals. More specifically, it is recommended to collect nasal or nasopharyngeal swab materials from animals with respiratory diseases; fecal samples from animals with enteric diseases; cerebrospinal fluid, fecal samples, and nasal swab materials from animals with central nervous system signs; genital swab materials from animals with genital diseases; conjunctival swab materials from animals with eye diseases; and vesicle swab materials and biopsy samples from animals with skin lesions [37,87,108]. More recently and especially for swine, alternative noninvasive sample types have become popular. For example, pen-based oral fluid samples, using three-strand twisted cotton ropes that hang into the pens [127], are collected on a routine basis in many large swine operations. The pigs are usually allowed to chew on the ropes for about 30 min and the absorbed oral fluid is later mechanically extracted from the ropes and used for diagnosis [126]. As a general practice, blood samples should always be collected from animals with suspected microbial diseases. Whole blood should not be frozen, as this will cause complete hemolysis and render the samples unsuitable for serological diagnoses. The whole blood should be allowed to clot at room temperature, and the serum and buffy coat cells separated by centrifugation. In most cases, paired serum samples collected several weeks apart are needed for a definitive diagnosis of a microbial disease. Cross-sectional blood collection from animals manifesting different stages of the disease is also an alternative and often very effective diagnostic approach for farm animals housed in larger groups. A fourfold or greater increase in serum antibody titer to a particular

agent is indicative of current infection associated with the suspected microorganism. However, a very high single titer to a specific pathogen that is supported by additional testing is also of diagnostic value. The amount of clinical material collected is also important for a definitive diagnosis. Whenever possible, adequate amounts of the clinical samples should be obtained because insufficient amounts of the sample could lead to an inconclusive or false-negative result.

Many organisms are labile and could quickly be inactivated if stored or transported improperly. Nonbleached swabs should be used to collect swab materials in order to avoid toxicity to either the microorganism or to cell cultures [70]. Samples collected for the detection of most microbial agents should be kept cold and moist, and in the appropriate transport medium for that agent. For viruses, the medium usually contains a buffered salt solution, proteins to prevent virus inactivation, and antibiotics to prevent bacterial and fungal growth. A variety of transport media are suitable for virus transport [70]. Commercial swabs (such as from Baxter, Deerfield, IL) or transport systems (such as from Becton, Dickinson and Company, Franklin, NJ) are available for collecting, storing, and transporting clinical materials containing infectious agents. Samples intended for bacterial identification should be collected separately in transport medium that does not contain antibiotics. Clinical materials intended for microbial identification should be refrigerated at 4°C after collection and transported to a diagnostic laboratory as soon as possible. During transportation, samples should be carefully packed in an appropriate shipping container with an adequate amount of commercial refrigerant packs. Care should be taken to prevent any potential leakage and safety hazards. Most microorganisms are stable at 4°C for up to 2–3 days [70]. However, if samples cannot be submitted in a timely manner, clinical materials should be immediately frozen at –70°C. Storage of samples intended for virus detection at –10°C to –20°C, such as the freezer sections of standard refrigerators, is detrimental and should be avoided as most agents rapidly lose infectivity when stored at this temperature due to water crystal formation [37,101]. Frozen samples should be transported to a diagnostic laboratory on dry ice. Special import and export permits are required for international and some domestic transportation of infectious materials, and appropriate authorizations from respective countries should be obtained prior to transportation. Interstate transportation of certain veterinary organisms within the United States also requires a permit from the US Department of Agriculture's Animal and Plant Health Inspection Service (4700 River Road, Riverdale, MD 20737; phone: 301-734-8695; <http://www.aphis.usda.gov/permits/>). Readers are referred to published federal guidelines and regulations (Title 42 CFR Part 72; Title 49

CFR Part 173.386-388) for details regarding packaging, labeling, and interstate shipping of infectious agents.

Proper labeling of samples and recording relevant clinical information prior to submission is as important as sample collection and transportation. Each veterinary diagnostic laboratory has its own standard sample submission form, which is usually available through the diagnostic laboratory's web site. Submitting veterinarians are usually asked to provide information such as animal species or source, age, body weight, sex, herd or flock size, number of animals in the herd or flock affected or dead, location of animals, major clinical signs, date of disease onset, date of samples collected, presumptive clinical diagnosis, vaccination history, types and numbers of samples submitted, and type(s) of diagnostic tests requested. The clinical information, especially the presumptive diagnosis, is important for laboratory diagnosticians not only to select the most appropriate and sensitive tests for the submitted specimens, but to evaluate and interpret the test results as well.

19.2 Selection of diagnostic laboratories and tests

Most states in the United States have their own veterinary diagnostic laboratory system, many of which are associated with their respective veterinary school. Each of these laboratories usually specializes in one or more areas such as poultry diseases, food animal diseases, fish diseases, companion animal diseases, and laboratory animal diseases. A few national laboratories such as the US Department of Agriculture's National Veterinary Service Laboratories (NVSL) have the capacity to provide more comprehensive diagnostic services and reagents. NVSL-Ames (1920 Dayton Avenue, Ames, IA 50010; phone: 515-337-7266; fax: 515-337-7397) provides reagents and comprehensive diagnostic services for most animal diseases. NVSL-Foreign Animal Disease Diagnostic Laboratory (FADDL) has the capacity of diagnosing animal diseases that are foreign to the United States (NVSL-FADDL, P.O. Box 848, Greenport, NY 11944-0848; phone: 515-323-3256; fax: 631-323-3366). The selection of a particular diagnostic laboratory depends largely on the submitting veterinarians' preference, test availability, animal species involved, and type of disease suspected. Most veterinarians tend to use local or regional diagnostic laboratories for convenience, but such preference is sometimes limited by the availability of certain tests at local laboratories. For lists of available diagnostic tests from veterinary diagnostic laboratories in the United States, readers are referred to nearby veterinary schools for locations of reference laboratories and their available diagnostic services. Each laboratory usually has a list of diagnostic services that are available on their diagnostic laboratories web site (see Appendix).

Selection of a diagnostic test for an infectious agent depends on many factors, such as the clinical specimens available for submission, the type of disease suspected, test availability, and cost consideration. For a given disease, multiple diagnostic tests may be available. The principles and selection of these tests are discussed below. In many cases, a confirmatory test is also performed on the same sample or different samples from the same diseased animal. Many traditional tests and procedures are still important in the diagnosis of veterinary microbial diseases, such as pathological and histopathological examination. However, electron microscopy and virus isolation are being used less frequently for diagnosis of viral diseases. Many modern diagnostic tests are rapid assays [58,67,87,108] aimed at detection of antimicrobial antibodies (enzyme-linked immunosorbent assay (ELISA), Western immunoblot analysis, immunofluorescence, serum virus neutralization, hemagglutination inhibition, agar gel immunodiffusion, complement fixation), detection of antigens (immunohistochemistry, antigen-capture ELISA, frozen tissue immunofluorescence), or detection/amplification of nucleic acids (PCR and its related techniques, *in situ* hybridization, Southern blot hybridization, dot blot hybridization). In addition, modern molecular tests and procedures (such as PCR-restriction fragment length polymorphism, sequencing and sequence analysis) are being used more commonly for further genetic characterization of viruses and highly fastidious bacteria, and for differential diagnosis. The basic principles and general application of the commonly used laboratory procedures and diagnostic assays in veterinary diagnostic microbiology are discussed below.

19.3 Pathology and histopathology

Pathology and histopathology form integral parts of laboratory procedures in veterinary diagnostic laboratories. Veterinary pathologists play an important role in the diagnosis of microbial diseases, especially in the identification of previously unrecognized agents. Live diseased animals with acute clinical signs and dead animals with no postmortem autolysis can be submitted for pathological evaluation. Specimens submitted for histopathological examination should be fixed in 10% neutral-buffered formalin. Veterinary pathologists can evaluate fresh tissues for the presence of macroscopic lesions and formalin-fixed tissues for microscopic lesions. Proper selection and preservation of samples by the submitting veterinarian is critical for histopathological examination. Generally, samples submitted for histopathological examinations should be fresh slices (about 0.5 cm) of affected organs fixed in 10% neutral buffered formalin. The organ slices should include the lesion and transitional areas, as well as the adjacent normal tissues. Samples from multiple organs

should be collected and submitted in case the submitting veterinarian is unsure of the organ type to be sampled, and this will allow the veterinary pathologist to select the most appropriate samples for examination. Based on characteristic lesions, the veterinary pathologist can make a tentative diagnosis or come up with a differential list of potential pathogens, which can then be confirmed by laboratory diagnostic tests.

19.4 Quality control of veterinary diagnostic assays: sensitivity and specificity

Accurate diagnosis of a veterinary infectious disease is critically important for effective and timely treatment of diseased animals, for prevention of disease spread within and between herds, and, in cases where the microorganism may have zoonotic potential, for prevention of disease spread to humans. The evaluation and interpretation of test results is largely dependent upon the sensitivity and specificity of a diagnostic test [21]. In general, the diagnostic test must be sensitive enough to detect its target at an acceptable detection threshold (analytical sensitivity) and minimize false-negative results (diagnostic sensitivity). The test must also be specific enough to minimize nonspecific detection resulting in false-positive results. Factors affecting the specificity and sensitivity of a diagnostic test may include the purity of the reagents, the cutoff values used for immunoassays, the quality of clinical samples, the instrument used, and the technical skill of the technicians. The sensitivity and specificity also vary from test to test. Therefore, the selection of a particular diagnostic assay for a given organism is important. A confirmatory test, if available, may also need to be performed.

The commercial diagnostic kits in veterinary use have set sensitivities and specificities that are standardized from lot-to-lot, thereby rendering quality control relatively easy. Since fewer commercial kits are available for veterinary use than for human diagnostics, veterinary diagnostic laboratories frequently use in-house assays in which the sensitivity and specificity may not be known. Each laboratory usually follows its own standard for quality control, evaluation, and interpretation of test results. Therefore, reproducibility of test results from laboratory to laboratory is often not known for the in-house assays. The specificity and sensitivity of a test can usually be improved by including adequate positive and negative controls for each run of specimens. Even for commercial kits that come with standard controls, inclusion of additional in-house positive and negative controls is still necessary for quality assurance. In addition to the routine quality control procedures for a clinical microbiology laboratory, extra care must be taken in a molecular

diagnostic laboratory setting. Many factors may affect the quality control of a molecular diagnostic test such as routine calibration of pipettors, the temperatures and ramping times of thermal cyclers, and monitoring for accurate temperatures of water baths, heating blocks, and incubators [112]. To minimize cross-contamination, supplies and reagents intended for PCR use should be delivered directly to a designated area of a laboratory, and stored in a separate refrigerator or freezer. Nonspecific amplification due to carry-over or cross-contamination in PCR-related methods can be significantly reduced by performing procedures (such as reagent preparation, RNA extraction, cDNA synthesis, and PCR assembly) in a laboratory that is physically separate from the one in which PCR products are analyzed.

19.5 Veterinary virology

19.5.1 Assays for detection of virus

19.5.1.1 Electron microscopy

Due to its low sensitivity (usually > 10⁵–10⁶ virus particles/mL of sample are required), high cost of equipment purchase and maintenance, necessity of a skilled technician, and long turn-around time, electron microscopy (EM) has generally lost its importance as a diagnostic tool for virus detection in veterinary medicine, although it affords an opportunity for direct visualization of viruses in clinical materials [116]. Nevertheless, EM is still useful for the identification of viruses that cannot be propagated *in vitro*, for identification of viruses from clinical samples that are toxic to cell cultures (such as urine, feces, and semen), or for further characterization of suspected viruses. Based on the morphological properties of viruses, a skilled diagnostic virologist can readily identify the virus family it belongs to, which can help make a tentative diagnosis or establish a differential list of potential pathogens. However, a definitive diagnosis usually cannot be made since EM is generally considered a nonspecific test. Samples appropriate for EM examination include, but are not limited to, secretions, excretions, thin tissue sections, vesicular fluids, and cell culture materials.

Negative staining is the most commonly used EM technique for viral detection. Viruses present in clinical materials have little contrast with their surroundings. The negative staining technique overcomes this problem by staining the surrounding background with a heavy metal stain such as phosphotungstate or uranyl acetate to increase the contrast between viruses and the background. After negative staining, the structural outlines and surface projections of viruses can be visualized using an electron microscope. Negative staining EM is useful for detecting enteric viruses [12,19,23,28,34,96,137]. As mentioned above, EM generally can detect, but cannot definitively identify, viruses. Additional tests such as immuno-EM and immunogold-EM

are usually required for a definitive diagnosis. Negative staining immuno-EM is achieved by adding an antiviral antibody specific to the suspected virus in the clinical specimen. The antibody will aggregate the virus and form virion-antibody complexes that can be visualized by negative staining EM. For immunogold-EM, gold (5-nm particles)-labeled protein G (or protein A) is usually added to the virion-antibody mixture. The gold-labeled protein G (or protein A) binds to the Fc fragment of the antibody. The gold-labeled virus particles are pelleted by centrifugation, washed to remove unbound gold particles, sprayed on EM grids, and visualized using an electron microscope. The immunogold EM technique has been used for the identification of many viruses of veterinary importance such as porcine rotavirus [94], porcine reproductive and respiratory syndrome virus [94,103], and bovine coronavirus [55].

19.5.1.2 Virus isolation and identification

Virus isolation (VI) followed by virus identification as a diagnostic tool is usually slow and laborious, but is still considered the gold standard in veterinary viral diagnosis. Virus isolation is still used in veterinary diagnostic laboratories to recover virus isolates from various diseases for further genetic characterization or autogenous vaccine production. Virus isolation is particularly useful for isolation of viruses from previously unrecognized diseases, for detection of viruses for which there are no other available assays, and for production of an adequate amount of virus required for further genetic analyses. The clinical materials appropriate for VI include samples of biopsy and necropsy tissues, blood, secretions and excretions from diseased animals, although some clinical samples (such as urine, feces, and semen) may be toxic to cell cultures. When collecting tissue samples for VI, it is recommended to collect the cleanest tissues (such as lymph nodes and liver tissues) first and the dirtiest tissues (such as enteric tissues) last in order to prevent bacterial contamination of the clean samples. The amount of material required for VI is usually more than that needed for rapid diagnostic assays; therefore a sufficient amount of clinical material intended for VI should be submitted.

The choice of cell culture for VI will largely depend upon the particular suspected virus [108], although some viruses cannot be propagated in cell culture. At least three major types of cell cultures are routinely used for VI: primary cultures, diploid cell lines, and established cell lines [101]. Primary cell cultures are prepared from organs of freshly killed animals. Diploid cell lines usually have a definite life span of 50–60 *in vitro* passages. A variety of established cell lines from various animal species can propagate various veterinary viruses, and are commercially available from the American Type Culture Collection (ATCC, Manassas, VA). The capability of cell cultures to isolate certain viruses can be greatly enhanced

by biochemical or physical treatments [64]. For example, the growth of rotaviruses and reoviruses can be enhanced in the presence of trypsin-containing culture medium and by treatment of clinical samples with trypsin. Isolation of an unrecognized virus in cell cultures often represents a tremendous challenge to veterinary virologists, and primary cell cultures derived from tissues of the same species as the diseased animal are usually the first choice for attempting to isolate an unknown virus.

Many viruses produce characteristic cytopathic effects (CPEs) in cell cultures that can be recognized under a microscope. However, for viruses that are noncytopathic, recognition of virus growth in cell cultures is sometime difficult. The observation of a CPE in cell cultures is not sufficient for a definitive viral diagnosis, and additional tests such as hemagglutination inhibition, hemadsorption, virus neutralization, immunodetection techniques, or other antigenic and genetic characterizations must be performed for identification of viruses growing in cell cultures. In addition, chicken embryos and live animals are also used for VI. For example, chicken embryos are often used to isolate avian viruses and influenza virus. Evidence for virus growth in embryonated chicken eggs can be visualized on the chorioallantoic membrane for certain viruses such as poxviruses and avian herpesviruses, although additional confirmatory tests such as hemagglutination, immunofluorescence, and PCR are usually required to verify virus growth. In mice inoculated with virus, clinical signs and characteristic pathological lesions are often indicative of virus growth, but additional laboratory tests such as serology, immunofluorescence, and immunohistochemistry must also be performed for a definitive diagnosis. Natural host animals may also be used to isolate viruses when other methods fail to produce results.

19.5.2 Assays for detection of antiviral antibodies

19.5.2.1 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay is commonly used for detection of antibodies to infectious agents in veterinary diagnostic laboratories. The basic principle of ELISA is an antigen-antibody reaction measured by colorimetric reaction (optical density or OD) between enzyme-labeled antibody and substrate. The assay is typically inexpensive, sensitive, and rapid, and a diagnosis can usually be made within hours. A large number of samples can be processed in a single run in a fully automated fashion using an automated plate washer and reader. With a known viral antigen coated on the ELISA plates, serum viral antibodies can be measured both qualitatively (positive or negative) and quantitatively (antibody titers). Competitive ELISA (cELISA) or blocking ELISA utilizes an antibody to a known specific agent to compete against

specific antibodies in the test serum sample. An antigen is coated onto a solid-phase surface, the test serum and an enzyme-labeled antibody specific for the coated antigen are added together, and they compete for binding to the immobilized antigen. The color developed (by adding a chromogenic enzyme substrate) is inversely proportional to the amount of viral antibody present in the test serum. A serum sample is considered positive in the competitive or blocking ELISA if the OD value is reduced by a defined percentage compared with an unblocked sample. The availability of commercial kits renders quality control relatively simple. ELISA kits for detection of viral antibodies are commercially available for a variety of veterinary viruses (Table 19.1). These commercial ELISA kits are approved by regulatory agencies such as the Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) in the United States, and the results are usually reproducible from laboratory to laboratory.

19.5.2.2 Serum virus neutralization

Serum virus neutralization (SVN) is used to detect and quantify serum virus-neutralizing antibodies, and is usually performed by mixing a standard amount of virus with serial dilutions of a serum sample. Alternatively, a standard dilution of a serum sample is mixed with serial dilutions of the test virus. The serum-virus mixtures are then inoculated onto monolayers of susceptible cell cultures, and the inoculated cells are monitored for evidence of virus infection. The highest dilution of serum that protects susceptible cells from virus infection is defined as the SVN titer. A susceptible cell culture is required to perform SVN for the test virus. Therefore, for viruses that do not replicate in cell cultures, antiviral-neutralizing antibodies can be detected by inoculating the *in vitro* serum-virus mixtures into live animals or embryonated eggs for evidence of virus infection. However, the *in vivo* neutralization approach is expensive and time-consuming, and is of little practical value. Commercial SVN kits are not readily available and veterinary diagnostic laboratories usually have in-house SVN procedures for each test virus. Neutralizing viral antibodies or reference viruses used for SVN controls are commercially available from many veterinary diagnostic laboratories (such as NVSL) or commercial vendors such as VMRD, Inc. (Pullman, WA), as well as the ATCC.

19.5.2.3 Agar gel immunodiffusion assay

Agar gel immunodiffusion (AGID) is a simple and easy assay to detect viral antibodies in serum samples. Also, with a known reference viral antibody, AGID can be used to detect viral antigen as well. The basic principle of AGID is the reaction between diffusing viral antibody and viral antigen on an agar plate. Viral antigen preparations are placed in a central agar well, and positive control

Table 19.1 Selected commercial diagnostic kits for veterinary viruses of major domestic animal species

Animal	Virus	Assay	Vendor*
Avian	Newcastle disease virus	ELISA [†]	IDEXX, Synbiotics
		PCR	Life Technologies
	Infectious bursal disease virus	ELISA	IDEXX, Synbiotics
	Infectious bronchitis virus	ELISA	IDEXX, Synbiotics
	Avian Reovirus	ELISA	IDEXX, Synbiotics
	Avian encephalomyelitis virus	ELISA	Synbiotics, IDEXX
	Chicken anemia virus	ELISA	Synbiotics, IDEXX
	Reticuloendotheliosis virus	ELISA	IDEXX
	Avian leukosis virus	ELISA, Antigen-capture ELISA [‡]	Synbiotics, IDEXX
	Avian pneumovirus	ELISA	Svanova, IDEXX, Synbiotics
	Avian influenza virus	ELISA	Synbiotics, IDEXX
		Antigen-capture ELISA	Alere Scarborough, Inc.
		PCR	Life Technologies
Bovine	Bovine viral diarrhea virus	Antigen-capture ELISA	IDEXX
		ELISA	IDEXX, Svanova, VMRD, Synbiotics
		PCR	Life Technologies
	Bluetongue virus	AGID	VMRD, VDT
		cELISA	VMRD
		ELISA	Synbiotics
		CF	VDT
		PCR	Life Technologies
	Bovine leukemia virus	ELISA	Svanova, IDEXX, Synbiotics
		AGID	Synbiotics
	Infectious bovine rhinotracheitis virus (bovine herpesvirus-1)	ELISA	Svanova, IDEXX, Synbiotics
		PCR	Life Technologies
	Bovine coronavirus	ELISA	Svanova
	Bovine respiratory syncytial virus	ELISA	Svanova
	Parainfluenza virus (PIV-3)	ELISA	Svanova
	Epizootic hemorrhagic disease virus	AGID	VDT
	Canine	Canine parvovirus	Antigen-capture ELISA
		ELISA	Synbiotics
Canine distemper virus		ELISA	Synbiotics
Caprine Ovine	Rabies	ELISA	Synbiotics
	Border disease virus	ELISA	Svanova
	Caprine arthritis encephalitis virus	AGID	VDT
Equine		Antigen-capture ELISA	VMRD
	Ovine progressive pneumonia virus	AGID	VDT
	Equine influenza virus type A	Antigen-capture ELISA	Centaur
	Equine infectious anemia virus	AGID	VMRD, IDEXX, Synbiotics
		ELISA	Centaur, Synbiotics, VMRD
Feline	Equine herpesvirus	ELISA (type 1 and 4 differential test)	Svanova
	West Nile Virus	ELISA	IDEXX
	Feline leukemia virus	Antigen-capture ELISA	Synbiotics
	Feline immunodeficiency virus	ELISA	Avecon, IDEXX
	Feline infectious peritonitis virus	ELISA	Avecon, Synbiotics
	Rabies	ELISA	Synbiotics
Porcine	Porcine reproductive and respiratory syndrome virus	ELISA	IDEXX
		PCR	Life Technologies
	Pseudorabies virus	ELISA	Svanova, IDEXX, Synbiotics
	Porcine circovirus type 2	ELISA	Synbiotics, Ingenasa
	Classical swine fever virus	Antigen-capture ELISA	Synbiotics
		ELISA, antigen-capture ELISA	IDEXX, Synbiotics
	Swine influenza virus H1N1	ELISA	IDEXX
	Swine Influenza virus H3N2	ELISA	IDEXX
	Influenza virus type A	Antigen-capture ELISA	Centaur
		PCR (detection or subtyping)	Life Technologies
		RIM test	Synbiotics
	Transmissible gastroenteritis virus/porcine respiratory coronavirus	ELISA (differential test)	Svanova

* Vendor information: Alere Scarborough Inc. – 10 Southgate Road, Scarborough, ME 04074; Centaur – Centaur, Inc., P.O. Box 25667, Overland Park, Kansas 66225-5667; IDEXX – IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, ME 04092; Ingenasa – Hermanos Garcia, Noblejes, 39, 28037 Madrid, Spain; Life Technologies – Life Technologies, 3175 Staley Road, Grand Island, NY 14072; Meridian – Meridian Bioscience, Inc., 3471 River Hills drive, Cincinnati, OH 45244; Svanova – Boehringer Ingelheim Vetmedica, Svanova, Box 1545, SE-751, 45 Uppsala, Sweden (Svanova Biotech's US distributor – Diagnostic Chemical Limited, Marketing and Product Manager, Veterinary Diagnostics, 160 Christian Street, Oxford, CT 06478); Synbiotics – Synbiotics, Inc., subsidiary of Pfizer Corp, 812 Springdale Drive, Exton, PA 19341; VDT – Veterinary Diagnostic Technology, Inc., 4890 Van Gordon St. Suite 101, Wheat Ridge, CO 80033; VMRD – VMRD, Inc., 4641 Pullman-Albion Road, PO Box 502, Pullman, WA 99163.

[†] Antigen-capture ELISA: for detection of viral antigen.

[‡] ELISA: for detection of viral antibody (serology).

and test sera are placed in peripheral wells. Precipitin lines are formed when the diffusing viral antibodies meet and react at optimal conditions with the diffusing viral antigen on the agar plate. Commercial AGID kits are available for antibody detection of many viruses such as equine infectious anemia virus and bluetongue virus (Table 19.1). AGID has also been used in many diagnostic laboratories to detect antibodies to viruses for which commercial AGID kits are not yet available, such as caprine arthritis-encephalitis virus [135], swine encephalomyocarditis virus [75], avian influenza virus [190], and bovine leukemia virus [151]. AGID is usually considered a relatively specific test, but the sensitivity is usually low resulting in false-negative results. A confirmatory test is often needed to validate AGID results.

19.5.2.4 Hemagglutination inhibition assay

Hemagglutination inhibition (HI) is a simple and inexpensive assay for the detection of serum antiviral antibodies of viruses that hemagglutinate red blood cells (RBC), such as influenza virus and paramyxovirus. The test serum is diluted serially in 96-well plates and incubated with a standard amount of virus. After addition of RBCs from certain animal species, hemagglutination is inhibited in the presence of sufficient amount of serum antibodies that react with the virus. The serum HI titer is expressed as the highest dilution of test serum that inhibits the aggregation of RBCs. The HI reagents are commercially available from diagnostic laboratories (such as NVSL) and commercial vendors such as ATCC and VMRD, Inc. With a known reference viral antibody, HI can also be used to detect viral antigen.

19.5.2.5 Fluorescent antibody assay

Fluorescent antibody assay (FA) is a rapid and specific test for detection of viral antibody. A known viral antigen is immobilized on a slide or plate, and the test sera containing viral antibody is then added to the wells, and the antigen-antibody complex is visualized by adding a fluorochrome (such as fluorescein isothiocyanate (FITC) or Texas-red)-conjugated secondary antibody that reacts with the specific viral antibody. A few commercial FA kits for human and zoonotic viruses (such as Western and Eastern equine encephalitis viruses, parainfluenza virus, respiratory syncytial virus, herpes simplex virus, and adenovirus) are available. Commercial FA kits for veterinary viruses are rare, but FA reagents are commercially available from vendors such as VMRD, Inc., ATCC, and KPL (Gaithersburg, MD), and from veterinary diagnostic laboratories (such as NVSL). Each veterinary diagnostic laboratory usually has its in-house FA procedures for each virus. An immunofluorescence assay can also be used to detect viral antigens if a known specific viral antibody is available (see below).

19.5.2.6 Complement fixation test

The ability of complement to lyse RBC's in the presence of anti-RBC antibody (hemolysin) forms the basic principle of the complement fixation (CF) test [21]. The test serum is mixed with defined amounts of a known viral antigen, guinea pig complement, sheep RBCs, and hemolysin (rabbit anti-sheep erythrocyte stromata serum). In the absence of specific viral antibody, sheep RBCs and hemolysin activate complement leading to the lysis of RBCs. If the test serum contains viral antibody, the antigen-specific viral antibody forms immune complexes with the viral antigen. The antibody-antigen complexes bind to and activate complement, thus preventing RBCs from lysis by hemolysin. The amount of CF viral antibody in the test serum is inversely proportional to the amount of RBC lysis. The CF test has been used to detect antibodies of many viruses such as bovine leukemia virus [89], bovine respiratory syncytial virus [46], bovine herpesvirus [48], and malignant catarrhal fever virus [149]. The CF test is usually laborious and time-consuming [21], and therefore is not routinely used in modern veterinary diagnostic laboratories. Nevertheless, CF test kits for certain veterinary viruses are commercially available (Table 19.1). The CF test can also be used to detect viral antigen, such as foot-and-mouth disease virus antigen, when a known antiviral antibody is available [114].

19.5.2.7 Western immunoblot analysis

Western immunoblot analysis can detect viral antibodies in serum specific to a viral protein. A purified known viral protein or the entire purified virion is denatured with sodium dodecyl sulfate (SDS), separated by SDS-polyacrylamide gel electrophoresis, transferred and immobilized to a nitrocellulose or nylon membrane, and hybridized with the test serum containing viral antibodies. The presence of an antibody-antigen complex is demonstrated by adding an enzyme-, chemiluminescent-, or radioisotope-labeled secondary antibody. Western immunoblot analysis is often used as a confirmatory test to verify the results of serological assays. This assay has been used for detection of viral antibodies of many viruses such as equine lentivirus [20], bovine immunodeficiency virus [188], African horse sickness virus [17], and bovine leukemia virus [71]. With a known specific viral antibody, Western blot analysis can also be used to detect virus antigens in clinical samples. Supplies and reagents for Western immunoblot analysis are available from commercial companies such as Bio-Rad Laboratories (Hercules, CA). Reference viral antigens and viral antibodies for Western blotting can be purchased from commercial vendors such as VMRD, Inc. and veterinary diagnostic laboratories (such as NVSL).

19.5.2.8 Fluorescent microbead-based immunoassay

A modification of the conventional ELISA platform is the microsphere-based technology that is based on a liquid suspension array and is designed for multiplex testing [32]. This technology utilizes multiple microspheres (up to 100 sets) and each one is conjugated to a different antigen for uniform detection of multiple antibodies in a small sample volume. Commercial assays using this technology have been introduced for chickens (Biovet Inc., Saint-Hyacinthe, Québec, Canada) and allow simultaneous detection of antibodies to Bursal disease virus, Newcastle disease virus, infectious bronchitis virus, and reovirus in serum samples.

19.5.3 Assays for detection of viral antigen

19.5.3.1 Antigen-capture ELISA

Antigen-capture ELISA detects viral antigen (both infectious and inactivated viruses) from a variety of clinical materials such as tissues, secretions, and excretions. Viral antigen-specific antibody is coated to a solid-phase surface (usually a 96-well plate), and the clinical specimen containing viral antigen is then added. After addition of an enzyme-labeled antibody, a chromogenic enzyme substrate is added for color development. The color developed is proportional to the amount of viral antigen present in the specimen. The antigen-capture ELISA is very useful for rapid diagnosis of veterinary viral infections because it is simple and easy to perform. Antigen-capture ELISA kits are commercially available for many veterinary viruses (Table 19. 1). The assay has also been used in veterinary diagnostic laboratories for detection of viral antigens of many other viruses for which commercial antigen-capture ELISA kits are not yet available, such as bluetongue virus [53], bovine coronavirus [146], bovine viral diarrhea virus [144], bovine respiratory syncytial virus [180], and infectious bursal disease virus [52].

19.5.3.2 Immunofluorescence assay for antigen detection

Direct immunofluorescence assay utilizes a specific viral antibody labeled with a fluorochrome such as FITC to detect viral antigen. For indirect immunofluorescence assay, the specific viral antibody is not labeled and the antibody-antigen complex is visualized by adding a fluorochrome (such as FITC)-labeled secondary antibody that reacts with the specific viral antibody. Many clinical materials, such as frozen tissue sections, cell smears, or monolayers, are appropriate for this assay, but fresh clinical samples are the best. The limitation of the immunofluorescence assay for viral antigen detection is that cells or tissues containing viral antigens are required, and other

samples such as serum or feces cannot be used for this assay. Immunofluorescence assay is routinely used in combination with VI for identification of viruses growing in cell cultures. The reagents needed for immunofluorescence assay are commercially available from many vendors such as VMRD, Inc., and ATCC, or from veterinary diagnostic laboratories (such as NVSL). Each veterinary diagnostic laboratory usually has its in-house immunofluorescence assay procedures for each virus.

19.5.3.3 Immunohistochemistry

Immunohistochemistry (IHC) can utilize convenient formalin-fixed tissues for detection of viral antigen, and affords the opportunity to visually localize specific viral antigens within the lesions. The specific viral antigen within tissue sections can be visualized by adding a viral-specific antibody, followed by a biotinylated secondary antibody, an avidin-biotin-peroxidase complex, and a substrate such as DAB (diaminobenzidine tetrahydrochloride) for color development (Figure 19.1). IHC has been widely used among veterinary pathologists to confirm histopathological diagnoses, since the formalin-fixed tissue sections can be used for both histopathological examination and IHC. Most IHC tests employ the avidin-biotin-peroxidase complex method, although other systems such as peroxidase-antiperoxidase complex, and streptavidin-biotinylated-alkaline phosphatase complex are also used. Nonspecific reactions in IHC due to the presence of endogenous peroxidase activity in tissues are a major concern, but can be minimized by blocking the endogenous peroxidase activity with hydrogen peroxide and

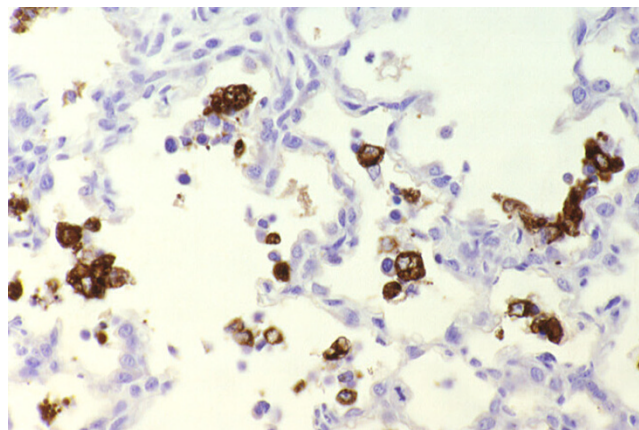


Figure 19.1 Immunohistochemical detection (IHC) of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the alveolar macrophages of a lung from a pig infected with PRRSV. Alveolar macrophages show dark brown staining of PRRSV antigen. Avidin-biotin peroxidase complex staining, hematoxylin counterstain. (Courtesy of Dr. Patrick G. Halbur, Iowa State University.) (See insert for color representation of the figure.)

by including adequate controls. IHC has been successfully used to detect antigens of many veterinary viruses, including but not limited to, feline leukemia virus [66], porcine reproductive and respiratory syndrome virus [50], eastern equine encephalomyelitis virus [120], swine vesicular disease virus [107], swine influenza virus [168], and bovine coronavirus [28]. Many commercial companies, such as DAKO (Carpinteria, CA), Vector Laboratories, Inc. (Burlingame, CA), and KPL, manufacture universal IHC kits that usually contain all necessary IHC reagents except for the specific viral antibody. Specific viral antibody can be purchased separately from veterinary diagnostic laboratories (such as NVSL) or commercial vendors such as VMRD, Inc. A searchable database of available IHC assays, currently being offered at different United States veterinary diagnostic laboratories, is available at <http://ihc.sdstate.org/>. The intended users of this database are veterinary diagnosticians (i.e., pathologists, microbiologists, virologists, and others).

19.5.3.4 Latex agglutination test

The latex agglutination test (LAT) is a simple test to detect viral antigen present in clinical specimens by agglutination with small latex beads coated with known specific viral antibody. The antibody-coated latex beads are stable, and the results can be read by eye or can be measured photometrically within 30 min. This assay is traditionally used to detect the presence of viral antigen in feces of many veterinary viruses such as porcine rotavirus [145], bovinerotavirus [51,113], and canine parvovirus [15]. With a known viral antigen, LAT can also be used to detect viral antibody. LAT kits are commercially available for a few veterinary viruses (Table 19.1). The major concern for the use of LAT is its low sensitivity compared to other immunoassays.

19.5.4 Assays for detection of viral nucleic acids

19.5.4.1 Southern blot hybridization

Edwin Southern in 1975 [153] pioneered the Southern blot to detect specific sequences among hundreds and thousands of restriction fragments of genomic DNA. The technique utilizes restriction enzymes that recognize specific cleavage sites of DNA and cleave the DNA into smaller fragments. After separation of the cleaved DNA fragments by gel electrophoresis, the DNA fragments are transferred to a membrane such as nitrocellulose, denatured, and hybridized with a labeled specific probe. Southern blot analysis is useful for detecting nucleic acids of DNA viruses such as porcine parvovirus [79], bovine leukemia virus [43], ovine papillomavirus [95], and bovine herpesvirus [91]. Traditionally, ^{32}P was used to label probes by methods such as nick translation and end-labeling. However, due to safety

concerns, many diagnostic laboratories now use nonradioisotope labeling methods such as biotin [80], digoxigenin [73], or chemiluminescence. The digoxigenin system is particularly attractive because nonspecific background can be minimized. The modified digoxigenin only occurs in nature in the *Digitalis* plant. Thus, nonspecific reactions are greatly reduced. Several companies such as DAKO, Roche Diagnostics (Indianapolis, IN), Thermo Scientific (Rockford, IL), Applied Biosystems (Bedford, MA), and GE Healthcare Life Sciences (Amersham Pharmacia Biotech, Inc.; Pittsburgh, PA) manufacture commercial reagents and/or kits for the biotin, digoxigenin, and radioisotope labeling systems. However, the specific viral genomic fragments to be labeled as probes have to be generated in the research or diagnostic laboratories for each virus of interest. The popularity of Southern blot, dot blot, or *in situ* hybridization is diminishing due to the widespread use of PCR diagnostic technologies.

19.5.4.2 Dot blot hybridization

Dot blot hybridization can detect viral DNA or RNA immobilized onto a filter such as a nitrocellulose membrane. Total nucleic acids extracted from clinical samples are immobilized, denatured, and hybridized with a radioisotope-, chemiluminescent-, or enzyme-labeled single-strand viral nucleic acid probe. After washing away the unbound probe, the membrane is exposed to a film for autoradiography or, in the case of an enzyme-labeled probe, the signal of bound probes in the membrane is measured by color development with a substrate. Dot blot hybridization has been used to detect nucleic acids of many viruses such as porcine and bovine enteric coronavirus [150], infectious bursal disease virus [56], bovine rotavirus [118], swine encephalomyocarditis virus [104], feline infectious peritonitis virus [97], and avian reoviruses [93]. Like Southern blotting, virus-specific probes have to be generated, but reagents for probe labeling and hybridization are commercially available from companies such as Amersham (now GE Healthcare Life Sciences).

19.5.4.3 *In situ* hybridization

In situ hybridization (ISH) can detect viral nucleic acids in affected tissues at the genomic level. The principle of ISH is base-pairing (hybridization) between a labeled viral nucleic acid probe and the complementary base sequences of the target viral genomic DNA or RNA present in tissues or cells. Non-radioactive methods (such as biotin, digoxigenin, or chemiluminescence) have now gradually replaced the radioactive methods in labeling nucleic probes in ISH. For example, biotin-labeled viral DNA or RNA probes are now commonly used to detect viral nucleic acids in clinical samples by adding enzyme-labeled avidin and a substrate (Figure 19.2). Under a light microscope, a colorimetric reaction within a particular virus-infected

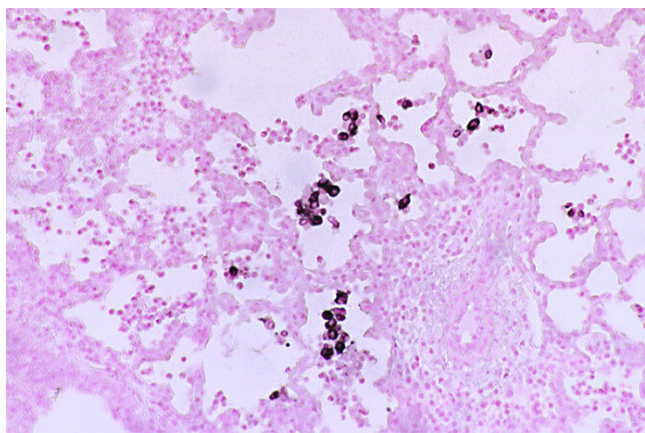


Figure 19.2 *In situ* hybridization detection (ISH) of porcine reproductive and respiratory syndrome virus (PRRSV) nucleic acid in the alveolar macrophages of a lung from a pig infected with PRRSV. PRRSV nucleic acid is detected in the cytoplasm of macrophages. ISH with a digoxigenin-labeled PRRSV riboprobe detected by anti-digoxigenin–alkaline phosphatase complex, nuclear fast red counterstain (Courtesy of Dr. Patrick G. Halbur, Iowa State University). (See insert for color representation of the figure.)

cell can be visualized in the context of other surrounding microscopic structures. Commercial ISH kits are available from many vendors such as Roche Diagnostics and DAKO, and these kits usually contain all the necessary reagents for probe labeling and ISH procedures. However, a specific viral genomic fragment or a synthetic oligonucleotide for each virus of interest has to be generated in a research or diagnostic laboratory for labeling as probes. ISH has now been widely used to detect nucleic acids of many veterinary viruses such as porcine reproductive and respiratory syndrome virus [54,83], porcine circovirus [102], porcine epidemic diarrhea virus [76], avian reoviruses [92], chicken anemia virus [141], avian adenovirus [85], bovine coronavirus [189], and rabbit hemorrhagic disease virus [44].

19.5.4.4 Polymerase chain reaction

The unmatched sensitivity and quick turnaround time of PCR is very useful for rapid detection of low levels of viral nucleic acids from virtually any clinical sample. PCR-based viral diagnosis is now being widely used in veterinary diagnostic laboratories, and many commercial PCR diagnostic kits are now available for detection of viral nucleic acids.

The basic principle of PCR is the enzymatic synthesis of a specific DNA sequence by using two short synthetic oligonucleotide primers, usually 18–24 base pairs (bp). The primers bind to the complementary sequences of the target DNA, and the viral genomic region flanking the two primers is amplified repeatedly by a thermostable *Taq* polymerase in an automated cycling fashion

in a Thermocycler. A typical PCR cycle consists of denaturation of target DNA usually at 94°C for 30–60 s, annealing of primers to the target DNA at an appropriate temperature (usually between 42 and 60°C) for 30–60 s, and extension of the products at 72°C for a certain length of time depending upon the size of the amplified fragments. After 25–35 cycles, the target DNA is amplified to a level that can be detected as a visible band by gel electrophoresis. The identity of an expected size of PCR product usually can be further confirmed by restriction enzyme digestion or nucleotide sequencing. The design of PCR primers is a key to the successful amplification of the target viral nucleic acids. The target virus nucleotide sequence must be known in order to design primers for PCR detection, and the primers need to be specific to the virus in question, but common to all strains of that virus. Since the introduction of PCR in 1983, numerous modifications of the original method have been applied for the diagnosis of infectious diseases such as reverse transcriptase PCR (RT-PCR), nested PCR, multiplex PCR, quantitative PCR, *in situ* PCR, and real-time quantitative PCR. Reagents for these PCR-related assays are readily available from many commercial vendors. A searchable database for veterinary diagnosticians that includes currently available commercial and in-house PCR tests offered at different US veterinary diagnostic laboratories can be found at <http://pcr.sdstate.org/>.

19.5.4.4.1 RT-PCR

RT-PCR detects viral RNA from clinical samples. The first step of RT-PCR is to extract viral genomic RNA or total RNA from clinical samples. RNA extraction kits and reagents from a variety of clinical specimens are available from many commercial companies, such as Qiagen RNA extraction kits (Qiagen Inc., Valencia, CA) and TriZol Reagents (Life Technologies, Grand Island, NY). The RNA template is reverse-transcribed into cDNA with a reverse transcriptase, which is then amplified by PCR with specific viral primers. A limitation of PCR has been that the enzymatic activity of the traditional retrovirus reverse transcriptase does not function in temperatures over 42°C. Therefore, the presence of strong secondary structures in the single-strand RNA template during cDNA synthesis at 42°C may limit specific cDNA synthesis. The discovery of a DNA polymerase with reverse transcriptase activity from a thermophilic bacterium has overcome this problem, and cDNA synthesis can now be performed at up to 65–70°C. Commercial RT-PCR kits in which cDNA synthesis is carried out at up to 70°C are now available, such as the rTth RT-PCR kit (Perkin Elmer, Waltham, MA). RT-PCR has been widely used to detect nucleic acids of many RNA viruses such as porcine epidemic diarrhea virus [65], infectious bronchitis virus [172],

avian pneumovirus [2], porcine reproductive and respiratory syndrome virus [170], and bovine viral diarrhoea virus [132].

19.5.4.4.2 Nested PCR and nested RT-PCR

Nested PCR and nested RT-PCR are extremely sensitive assays that are used for detection of nucleic acids of both DNA viruses, such as canine herpesvirus [147] and bovine herpesvirus [136], and RNA viruses, such as feline infectious peritonitis virus [40] and canine coronavirus [125]. The assays utilize two sets of specific PCR primers. In the first round of PCR, the external sets of primers amplify a product that serves as the template for the second round of PCR amplification. The second round of PCR (nested PCR) utilizes an internal set of PCR primers within the region of the first round PCR product. Therefore, the second round of PCR amplification often serves as a confirmation for the specificity of the PCR products amplified in the first round. Because of the extreme sensitivity, care must be taken to avoid carry-over or cross-contamination, which could lead to false-positive results.

19.5.4.4.3 Multiplex PCR

Multiplex PCR detects two or more target viral sequences in a single PCR reaction. Therefore, multiplex-PCR is more cost effective than other PCR methods. Usually two or more sets of primers specific for different target viral sequences are included in the same amplification reaction, and each specific set of primers produces different sized PCR products that can be distinguished by gel electrophoresis. Multiplex PCR has been used for the diagnosis of multiple virus infections from the same specimen, and for simultaneous detection and differentiation of different genotypes or serotypes of related viruses [45,82,133,159,162,173,186].

19.5.4.4.4 In situ PCR and in situ RT-PCR

In situ PCR and *in situ* RT-PCR can directly detect viral nucleic acids in paraffin-embedded, formalin-fixed tissue sections by directly incorporating a labeled dNTP, such as digoxigenin-11-deoxyuracil triphosphate (dUTP), into the amplicon during the *in situ* PCR reaction to visualize viral DNA or RNA in tissues by a colorimetric reaction. *In situ* PCR and *in situ* RT-PCR have been successfully used to detect nucleic acids of many viruses such as equine herpesvirus [16], avian reoviruses [92], infectious bursal disease virus [93], and porcine circovirus type 2 (PCV2) [90].

19.5.4.4.5 Negative-strand RT-PCR

Negative-strand RT-PCR detects nucleic acids of replicating RNA viruses from clinical samples. Positive-strand RNA viruses produce an intermediate negative-strand RNA during virus replication. A specific primer complementary to the negative-strand RNA of the target virus is

used for reverse transcription and cDNA synthesis. The resulting cDNA is then amplified by PCR. This method allows for distinction between virus replicating in the tissues and circulating virus. A positive result of a negative-strand RT-PCR indicates the presence of nucleic acid from a virus replicating in the particular tissue, and not due to contamination of tissues by circulating virus through the bloodstream. Negative-strand RT-PCR is primarily used for viral pathogenesis studies, for identification of virus replication sites [14,183], and for confirmation of virus replication in cell cultures in combination with VI.

19.5.4.4.6 Real-time PCR

Real-time PCR combines PCR amplification and detection into a single step, thereby allowing for rapid, high-throughput quantitative analysis of target nucleic acids of interest. The capability of using real-time quantitative PCR for large-scale rapid detection and quantification of pathogens in clinical samples makes it particularly appealing to clinical diagnostic laboratories. The accumulated PCR product is monitored continuously during amplification, and detection in the early exponential phase of the reaction permits rapid and reproducible quantification of the template. The basic principle of real-time quantitative PCR is the detection of target nucleic acid sequences using a fluorogenic 5' nuclease assay. A fluorogenic probe (often called the TaqMan probe) complementary to the target sequence is added to the PCR reaction mixture. If the target nucleic acids of interest are present, the probe anneals specifically between the forward and reverse primer sites during PCR. This annealing and detection process occurs in every cycle (thus the name "real-time" quantitative PCR). The fluorescence intensity of the annealed probe provides a direct measurement of the number of target molecules in the reaction mixture and the results are instantly displayed on a computer screen (Figure 19.3). Other advantages of real-time quantitative PCR for clinical diagnoses include reproducibility from sample to sample and from laboratory to laboratory, increased capacity for screening large numbers of clinical samples, and significantly decreased time for reporting results. Real-time quantitative PCR can detect the nucleic acid of a pathogen from clinical samples in a matter of minutes [10]. Many commercial companies now manufacture real-time quantitative PCR instruments, and real-time PCR has now become the standard procedure to detect viruses and some fastidious bacteria in most veterinary diagnostic laboratories. For example, real-time quantitative PCR has been used to detect nucleic acids of almost all known veterinary viruses, such as classical swine fever virus [100], feline coronaviruses [49], feline immunodeficiency virus [88], infectious bursal disease virus of chickens [105], porcine reproductive and respiratory syndrome virus [178], porcine circovirus type 2 [169], and torque teno sus virus [62].

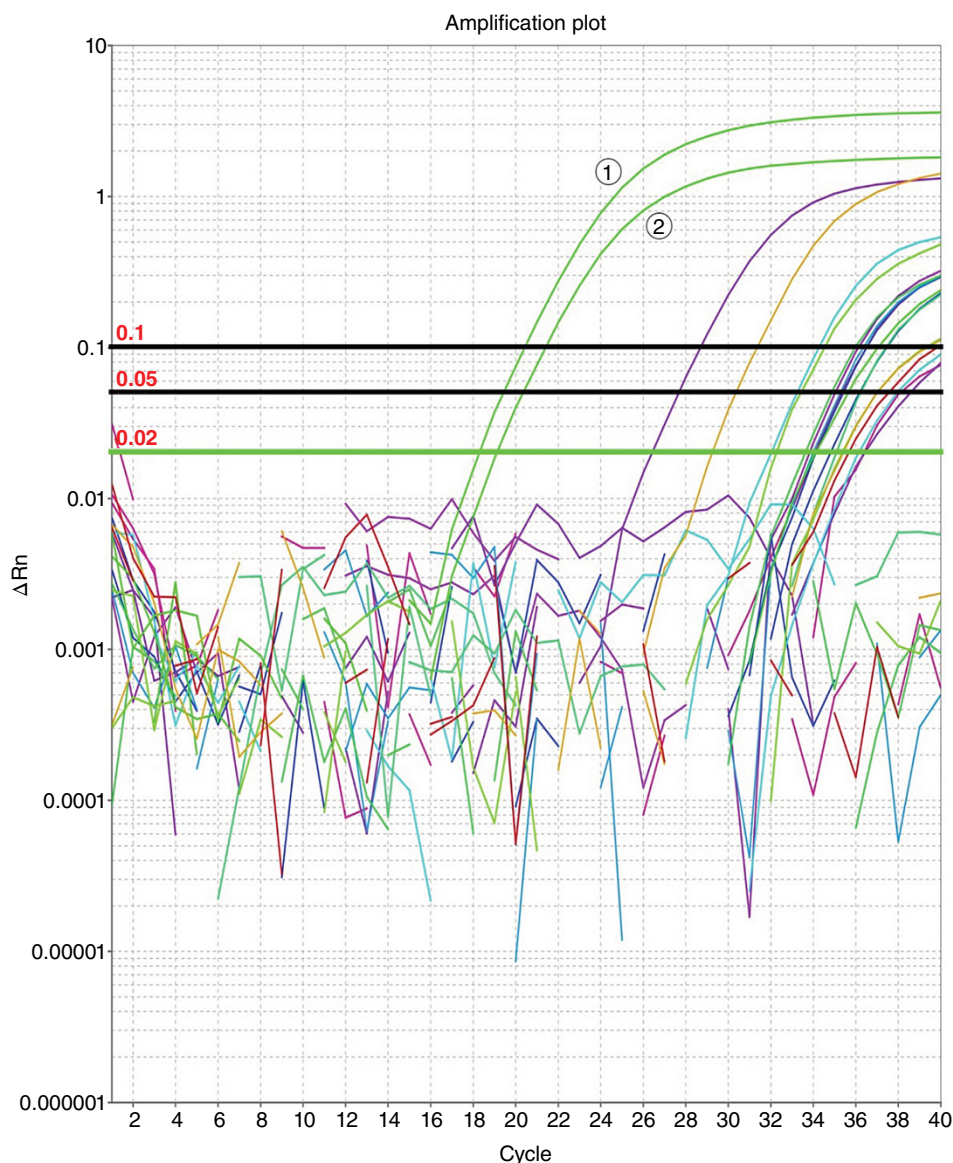


Figure 19.3 Detection and differentiation of type 1 and type 2 porcine reproductive and respiratory syndrome virus (PRRSV) nucleic acids by real-time PCR using a commercially available PRRSV RT-PCR kit. The lines indicate different thresholds: black 0.1 line = type 2 PRRSV; black 0.05 line = type 1 PRRSV; green 0.02 line = internal control. 1 = type 2 PRRSV positive control curve; 2 = type 1 PRRSV positive control curve. (See insert for color representation of the figure.)

19.5.5 Assays for genetic characterization and differentiation

19.5.5.1 PCR-restriction fragment length polymorphism

PCR-restriction fragment length polymorphism (PCR-RFLP) combines PCR amplification and restriction enzyme digestion techniques, enabling two related viruses to be distinguished at the molecular level without having to determine their nucleotide sequences. By carefully selecting unique restriction enzymes that recognize specific sites in the target viral genomes, PCR-RFLP can differentiate minor genomic differences between two virus isolates or genotypes that would otherwise not be

distinguished by other assays except by sequencing. PCR-RFLP requires post-PCR manipulation in which the amplified PCR products are digested with a chosen restriction enzyme(s) and separated by electrophoresis. A definitive or differential diagnosis can often be made on the basis of the restriction fragment patterns. Reagents for PCR and RFLP restriction enzyme digestions are commercially available. PCR-RFLP has been used to detect and differentiate the genomic sequences of many veterinary viruses such as feline panleukopenia virus [60], infectious bursal disease virus [163], porcine reproductive and respiratory syndrome virus [179], feline immunodeficiency virus [59], and porcine circovirus [33]. The assay enables

reports to be generated rapidly and is less expensive than other post-PCR manipulations, such as sequencing and sequence analysis.

19.5.5.2 Sequencing and sequence analysis

PCR amplification coupled with sequencing and sequence analysis is generally used to confirm the specificity of the amplified PCR products, differential diagnosis, genotyping, and genetic characterization of the target viruses. This method has become affordable and rapid in recent years and is now more frequently used in veterinary diagnostic laboratories to determine virus characteristics and sequence changes.

19.6 Veterinary bacteriology

19.6.1 Bacteriology identification systems

Many of the assays and techniques described above for veterinary viruses also apply to bacteria isolated from veterinary specimens, and therefore will not be repeated here. This section will focus primarily on reagents and systems designed specifically for bacterial identification. Many bacteria isolated from animals are identical to or are of the same genus as those that cause disease in humans. However, there are few commercial bacterial identification systems capable of adequately identifying most veterinary-specific pathogens because the number of veterinary-specific pathogens is inadequate to generate the large databases needed for their accurate identification. Furthermore, the cost to develop systems needed to identify the relatively few host-specific bacteria that are isolated in clinical veterinary laboratories is relatively high. Therefore, systems developed for isolation of bacteria from human clinical specimens are often used for the identification of bacteria from animal specimens. For a detailed discussion of rapid kits and instruments used in bacteriology, please refer to other chapters in this text. This section will focus on reviews of studies that have evaluated the application of available systems for identification of bacteria isolated from animal specimens.

Knowledge of the animal species and body site from which a bacterial isolate originated is an important key in the identification process. Although most state animal diagnostic laboratories and teaching hospital laboratories will use automated systems and kits for identification, many veterinary diagnostic laboratories still use conventional macrotube biochemical tests [128]. The cost of rapid identification systems compared to the cost of macrotube biochemicals may prohibit the use of the former in smaller veterinary laboratories. However, most veterinary diagnostic laboratories will also maintain the conventional macrotube tests because, as mentioned above, kit and automated systems usually lack data from a sufficient number of animal isolates to generate accurate biocodes

within their databases [1,27,139]. Many studies have evaluated the miniaturized biochemical test systems and semi-automated systems for their ability to identify isolates of veterinary origin. A review of these has been written [177], but an update is needed.

If accessible, 16S rRNA and/or *rpoB* gene sequencing is becoming the gold standard by which to identify the less common bacterial isolates encountered in the clinical laboratory. However, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF/MS) is rapidly becoming a popular identification system in clinical microbiology laboratories. MALDI-ToF/MS utilizes a laser beam to ablate the upper layers of the bacterial cells of a single colony to generate a matrix of large ionized and neutral particles, particularly proteins that can be used as a molecular signature. Following laser desorption, these molecules are ionized and analyzed by mass spectrometry to generate a spectrum (proteomic fingerprint) that is used to identify the bacterial species. A score of 2 using the Bruker Biotyper 3.0 system software (Bruker Daltonics, Inc.) is considered good correlation with bacterial identification to species level [99]. Recently, Thompson et al. [161] reported testing of almost 3000 bacterial isolates from a variety of animal species and specimens that were compared by MALDI-ToF/MS and conventional biochemical methods and/or 16S rRNA sequencing. MALDI-ToF/MS identification matched that of conventional testing to group, genus, and species level at 91%. The matching level score of MALDI-ToF in comparison to 16S rRNA sequencing was 1.9 and to conventional methods was 2.3. Overall, MALDI-ToF/MS results agreed with 16S rRNA sequencing more often than conventional methods, indicating this method may be highly applicable to veterinary clinical and diagnostic laboratories. Another advantage of MALDI-ToF/MS is that the identification process is simple and rapid (~6 min). However, the initial cost of the equipment is likely to place this technology outside the realm of many veterinary laboratories.

19.6.2 Automated systems

Of the automated systems, only Sensititre (TREK Diagnostic Systems, Thermo Scientific) designs identification and antimicrobial susceptibility plates specifically for the veterinary market, although Biolog claims that their system is broad enough to apply to animal pathogens. The Vitek Gram-positive identification system (bioMérieux, Vitek, Durham, NC) was evaluated using staphylococci and streptococci of bovine origin and had overall accuracy rates of 44.6 and 94.4%, respectively [69,98]. The authors felt that the poor performance of the system in the identification of the staphylococci could be attributed to the low number of veterinary-specific strains in the database. No recent updates of the Vitek system for

identification of staphylococci from animals have been reported. Patten et al. [119] examined the capability of the Sensititre system to identify Gram-negative isolates from animal samples with emphasis placed on the genera *Actinobacillus*, *Bordetella*, and *Pasteurella* and the family *Enterobacteriaceae*. After 18 h of incubation in test plates, 85% of the bacteria were correctly identified to the species level. Fritsche et al. [39] undertook a comparison of the current Sensititre ARIS 2X (TREK Diagnostic Systems) and Vitek 2 (bioMérieux) systems. Of 1377 isolates analyzed, 93.5% and 85.1% of Vitek 2 identifications were correct at genus and species levels, respectively, compared with 93.5% and 83.4% for Sensititre ARIS 2X. The systems were most accurate for identifications at the species level for *Escherichia coli*, *Klebsiella* spp., *Proteus mirabilis*, and *Enterococcus* spp., but species level accuracy was lower for other *Enterobacteriaceae*, nonfermentative bacilli, and *Staphylococcus* spp. As with previous studies, both systems had difficulty identifying *Pasteurella/Actinobacillus* and nonfermentative bacilli other than *Pseudomonas aeruginosa*. However, the same authors found that the two systems were similar and acceptable (>90% categorical agreement) in susceptibility testing of over 3700 Gram-negative isolates of veterinary origin [158]. In comparison to agar disk diffusion, the Sensititre system has been shown to be moderately to highly accurate in determining the susceptibility of Gram-negative and Gram-positive pathogens responsible for bovine mastitis [138]. The BBL Crystal system (Becton, Dickinson and Company, Sparks, MD) was compared with conventional macro-tube identification tests for identification of 132 isolates from bovine mastitis [47]. There was agreement between the commercial kit and the macro-tube tests of 86.4% and 63.6% at the genus and species level, respectively.

In conclusion, none of the commercial bacterial identification systems, either microbiochemical, semi-automated, or automated appear to be capable of identifying the diverse array of bacteria that may be encountered in a clinical veterinary setting. Many of these systems can be helpful in the identification process when used in conjunction with supplemental tests to identify a particular species. A bacterial identification with a relatively low degree of confidence should alert the veterinary bacteriologist to perform ancillary tests. Although it is possible to generate new bacterial identification codes with some commercial systems, comparative tests of automated systems and kits designed for the human market are less commonly being tested in veterinary laboratories as in-house molecular tests become more common.

19.6.3 Enterobacteriaceae

Studies are occasionally performed to evaluate the accuracy of commercial miniaturized biochemical test systems to identify Gram-negative bacilli of veterinary origin.

Identification of members of the family *Enterobacteriaceae* has been evaluated in two older studies, both of which used the API 20E kit (bioMérieux). Swanson and Collins found that the API 20E kit was accurate in determining 96% of veterinary enteric pathogens [156], while Peele et al. [122] found that 66% of veterinary enteric bacteria were correctly identified to genus and species level. The latter study evaluated primarily ATCC-derived strains and 38 members of the family *Enterobacteriaceae*, whereas the former study utilized 503 clinical isolates. Peele et al. [122] also evaluated the Crystal E/NF (Becton, Dickinson and Company), which correctly identified 68% of the enteric, bacterial isolates from animals.

Enterotoxigenic *E. coli* strains that express pili are common pathogens of newborn calves, and assays that detect pili are commonly used for diagnosis of these pathogens. An ELISA is available from IDEXX that is a combination test to detect and differentiate rotavirus, coronavirus, or *E. coli* K99 pili. Although not an identification test, exposure of poultry to *Salmonella* spp. can be determined by an ELISA for antibodies to *Salmonella flagella* (manufactured by IDEXX). Evaluations of these kits have not been reported. Biovet has a Luminex-based *Salmonella* serotyping kit (xMAP) which is a multiplex nucleic acid assay that will identify $\geq 90\%$ of *Salmonella* serotypes in <4 h.

19.6.4 Weakly fermentative and nonfermentative Gram-negative bacteria

Few studies have evaluated the capability of miniaturized biochemical test systems to correctly identify the Gram-negative, weakly fermentative and nonfermentative bacilli isolated from animals. This diverse group includes the genera *Actinobacillus*, *Pasteurella*, *Haemophilus*, *Mannheimia*, and *Bordetella*, among others. Some of these kits are no longer available. Collins et al. [27] reported that none of the systems they evaluated adequately identified *Pasteurella multocida* and *Mannheimia haemolytica*. In another study conducted by Collins and Swanson, the capability of the API 20E system to correctly identify 272 nonfermenting and weakly fermenting Gram-negative bacilli was evaluated [26]. This system was determined to be 62% accurate in identifying this group of organisms. Salmon et al. [140] conducted two studies to evaluate systems designed to identify weakly fermentative or nonfermentative isolates from animals. The RapID NH system (Remel, Thermo Scientific) database did not include *Histophilus somni*, *P. multocida*, *M. haemolytica*, and *Actinobacillus pleuropneumoniae*, but by including modifications, such as animal source and growth requirements, correct identification of these isolates could be obtained. Following a study of the RapID NF Plus system (Remel, Thermo Scientific, Lenexa, KS) that included 273 bacterial isolates in the genera *Actinobacillus*,

Bordetella, *Haemophilus*, *Avibacterium*, *Gallibacterium*, *Mannheimia*, and *Pasteurella* [139], 20% of the isolates were correctly identified. However, if the unique biocodes that were generated by the study were added to the system database, 85.5% of the isolates could be correctly identified. An evaluation of the RapID NF Plus system to identify the avian respiratory pathogen *Ornithobacterium rhinotracheale* was also conducted [123]. Although the identification kit did not include this organism in its database, five unique biocodes were generated. The authors concluded that the system could be used to identify this pathogen if these biocodes were added to the database. More recently, Sellyei et al. [148] reported the capability of the Biolog Microstation ID System (Biolog, Hayward, CA) for identification of closely related *Pasteurella* spp. Although 38 isolates of *P. multocida*, *Pasteurella dagmatis*, and *Pasteurella canis* were examined, the authors concluded that the Biolog system could not adequately discriminate *P. dagmatis*, *P. dagmatis*-like genomospecies, and *P. canis*.

Commercial ELISAs for the detection of antibodies to *P. multocida* in chickens and turkeys, and antibodies to the *A. pleuropneumoniae* RTX toxin IV for identification of infection by any serotype of *A. pleuropneumoniae* in pigs are available from IDEXX, but have not been independently evaluated. An indirect ELISA for detection of antibodies to *A. pleuropneumoniae* serotypes 1–12 is available through IDVet (Montpellier, France). Using immunofluorescence a reagent for identification of *Taylorella equigenitalis* (Pourquier IFI pool of monoclonal antibodies, IDEXX) is available for use with genital swabs, but is available only outside the United States for screening of imported horses for contagious equine metritis.

19.6.5 Gram-positive bacteria

Several studies have evaluated some miniaturized biochemical test systems to identify Gram-positive bacteria of veterinary significance. Again, studies of older systems, many of which are no longer in production, were overall inaccurate at identification of staphylococci [68,81,130,175,176]. A major reason attributed to the inaccuracy of these systems was the inability to adequately identify isolates more specific to animal specimens. In a study by Park et al. [117], 263 coagulase-negative staphylococci isolated from intramammary infections were identified by partial 16S rRNA gene sequence analysis and compared to the API Staph test (bioMérieux). Seventy-six percent of isolates were correctly identified by the API Staph assay, but 97% were correctly identified by a gap PCR-RFLP assay. Recently, the overall capability of the API Staph test to speciate coagulase-negative staphylococci from goat milk was determined to be low (72%), in comparison

to transfer RNA-intergenic spacer PCR. The API Staph assay was capable of adequately identifying most isolates of *Staphylococcus epidermidis* and *Staphylococcus caprae*, but not *Staphylococcus simulans* [78].

The efficacy of commercial miniaturized biochemical systems to identify streptococci from animal specimens has also been examined. Several investigators examined the capability of the API 20 Strep system (bioMérieux) to identify streptococci of bovine origin [69,124,174]. The success of this system to identify the isolates to species level was highly variable (71.4–96.5%). Several *Streptococcus* species were particularly difficult to identify, including *Streptococcus uberis*. A study by Wyder et al. [185] showed that the API 20 Strep test was comparable to sequencing of the 16S rRNA gene for identification of *S. uberis*, *Streptococcus dysgalactiae* subsp. *dysgalactiae*, and *Streptococcus agalactiae*, but there was a high degree of identification discordance for *Enterococcus* spp., *Aerococcus viridans*, and the viridans streptococci. In addition, Reinoso et al. [131] reported that the API 20 Strep system identified 59% of Gram-positive, catalase-negative isolates from bovine mastitis to species level. The API 20 Strep system has also been utilized for the identification of *Trueperella* (*Arcanobacterium*) *pyogenes* [106]. Although this organism is not currently in the database of the system, 62 strains from bovine milk samples were tested, and four unique biocodes for this organism were generated. The authors suggested that the API 20 Strep system could be used along with characteristic microscopic and colonial morphology for the routine identification of this organism.

The rapid latex agglutination kits designed to identify streptococci from human specimens are also applicable for the identification of streptococci from animals. These kits include reagents for identification of groups C and G streptococci, which are commonly isolated from horses and dogs, respectively. Isolates of groups A, B, D, and F are also occasionally isolated from animal specimens. Once the bacteria are isolated in pure culture, these kits are very accurate for group typing of streptococci. Similar agglutination tests are available for identification of *S. aureus*, but have not been tested on isolates specifically from animals.

19.6.6 Anaerobic bacteria

Few studies have evaluated commercial microbiological systems for the identification of veterinary anaerobic bacteria. Adney and Jones [1] evaluated the RapID ANA system (Remel, Thermo Scientific) using 183 clinical isolates. With the additional tests recommended by the manufacturer, the system correctly identified 81% of isolates to the genus level. The majority of the errors that were incurred were a result of biocodes not appearing within the codebook of the system.

Four commercial systems, the RapID ANA (Remel, Thermo Scientific), An-Ident (no longer available; bioMérieux), API 20A (bioMérieux), and Minitek (no longer available; Becton, Dickinson and Company), have been evaluated for their usefulness in identifying *Actinobaculum (Eubacterium) suis*, a cause of cystitis and pyelonephritis in sows, by Walker et al. [171]. It was concluded that the RapID ANA was a useful adjunct for identification of *A. suis*, but that colonial morphology and Gram-stain reaction must be taken into account.

Although none of the numerical codes generated were in the current database, there were no discrepant results in 12 of 18 tests. Reactivity of *A. suis* in the API 20A was limited, with only urease production being consistently positive and 16 of 21 of the reactions being consistently negative. Fluorescent antibody reagents are available from VMRD to identify *Clostridium chauvoei*, *Clostridium novyi*, *Clostridium septicum*, and *Clostridium sordellii*.

An Amies agar-gel-based anaerobic transport swab system (BD Culture Swab MaxV(+); Becton, Dickinson and Company), was evaluated for the capability to maintain viability of strict anaerobes (*Propionibacterium acnes*, *Peptostreptococcus anaerobius*, and *Clostridium sporogenes*), and facultative anaerobes and strict aerobes (*Stenotrophomonas maltophilia*, *E. coli*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus saprophyticus*, and *Lactobacillus casei*) typically isolated from psittacine feces and cloacal samples of birds. After storage at -80°C for 40 days there was no substantial loss of viability compared to baseline cultures for any of the species except *P. anaerobius*, for which there was significant ($P < 0.001$) reduction in growth [109]. Although storage in the transport swab medium was considered useful if freezing of the specimen was necessary, tests were not carried out with freezing the specimen at -20°C , which is likely to be more common.

In addition to being an important nosocomial pathogen in humans, *Clostridium difficile* is also the most common cause of neonatal diarrhea in piglets in the United States and can cause disease in other animal species. Keessen et al. [72] evaluated three commercial immunoassays (Premier Toxins A&B, Meridian Biosciences, Inc., Cincinnati, OH; ImmunoCard Toxins A&B kit, ICTAB, Meridian Biosciences, Inc.; VIDAS, bioMérieux) and a commercial real-time PCR assay (Becton, Dickinson and Company) in comparison to cytotoxicity assays and toxigenic culture for detection of *C. difficile* in 139 diarrheic and 33 normal specimens from piglets. The authors concluded that the performance of all the assays was unacceptable as a single test and recommended that a two-step algorithm be used. However, the real-time PCR assay had the highest negative predictive value in comparison to standard tests, and was acceptable for screening for the absence of *C. difficile*, with toxigenic culture as a follow-up assay.

19.6.7 Mycoplasmas

The API-ZYM (bioMérieux) and the An-Ident (no longer available) systems are enzyme activity assays. Their ability to identify seven species of mycoplasma isolated from swine (*Mycoplasma hyosynoviae*, *Mycoplasma arginini*, *Mycoplasma solivarium*, *Mycoplasma hyorhinis*, *Mycoplasma flocculare*, *Mycoplasma hyopneumoniae*, and *Acholeplasma laidlawii*) based on generated enzyme profiles was evaluated [74]. *Mycoplasma hyorhinis*, *M. hyosynoviae*, and *A. laidlawii* could easily be identified by these assays, but *M. flocculare* and *M. hyopneumoniae* could not be differentiated. Although the systems appeared promising for identification of mycoplasmas, confirmation by FA assay may be required. Synbiotics has an ELISA for detection of antibodies to *M. hyopneumoniae* in pigs. However, the primary methods currently being used for diagnosis of mycoplasma infections in animals are real-time PCR assays, and these may be commercially available for several animal species from IDEXX Laboratories; and many are developed in-house. Standardized reagents for PCR are available for detection of *Mycoplasma gallisepticum*, *Mycoplasma meleagridis*, and *Mycoplasma synoviae* genomic DNA from chicken and turkey tracheal swab samples (IDEXX). The company has reported that the assay has the potential to detect less than 10 copies of DNA/reaction [5].

19.6.8 Mycobacterium spp.

Mycobacterium avium subsp. *paratuberculosis* is the etiological agent of Johne's disease in cattle, and has been implicated as an agent of Crohn's disease in humans. Animals infected with *M. avium* subsp. *paratuberculosis* can be difficult to identify in the early stages of infection because they will appear clinically healthy, and will not have mounted an immune response or begun to shed bacteria. However, animals with *M. avium* subsp. *paratuberculosis* infection do not recover. Collins [25] has reviewed the methods currently available for the diagnosis of *M. avium* subsp. *paratuberculosis*. The standard for paratuberculosis diagnosis has been solid medium culture methods, which have been reviewed by Whipple et al. [181], and will not be discussed here. Standard culture methods for *M. avium* subsp. *paratuberculosis* require 12–16 weeks or more due to the slow generation time (22–26 h) of this organism. This bacterium lacks the siderophore mycobactin, making *M. avium* subsp. *paratuberculosis* an obligate pathogen of animal cells. Addition of mycobactin to the medium is required for culture of this agent. Therefore, culture of the agent is 100% specific, but is only about 50% sensitive [152]. However, a liquid fecal culture test is available from TREK (TREK Fecal Culture, Thermo Scientific), which claims recovery of the pathogen in as little as 2–3 weeks. An automated culture method is the commercial BACTEC MGIT (Mycobacterial Growth Indicator

Tube) (Becton, Dickinson and Company) 960 paraTB culture medium and MGIT 960 instrument, which uses fluorometric technology to detect oxygen consumption. This system was compared to the previous BACTEC 460TB system on 1128 clinical samples (feces and tissues), and positive samples were confirmed by culture and staining [160]. The authors concluded that the MGIT system was significantly more sensitive and faster (time to detection 23 days compared to 33 days) than the 460TB system. However, contamination rates were slightly higher for the MGIT system (6.8% compared to 5.9%).

A genetic probe that is specific to insertion element IS900, which is unique to *M. avium* subsp. *paratuberculosis*, is available through IDEXX to identify this organism following PCR to amplify the specific DNA. Identification using the DNA probe requires only 3 days and is 100% specific, provided that false-positive results do not occur due to contamination of samples by gene products present in the laboratory. Drawbacks are the probe is less sensitive than culture (limit of sensitivity is 104 *M. avium* subsp. *paratuberculosis* bacteria/g of specimen), the assay is relatively expensive, and skilled technicians and specialized equipment are required. However, the use of both culture and the DNA probe could overall increase sensitivity and shorten the time of presumptive diagnosis [24]. Tetracore (Rockville, MD) has an USDA-approved real-time PCR test kit for detection of *M. avium* subsp. *paratuberculosis* for direct fecal detection and culture confirmation. However, a recent comparison of the Tetracore assay with TREK fecal culture indicated the PCR test was positive on 31.3% of culture-positive fecal samples and 8.5% false-positive results were obtained [182].

Several assay formats have been developed for detecting serum antibodies to *M. avium* subsp. *paratuberculosis* in cattle, such as ELISA, AGID, and CF. ELISA is considered the most sensitive and quantitative, whereas CF is the least sensitive. However, all assays that are licensed by the USDA are > 99% specific. Adsorption of the serum with an extract of *Mycobacterium phlei* will optimize the sensitivity and specificity of the ELISA by removing potentially cross-reacting antibodies [24]. The ELISA is available through IDEXX, Synbiotics, and ImmuCell (Portland, ME). A recent evaluation of the IDEXX assay with revised test cutoff levels indicated that the sensitivity of the assay could be increased from 58% to 63% for serum and up to 75% for milk. Specificity remained the same at > 99% [121]. A commercial AGID kit is also available through ImmuCell. However, the sensitivity of any antibody-mediated test depends on the time course of the infection. In the early stage of infection, the sensitivity of ELISA in low-level fecal shedders was 15%, whereas in animals with clinical signs of disease it was 87%, with an overall sensitivity of 45% [157]. The agreement of AGID with ELISA or PCR tests has been considered poor [22].

Infections due to mycobacteria can also be identified by the skin reaction elicited following inoculation of the skin with an extract of the bacteria, and is due to the cell-mediated immune response that follows mycobacterial infection. However, skin testing is not recommended for the diagnosis of paratuberculosis, due to low specificity. The USDA-licensed ELISA test, followed by fecal culture, is considered the most effective way to diagnose clinical paratuberculosis in regard to overall speed, accuracy, and cost [24].

Mycobacterium bovis is the etiological agent of tuberculosis in cattle, and this pathogen is still a concern in domestic animals and for wildlife. Rapid serological antibody detection tests are available from Chembio Diagnostic Systems, Inc. (Medford, NY), and include the CervidTB Stat-PakR and the Dual Path PlatformR VetTB. Both appear to be useful in diagnosis of tuberculosis in elk, reindeer, and white-tailed deer. The sensitivity and specificity of these tests are comparable to the standard skin assays single cervical tuberculin test (SCT) and comparative cervical tuberculin test (CCT) [110].

The Caudal Fold Test is currently the standard skin test used in the United States for diagnosis of bovine tuberculosis. BOVIGAM (Prionics Switzerland) is a gamma interferon test that received approval by the USDA in June of 2003 as an official supplemental test for diagnosis of bovine tuberculosis in cattle [129]. A retrospective study of 16,664 animals sourced from more than 175 herds in eight different countries and published between 1991 and 2006 indicated that overall BOVIGAM was significantly better than skin testing for diagnosis of bovine tuberculosis, and should be included in bovine control programs in the United States.

A rapid, lateral flow assay for detection of feline antibodies to a cocktail of *M. bovis* antigens is available from Chembio Diagnostic Systems, Inc. A multiple antigen print immunoassay, which is a Western blot-type assay, is also available from the same company.

19.6.9 *Chlamydia* species

Although the genus *Chlamydia* had been divided into two genera (*Chlamydia* and *Chlamydophila*), the genus name *Chlamydophila* has not been accepted [154], and therefore only the genus name *Chlamydia* is now used for all nine recognized species [61]. These species include *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Chlamydia muridarum*, *Chlamydia suis*, *Chlamydia abortus*, *Chlamydia caviae*, *Chlamydia felis*, and *Chlamydia pecorum*.

Culture of *Chlamydia* spp. in McCoy cells is the most sensitive method for diagnosis of these agents in veterinary laboratories. However, if laboratories with cell culture facilities are not available locally, specimens containing viable organisms would need to be transported through the mail.

This may result in loss of viability and/or contamination of specimens, reducing sensitivity and specificity. The use of FA reagents can improve the speed and specificity of culture. Lipopolysaccharide (LPS) is a genus-specific antigen, so antibodies to LPS will react with any species in the genus. Other diagnostic methods include serology, histochemistry of tissues using various pathology stains (hematoxylin and eosin, Giemsa, or Gimenez methods), IHC and a colorimetric substrate, or direct FA testing of tissue. The most sensitive nonculture method for direct detection of *C. psittaci* in specimens from psittacine birds is IHC using a monoclonal antibody to the LPS in combination with avidin–biotin alkaline phosphatase [31]. Diagnosis of *Chlamydia* spp. based on antigen detection is an attractive alternative. Commercial kits to identify specific *Chlamydia* spp. from animals are not available, but ELISAs and FA assays for detection of LPS have been developed for detection of *C. trachomatis* from urogenital and conjunctival swabs from humans. These kits are not approved for diagnosis of other *Chlamydia* spp., but several studies have evaluated the capability of some kits to detect *Chlamydia* in various specimens from animals [142,143,166,184]. Overall, most kits had good sensitivity and specificity in diagnosis of *Chlamydia* infections, particularly from placenta and fetal tissues from abortions. In some cases, the ELISA kits were more sensitive than culture, demonstrating the difficulty of maintaining viability of *Chlamydia* in some animal specimens. Unfortunately, many of these kits are no longer available, and the kits that are currently available have not been evaluated. A complete list of these kits can be found in other chapters of this text.

IDEXX provides an ELISA for detection of antibodies to *C. abortus*, which is responsible for enzootic abortion in sheep and goats, and less commonly in cattle, pigs, and deer. However, most diagnostic testing for *Chlamydia* spp. is currently carried out by PCR, which is often an in-house assay.

The Avian and Wildlife Laboratory at the University of Miami Miller School of Medicine can test for *Chlamydia* antigen by ELISA and for avian antibodies to *Chlamydia* by IFA.

19.6.10 Spirochetes and spiral organisms

Leptospira species are important pathogens of many animal species and are zoonotic pathogens for humans. VMRD provides monoclonal antibodies for use in assays to detect *Leptospira interrogans* serovars *australis*, *canicola*, *copenhageni*, *hardjo*, *icterohaemorrhagiae*, and *pomona*. A standard serological assay is the microscopic agglutination test (MAT), which is usually done in reference laboratories due to the requirement to maintain live cultures of *Leptospira* organisms.

IDEXX sells a lateral flow ELISA-type assay (Snap 4Dx Plus Test) (Figure 19.4) for detection of antibody to the C6 surface antigen of *Borrelia burgdorferi*. Antibody to



Figure 19.4 SNAP 4Dx Plus Test. (Courtesy of IDEXX Laboratories. Copyright © 2012, IDEXX Laboratories, Inc. All rights reserved. Used with permission.)



Figure 19.5 The LACTELISA Brucella Ab Mono Indirect ELISA. (Courtesy of Zoetis.)

C6 wanes quickly after successful treatment, providing a way to monitor response to therapy. Synbiotics Corp. sells a micro-ELISA for *B. burgdorferi* in dogs. VMRD sells Lyme disease fluorescent antibody substrate slides for detection of antibodies to *B. burgdorferi* by indirect FA; control sera are provided.

19.6.11 Miscellaneous agents

ELISA and FA assays are available from IDEXX for the detection of bovine antibodies to *Brucella abortus* in milk and serum, and ovine antibodies in serum, but are only distributed outside the United States. Synbiotics (Pfizer Animal Health, now Zoetis, Madison, NJ) provides antigen for detection of bovine antibodies to *B. abortus* by CF, agglutination, and indirect ELISA (Figure 19.5) tests, and killed cells to *Ehrlichia canis* for detection of canine antibodies; a rapid immunomigration assay for antibodies to the same pathogen is also available. Svanova has an ELISA for detection of bovine antibodies to *Brucella* and *Anaplasma marginale*, and VDT sells a kit that contains antigen for detection of antibodies to *A. marginale* by CF in cattle. FA substrate slides by the

indirect FA technique are available from VMRD for detection of antibodies to *B. canis*, *Rickettsia rickettsii*, and *E. canis* in dogs, *Bartonella henselae* in cats, *B. abortus* in cattle, and *Anaplasma phagocytophilum* in horses. These tests may also be used to differentiate the antibody class (IgG or IgM) with a suitable fluorescein-conjugated second antibody. Synbiotics Corp. sells a micro-ELISA for detection of antibodies to *B. burgdorferi* in dogs, and a slide agglutination test for canine antibodies to *B. canis*. A competitive ELISA is also available from VMRD to detect antibodies to *A. marginale*, *Anaplasma ovis*, and *Anaplasma centrale* in cattle. VMRD provides a monoclonal antibody to *A. marginale* that can be used in various assays for antigen detection, such as Western blotting, ELISA, immunoprecipitation, and immunofluorescence. The Snap 4Dx Plus kit from IDEXX (described above) is a point-of-care ELISA for detection of antibodies to *Ehrlichia ewingii*, *Anaplasma platys*, *A. phagocytophilum*, and *E. canis*, as well as to *B. burgdorferi* and heartworm.

19.6.12 Veterinary state and university-associated diagnostic laboratories and others

Many state diagnostic laboratories associated with university veterinary medical schools and teaching hospitals also provide in-house diagnostic testing for many fastidious, intracellular, and tick-borne pathogens based on host species. A fact sheet for such testing at some laboratories in the east and mid-west United States is provided by the Animal Health Diagnostic Center at Cornell University (AHDC) for tests they and their reference laboratories provide (http://ahdc.vet.cornell.edu/docs/Ehrlichia_Anaplasma_Babesia_Testing.pdf). Many such laboratories are accredited by the American Association for Veterinary Laboratory Diagnosticians, and a list of these laboratories is available: <http://www.aavld.org/accredited-laboratories>. Specific tests run by these laboratories can be found at their respective web sites. It is the responsibility of the hospital or service user to determine which laboratories offer tests of interest to them, and if such laboratories are regionally or nationally located.

Some commercial companies, including IDEXX, VMRD, American BioResearch, and AnTech Diagnostics, provide specific diagnostic services, such as PCR, serology, or culture, for some agents. The specific diagnostic test services offered are listed on their web site (see the Appendix).

19.7 Veterinary mycology

Many of the fungi that cause diseases in animals are responsible for the same infections in humans. *Malassezia pachydermatis*, which is an important cause of otitis media and interdigital dermatitis in dogs, is an exception in that it is an uncommon cause of catheter-associated sepsis in neonatal intensive care units. Therefore, the reader is referred

to the Mycology chapter of this text for isolation and identification of most fungi from animal specimens. For example, all the tests described for cryptococcal capsular polysaccharide antigen testing, identification, and susceptibility testing would be applicable to isolates from animals. However, the same limitations of commercial systems for identifying veterinary-specific pathogens seem to apply to the identification of more veterinary-specific fungi. The MIDI Sherlock yeast identification system, which is based on fatty acid analysis, identified less than 50% of animal yeast isolates to species level, and was inferior to substrate assimilation testing (54% accuracy) and sequence analysis (87% accuracy) [41].

The identification of dermatophytes from animal specimens will be emphasized in this chapter because dermatophytes are responsible for zoonotic infections as well as skin infections in animals. Some dermatophyte species predominate, but are not specific to a given host, such as *Microsporum canis* in cats and dogs, *Microsporum nanum* in swine, *Trichophyton verrucosum* in cattle, and *Trichophyton equinum* in horses. Commercial methods are primarily limited to culture systems, with identification still based primarily on recognition of specific spores and hyphae or nutritional requirements. Culture is carried out most effectively by inoculating skin scrapings from the edge of the lesion and a few short, plucked hairs onto dermatophyte test medium (DTM). Although DTM is selective and differential, too much hair inoculated into the DTM may result in contaminant molds growing and the medium eventually turning red. The overgrowth by contaminants, particularly dematiaceous ones, makes it difficult to determine if a more slowly growing dermatophyte is present. DTM is available from multiple sources, including Remel (Thermo Scientific) and Becton, Dickinson and Company. Hardy Diagnostics (Santa Maria, CA) also provides Derm-Duet II (Figure 19.6), which is a biplate medium consisting of dermatophyte test medium and rapid sporulation medium. The InTray culture system from Tridelta Development Ltd (Maynooth, County Kildare, Ireland) allows dermatophytes to be cultured and identified in a completely closed culture system. Once the InTray is inoculated with hair, skin, or nails it is resealed and does not need to be reopened. A dermatophyte will turn the medium from yellow to red, and the tray can be placed directly on a microscope stage for identification of the agent through a clear plastic window. A similar assay (Mykodermoassay ESA) is available from Megacor Diagnostik GmbH (Hoerbranz, Austria), but the medium turns from yellow to green/blue and enhances sporulation. FUNGASSAY Dermatophyte Test Medium is available from Symbiotics (Orange, CA). In Australia, the AgriMart BVT Dermatophyte Test is available in six and 18 tests per kit. However, subsequent identification of any fungi isolated must still be undertaken in-house or

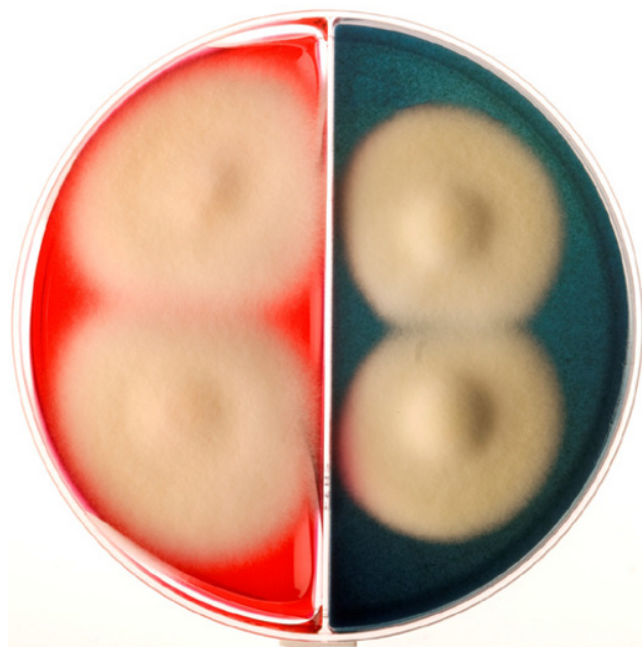


Figure 19.6 *Trichophyton* culture on Derm-Duet II, DTM/ESA (Dermatophyte Test Media/Enhanced Sporulation Media). (Courtesy of Hardy Diagnostics, www.HardyDiagnostics.com, used with permission.) (See insert for color representation of the figure.)

by laboratories that offer fungal identification. Most dermatophytes of the genus *Microsporum* can be identified by distinctive macroconidia. For *Trichophyton* spp. the species-specific nutritional requirements of some species can be determined using *Trichophyton* media (Remel and Becton, Dickinson and Company). These media consist of seven different agar preparations that are nutritionally distinct. Three zoophilic agents that can be identified using these media are: *T. verrucosum*, which requires inositol and/or thiamine-HCl for growth; *T. equinum*, which requires nicotinic acid for growth; *Trichophyton gallinae*, which requires ammonium nitrate for growth [6]. The appearance of the distinctive conidia of dermatophytes has been described [84].

Serological tests may also be useful for confirming a questionable systemic fungal diagnosis, but commercial kits are not available that will test for animal antibodies to these fungi. Several state diagnostic laboratories can provide testing for antibodies to systemic fungal pathogens (see above). However, serological tests for some fungal pathogens are subject to cross-reactivity [77], and may not differentiate previous exposure from active disease [18].

19.8 Veterinary parasitology

Advancements in veterinary diagnostic parasitology, in some ways, have brought about significant changes in the diagnostic approach to the detection of parasites and

parasitic diseases. Commercial test kits specific for animals, as well as commercial veterinary testing laboratories, have increased in numbers over the past decade. PCR methods focusing on parasites have also become commonplace. However, despite these advancements veterinary diagnostic parasitology still relies primarily on less expensive means of detection, the most common of which is the examination of fecal flotations and the microscopic identification of parasite eggs, cysts, or oocysts in fresh, unstained preparations [8,187]. This is very different from human diagnostic parasitology where fecal flotation is a seldom-used technique and samples are usually preserved, often in 10% formalin. For veterinary diagnostic parasitology, preservation of samples can negatively impact the ability of parasite eggs to float [35]. Preservation of human samples does not have the same problem because the sedimentation technique, which does not rely on the ability of eggs, cysts, and oocysts to float, is the more commonly used technique. In veterinary diagnostic parasitology, the sedimentation technique is primarily limited to examination of fecal samples for trematode eggs.

Heartworm (*Dirofilaria immitis*) testing and detection of antibodies to tick-borne organisms are other common parasitological diagnostic tests conducted in veterinary clinics and diagnostic laboratories. The diagnostic approach to heartworm differs with dogs compared to cats [3,4] and is dependent on the serologic detection of antigens of (dogs) and/or antibodies to (cats) *D. immitis*. The examination of blood for microfilariae is no longer the diagnostic test of choice for heartworm, but should be performed in antigenemic dogs as part of the overall pre-treatment workup.

Ectoparasitology is much more important in veterinary medicine than in human medicine. Examination of skin scrapings or ear wax for detection and identification of mites and the identification of fleas, lice, and ticks from the host animal are commonplace in veterinary practices and diagnostic laboratories [8,57,187]. Although several serologic tests are available for tick-transmitted diseases (i.e., *B. burgdorferi*), the proper identification of ticks is extremely important to the continued efforts at control of eradicated diseases (e.g., bovine babesiosis).

19.8.1 Equipment

A quality microscope with a calibrated ocular micrometer is essential for veterinary diagnostic parasitology. Differentiation of most coccidial oocysts and some helminth eggs depends on measuring their size. Objectives of 10 × and 40 × are critical for scanning slides, usually performed at 10 ×. The 40 × objective is generally used for closer examination of specific organisms such as trophozoites of *Giardia*, *Tritrichomonas foetus*, or oocysts of *Cryptosporidium*. The oil immersion objective is not

used for fecal flotations as the coverslip is not permanently mounted to the slide and will move. Rather, the oil objective is generally used for the examination of blood smears for protozoal parasites and other intraerythrocytic organisms or fecal smears for oocysts of *Cryptosporidium*. A stereomicroscope is needed for the specific identification of most helminths and arthropods.

Although several simple flotation techniques/kits have been described, most agree that the convenience of these techniques and kits are offset by the loss of diagnostic sensitivity. However, centrifugation techniques have increased diagnostic sensitivity, which is particularly important when the diagnostic stages of the parasite are present in low numbers [11,29,187]. A swinging bucket centrifuge is preferred, but a fixed-angle centrifuge can be used with some modification of the procedure [29]. Appropriate test tubes, sieves, disposable gloves, applicator sticks, tongue blades, solution containers and bottles, scalpel blades, microscope slides, coverslips, chemicals, weigh boats, scales, and a refrigerator are also needed in the laboratory. An epifluorescence microscope is required to examine feces for some of the zoonotic protozoa when using the direct immunofluorescence technique. Historically, because of their expense and specialized use, these microscopes have only been available in veterinary diagnostic laboratories or research laboratories. Recently, more affordable alternatives have become available, such as the Lumin offered by LW Scientific. A funnel, hose clamp, and ring stand are the traditional materials needed to conduct the Baermann technique (described later in this chapter) for lungworm and other nematode larvae [164,187]. Depending on the parasite of interest, other setups for performing the Baermann technique may be more appropriate [38].

19.8.2 Fecal specimen collection and examination

Fecal samples are used primarily to detect those parasites that live in the gastrointestinal tract and liver. Parasites that live in the lungs and other organs can also be detected through fecal examination if their diagnostic stages pass out of the body via the digestive tract [8]. Fecal collection systems and fecal flotation solutions used for human parasitology (see the relevant chapter in this text) could be used in veterinary diagnostic parasitology laboratories, but have not gained wide usage in veterinary medicine because of the preservatives used in these products and their higher costs.

Fecal samples should be examined as fresh as possible. When possible, up to 10 g of fecal material should be obtained. This is often not possible for companion animals, but is usually readily obtainable from livestock. Fecal loops should be avoided in companion animals unless there is no other alternative available. Samples

from companion animals should be collected immediately after defecation whereas those from livestock are usually obtained from the rectum using a gloved hand. The diagnostician's choices of procedures are limited if less than 1 g of feces is available. If collecting samples from feces that have fallen to the ground, care must be taken to avoid picking up soil or vegetation as the samples may become contaminated with free-living nematodes or their eggs. If possible, samples should not be collected and submitted in examination gloves (i.e., palpation gloves). Samples should be placed in sealed containers from which as much air as possible is removed and identified as to animal name or number, species, age, date, owner's name, and veterinarian's name [8,57,187]. Knowledge of the animal's age and species is important because the prevalence of parasites can differ in different age groups of different animals. Generally, samples should be kept on ice until processing or until they can be refrigerated. However, diarrhetic feces to be examined for living trophozoites should not be refrigerated and should be examined within 30 min of collection [187]. Samples shipped to a diagnostic laboratory should be packed on ice or other coolant and shipped using a 24- to 48-h transport service, or split and half shipped without ice if trophozoite examination is to be conducted.

Four common methods are used in veterinary diagnostics to examine feces for parasite eggs, cysts, and oocysts: direct smear/stained fecal smear, the Baermann technique, fecal sedimentation, and fecal flotation. Direct smears (also called wet mounts) and fecal flotations are the two most common procedures used. Direct smears are used primarily for the detection of motile stages in feces, such as the trophozoites of *Giardia* and *T. foetus*. However, because very little fecal material is used, the diagnostic sensitivity of this technique is very low and is not recommended for routine fecal examinations [187]. Materials needed for wet mounts are an applicator stick, microscope slide, coverslip, and normal saline. A drop of Lugol's iodine can be added to the smear to enhance internal structures, but the trade-off is that it will eliminate motility because it kills the trophozoites. Fecal smears can also be stained for identification of intestinal protozoa. Several stains have been used, including acid-fast stains for the detection of *Cryptosporidium* oocysts [164].

Numerous fecal flotation techniques have been described, but they all depend on the difference between the specific gravity of the flotation media and that of the parasite eggs, larvae, cysts, or oocysts being floated [8]. Therefore, the specific gravity of the flotation media will directly impact the ability to detect parasites [29]. Flotation solutions usually have a specific gravity of between 1.18 and 1.3 [164,187]; solutions of at least 1.25 are generally recommended for routine use. Sugar, zinc sulfate, and sodium nitrate are common solutions used for flotation. These are

commercially available either already in solution or pre-measured in dry form, to which water is added; however, not all have a specific gravity of at least 1.25. Commercially available zinc sulfate preparations include OvaSol (Vedco Inc., St. Joseph, MO) and Ova-Mix (VetOne, vetone.net). Sodium nitrate preparations include FECA-MED, FECA-MIX (Vedco Inc., VetOne), and Fecasol (Vetoquinol, Lure cedex, France). Sugar solutions available include Sheather's Sugar Flotation Solution (Jorgensen Laboratories, Inc., Loveland, CO) and Sugar-Med (VetOne). Whether purchased through commercial sources, or produced in-house, the specific gravity of the flotation media should be routinely (e.g., monthly) checked with a hydrometer to detect changes in specific gravity associated with evaporation. If the specific gravity becomes too high, eggs, cysts, and /or oocysts can rupture [164]. Hydrometers are available through many commercial sources including Cole-Palmer, Fisher Scientific, VWR International, Jorgensen Laboratories, and Wards Natural Science.

Zinc sulfate is often recommended as the medium of choice for detection of *Giardia* cysts [164]; however, it should be noted that these cysts will float in other solutions, such as sugar. Because flotation in higher specific gravity media will distort the cysts [187], the inability to detect cysts is more likely associated with observer skill in recognizing the cysts, than the ability of the medium to float them.

Whether or not a centrifuge is used will also greatly impact the diagnostician's ability to detect parasitic infections [11,29,42]. Several gravity-based (also called simple) flotation devices are commercially available, including Fecalizer (Vetoquinol) Fecal Diagnostic Kit (VetOne), Fecal Flotation System (Phoenix Pharmaceutical, Inc., St. Joseph, MO), OvassayPlus (Synbiotics-Pfizer), Fecal Ova Float (Jorgensen Laboratories, Inc), and Feca Flotation Device (Vedco Inc.). These kits usually have a built-in specimen container, a flotation tube, and a sieving device to remove large fecal particles. Because these kits are not subjected to centrifugation, they must stand undisturbed for a minimum of 10 min, although longer times are actually required, before the coverslip can be examined. A special type of simple flotation device is the McMaster slide (McMaster Worm Egg Slide [Jorgensen Laboratories, Inc], McMaster Counting Slides [Chalex Corp., Wallowa, OR]). This is a two- or three-chambered slide that utilizes a dilution technique to estimate eggs per gram of feces and is particularly useful when high egg counts are expected.

While simple flotation devices are popular, centrifugal flotation has been shown to be the superior method [11,29,42]. There are several benchtop centrifuges available; however, it may not always be easy to find an appropriate machine. Key features to look for is a swinging bucket centrifuge with a slow start, slow stop, and capable of holding the appropriate sized test tube. Fixed-angle centrifuges can be substituted with appropriate changes in the

procedure [29,164]. Refurbished centrifuges, such as IEC HN-SII, can be an inexpensive alternative to new; however, the popularity of these workhorses can make finding used machines challenging. New machines that can be less expensive alternatives include the Swing Arm Centrifuge series (Jorgensen Laboratories, Inc), one of which has the advantage of preset speeds for centrifugation of blood, urine, or feces. Finally, traditional scientific supply houses (e.g., ThermoFisher Scientific, VWR International) may have suitable centrifuges. Attempts to combine the advantages of sieving offered by the simple flotation devices with centrifugation have resulted in development of devices such as the Contrate fecal filter, the Squeeze Test (Jorgensen Laboratories, Inc), and the StatSpin OvaTube (Iris International, Inc., Chatsworth, CA).

There are several coproantigen tests available for the detection of *Giardia* and *Cryptosporidium* in humans, many of which have been adapted for use in a variety of other animal species. However, it is generally preferable to use kits that have been tested and approved for use in the animal species of interest. Those currently available are traditional ELISAs or ELISA-based membrane tests and include the *Cryptosporidium* Immunoassay, *Giardia* Immunoassay (SafePath Laboratories, Carlsbad, CA), and the SNAP *Giardia* Test (IDEXX). Use of a direct immunofluorescent antibody test (MERIFLUOR [Meridian Bioscience, Inc]) for detection of cysts and oocysts of *Giardia* and *Cryptosporidium* in many animal species have been described [9,63,134,167].

The Baermann technique is used to recover nematode larvae in the feces and is primarily used for the detection of lungworms in ruminants and cats, and *Strongyloides stercoralis* and *Angiostrongylus vasorum* in dogs. Fresh, uncontaminated feces are essential. Larvae hatching from nematode eggs in aged feces or free-living soil nematodes in contaminated feces may overwhelm the sample and hinder the diagnostician's ability to even find the larvae of interest. At least 5 g of feces is recommended for use. The traditional method places the feces in cheesecloth, which is tied at the top with a rubber band. This fecal pack is suspended in a funnel that has a collecting tube and a clamp attached. Alternatively, the cheesecloth is placed on a suspended piece of wire mesh or sieve and the feces set on top. Lukewarm water (25–30°C) is added to the funnel, the nematode larvae migrate out of the feces and gravity pulls them to the bottom of the collecting tube. The amount of time the Baermann apparatus sits undisturbed depends on the height of the tube; however, 6–8 h is usually recommended, although most are allowed to sit overnight. Afterwards, the liquid in the collecting tube is obtained, centrifuged, and examined for larvae [8,57,164,187]. The procedure is essentially the same when using straight-sided beakers or jars in place of the funnel, as recommended for certain parasites [38].

Table 19.2 Heartworm tests available for dogs and cats

Test (manufacturer)*	Canine/feline	Antibody/antigen	Sample type
DiroCHEK (S)	Both	Antigen	Serum, plasma
WITNESS HW (S)	Both	Antigen	Serum, plasma, whole blood†
VetScan Canine Heartworm Rapid Test (A)	Both	Antigen	Serum, plasma, whole blood†
SNAP Feline Triple‡ (I)	Feline	Antigen	Serum, plasma, whole blood†
PetChek Heartworm PF(I)	Canine	Antigen	Serum, plasma
Solo Step CH (H)	Canine	Antigen	Serum, plasma, whole blood†
SNAP Heartworm RT (I)	Canine	Antigen	Serum, plasma, whole blood†
Canine Heartworm Antigen Test Kit (Sa)	Canine	Antigen	Serum, plasma, whole blood†
Solo Step FH (H)	Feline	Antibody	Serum, plasma, whole blood†

* Manufacturer: S, Synbiotics Corporation (Zoetis); A, Abaxis, Union City, CA; I, IDEXX Laboratories; H, Heska Corporation, Loveland, CO; Sa, SafePath Laboratories.

† Whole blood means anticoagulated whole blood.

‡ Coupled with feline immunodeficiency virus and feline leukemia virus.

Fecal sedimentation techniques are used to detect those parasites whose eggs do not readily float in the standard flotation media, including those of the ruminant liver fluke *Fasciola hepatica* and the canine schistosome *Heterobilharzia americanum*. Test tubes, beakers or cups, scale, water or normal saline (*H. americanum*), tongue depressor or other spatula, and a timer are needed for this procedure. A sieve-based apparatus (FlukeFinder; Visual Difference, Soda Springs, ID) is available, which shortens the overall length of the procedure by removing larger debris with one screen while trapping the eggs on the second screen [8].

19.8.3 Canine and feline heartworm (*D. immitis*)

The diagnostic approach to heartworm has changed over the past 10 years and differs between dogs and cats [3,4]. This change in dogs was primarily brought about by the development of serologic tests capable of detecting parasite antigens with a high degree of accuracy. The examination of blood for microfilariae is no longer the diagnostic test of choice for heartworm, but should be performed in antigenemic dogs as part of the overall pre-treatment workup. In cats, advances in our understanding of the biology of the parasite and the disease it produces relative to the available serologic tests has shifted our approach. Infections in cats generally do not mature, but if they do, few worms are normally present. In addition, clinical disease can occur with either juvenile or adult worms. Thus, both antibody- and antigen-based serologic testing is recommended [4,13]. Antigen-based tests detect proteins produced by the female reproductive tract; thus, the accuracy of these tests decreases when there are (i) male-only infections, (ii) infections of one to three females (usually one) only, or (iii) females are still immature. Antigenemia may also be suppressed/delayed in infected dogs placed on macrocyclic lactone preventives or may persist for up to 4 months after the heartworms have died. Antibody-based tests have the advantage of being able to detect antibodies to both male and female infections with a detectable immune response

as early as 2 months after infection. However, antibody tests cannot confirm the presence of an existing infection, only that an infection has occurred. Thus, correct interpretation of test results requires additional information.

Formats for heartworm serologic tests include ELISA and immunochromatographic test systems. Depending on the test utilized, serum, plasma or whole blood may be used (Table 19.2). All assays have specific directions on specimen handling and procedural instructions. In order to obtain reliable and reproducible results, testing must be performed in strict compliance with these instructions. In addition, some manufacturers offer follow-up consultation/confirmatory testing for those clinics that integrate their tests into patient assessments.

Microfilariae can be found in the blood of dogs with patent heartworm infections that are not on macrolide heartworm preventives. In North America, the microfilariae of *D. immitis* must be differentiated from microfilariae of *Acanthocheilonmea (Dipetalonema) reconditum*, a filarial parasite of connective tissue. Tests include direct examination, the hematocrit test, and concentration techniques. For direct examination, a drop of blood is placed on a microscope slide, covered with a coverslip, and scanned for microfilariae with the 10× objective. Microfilariae will be motile in fresh samples as well as in samples that have been collected in anticoagulant, refrigerated, and warmed to room temperature. Direct examination is the least sensitive method of detecting microfilariae of *D. immitis*. This technique can be used as a rapid screening test, but should be followed by a concentration technique when negative. The hematocrit test is slightly more sensitive than direct examination. For this test, fresh, whole blood is drawn into a microhematocrit tube, which is sealed and then centrifuged for 3 min. Afterwards, the plasma portion of the separated blood is examined within the tube using the 10× objective. Microfilariae can be found moving in the plasma above the buffy coat [187].

Concentration techniques such as Knott's test are far superior to direct examination, particularly in cases where

few microfilariae are present in the sample. Concentration tests depend on the lysis of red blood cells, which allows for a larger volume of blood to be examined. The Knott's test uses 2% formalin (2 mL of formalin plus 98 mL of distilled water) to cause lysis of the red blood cells and preservation of the microfilariae. In this procedure, 9 mL of 2% formalin is added to 1 mL of EDTA- or heparin-treated blood, mixed thoroughly and allowed to stand for 3 min for lysis to occur. The sample is then concentrated by centrifugation and the supernatant discarded. A drop of 0.1% methylene blue can be mixed with the sediment if desired. The sediment is then examined for microfilariae. Membrane filter tests are similar to the Knott's test; however, production of membranes specifically for these tests has ceased.

Size is an important characteristic in the differentiation of microfilariae, so a calibrated ocular micrometer for the microscope is necessary. Examination of relatively fresh, whole blood is important since storage for more than 3 days may cause *D. immitis* microfilariae to shrink [187]. The size of fixed specimens obtained in the Knott's procedure will also vary from that of fresh specimens [36].

19.8.4 Detection of other nematode parasites

Antibodies to *Trichinella spiralis* can be detected in the serum and meat juice of swine by ELISA (Trichinae ImmunoassayKit [SafePath Laboratories] or *Trichinella spiralis* Antibody Test Kit [Bio-Rad]). Because *T. spiralis* does not cause clinical disease in pigs, this test is primarily used to ensure the safety of pork.

19.8.5 Serological tests for protozoal parasites: *Neospora caninum*, *Toxoplasma gondii*, *Trypanosoma cruzi*, *Leishmania* spp., and *Babesia* spp.

Commercially available serological tests for *N. caninum* in cattle and dogs are available, and include an ELISA (IDEXX *Neospora*X2 Ab Test [IDEXX]), a competitive ELISA (*Neospora caninum* Antibody Test Kit [VMRD, Inc.], and an indirect FA test (*Neospora caninum* fluorescent antibody substrate slide and positive and negative control serum [VMRD]). The ELISAs detect IgG antibodies, as does the indirect FA test. However, the latter can be modified to detect IgM. The tests are intended for use in cattle (all three tests) and dogs (indirect FA test). Modifications to detect IgG antibodies of other species have been described [30,155].

Available tests include the serologic detection of IgG antibodies against *T. gondii* in domestic animals: the Toxotest MT (Tanabe U.S.A., Inc., Las Vegas, NV), the *Toxoplasma* Immunoassay kit (SafePath Laboratories), and the *Toxoplasma gondii* Antibody Test Kit (Bio-Rad). The Toxotest MT is a latex agglutination test for use in cats and swine, whereas the other two tests are ELISA kits for

detection of IgG in serum, plasma, or tissue fluids of swine. The modified agglutination test, which is the diagnostic technique of choice for animal species other than swine [155], is only available through some veterinary diagnostic laboratories. Slides labeled with tachyzoites of *T. gondii* are also available for use with animal sera in conducting indirect FA tests (*Toxoplasma gondii* fluorescent antibody substrate slide [VMRD]). Kits for use in humans can be used in other animals as long as they are of the agglutination type, as other methods require species-specific reagents [155,164]. ELISAs specific for dogs and cats have been developed that are available through some veterinary diagnostic laboratories listed elsewhere in this chapter.

Detection of *T. cruzi* and *Leishmania* spp. in dogs is problematic, primarily due to their cross-reactivity in many tests. One test is available for *T. cruzi*: Canine Chagas Dipstick (InBios, Seattle, WA). A second test for use in humans is Chagas STAT-PAK Assay (Chembio Diagnostic Systems, Inc.), which has been evaluated in dogs [111]. Both tests show a high degree of sensitivity and specificity and may address the inconclusive and false-positive results associated with serologic tests that use epimastigote antigens. Either one of these tests could be a useful screening test. However, results from a single test should not be used as a sole criterion for the diagnosis of *T. cruzi* infection [111]. Available tests for detection of antibodies to *Leishmania* spp. include the *Leishmania donovani* ELISA canine IgG antibody detection kit (Helica Biosystems Inc., Santa Anna, CA) and the Canine Visceral Leishmaniasis Dipstick (InBios). A human test, *Kalazar Detect* Rapid Test (InBios), may be useful in dogs [86]. Slides are also available for use in conducting indirect FA tests (*Leishmania infantum* FA Substrate Slide, and *Leishmania infantum* negative and positive control [VMRD]).

Two competitive ELISA kits exist for the serologic detection of *Babesia* spp. in the equine: one for *Babesia caballi* (*Babesia caballi* Antibody Test Kit) and a second for *Babesia equi* (*Babesia equi* Antibody Test Kit; both through VMRD). Antigen slides and control sera for indirect FA testing are also available commercially for *Babesia bigemina* (*Babesia bigemina* positive blood slide [VMRD]) and *Babesia bovis* (*Babesia bovis* positive blood slide [VMRD]). However, as there are federal regulations regarding these organisms, these supplies may have limited availability.

19.8.6 *Tritrichomonas foetus*

Several *in-vitro* culture media have been described for use in the detection of this parasite in infected cattle, most of which have to be made in-house. While this is relatively easy to do, the advent of a self-contained pouch system (InPouch TF Bovine; BIOMED, White City, OR) for the transportation and culture of samples for *T. foetus* has simplified the procedure. Samples (preputial scrape/wash, cervical mucus, fetal abomasal contents) are placed into the top chamber

and mixed with the media. The contents are then squeezed into the bottom chamber; the top folded down, rolled twice, and the wire tabs folded over to prevent the pouch from opening. The pouches are then incubated upright at 37°C. The pouches are transparent, enabling them to be examined directly on the microscope for the presence of trichomonads. Growth of trichomonads within the pouch is not confirmation of *T. foetus*, as other intestinal trichomonads that may contaminate the sample will also grow in the media. Therefore, another method (usually PCR) must be used to confirm the organism's identity. Diagnosis of bovine trichomoniasis is regulated by many states, and therefore specific rules apply that must be strictly adhered to.

A similar pouch system, the InPouch TF Feline (BIOMED), has been developed for the culture of *T. foetus* from the feces of cats. Approximately 0.05 g of feces is added to the upper chamber, mixed, and then squeezed into the lower chamber for incubation. An initial incubation at 35–37°C for 18–24 h is recommended, after which time it is left to stand vertically at room temperature. The pouch is examined at least every other day until organisms are seen, or for a total of 12 days. In feline feces, *Pentatrichomonas hominis* and *Giardia* spp. may be present as well. However, these organisms do not survive in the media beyond 24 h. As with bovines, final confirmation of the organism's identity generally relies on PCR. Real-time PCR amplification reagents and protocol for *T. foetus* are available for use in cattle (VetMax *T. foetus*; Life Technologies). PCR testing using this or conventional PCR techniques can be performed by many veterinary diagnostic laboratories.

19.8.7 IHC staining

Primary antisera (goat origin) for use in IHC for the detection of *N. caninum* and *T. gondii* in animal tissues are available (VMRD).

19.8.8 Ectoparasitology

Specific identification of ticks, mites, fleas, lice, and fly larvae infecting animals is commonly requested of veterinary parasitologists. Macroscopically visible ectoparasites are manually removed from the host. If organisms are to be preserved, storage in 70% ethanol is preferred. Dichotomous keys are valuable in the identification of ectoparasites [7]. Skin scrapings are the technique of choice to demonstrate mites. A small quantity of mineral oil is placed on a microscope slide. After clipping the hair, a blunt blade is dipped in the oil and then scraped across the affected area. The sample is mixed in the mineral oil on the microscope slide, which can then be examined with or without a coverslip [57,164,187].

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Microbiology Laboratory Information Systems

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A microbiology information system is focused on the particular information processing needs of the clinical microbiology laboratory, and should be distinguished from a general laboratory information system, which has been configured to attempt to provide functionality to support the operation of a microbiology section. However, in some cases the designers of a general clinical laboratory information system (LIS) have undertaken a separate design effort to create a comprehensive microbiology system. Our understanding of microbiology laboratory information systems has continued to evolve since the previous edition of this book [13].

20.1 In general, microbiology laboratory information systems fit one of three categories

- 1 Complete microbiology LISs provide the entire range of functionality needed in a clinical microbiology laboratory from accessioning, receiving, and processing to reporting of specimens for direct examinations and cultures. It includes clinical workup and reporting as well as management analysis such as quality control and quality assurance monitoring.
- 2 Instrument management and middleware modules focus on connectivity between analytical instruments and a general laboratory information system. Typically, these provide additional capabilities in the form of decision rules, report formulation, and many other features that a general laboratory information system may lack.
- 3 Specialized modules for a broad spectrum of particular tasks. Examples include infection control management and more advanced “business intelligence” management reports. These are in addition to reporting, which is

standard in a comprehensive microbiology system, such as antibiogram and trend analysis reports.

In any consideration of microbiology information systems, it is important to recognize how the data generated and used in microbiology differ from other laboratory disciplines such as chemistry and hematology. We list here a few contributing factors. There are doubtless others:

- 1 A very high level of technical skill is required to perform bacteriology, mycology, mycobacteriology, parasitology, and virology. A huge knowledge base is required to transform raw observations in the microscope and on the culture plate into putative organism identifications.
- 2 Microbes evolve, while chemistries do not. Once rules are established to accept and validate glucose or creatinine results, those remain very stable over years, but knowledge around microorganisms changes. For example, the FDA (Food and Drug Administration) updates recommended antimicrobial susceptibility testing breakpoints for the commercial assays it approves and reviews. In addition, the CLSI (Clinical and Laboratory Standards Institute) annually publishes new criteria for what levels of antibiotic response constitute sensitive, intermediate, and resistant organisms. An issue arises when CLSI differs from FDA breakpoints. Information system decision tables must be updated for each of these, each time new criteria are published.
- 3 Timing: most chemistries produce results in a couple of hours. A microbiology culture may “hang around” for days, or even weeks. While a culture is being monitored, there is often more than one stage of reporting (preliminary, final). Therefore, the information system must have multiple “slots” into which to insert results, and the significance of those “slots” (preliminary, final) must be clearly distinguished.

- 4 Textual versus numeric: chemistry and hematology values are rather standard from one laboratory to the next, and follow well defined rules. Microbiology organisms, on the other hand, are represented with idiosyncratic abbreviations, and these textual codes will often differ between laboratories even within the same health system. Microbiology results take a variable number of characters. Often, a block of text commentary may be included.
- 5 Clusters of data are linked together in structured ways from specimen type, to culture focus (e.g., aerobic vs. viral), to colony count, to microorganisms, and to sets of susceptibilities.
- 6 There is a hierarchical arrangement of results rather than a simple linear listing of values (as you would have in a chemistry panel).
 - (a) The overall specimen is at the top level.
 - (b) For each specimen/source, there may be a series of specific types of cultures (aerobic, anaerobic, fungal, acid-fast bacteria (AFB), viral).
 - (c) For each culture there are a series of observations, which can include direct examinations or smear results (i.e. Gram stain, Ziehl Nielsen, or other stain pertinent to the culture type), plate observations (colony count and colony morphology), and isolated organisms.
 - (d) Identification tests for those organisms.
 - (e) Susceptibility testing of an organism to various antibiotics.
- 7 Need to aggregate data across microorganisms, and across clusters over time.
 - (a) Tracking the same organism across multiple specimens from the same patient helps assess the clinical significance of those isolates.
 - (b) If multiple bacteria are of concern, a collation of the susceptibility pattern for all of them is needed to select an antibiotic that will be active against all.
 - (c) Tracking the same organism across multiple patients is essential for infection control systems.
- 8 Many specialized rules must be applied. Certain combinations of organism identification and susceptibility dictate a changed interpretation. For example, if an organism is resistant to certain antibiotics, then certain antibiotics that appear effective may be contraindicated.
- 9 Limits on frequency of repeating certain assays: some laboratory assays are quite expensive, and are needed clinically only at a certain frequency. For example, it would be a waste of resources to perform an HIV viral load daily. A set of maximum ordering frequencies should be built into the order entry subsystem.
- 10 What constitutes an “abnormal” result as we feed information to the electronic health record (EHR)? This has been an issue, also, as we send results to the

public health surveillance system. Typically, microbiology systems have not marked results as “normal” or “abnormal”. As long as only humans were reviewing and acting on the results, this worked fine. But in the age of automated case triage, we need to build in algorithms that will classify the typical textual results into “review not needed” and “need to review” buckets (if one prefers to avoid the “abnormal” terminology). Further, any textual result that the algorithm does not recognize should be routed to the “need to review” category. Furthermore, the microbiology section is often responsible for performing and reporting immunology and serology assays. Since the characteristics of these test results are more akin to chemistry and hematology, it may be advisable to use the “general laboratory” section of the LIS. However, this requires that microbiology staff be comfortable in rather different segments of the LIS.

- 11 Other laboratory sections have a finite list of possible outcomes versus the almost infinite scope of Bergey’s Manual for microbiology results. This implies that the automated rules will handle the common cases, and the cases not recognized would be flagged. Not only is there a wide scope of names, but also the taxonomists frequently change the name of common (and rare) bacteria and other microorganisms. While this may make good sense for molecular genetic purists, it can wreak havoc in a system that is not configured to deal with three or four differently named organisms as equivalent entities. Of course, this also adds complications to reporting of cultures, as we try to explain to our clinician colleagues that this is not a new, unfamiliar organism, but rather one that they know well, but under a different name.

20.2 What are the key features of software to support management of microbiology?

Systems must produce and handle the clustered data described above. Even if a comprehensive system has been designed, it will only evolve to usability by implementing a prototype in a real laboratory, and then incrementally improve the ability of that prototype to effectively handle microbiology data. The concept of developing theoretical specifications, writing a final system to those specifications, then implementing is ludicrous.

20.2.1 Reflexive action based on results

A variety of regulatory and accrediting bodies have promulgated regulations and guidelines for microbiology practice to include reflexive action based on results (Logical Progression, Susceptibility Rules). These expectations should be built into the logic of the microbiology

information system. Principal among these are the CLSI, the FDA, and the College of American Pathologists (CAP). The software should immediately alert technologists, management, and/or treating clinicians to sentinel circumstances (e.g., methicillin resistant *Staphylococcus aureus*, or MRSA). These may require patient isolation, special antibiotic cocktails, or other uniquely designed interventions.

20.2.2 Quality control management

There is an extensive array of quality control (QC) checks inherent in running a microbiology laboratory, from culture media sterility and growth promotion to incubator temperatures and its atmospheric conditions. While it is possible to monitor and follow these with paper records, it is far more efficient and complete to rely on your microbiology laboratory information system to monitor and track these results, and remind the manager/supervisor if a required QC check has been missed.

20.2.3 Inventory management

Given the myriad of diverse supplies and reagents required in a complex microbiology laboratory, it is helpful for your primary LIS to assist in tracking usage and stocks of these supplies, and to ensure that they are re-ordered in plenty of time.

20.2.4 Report Module

The Report Module may include ad hoc or user-specified database queries, antibiograms, contamination rates, among others. After patient reports have been issued, there are many other important applications of the data.

- 1 What is the pattern of antimicrobial resistance as a guide to empiric antibiotic treatment and also to detect changes (such as emergence of resistant strains)?
- 2 How often are cultures contaminated? For blood cultures, is there a pattern of contamination that might be improved by retraining certain groups of staff collecting these cultures?

A typical system will incorporate dozens of such special reports. In addition, management and medical staff will ask other questions about microorganism patterns, and these can be addressed by “ad hoc” or “report writer” functions asking arbitrary questions of the database.

20.2.5 Instruments

What types of analytical instruments are key? We will not attempt to duplicate the extensive coverage elsewhere in this book of the variety of new instruments and measurement technologies. For each instrument, a method must be devised to convey the results produced by that instrument (identified by a specimen number) into the microbiology information system. Often, the results will be more than a short text field. Automated plate

readers will send images. Susceptibility testing devices will transmit arrays of antimicrobial results. Genetic-based assays may send tens of thousands of base pairs per organism. With the growing array of instruments being developed for the clinical microbiology laboratory, which may have capabilities from specimen plating to organism identification and antibiotic testing, it will become even more important to optimally interface these instruments to the LIS.

The types of instrumentation to be considered include (but are not limited to):

- 1 microbial identification
 - (a) biochemical based
 - (b) newer technologies, such as mass spectrometry, MALDI-ToF, and others
 - (c) genetic markers and sequencing;
- 2 susceptibility testing
 - (a) traditional
 - (b) genetic;
- 3 plate inoculators/streakers;
- 4 automated plate readers;
- 5 automated growth and detection systems (Bactec, BacT/ALERT, MGIT, TREK, and others);
- 6 total laboratory automation systems – incorporating automated plate transporters, automated plate streakers, and automated incubators.

20.2.6 Interfaces and additional features

The laboratorian must learn from their colleagues in other organizations to beware of the caveats and lessons learned in connecting a microbiology information system with the “typical” hospital information system (HIS). Often the HIS or the EHR does not have internal structure to properly store microbiology results. They only know how to store short phrases. Therefore, it is common for microbiology laboratory information systems (MLIS) to formulate a page image report, and transmit that to the HIS, which then displays it as such. This is known as a “text blob”. The advantage of a page image is that HIS/EHR does not garble display. On the other hand, the downside of a page image is that it obscures granular data on the MLIS report (e.g., susceptibility results) that cannot be trended, or individually analyzed (“sliced and diced”) by applications on the HIS/EHR.

In site visits to health systems that implemented the “text blob” microbiology interface, this inability to trigger on individual microbiology data was a major source of dissatisfaction. Instead, use of the standard HL7 2.5.1 OBR/OBX structure more effectively represents individual microbiology results. The HL7 is the industry standard tool for communication between medical information systems, and has been implemented, worldwide, by essentially every developer of clinical systems. Implementation guides developed by the CDC make use

of HL7 2.5.1 for transmission of communicable disease data. The Meaningful Use regulations, as published by the US Federal Office of the National Coordinator for Health Information Technology, specify use of HL7 2.5.1 in order to qualify for Meaningful Use incentive payments. When the implementers have followed the rules, HL7 standards have been highly successful in conveying discrete results to the HIS. However, this does imply that the HIS/EHR have internal programming to display the discrete results to the clinician in a meaningful way. For more complex report types, and if the EHR has shown its inability to display properly formatted reports, we advocate sending both page image reports (for clinicians review) and fielded detail data (for trending and granular data analysis). For transmission of page image reports, the most widely used mechanism has been to use PDF (portable document format), although some systems can now support HL7 CDA (clinical document architecture).

Whenever fielded data are transmitted, it is important to represent test names, and organism identifications, using standard LOINC (logical observation identifier names and codes) and SNOMED (systematized nomenclature of medicine) codes. In general, LOINC is used to identify what test was run (e.g., a viral culture, or imipenem susceptibility), while SNOMED is used to provide organism identification (e.g., *S. aureus*) and in some cases specimen identification (sputum, urine). Use of LOINC and SNOMED is not only responsible laboratory practice, but it is now required in US Federal Meaningful Use regulations.

20.3 Microbiology information systems have evolved over several decades

One of the earliest “homegrown” systems was created in the mid-1970s, at the University of California, San Francisco. Dr. Stephen Cohen led a successful effort to create a MLIS. The small computer used had only two cathode-ray tube (CRT) workstations, yet data entry was to be performed by eight technologists. This was accomplished by implementing all technologist data entry using mark sense forms. When a specimen was received in the laboratory, the receiving clerk would type patient name, specimen description, and other demographics into a CRT. He/she would then print a mark sense form, which would go with the plates for Gram stain, incubation, and reading. As the technologist examined the smears/plates, they would mark on the form their observations. As each stage of examination was completed, the mark sense forms were scanned into the computer. This information was then immediately available for telephone inquiries and consultation. When the culture examination was complete (including susceptibilities), the technologist would mark the “final” box, scan the form, and the computer would

automatically print out the reports for the patient chart. Another important early system was developed at the National Institutes of Health Clinical Center [14].

20.3.1 Modules of commercial laboratory information systems

While home-grown systems were instructive, the systems with the most impact were those incorporated into commercial laboratory information systems. Although a number of the LISs of the late 1970s included some level of logic to handle microbiology, the model introduced in 1980 by Peebles and Ryan [15], and still used in the Sunquest LIS of today, incorporated several key advances.

- 1 Highly efficient data entry by technologists using designated single keys to enter common (but lengthy) utterances.
- 2 A database structure that accurately represented microbiology data (as opposed to shoehorning microbiology into a chemistry model).
- 3 Use of a database tool that efficiently handles sparse array data (M, now Cache).

Other commercial laboratory information systems have since created microbiology modules as parts of their comprehensive LISs. In some cases, these have been well fit to efficient microbiology practice. In other cases, they were more of an afterthought to chemistry, and required voluminous keystrokes to enter common results. Fortunately, it was possible to apply front-end reengineering to these legacy systems to streamline data entry [3]. Healthcare systems have advanced the integration of microbiology data in hospital-wide clinical systems [12]. Vendors continue to newly release software focused on microbiology [16].

Although most MLISs are now a module of a larger scale LIS, some vendors have developed, and support, standalone microbiology information systems. A niche particularly receptive to microbiology only software is the public health laboratory (PHL), since a typical public health laboratory will have a relatively restricted test mix. Unless the PHL doubles as a general clinical laboratory for community health clinics, its patient-specific testing will exclude hematology, chemistry, or anatomic pathology. In some cases, almost all patient-specific testing will be microbiology focused.

Also evolving have been vendors of middleware, including automation controllers. Some of these tools are now full-fledged microbiology LISs, but are often implemented in a laboratory that already has a general purpose LIS. The middleware handles functions that the general purpose LIS is not quite ready for. Typically, a laboratory will use the middleware software provided by the vendor of the instrumentation mainly used by the laboratory. Thus, if you operate a particular, commonly

Table 20.1 Vendors of microbiology laboratory information systems*

Vendor name	Product	Year first installed	Number of non- PH micro contracts in US	Number of PH micro sites	Website (add www. to each)
Aspyra	CyberLAB	1982	45	0	aspyra.com
Becton Dickinson	Epicenter	NA	NA	NA	bd.com/ds
bioMérieux	Myla	2010	300	NA	biomerieuxperformancesolutions.com
BtB	BtB LIMS	1990	0	4	btbsoftware.com
Cerner	Millennium Pathnet	1982	632	3	cerner.com
Clinical Information Systems	CIS Laboratory	1981	39	1	cislab.com
Clinical Software Solutions	CLIN1 Laboratory	1987	55	0	clin1.net
ClinLab	LIS	1987	10	2	clinlabinc.com
Common Cents [†]	ApolloLIMS	1987	12	12	apollolims.com
Comp Pro Med	Polytech	1981	21	0	compromed.com
CompuGroup/Antek	LabDAQ	1991	300	5	cgmus.com
CPSI		1986	454	0	cpsinet.com
CSS	CLS2000	1980	86	2	csslis.com
Diamond	LabGEM	1984	40	5	diamondcomputing.net
Data Innovations	Instrument manager	1993	NA	NA	datainnovations.com
Dawning Technologies	JResultNet	1984	NA	NA	dawning.com
Entre Technology	Genesys NonStopLab	1984	3	0	genesysnet.com
EPIC	Beaker	1984	17	1	epic.com
Hex	Lab/Hex	1981	145	1	hexlab.com
LabSoft	LabNet	1992	60	3	labsoftweb.com
LigoLab		2006	NA	0	ligolab.com
McKesson	Horizon	1972	NA	0	mckesson.com/laboratory
Meditech	Magic,C/S,6.0	1970	NA	NA	meditech.com
Netlims	AutoLIMS	1996	35	5+	netlims.com
Orchard	Harvest	1993	300	18	orchardsoft.com
Psyche	WindoPath/LabWeb	1976	21	1	psychesystems.com
Quality Software	LabHealth	1984	17	0	labhealth.com
SCC Soft	SoftLab	1985	310	3	softcomputer.com
Schuyler House	SchuyLab	1994	560	0	schuylerhouse.com
Siemens	Novius Laboratory	1983	47	1	usa.siemens.com/medical
StarLIMS	Clinical LIMS	2001	10	26	starlims.com
Sunquest	Microbiology	1979	400	3	sunquestinfo.com
Technidata	TD-Synergy	1972	8	1	technidata-web.com

Sources: Some data adapted with permission from references 1 and 2, plus additional unpublished data.

NA, data not available at the time of this writing.

*Information contained in this table consists of information based on the authors experience, contacts with vendors, and data available at the time of this writing.

[†]Also consult vendor for M/MGMT system.

used brand of equipment, you are unlikely to get the most benefit from their competitor's middleware (which is optimized for that competitor's equipment). Three vendors/microbiology-specific systems in this space include (but may not be limited to):

- 1 bioMérieux Myla [11]
- 2 SiemensLabPro Information Manager/Connect (Micro-scan Systems)
- 3 Becton Dickinson Epicenter.

General-laboratory middleware products provided by Dawning and Data Innovations have also connected to some microbiology instrumentation. This discussion reflects the scope of microbiology-relevant middleware that we have encountered in some decades of laboratory informatics practice. However, there are certainly other offerings in this category: as with other products, the potential user must evaluate and investigate, through multiple sources and colleagues, all products available at

the time, including all vendors and their web sites, since this is a rapidly advancing field.

Since 1987, one of us (RDA; with colleagues M. Weilert, O. Pasia, K. Carey, H. Weiner and others) has published in *CAP Today* a yearly tabular review of LIS software available. Most recently, we published a table of general LIS products (which includes many microbiology applications), and tables of middleware systems (including some with microbiology capability). Data adapted from both of these, and other sources, is presented in Table 20.1. As discussed earlier, almost all commercial LISs offer "microbiology functions". Caveat emptor: one must evaluate their usability, completeness, and efficiency. If for institutional organizational reasons, you are required to use a particular system, look into reengineering techniques [3,18] that will permit you to improve efficiency. Also, you may want to incorporate middleware to provide some of the database capability missing from the "complete

LIS”. At one time, there were a sufficient number of dedicated MLIS offerings that we published a separate tabulation of these in the May issues of *CAP Today* [1,4–6,17]. However, more recently the number of nonmiddleware focused MLISs has decreased, so we have included those few microbiology focused vendors into our November general LIS tables [7].

20.4 Criteria for comparison of current systems

- Usability/productivity is a criteria typically missing from exhaustive listings of “functions”. Sure, the system permits you to enter an organism ID, but is it one key-stroke or 35? How many screen flips are required in routine workup of a common organism? How long do those take? The emphasis should be on efficient, intuitive operation, not on “pretty screens”. In one recent visit, we were distressed to hear the technical staff comment: “in our lab, we work for the computer”.
- Support for LOINC and SNOMED: as noted above, this is increasingly important for integration with clinician’s EHRs, as well as reporting of cases to Public Health Disease Surveillance (PHDS).
- Ability to integrate with other vendor’s systems: all vendors will claim “sure, we can connect to any system”, but this is a common source of failure. It is best to call contacts at sites that actually have the interface you have in mind up and running, and verify that it is a reality. Furthermore, what is the nature of the interface? Is it via a text blob, where the receiving HIS/EHR can only display a page of all results, but not look at patterns of individual results (such as imipenem susceptibility)?

- Upsides and downsides of a system focused on microbiology versus a generic one designed for a general electronic health record (or LIS). Unless you are a PLH, or a specialized laboratory that only performs microbiology, you are likely to be running a general purpose LIS. You may have to reengineer portions of this to improve efficiency or effectiveness for the microbiology laboratory. This may be doubly the case if your hospital has decided that you will run the associated LIS provided by its EHR vendor.

20.5 Specialized software

We discuss here several types of tools that are beyond the scope of a general microbiology information system. Typically, these functions are not provided by the general MLIS, but instead by a separate group of vendors. We discuss them here because it is important to be aware of, and understand, their interactions with MLIS.

- 1 Infection control software: this is a large class of software (Table 20.2) that draws data from a number of hospital systems (laboratory, admitting, surgery, and pharmacy) and collates these data to guide infection control professionals in their work to detect and arrest the spread of infections within the hospital. Such software has been used since the mid-1980s, and it has made significant progress in the decades since then.
- 2 Reporting disease to PHDS: every microbiology information system should have the capability of identifying cases of importance to PHDS, encoding those with LOINC and SNOMED, and securely sending those to the appropriate PHDS jurisdiction [2,9,10]. Increasingly, EHR systems are being asked to do the same but we strongly encourage this selection/sending

Table 20.2 Infection control systems*

Vendor name	System	Website
Atlas Development	Guardian	atlasdev.com
Becton Dickinson	ICPA Redbat (consult vendor for availability)	bd.com
Carefusion	MedMined	Carefusion.com
Cerner	Millennium	Cerner.com
CKM Healthcare	Infection Prevention and Control	Ckmhealthcare.com
EpiQuest	EpiQuest	Epiquest.com
ICNet International	Antimicrobial stewardship, infection prevention	Icnetplc.com
Intersystems	Cache, Ensemble, TrakCareLab	Intersystems.com
ACS	Midas+	Acs-inc.com
RL Solutions	RL6: Infection	RLsolutions.com
Premier	SafetySurveillor	Premierinc.com
PharmacyOnesource	Sentri7	Pharmacyonesource.com
System Services Inc	(under development)	Ssinow.com
Hospira	Theradoc	Hospira.com
Thomson Reuters	Infection Xpert	Thomsonreuters.com
Vecna Technologies	QCPathfinder	Vecna.com
Vigilanz Corporation	Dynamic Infection Control Monitor (ICM)	Vigilanzcorp.com

Source: modified from the website www.apic.org – of the Association of Practitioners of Infection Control; as of May 1, 2013.
*Consult vendors for current information.

to be done by a system more focused on microbiology. At the present time, granular understanding of complex microbiology results is too far outside the core expertise of an EHR.

- 3 Analysis of DNA sequencing: this important class of software is quite distinct from a general microbiology LIS. It operates on a different type of data set (sequencing of an organism vs. a library of sequences of known organisms) and we believe is best viewed as a type of analytical instrument.

20.6 Selecting an information system for your laboratory

Given the variety of capabilities required in an information system, it would be logical to think that a selection should be based on searching for a system with the most functions, then choosing that one. Surprisingly, that is not the optimal approach. This arises because the tool must be continuously adapted to your laboratory needs, and you are choosing a tool for long-term use, perhaps over decades. It is much more important to choose a business partner who will be responsive to your current needs, and will be focused on your needs long term, not just in the 6 months after you install it.

The LIS functioning as your knowledge engine, commonly known as software, encapsulates the business rules by which your laboratory operates. In the past, these were sometimes ancillary, with the real process engine being operated in the minds of the laboratory staff, using the software for support and documentation. Increasingly, though, the software is configured to actually guide and drive the professional work of the laboratorians. Together with the skills and dedication of the staff, the robustness, reliability, and accuracy of the software are the two irreplaceable success factors in laboratory informatics.

20.6.1 The preferred strategy for selecting software

First, the general functional characteristics of the system you seek to install should be defined. Then, identify several commercial suppliers/vendors who provide such software, and obtain from each a complete listing of all their clients, and contact information. Information on which vendors are likely to provide such listings is difficult to obtain. Speak with several clients (your choice, not the vendor's) for each prospective vendor. Then enter into discussions with the vendor(s) with the highest client satisfaction. Evaluate which will be the best long-term (10+ years) business partner. Unfortunately, many organizations ignore this tested methodology, and instead prepare a basic "Request for Proposal" (RFP) listing a myriad of detailed functionalities, numerically score the responses, and select a company that claims the needed functionality,

but may be the worst long-term business partner of the lot. Thus, we prefer the acronym RFP to be translated "Request for Problems".

20.6.2 Avoiding the flawed RFP strategy

In the traditional RFP approach, the organization lists all the functions needed in a new system, the department asks prospective vendors to respond, and then scores the RFPs and selects the highest scoring proposal. However, this procedure has significant flaws and risks. As noted above, there is a far more productive way to determine what vendor or system should be selected. The most important activities are to talk with many of the current clients of a vendor under consideration (making their own selection of which clients to check from a complete list of clients). The department should carefully evaluate the quality of support offered by the vendor, and remember that this selection involves choosing a long-term (10+ years) business partner rather than a vendor to perform a one-time task of installation. The minutiae of functionality are much less important, and will evolve, given a vendor who is dedicated to supporting the business priorities of your organization.

20.7 Cases

We present several scenarios, based on personal experience, and on discussions with colleagues. These are intended to illustrate important principles, and to help the reader avoid common pitfalls.

20.7.1 Case 1: Did you actually check the references?

A large government department prepared a detailed RFP, placing particular emphasis on a commonly used database platform (SQL) and how knowledge should be represented (ontology, XML, etc.). Previously, one of their local affiliates had developed a system that was functioning well in several departments, but that system did not match the large department's preconceived specifications. The local affiliate spoke glowingly about their business partnership with the vendor they had selected to develop their system. However, the large department instead chose a vendor that purported to meet almost all of the departments' desired functionality, and was reported by the vendor to have been installed in two other departments. Three years later, installation progress had been exceedingly slow and the vendor declared bankruptcy. At that point, it was discovered that the department selection team had never called the two other reference departments to personally verify the vendor statements. One reference department had been unable to advance the system to

production status, and the other had never begun testing the software. The department lost several million dollars in the venture, as well as its credibility with the local affiliates. A few years later, that department contracted with another LIS vendor.

20.7.1.1 Lessons learned

- 1 The decision to purchase an information system is not primarily a matter of IT requirements, and the RFP must not be the primary selection tool. A key activity in systems selection is investigating customers of the vendor, specifically the existing users of the system in question. The relationship between the vendor and its customers is of much greater importance than a particular technical architecture.
- 2 Usage of a department, laboratory, or company-preferred technology platform may not be an appropriate method for vendor selection. The effectiveness of a system, and the long-term growth trajectory of the vendor behind it, is far more important than use of a particular proprietary technology, no matter how fashionable.

20.7.2 Case 2: Demonstrated vendor performance and reputation outweighs an RFP score, even a score of zero

In selecting a LIS, a hospital prepared an extremely detailed RFP (almost 200 single-spaced pages), to which multiple vendors responded, each stating that most criteria were “standard in our system.” However, the hospital had been on site visits to other laboratories running these systems and had seen that the systems did not provide the claimed functionality. Another vendor had not responded to the RFP at all (and therefore had an initial RFP score of “zero”), but upon hearing good reviews of that vendor, the hospital initiated contact. Despite being a small company with limited resources, the vendor sent a brief handwritten response. Although many responses were stated as not “standard in the system”, the hospital continued to investigate this vendor. But after a demonstration of that vendors system, as well as two site visits to extremely satisfied clients, the hospital decided to select this initially nonresponding vendor. The hospital had a successful installation of that LIS and, decades later, is still running that same system (much improved in the ensuing upgrades). Meanwhile, three of the original four vendors who provided voluminous RFP responses had declared bankruptcy within 5 years.

20.7.2.1 Lessons learned

This case illustrates another cautionary note for laboratories using RFPs for system procurement. The results of RFPs may not only be misleading, but may lead to

overlooking qualified candidates. Factors additional to the RFP, such as site visits, are crucial in the decision process; these additional factors are of greater importance in making a correct choice.

Focus on implementation and management: while many “good” information systems have been unsuccessful due to factors such as poor implementation or management, it is quite possible for a “bad” information system to support the needs of the laboratory very well, if managed by a stellar staff and a supportive vendor. A successful system is principally dependent on two factors: (i) talented, motivated, and respected staff in the organization; and (b) a software supplier (vendor) culturally dedicated to partnership with the client and support of the end user.

20.7.3 Case 3: The bandwagon effect

A large group of laboratories chose a particular vendor to install their categorical software system, solely on the basis that one large, respected laboratory had chosen that vendor. This entity was using this vendor’s system in a very complex environment with a myriad of specialized laboratory divisions, versus the group of independent laboratories that had a very different requirement, each encompassing all these activities in a single laboratory. The fact that the large entity had an entirely different business model and information processing needs was not considered. Four years later, many of those laboratories were still struggling to achieve basic functionality from what turned out to be a “tool kit” rather than a functioning system.

20.7.3.1 Lesson learned

Look at the needs of your entity, not what some unrelated (though respected) organization has chosen to do.

20.8 Management of the laboratory information system

After installing your new software package, it must be managed. This entails much more than would be required for a typical laboratory instrument. The CAP, in their General Laboratory Accreditation checklist, provides a very useful checklist of many measures that should be in place for your LIS.

20.9 Personnel management

Key to the successful implementation and management of your laboratory information system are your staff. Organizations that take for granted the skills and dedication of these team members will learn to regret it.

Providing all of the other funding and infrastructure described here to an inadequately trained, underrecognized,

or unmotivated workforce may be actively counterproductive. Conversely, a motivated and talented team can produce remarkable accomplishments with “inadequate” equipment and funding.

Knowledge of your professional team depends on both formal training courses, as well as ongoing participation in peer interaction, through national and regional laboratory informatics groups and meetings, webinars, and user-group meetings. One of the hallmarks of the most successful informatics programs is sharing, and receiving, innovations from other programs.

In a small laboratory, it is even more important than in a large program to encourage your staff to participate in national training, and user-group and informatics conferences. Much problem solving in informatics depends on creative and nonintuitive solutions to implementation and connectivity conundrums. Contacts to enable these solutions are made at the national level.

The need for effective personnel management is not unique to the microbiology laboratory, or to the informatics section but is especially important when trying to attract and retain scarce resources such as laboratory informaticists. Some laboratory organizations have been guilty of treating their informatics resources as lower level “computer technicians,” thus denying them the support and flexibility that are needed in order to achieve the full benefit from their skills.

Those who simply assume “we will install the computer system, and it will solve our problems” are making a very serious mistake. Informatics-aware professionals are more difficult to recruit than a typical clinical laboratory scientist. It may be necessary to accommodate flexible work schedules, and keep in mind that promotions and advancement must be available.

In summary, recruiting, motivating, and retaining your informatics staff is every bit as important as selecting the right vendor, or system, to begin with.

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Emerging Infectious Diseases

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21.1 Introduction

Over the past 40 years the field of infectious diseases has undergone significant changes. We have witnessed the emergence of new infections such as *Legionella*, HIV, Hantavirus, and severe acute respiratory syndrome (SARS). In some cases, significant technological strides in diagnostics and molecular amplification have allowed us to identify new pathogens. In addition, with the development of resistance to antimicrobial therapy, diseases caused by previously well-characterized pathogens have presented new challenges to clinicians, as is the case with extremely drug resistant (XDR) tuberculosis. Thus, humans are constantly experiencing newly recognized infectious diseases and encountering previously known infections with new characteristics. Recently, emerging diseases have been defined as “Diseases of infectious origin whose incidence in humans has increased within the past 2 decades or threatens to increase in the near future”, by the Institute of Medicine. Although the definition may appear too restrictive, it gives us sound guidance in describing emerging infections.

In the previous edition, we discussed ehrlichiosis, bartonellosis, bovine spongiform encephalopathy, HHV-8, West Nile Virus (WNV) encephalitis, and *Escherichia coli* O157:H7 among others [249]. In this chapter we review some of the most important organisms that have been identified as new human pathogens in the past 10 years as well as some previously known pathogens that have undergone significant changes in the same period.

21.2 *Plasmodium knowlesi*

Four species of *Plasmodium* (*falciparum*, *vivax*, *ovale*, and *malariae*) have long been known to cause malaria in humans. *Plasmodium knowlesi* was discovered as a distinct

malaria species in long-tail macaques in 1931. The following year, researchers demonstrated that the parasite could infect humans. However, in 2004, 120 cases of malaria due to *P. knowlesi* were reported in patients from Malaysia [245]. Since then, this pathogen has been increasingly recognized as a human malaria parasite in Southeast Asia.

Infection with *P. knowlesi* has been studied extensively in primates, including in rhesus monkeys that develop fulminant disease from the parasite. The asexual cycle lasts 24 h, which is the shortest cycle among all types of primate malaria. Similar to *P. falciparum*, it can infect any age red blood cell [126] and there is no evidence of a latent stage in the liver [137]. In Malaysia, the primary natural vector in macaques is *Anopheles hackeri*, although many other species of *Anopheles* have been shown to transmit *P. knowlesi* as well.

In 1965, the first natural infection of a human with *P. knowlesi* was reported in a traveler returning from what is now Malaysia, with the diagnosis being made on the basis of the quotidian fever pattern and the ability of the isolated parasite to cause overwhelming infection in rhesus monkeys [30]. In 1971, another suspected *P. knowlesi* infection was described in a patient returning from fieldwork in the jungle in Malaysia. The diagnosis was based on the morphology of the parasite on blood smear and convalescent serum for antibody titers [73]. Thus *P. knowlesi* was viewed as a malaria parasite of primates that was rarely documented to cause human disease until the report by Singh et al. in 2004 [245]. Subsequently, *P. knowlesi* infections have been reported throughout Southeast Asia [19,42,46,61,70,104,118,122,124,127,151,154,162,188,196,216,250,272]. Furthermore, archived blood samples from patients in Malaysia diagnosed with *P. malariae* infection in 1996 were reexamined and found to contain *P. knowlesi* DNA in 97% of the samples [153]. Since the growing recognition of

P. knowlesi as a cause of human malaria, one case has been reported in the United States in a traveler returning from the Philippines in 2008 [39].

The clinical presentation of *P. knowlesi* infection is similar to that of the other human malarias, with fever, chills, headache, and malaise being the predominant symptoms. Gastrointestinal symptoms including nausea, vomiting, abdominal pain, and diarrhea were reported in 30–50% of patients in one case series [46] but were less common in another series, occurring in 5–15% of patients [245]. Disease can be severe, with 11 deaths reported in the literature to date [41,42,281]. The level of parasitemia with *P. knowlesi* infection can be quite high. In the first series reported by Singh et al. [245], approximately one-third of patients presented with parasitemia of over 5000 parasites/ μ L in blood with a maximum level of 117,600 parasites/ μ L in blood. All fatal cases reported have been associated with high levels of parasitemia, including one patient with 764,720 parasites/ μ L in blood [41,42,281]. Laboratory findings are notable for thrombocytopenia while anemia is uncommon [46]. Reinfection with different strains of *P. knowlesi* has been reported [148].

Transmission of *P. knowlesi* infection appears to occur primarily from primate to humans via the *Anopheles* mosquito. Human to human transmission has been documented under experimental conditions [30].

The mainstay of diagnosis of malaria in the community continues to be through the examination of thick and thin peripheral blood smears and this is the case for *P. knowlesi* as well. However, distinguishing *P. knowlesi* from other types of malaria by blood smear alone is very difficult. It is often

mistaken as *P. falciparum* or *P. malariae*. The morphology of *P. knowlesi* malaria has been described in 10 patients in whom mono-infection with this species was confirmed by nested polymerase chain reaction (PCR) [154]. In nine of the ten patients, the infection was asynchronous at the time of presentation. Similar to *P. falciparum* and *P. malariae*, erythrocytes infected with *P. knowlesi* were not enlarged. Faint irregular dots, known as Sinton and Mulligan's stippling, which is distinct from Schuffner's dots sometimes seen in *P. vivax* infection, were observed in some infected erythrocytes. This appearance is similar to that of Ziemer's stippling sometimes observed in *P. malariae* infection. The early trophozoite form of *P. knowlesi* closely resembles *P. falciparum* due to the presence of double chromatin dots, multiple parasites in a single erythrocyte, and appliqué forms. From the late trophozoite stage onward, there were no morphologic characteristics of *P. knowlesi* infection that distinguished it from *P. malariae*. (Table 21.1 and Figure 21.1) [154].

Given the difficulty in distinguishing *P. knowlesi* infection from other types of human malaria by microscopy, molecular methods for the detection of this infection have been pursued. PCR primers for diagnosis of malaria typically use small subunit rRNA genes that are distinct to each species of *Plasmodium* [277]. Primers with 100% specificity for *P. knowlesi* and the ability to detect as few as ten copies of *P. knowlesi* DNA/ μ L in blood have been developed [4,52,110,193]. Another molecular diagnostic method, loop-mediated isothermal amplification (LAMP), requires a less specialized laboratory set-up and therefore may be a better option to implement in the field. Two LAMP assays,

Table 21.1 Morphologic description of *P. knowlesi* compared to *P. malariae*

	<i>P. malariae</i>	<i>P. knowlesi</i>
Host erythrocyte		
size	Not enlarged	Not enlarged
shape	Rounded, not distorted	Rounded, generally not distorted
stippling	Ziemer's stippling under certain staining conditions	Irregular dots or stippling in some erythrocytes with mature trophozoites, schizonts and gametocytes
Parasite		
early trophozoite (ring form)	Ring form with dense cytoplasm and single chromatin; sometimes with accessory dot	Ring forms are compact with dense cytoplasm: single or double chromatin and rarely triple chromatin; appliqué forms: multiple parasites in single erythrocyte
late trophozoite	Regular and compact cytoplasm: appearance of pigment	Dense and thick cytoplasm; cytoplasm slightly amoeboid and irregular: band forms; varying pigmentation
mature trophozoite	Compact, rounded, heavily pigmented: band forms: not amoeboid	Compact and dense cytoplasm, rounded shape with dark brown pigment: band forms: not amoeboid
Schizont	Occupies whole erythrocyte. Contains 6–12 merozoites, usually 8: merozoites clustered around dark-brown malaria pigment forming rosette pattern	Occupies whole erythrocyte; contains up to 16 merozoites; merozoites irregularly scattered or grape-like cluster; malaria pigment scattered or collects into a single mass
Gametocyte	Round, compact. Occupies whole erythrocyte, scattered malaria pigment: early forms are very similar to mature trophozoites	Round, compact. Fills whole erythrocyte. Scattered or clumped malaria pigment: early forms are very similar to mature trophozoites

Source: Adapted from Lee et al. [154].

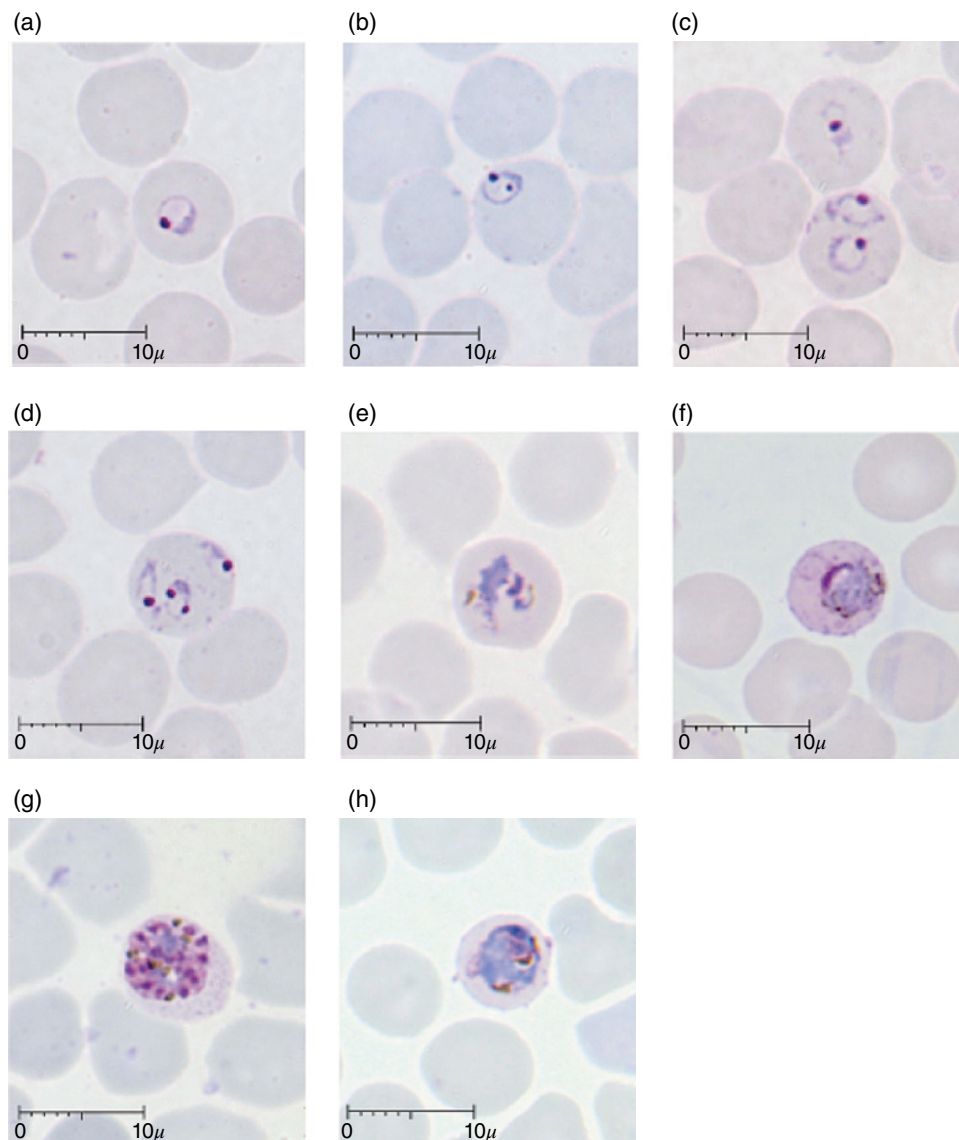


Figure 21.1 Giemsa-stained thin blood films of different stages of *P. knowlesi* infection in human red blood cells (RBC). Early trophozoite (a), early trophozoite with double chromatin dots (b), single RBC with multiple early trophozoites (c), appliqué forms of early trophozoites (d), late trophozoites (e), mature trophozoites (f), schizonts (g), and gametocytes (h). (Adapted from Lee et al. [154], <http://www.ncbi.nlm.nih.gov/pmc/articles/pmc2676309/>, used under cc-BY 2.0; <http://creativecommons.org/licenses/by/2.0/>.) (See insert for color representation of the figure.)

one targeting β -tubulin genes [112] and one targeting the apical membrane antigen-1 gene [149] have been developed to detect *P. knowlesi* infection. The specificity and sensitivity of this method is reported to be equivalent to nested-PCR assays [112,149].

Another approach to the diagnosis of malaria is the use of rapid diagnostic tests (RDT), which check for *Plasmodium* antigens in human blood samples. There is limited, conflicting published experience with the use of RDTs in cases of proven *P. knowlesi* infection: tests have been reported as negative for all malaria species [19], positive for *P. falciparum* only [196], positive for non-*falciparum* malaria only, or positive for both *P. falciparum*

and non-*falciparum* malaria [104,196]. Thus the currently available RDTs for human malaria are not reliable for use in the diagnosis of *P. knowlesi* infection.

While access to molecular diagnostics remains limited and rapid diagnostic tests remain unreliable, the diagnosis of *P. knowlesi* infection should be considered in patients living in or returning from Southeast Asia with symptoms consistent with malaria. Markers that strongly suggest a diagnosis of *P. knowlesi* in such patients are exposure to the forest or forest fringe, and morphologic evidence of *P. falciparum* in the early trophozoite stage or of *P. malariae* by microscopy combined with a higher level of parasitemia or more severe disease than expected with *P. malariae* infection.

There is limited data about the treatment of *P. knowlesi* infections. There are numerous case reports and small case series of different antimalarial agents successfully used for the treatment of *P. knowlesi* infection, albeit with varying degrees of follow-up: chloroquine [104,124,151,188,281], mefloquine [19], quinine with or without doxycycline [127,148,151,281], atovaquone/proguanil [39,70], sulfadoxine-pyrimethamine [245], and artemisinin derivatives [107,281]. In the first reported series by Singh et al., information about treatment was available for 94 patients with *P. knowlesi* infection. Two patients were treated with quinine and 92 were treated with chloroquine and primaquine. There was no sign of early treatment failure as evidenced by clearance of parasitemia in all patients and there were no deaths reported [245]. In another series of 87 patients, most were treated with chloroquine and primaquine with no deaths reported [46]. In Sabah, Malaysia, there were six deaths reported out of a group of 56 patients with *P. knowlesi* infection. All patients who died met World Health Organization (WHO) criteria for severe malaria. Five of these patients were treated with quinine and one was treated with artesunate. An additional 22 patients received quinine and 13 received an artemisinin derivative, all of whom survived [281].

One prospective observational study has investigated the response of *P. knowlesi* infection to oral chloroquine and primaquine in 60 patients with 28 days of follow-up. Only patients with uncomplicated infection were included (parasitemia of $\leq 100,000/\mu\text{L}$). All patients received four doses of chloroquine and, if normal glucose-6-phosphate dehydrogenase levels, two doses of primaquine. All patients responded to treatment. Follow-up blood samples at 28 days found no evidence of *P. knowlesi* infection by nested PCR, suggesting that there was no resistance to chloroquine or primaquine and that there were no episodes of reinfection or recrudescence [45]. However, the use of primaquine is probably unnecessary given the lack of evidence for a latent stage of *P. knowlesi* infection in the liver [137].

There are no studies investigating the role of chemoprophylaxis for the prevention of *P. knowlesi* malaria. Given the response of infection to chloroquine, doxycycline, mefloquine, and atovaquone/proguanil, these agents may be effective for chemoprophylaxis as well.

21.3 *Clostridium difficile*

Clostridium difficile, a toxigenic Gram-positive spore-forming organism, was identified as the primary cause of antibiotic-associated diarrhea and colitis in 1977 [9]. During the past decade, a startling increase in rates of *C. difficile* infection (CDI) was noticed in the United States and Canada, and has spread worldwide, with increased morbidity and mortality [80,185,208]. This was associated with the emergence of a so-called “hypervirulent” strain.

Three distinct features of this strain have led to a long and somewhat cumbersome description: (i) belonging to restriction endonuclease type BI; (ii) having North American pulsed-field type 1; and (iii) exhibiting PCR-ribotype 027. Hence this strain is called BI/NAP1/027 [159,175].

The reported incidence of CDI in the mid to late 1990s in acute care hospitals in the U.S. was stable at 30–40 cases per 100,000 population which rose to 50 cases in 2001 and further still, to 84 per 100,000 in 2005 [176,185]. In Canada, a retrospective study of all cases of CDI over a 13-year period in Quebec reported an increase in incidence from 35.6 cases per 100,000 population in 1991 to 156.3 cases in 2003 [208]. The clinical presentation was also more complicated, with increased 30-day mortality. This increase has been attributed largely to the spread of the BI/NAP1/027 strain, which prior to the year 2000 had accounted for fewer than 1% of US *C. difficile* isolates [175]. In England, a large outbreak caused by this strain occurred between 2003 and 2004 and again, in 2004 and 2005, at a single hospital, which resulted in a total of 334 CDI cases and 38 deaths [80]. Since then, cases of CDI caused by BI/NAP1/027 have been documented in multiple states in the USA, all provinces of Canada and many European nations, both causing outbreaks and sporadic disease [80]. Today, rates of CDI continue to rise, though not all are due to BI/NAP1/027 [10,28]. While healthcare-facility-associated CDI is well known, community-associated disease from the NAP1 strain is also recognized [10].

The virulence of *C. difficile* is due to the production of two toxins, TcdA and TcdB, originally described as an enterotoxin and cytotoxin, respectively [255]. The toxins are taken up by the colonic epithelial cells and they irreversibly glycosylate certain cellular proteins. This alters the actin cytoskeleton leading to cell death from impaired nutrient transport. The toxins are transcribed from a pathogenicity locus that comprises five genes: two toxin genes, *tcdA* and *tcdB*, and three regulatory genes, one of which (*tcdC*) encodes a putative negative regulator of toxin transcription [255]. Certain BI/NAP1/027 isolates carry *tcdC* gene deletion mutations that have been associated with an approximately 16-fold more toxin A and 23-fold more toxin B production *in vitro* than strains that do not have these mutations [276]. In addition, a third toxin is produced by the NAP1 strain; a binary toxin, CDT, which has enterotoxic effects *in vitro*. Its role, however, in the pathogenesis of clinical disease is not known, though speculation exists as to whether it may have synergistic effects with toxins A and B to cause severe disease [84]. Not all toxins are produced by all strains of *C. difficile*; in the Canadian outbreak, toxin A and B production occurred in about 65% of strains, binary toxin in 35% of strains while toxin B was produced by all strains [173].

Further studies have suggested that NAP1 strains have greater sporulation capacity *in vitro* than nonoutbreak strains, but other studies refute this claim [22,67]. Another unusual aspect of the NAP1 strain is the resistance to fluoroquinolones. All older isolates prior to 2000 were susceptible to the newer quinolones moxifloxacin, levofloxacin and gatifloxacin, but recent isolates are all resistant. The widespread use of fluoroquinolones in hospitals has likely promoted the spread of NAP1 strains due to selection pressure [159,175,185,207].

Patient risk factors for CDI have typically included hospitalization, advanced patient age, especially older than 65 years and antimicrobial exposure. Almost every antimicrobial class has been reported to lead to CDI due to suppression of the normal bowel flora, allowing *C. difficile* to flourish. A few are more common culprits, namely, clindamycin, cephalosporins, penicillins, and now fluoroquinolones due to the resistance patterns of the epidemic NAP1 strain [191]. The duration of exposure to antibiotics as well as exposure to multiple agents versus a single agent increases the risk for CDI [206]. Duration of hospitalization is also a risk factor, serving as a proxy for the duration and possibly degree of exposure to the organism from other patients with CDI [35]. A controversial area is the role of gastric acid suppressants in CDI. Various studies have shown an association between stomach-acid suppressing medications such as proton-pump inhibitors and histamine-2 blockers and CDI, but this association could be the result of confounding from underlying severe illness [48,49,159,185,206]. Other reported risk factors include cancer chemotherapy, HIV disease, gastrointestinal surgery or gastrointestinal tract manipulation, including tube feeding [35]. Potential sources of *C. difficile* in the community include soil, salt, tap and fresh water, pets, meats, and vegetables [191]. To date, there is no conclusive evidence that ingesting food contaminated with *C. difficile* has led to clinical disease in humans. Disease has also been reported among populations not previously at risk, such as healthy peripartum women and close contacts of patients with *C. difficile* infection with no other risk factor [29].

The primary mode of transmission is person-to-person spread through the fecal-oral route. The presence of spores on the hands of healthcare workers transiently contaminated could be a mode of spread given the lack of activity of alcohol-based hand rubs on spores that are routinely used in most hospitals [178]. Environmental contamination with the spores is also important in healthcare settings [53]. Prevalence of asymptomatic colonization with *C. difficile* varies from 7 to 26% in adult inpatient acute-care facilities, and between 5 and 7% among elderly patients in long-term care facilities [35]. Colonization risk increases during hospitalization indicating a daily cumulative risk of exposure. However, the risk of developing CDI appears to decrease rather than increase over

time, suggesting that progression to diarrhea develops early or not at all [231]. A recent prospective study in Canada found that the NAP1 strain was predominant among patients with CDI, while asymptomatic carriers were more likely to be colonized with other strains of *C. difficile* [158]. The usual incubation period between exposure and development of CDI is not known but is estimated to be between 2 and 3 days [35]. This is different from the increased risk of CDI that persists for weeks after exposure to, and cessation of, antimicrobial agents.

The clinical manifestations vary widely from a self-limited diarrheal disease that abates when the offending antibiotic is discontinued to fulminant disease that can result in death [255]. Watery diarrhea multiple times a day is usual with lower abdominal pain and cramping, low-grade fever and leukocytosis often over 15,000 cells/mm³ [255]. Physical examination can reveal lower abdominal tenderness. Sigmoidoscopy or colonoscopy may show patchy mild erythema and friability to severe pseudomembranous colitis. Unexplained leukocytosis in a hospitalized patient even without diarrhea may represent underlying CDI, though diarrhea often develops in the next 1–2 days [275]. Fulminant disease presents with diarrhea, severe lower quadrant or diffuse abdominal pain, abdominal distension, fever, hypotension, lactic acidosis, and a marked leukocytosis. Ileus may mask the diarrhea. Toxic megacolon and bowel perforation can also occur [275]. Small-bowel infection is very rare but enteritis may occur in the setting of prior colectomy [150].

Outbreaks caused by the BI/NAP1/027 strain have been associated with more severe disease and increased mortality. In Quebec, in 2003, the 30-day attributable mortality was 6.9%, four times higher than the previous national average in 1997 [159]. The proportion of complicated disease increased from 7.1% in 1991–1992 to 18.2% in 2003 [208]. A high leukocyte count ($\geq 20,000$ cells/mm³) and an elevated creatinine (≥ 2.3 mg/dL) were strongly associated with adverse outcomes, as was age ≥ 65 years [208]. In the outbreak in England where greater than 59% of the isolates were NAP1 strains, the attributable mortality was 11% [278]. Increased rates of recurrence have also been reported with the NAP1 strain [206].

The gold standard for the diagnosis of CDI had previously been either the cell culture cytotoxicity assay or toxigenic culture [35], but PCR is rapidly becoming the preferred test. Testing should be performed only on unformed stool unless ileus is suspected. Enzyme immunoassay (EIA) for toxins A and B is rapid but less sensitive (64–94%) [44]. Other useful tests include detecting the presence of GDH (glutamate dehydrogenase) in stool (sensitivity 88–100% with negative predictive value of 98.8–100%) or real-time PCR of feces to detect toxin genes (sensitivity 80–94%, negative predictive value $> 98\%$) [58]. Some laboratories utilize a two-step testing algorithm using an

initial screening test such as EIA combined with the GDH test, with further testing of positive samples by toxigenic culture, cell cytotoxicity assay or PCR [138]. Repeat testing during the same episode is of limited value [35].

Diagnosis of the BI/NAP1/027 strain, while important for epidemiological purposes, is not specifically important for the individual patient and clinical disease severity should guide treatment decisions. Stopping any offending antimicrobial agent is a key. However, some cases are not associated with any antimicrobial use and a good number do not appear until after the antibiotic has been stopped.

Metronidazole 500 mg orally three times daily for 10–14 days is suitable for mild–moderate disease, while vancomycin 125 mg orally four times daily for 10–14 days is recommended for severe CDI [44]. Severe, complicated disease should be treated with vancomycin either orally or rectally (if ileus is present) with or without intravenous metronidazole [160,295]. Patients with toxic megacolon, lactic acidosis, and hypotension may require a colectomy [144].

There are some newer approaches to CDI treatment. Fidaxomicin was approved in the United States in 2011 to treat CDI. At a dose of 200 mg twice daily, it had similar cure rates, but lower rates of disease recurrence for strains other than the BI/NAP1/027 strain [144]. Treatment of a first recurrence is usually with the same agent that was used for the initial episode; however recurrences after the first should not be treated with metronidazole due to the risk of cumulative neurotoxicity [129]. A vancomycin taper, or pulse and taper, regimen is usually employed to mop up cells that are still in spore form but germinate after the vegetative cells have been eliminated [177]. Other therapeutic agents that have shown variable efficacy include rifaximin and nitazoxanide. Toxin-binding regimens using cholestyramine and colestipol [123] have been utilized, although these drugs can bind antimicrobials in the colon as well. Probiotic agents such as *Lactobacillus* species and *Saccharomyces boulardii* may have some benefit in conjunction with standard treatments. Bioremediation or “fecal transplant” where a stool from a healthy donor is instilled by either nasogastric tube or enema, has been tried with high rates of success but there are several limitations to this method [1]. Human monoclonal antibodies against toxins A and B given in addition to antibiotics have shown a significant reduction in the recurrence of CDI, including patients infected with the NAP1 strain in a phase-2 trial [161].

Prevention strategies for CDI include those directed against patient to patient or environment to patient transmission and those minimizing patient risk of acquisition if exposure occurs. Isolation techniques (private rooms, contact precautions), rigorous environmental cleaning along with good hand hygiene with soap and water is recommended after contact with patients with CDI [35]. Antimicrobial stewardship programs to help decrease

antibiotic consumption also play an important role [185,262]. A *C. difficile* toxoid vaccine is currently being evaluated in a phase-2 trial for the prevention of CDI (clinicaltrials.gov NCT00772343).

CDI has evolved over the past decade and is a major cause of infectious diarrhea in healthcare settings. Most of this change has been due to the emergence of the BI/NAP1/027 strain. Progress has been made in regards to novel agents to treat disease and a better understanding of risk factors and measures needed to control outbreaks. However, reductions in the prevalence of the NAP1 strain will not prevent the emergence of new epidemic strains in the future and continued surveillance is warranted.

21.4 Pandemic H1N1 influenza

Influenza viruses are members of the *Orthomyxoviridae* family and are classified into types A, B, and C. All influenza types have segmented, negative-sense, single-stranded RNA genomes with host-derived cell envelopes, and surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA), which determine the specific subtype. HA is responsible for binding and fusion to host cell receptors, often on respiratory epithelium, while NA is responsible for release of viral particles from infected cells by catalyzing the removal of terminal sialic acids. The M2 ion channel, unique to influenza A, is also on the cell envelope and is necessary for viral uncoating. Influenza A can infect a variety of hosts in addition to humans, including: swine, equine, avian, marine mammals, canines, and feline populations [258].

Alteration in HA or NA structure can lead to new variants (antigenic variation). Antigenic drift is due to point mutations that can slightly alter structural antigens. This occurs during and between influenza seasons and may permit reinfection even in patients who have previously been infected or immunized by a parent virus. Antigenic shift is due to virus reassortment in an animal reservoir, resulting in a new virus and in some cases there may be little or no population immunity. While birds are easily infected by influenza viruses and shed the virus in their GI tracts allowing for easy human exposure, pigs have receptors for both avian and human influenza virus strains and thus create a crucible for the exchange of genetic information and the potential to create novel subtypes to which humans have little immunity [186].

In March 2009, a novel swine-origin influenza A virus (H1N1) was reported in Mexico with subsequent reports in the United States. Airline travel helped spread human infection to multiple continents, resulting in the WHO declaring a pandemic in June 2009, which continued through August 2010 [133]. The pandemic is thought to be due to a reassortment of two swine strains that served as reservoirs for multiple cross-species transmissions. The

novel virus resulted in an unusually high rate of morbidity and mortality in children, healthy young adults, and pregnant patients [298] and a comparatively low rate of morbidity for older adults (including the elderly). The Centers for Disease Control and Prevention (CDC) estimated that there were over 60 million cases of pandemic H1N1 (pH1N1) in the United States, with over 250,000 hospitalizations and 12,500 deaths. Pandemic H1N1 was less common in persons over 65 years likely due to preexisting cross-reacting antibodies in those born prior to 1930 [72,100,181].

Person-to-person transmission of influenza viruses occurs through contact with large-particle droplets and respiratory secretions, though fomites are also thought to play a role. In contrast to seasonal influenza epidemics that occur during the winter, with highest severity in the elderly and adults with co-morbid conditions, pandemics often affect multiple age groups outside of the winter season, with increased mortality in young adults. Pandemic H1N1 occurred in two waves, which peaked in June and October with a lower rate while children were out of school [120].

This pandemic H1N1 variant was shown to replicate to a higher degree than usual in tracheobronchial epithelium and alveolar epithelial cells, resulting in desquamation of respiratory epithelium, loss of cilia, and inflammation of the trachea and bronchi. Severe cases may progress to necrotizing bronchiolitis, diffuse alveolar damage with alveolar hemorrhage, edema, hyaline membrane formation, and organizing fibrosis [113].

The incubation period is 1.5–3 days, with viral shedding starting a day prior to symptom onset and persisting for 7 days. Immunosuppressed and transplant patients can shed virus for weeks [31]. Clinical symptoms of pH1N1 are consistent with other subtypes, including: fever, chills, sore throat, malaise, anorexia, myalgias, nonproductive cough, rhinorrhea, and headache. Infants may present with lethargy, poor feeding, irritability, tachypnea, or apnea. Gastrointestinal symptoms such as diarrhea and vomiting appear to be more common with pH1N1. Complications are most severe in children less than 2 years, the immunosuppressed, and those with underlying co-morbid conditions, such as chronic lung disease including asthma, cardiovascular disease, cancer, diabetes, and children with neurologic conditions, although a high proportion of otherwise healthy adults, obese, and pregnant patients were also affected. Pregnant patients are at increased risk of spontaneous abortion, preterm labor, and fetal distress. The highest number of hospitalization and deaths were in those < 50 years with chronic lung or cardiac conditions, pregnant patients in their third trimester, and children < 5 years with neurologic or pulmonary disorders (although many infected patients had no underlying medical condition) [25,75,115,116,120,140,152,155,246,289].

A high rate of complications was noted during the pandemic including: secondary bacterial pneumonia with *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae*, hypoxemic respiratory failure with acute respiratory distress syndrome (ARDS) requiring mechanical ventilation, renal insufficiency, multisystem organ failure, rhabdomyolysis, encephalopathy, seizure, and myocarditis. Laboratory abnormalities may include elevated liver transaminases and lactate dehydrogenase with lymphopenia and anemia. Chest radiographs in hospitalized patients commonly have mixed interstitial and alveolar infiltrates that vary on computed tomography from patchy consolidation or multilobar infiltrates to ground glass opacities [116,289].

Rapid influenza diagnostic tests (RIDT) such as enzyme-linked immunosorbent assay (ELISA), optical immunoassay, or lateral flow are commercially available and can be performed in < 30 min. While the specificity is very high, the sensitivity of RIDT ranges from 40 to 75% without any difference in accuracy during the recent H1N1 pandemic [27]. RIDT are able to distinguish influenza A and B viruses but are unable to distinguish subtypes of influenza A, as is the case with direct and indirect immunofluorescent antibody testing (DFA and IFA), which require a fluorescence microscope and take 1–4 h to perform. The sensitivity and specificity are over 90% and 95%, respectively, when compared to real-time reverse transcriptase PCR (rRT-PCR) [212]. Viral culture on a variety of cell lines, including Madin–Darby canine kidney (MDCK) cells and primary human airway epithelial cells, often reveals growth in 3 days [113]. rRT-PCR remains the most sensitive and specific testing modality and is able to differentiate influenza types and subtypes, although cost and availability may inhibit routine use [242]. Nasopharyngeal aspirates (NPA) have generally been considered the specimen of choice, although the diagnostic yield of combined nasal and throat swabs (CNTS) has been reported to have a higher diagnostic yield [197]. Testing can also be performed on bronchoalveolar lavage (BAL) fluid in those with lower respiratory tract disease. Serologic testing is more useful for influenza surveillance rather than diagnosis.

Pandemic H1N1 contains a mutation in the M2 protein that confers resistance to M2 inhibitors (adamantanes) [241]. Neuraminidase inhibitors remain the drugs of choice for pH1N1. Sporadic oseltamivir-resistance has occurred due to neuraminidase mutation H274Y and often in the setting of oseltamivir chemoprophylaxis or prolonged therapy [11,213]. Oseltamivir-resistant pH1N1 remains susceptible to zanamivir although the inhaled formulation can cause bronchospasm and clog ventilator tubing [213]. Intravenous peramivir is available with emergency use authorization (EUA) and although large clinical trials are lacking, it appears to be effective [289].

Peramivir requires dose adjustment for renal insufficiency and failure with peramivir has been reported in those with oseltamivir-resistant virus [106]. The addition of glucocorticoids to standard treatment has not demonstrated clinical benefit and may increase the risk of developing critical illness and death [95].

Postexposure chemoprophylaxis is reserved for those who have not been vaccinated against the current circulating strain, had a recent close influenza contact, and are at high risk for influenza complications. Preexposure prophylaxis is often reserved for patients in long-term care facilities who are at high risk for influenza complications. Contact and droplet precautions remain the standard of care for outbreak control [213]. Vaccine effectiveness is determined by the degree of match between vaccine strains and the circulating influenza strain, and has been shown to decrease incidence and duration of illness, missed work days, and physician visits. Different formulations of an inactivated, nonadjuvant, split-virion, monovalent pH1N1 vaccine have been developed and most have been safe and immunogenic. While young children and transplant recipients may require two doses, a single dose has been safe and immunogenic in elderly, HIV-infected, and pregnant patients with sufficient transfer of antibodies to newborns [114,156,224,254,261,290]. The 2009 pH1N1 strain was subsequently included in the 2010 trivalent influenza vaccine. A single dose is recommended for those at high risk for influenza complications and there have been no substantial increase in adverse events, such as Guillain-Barré syndrome, in those receiving the pH1N1 compared to the seasonal influenza vaccine [214].

Although it appears that the 2009 H1N1 pandemic strain has been controlled, there may be new pandemics at any time due to antigenic shift. It is possible that future pandemic strains will lead to significant morbidity and mortality in younger individuals, as was the case with the 1918 as well as the 2009 pandemic.

21.5 *Escherichia coli* O104:H4

In early May 2011 through July 2011, northern Germany was the epicenter of an epidemic of bloody diarrhea associated with the development of hemolytic-uremic syndrome (HUS) caused by a Shiga-toxin-producing *E. coli* (STEC). The clone of STEC classified as O104:H4 resulted in over 4000 cases of diarrheal illness, 900 cases of HUS, and 50 deaths in residents and travelers to northern Germany. The source is thought to be fecally contaminated sprouted fenugreek seeds. The strain was a novel bacterium containing a plasmid-encoded extended spectrum beta-lactamase (ESBL) and genes from enteroaggregative *E. coli* (EAEC), which enhanced survival and intestinal colonization. In contrast to the more common

Shiga-toxin-producing O157:H7 *E. coli*, which causes HUS in 5–10% of children and the elderly, O104:H4 had a longer incubation period and predominantly affected young adult women with a higher rate of HUS (approximately 22%) [2,15,20,89,103,220].

The principle reservoir of many STEC are cattle, with colonization rates of 0–23%, although other species including pigs, chickens, ducks, sheep, and goats have been known to be carriers [99,142,257]. The German outbreak strain O104:H4 has been traced back to ingestion of fenugreek sprouts and specific distributors, however, fecal studies of cattle in the vicinity of the outbreak area in northern Germany failed to isolate O104:H4 or other EAEC, suggesting that the German outbreak strain may have an alternate unidentified reservoir within or outside of Germany or is particularly adapted to humans [20,280].

Some enterohemorrhagic *E. coli*, including O157:H7, contain a chromosomal pathogenicity island called locus enterocyte effacement (LEE) responsible for intestinal mucosa attachment and microvilli effacement. LEE also produces the *eae* gene product intimin, which is essential for adherence and effacement in addition to colonization of animals [13,142]. The German outbreak strain lacked this pathogenicity island and was an enteroaggregative *E. coli* (EAEC) strain, meaning that organisms formed bundles firmly attached to the intestinal mucosa. In conjunction with the virulence potential of a Shiga toxin, this organism was able to cause an exceptionally high rate of morbidity and mortality. The Shiga-toxin gene was acquired from a bacteriophage [220]. STEC can release Shiga (Vero) toxins Stx1 (Shiga-toxin 1) and/or Stx2 (Shiga-toxin 2), encoded by lysogenic bacteriophages, in the gut lumen. When the toxin enters the bloodstream, systemic disease can follow [55,130,142]. Shiga toxin is composed of one A subunit, which leads to cell death by inhibiting protein synthesis, and five B subunits, which bind to glycosphingolipid globotriosylceramide (Gb3) receptors in human renal and cerebral vasculature [215,237]. Ruminants lack vascular Gb3 receptors and therefore can remain colonized without developing active disease [142]. Stx2 is more commonly implicated in development of bloody diarrhea and a higher rate of hospitalization compared to Stx1 [103]. The German outbreak strain acquired a variant Shiga-toxin 2 (*stx2a*) bacteriophage in addition to a large plasmid encoding a CTX-M-15 ESBL and a TEM-1 β -lactamase, and a gene cluster encoding aggregative adherence fimbria (AAF/I) [228]. In addition, a high pathogenicity island encoding an iron uptake system was also identified [14].

There has been a long association between STEC and HUS and more than 200 different *E. coli* O:H serotypes are associated with the production of Shiga toxins. Risk factors for development of HUS include infection with O157:H7, which contain higher rates of Stx2 and intimin,

exposure to antimotility agents, age ≤ 7 years, and possibly exposure to antibiotics, which may exacerbate Shiga-toxin release [12,13,64,130,240].

STEC is the main cause of renal failure in childhood as a majority of children that develop HUS will require dialysis, with a 3–7% mortality rate and 5–10% with permanent renal or neurologic sequelae [103,237]. Typical HUS consists of the triad: nonimmune hemolytic anemia, thrombocytopenia, and acute renal dysfunction. Pathologically, fibrin thrombi form within small vessels, resulting in ischemic damage accompanied by glomerular endothelial cell swelling, tubular epithelial cell damage and necrosis, and mesangiolysis [190,215,257]. A small proportion of patients can also develop neurologic manifestations such as seizure and encephalopathy [33].

The German outbreak STEC strain had a longer median incubation period of 8 days, compared to 3–4 days with STEC O157:H7, with symptoms of frequent watery stools that became bloody accompanied by severe abdominal pain and cramping and less commonly nausea or vomiting. Fever was not a predominant feature and most patients had normal leukocyte levels, although lactate dehydrogenase and C-reactive protein levels were elevated. The average interval from diarrhea to development of HUS was 5 days [78]. The German outbreak strain resulted in a 22% rate of HUS with 88% of cases affecting adults, with highest rates in adult women aged 30–34 years. Only 2% of case patients with HUS were children < 5 years of age and their course was historically similar to O157:H7 with a low mortality rate [132]. Severe neurologic complications were also common, manifesting as encephalopathy and seizure [14]. For patients with a fatal outcome, death occurred a median of 10 days after onset of disease, with the highest death rates in elderly women (median age 74 years) [78].

Many STEC can be readily identified on sorbitol-containing selective media. In contrast, the O104:H4 strain can ferment sorbitol on sorbitol–MacConkey agar (SMAC) or cefixime tellurite–sorbitol MacConkey agar (CT-SMAC), and like most strains of *E. coli* it will ferment lactose. Pure culture is then needed for further serotyping and molecular characterization, including pulsed-field gel electrophoresis (PFGE), useful in outbreak settings, and EIA or PCR for detection of Shiga toxin. EIA testing of cultures is preferred as the amount of free fecal Shiga toxin is often low, resulting in lower sensitivity and specificity. Cell cytotoxicity assays using Vero (African green monkey kidney) and HeLa cell lines are sensitive for detection of Shiga toxin as they contain high concentrations of Gb3. Cytopathic effect is confirmed using specific neutralizing antibodies to Shiga-toxin 1 and 2, although the test can take up to 72 h to complete [88].

Treatment of HUS with fresh frozen plasma (FFP), steroids, and commercial immunoglobulin preparations

are no better than supportive care [128,180]. Antimotility agents have been associated with increased risk of developing HUS and neurological complications. Antibiotics have been controversial with some studies showing progression of HUS due to bacteriolysis and bacteriophage induction with increased Shiga toxin gene expression *in vitro* and *in vivo*, specifically with quinolones and co-trimoxazole. Other studies have demonstrated no association while some have shown that early treatment with fosfomycin improved outcome and reduced the incidence of HUS [12,109,136,192,230,274,287,297]. Eculizumab, a recombinant humanized monoclonal antibody to C5 complement protein, commonly used in paroxysmal nocturnal hemoglobinuria, has been approved for HUS and case reports have shown success in STEC-HUS [146]. None of the above methods have been extensively studied in O104:H4 but a case series with early plasma exchange in five patients with HUS and central nervous system dysfunction has shown efficacy in the German outbreak strain [37].

The outbreak strain is resistant to trimethoprim-sulfa methoxazole, beta-lactam antibiotics and third-generation cephalosporins, partially resistant to quinolones (nalidixic acid), but remains sensitive to carbapenems and aminoglycosides, although antibiotic treatment has overall not been shown to alter the course [14]. Azithromycin has been shown to decrease long-term carriage of STEC O104:H4 but the clinical implication is not clear [189].

21.6 *Cryptococcus gattii*

Cryptococcus is an encapsulated, basidiomycetous yeast with worldwide distribution. It is often found in soil, decaying organic matter, and bird guano, including pigeons, turkeys, chickens, and parrots. Antigenic variability is based on the polysaccharide capsule, which is antiphagocytic, depletes complement, produces brain edema, and interferes with antigen presentation. *Cryptococcus* is composed of two species that consist of five serotypes including: *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), hybrid serotype AD, and *C. gattii* (serotypes B and C) [210,256]. *C. neoformans* serotype A has been the most common cause of human disease worldwide both before and after the HIV pandemic, predominantly causing meningitis although any organ can be infected, including lung, skin, eye, prostate, adrenal glands, and bone [47,210].

C. gattii has emerged as a pathogen of interest due to a recent outbreak on Vancouver Island, British Columbia, Canada and neighboring states in the Pacific Northwest in 1999. While some patients in this outbreak were immunocompromised, many were otherwise healthy non-HIV infected adults. *C. gattii* is classified into four discrete molecular types (VGI–VGIV) based on multilocus

sequence typing [23,79,135]. The VGII class represents the predominant type in the Vancouver Island outbreak and the Pacific Northwest and falls into three discrete genotypes: major (VGIIa), minor (VGIIb), and a novel, independent strain (VGIIc) [24,79]. The major genotype, VGIIa, was also isolated from a Seattle patient dating back to 1971 suggesting that a recent genetic or environmental change may be driving reemergence. VGIIa represents the predominant strain, causing over 90% of infections on Vancouver Island [134]. VGIIb molecular type is similar to those also found in Australia and is the second most commonly identified genotype from Vancouver Island. In contrast, VGIIc has only been recovered in Oregon and has never been found on Vancouver Island or outside the United States [23,101].

Cryptococcus spp. generally exist as asexual, haploid yeasts that reproduce by budding or haploid fruiting in which the yeast produce basidiospores by meiosis without mating. Infectious propagules are inhaled, deposit in the alveoli, and are taken up by alveolar macrophages where they are either eliminated, remain dormant in the lung-lymph node complex, or proliferate. Previously thought to be exclusive to the laboratory, sexual reproduction via recombination is also possible and increasing evidence suggests this occurs in the natural environment. Two mating types, designated α and α undergo conjugation. Same mating type recombination or monokaryotic fruiting has also been shown to occur and α - α mating may be responsible for the Vancouver Island outbreak and increased virulence [47,79,210,233]. Murine models infected via intranasal instillation with Pacific Northwest *C. gattii* outbreak isolates, including VGIIa and VGIIc, have demonstrated higher intracellular proliferation capacity, suggesting that the ability to survive and proliferate in host macrophages may contribute significantly to *C. gattii* virulence. Despite evidence that most isolates from the Pacific Northwest outbreak are exclusively α - α mating, the VGIIc genotype may have resulted from α - α mating, suggesting both mating events are contributing to recombinant genotypes and increased virulence [24,164].

The incidence rate among residents of Vancouver Island was 8.5 cases per million residents in 1999 and increased to 37 cases per million residents in 2003 [47,83,166]. In a preliminary study by Kidd et al. of 38 *C. gattii* human infections [134], the majority were immunocompetent with lung or central nervous system involvement. During the same time period, a variety of animals were diagnosed with cryptococcal infection, including dogs, cats, ferrets, squirrels, horses, and washed-up porpoises. Birds seem to be protected by their higher body temperature resulting in GI colonization without clinical illness, however, *C. gattii* has not been detected in bird guano [210].

From 1999 to 2007, a total of 218 cases were reported to the British Columbia Centre for Disease Control. The

majority of case-patients were immunocompetent adults living on Vancouver Island, there was no seasonal variation, and the median incubation period was 6–7 months [165]. Most patients presented with a respiratory syndrome consisting of cough, dyspnea, and chest pain accompanied by single or multiple lung nodules. A minority was asymptomatic or presented with cryptococcal meningitis consisting of headache, nuchal rigidity, photophobia, blurry vision, seizure, chills, nightsweats, and weight loss. The VGIIa strain was the predominant genotype, although clinical presentation did not differ by genotype. The case-fatality rate was 8.7% and death was more likely in those presenting with a central nervous system (CNS) syndrome, age greater than 60 years, and in those with an underlying medical condition. Case patients were more likely to have an underlying lung condition, systemic corticosteroid use, an invasive malignancy, or less commonly HIV. No clear environmental exposure was found and case-patients were actually less likely to report outdoor activities [83,101,165,166]. Human-mediated environmental disruption is thought to facilitate aerial dispersal [134].

Beginning in 2004, cases of *C. gattii* without exposure to Vancouver Island were detected in the British Columbia mainland, Gulf Islands, Puget Sound area, and the US Pacific Northwest. Most isolates were VGIIa and had identical multilocus sequence typing (MLST) profiles to isolates from Vancouver Island [166,260]. One case of the novel VGIIc genotype was also isolated [23,47].

From January 2004 through July 2010, a total of 60 cases were reported to the CDC from California, Idaho, Oregon, and Washington, with the majority having no exposure to British Columbia and at least nine reported deaths due to *C. gattii*. Half were of the VGIIa subtype but 32% were of the novel VGIIc subtype and most had an underlying chronic illness.

By July 1, 2011 a total of 96 Pacific Northwest cases were reported to the CDC, with the majority VGIIa and VGIIc, and 83 cases likely acquired in the Pacific Northwest or from recent travel to British Columbia. The mortality rate did not differ between groups but the overall death rate was higher than previous reports, with 19 deaths (20%), which may be attributable to the increased virulence of VGIIc [101].

Identification of *Cryptococcus* can be carried out using a variety of methods. White-creamy, mucoid colonies grow readily in 48–72 h on routine agar at 30–35°C. *Cryptococcus* spp. convert urea to ammonia resulting in a positive rapid urease test and also contain laccase, which converts diphenols (e.g. L-dopa, norepinephrine) into melanin, producing a brown-black color on media containing niger seed, caffeic acid, or dopamine. Cryptococcal antigen testing can be performed on serum and cerebrospinal fluid but does not distinguish species. Further identification to *C. gattii* can be elicited by growth

on L-canavanine, glycine, 2-bromothymol blue (CGB) agar as *C. gattii* is canavanine-resistant and able to use glycine as a sole carbon source, triggering a bromothymol blue color reaction. *Cryptococcus neoformans* is canavanine-sensitive and cannot use glycine as a sole carbon source [24,210]. Molecular typing of *C. gattii* can be accomplished by PCR fingerprinting, MLST, restriction fragment length polymorphism (RFLP), and random amplification of polymorphic DNA (RAPD) [210].

Traditionally, CNS cryptococcal disease is initially treated with combination amphotericin B and flucytosine, while extrameningeal disease is treated with azoles, most commonly fluconazole. *In vitro* susceptibility studies have demonstrated that older antifungal drugs such as amphotericin B, flucytosine, and fluconazole, while active, had lower levels of activity against *C. gattii* compared to itraconazole, voriconazole, posaconazole, and isavuconazole. VGII has been found to have significantly higher geometric mean minimum inhibitory concentrations (MICs) for most antifungal agents compared to VGI, with fluconazole and flucytosine having the lowest levels of activity. A study of 43 isolates from the Pacific Northwest typed by MLST showed that 23.3% had fluconazole MICs of 16–32 µg/mL, with VGIIc having the highest geometric mean MIC. The newer triazoles still demonstrate excellent potency against all genotypes, including those with reduced susceptibility to fluconazole [91,111,256].

21.7 *Borrelia miyamotoi*

Relapsing fever is a clinical syndrome often caused by tick-borne and louse-borne (*Pediculus humanus*) arthropod-zoonoses, more specifically, spirochetes of the genus *Borrelia*. Relapsing fever is characterized by recurrent bouts of febrile illness associated with spirochetemia. There are over 15 known *Borrelia* spp. associated with tick-borne relapsing fever (TBRF), which are distributed throughout North and South America, Europe, Africa, and Asia [87,225]. Within North America, most cases occur in British Columbia and the western states such as Washington, California, Colorado, Arizona, New Mexico, Nevada, and Idaho, especially in forested areas, low elevation mountainous areas, limestone caves, lakes, rustic cabins nears bodies of water, and state parks including Grand Canyon National Park [56,57]. Recreational activities such as camping and hiking also increase exposure risk, especially during the spring and summer months [56].

In 1995, Fukunaga et al. described a new *Borrelia* species in Hokkaido, Japan, using comparative 16S rRNA gene sequence analysis and DNA-DNA hybridization, termed HT31^T or *B. miyamotoi* isolated from *Ixodes persulcatus*. Relapsing fever borreliae often contain only a single copy of the 23S and 5S rRNA gene compared to two sets in

B. burgdorferi, a causative agent of Lyme disease. Further sequencing of the 38-kDa flagellar protein found more similarities to relapsing fever borreliae than Lyme disease borreliae [81]. The capacity for *B. miyamotoi* to infect humans was not known but would later be elucidated by Platonov et al. in 2011 [211].

Argasid soft-bodied and ixodid hard-bodied ticks transmit *Borrelia* spp. between reservoirs, which include rodents, mice, rabbits, voles, chipmunks, squirrels, birds, and lizards to human hosts [87,225,236]. All stages of the tick life cycle require blood feeding and are able to transmit the spirochetes between stages (trans-stadial transmission). *Borrelia* are taken up during nightly blood meals, multiply within the vector, and invade the salivary glands, which facilitates transmission to the host during feeding. Within the host bloodstream, *Borrelia* multiply and are sequestered resulting in specific antibody production [87,225].

Antigenic variation is the process that accounts for relapsing fever and evasion of the host immune system. *Borrelia* spp. produce several lipoprotein antigenic variants on the outer membrane called variable small proteins (Vsp) and variable large proteins (Vlp), formerly called variable major proteins (VMP). Encoded by linear plasmids, variation is mediated by recombination on the expression site, which causes a specific host antibody response. The process continues in a cyclical pattern, with high levels of spirochetemia and fever followed by recrudescence [7,8,248].

Further studies have identified *B. miyamotoi* outside of Japan. From 2001 through 2006 *B. miyamotoi* has been isolated from a variety of *Ixodes* ticks, including *I. ricinus* and *I. scapularis* in the United States (Connecticut, Rhode Island, New York, New Jersey, California, and Tennessee), Europe (Sweden, Germany, and France), and most recently Russia [21,77,184,211,227,235,236]. Further phylogenetic research indicated that *B. miyamotoi* forms a monophyletic relapsing fever group closely related to *B. lonestari*, the causative agent of southern-tick-associated rash illness (STARI) [226].

In 2011, patients in Yekaterinburg City, Russia with clinical symptoms and laboratory-confirmed *B. miyamotoi* infection were compared to patients with *B. garinii* in the same area and *B. burgdorferi* infections in the northeast United States. *B. miyamotoi* was found in 17% and the cause of borreliosis in almost 25% of the cases in Yekaterinburg City [211]. The mean incubation of TBRF is 7 days, although may be up to 15 days for *B. miyamotoi* [57,211]. Relapsing fever is characterized by episodes of fever interspersed with afebrile periods that correlate with levels of spirochetemia and the host immune response to lipoprotein antigenic variants. The first febrile period often lasts 3–6 days followed by milder episodes lasting up to 3 days and may be accompanied by fatigue, headache,

or myalgias, and less commonly chills, arthralgias, neck stiffness, abdominal pain, nausea, vomiting, or diarrhea [57,211]. Intervening afebrile periods often last 4–14 days [57]. Severe symptoms occur more commonly with untreated louse-borne relapsing fever (LBRF) and may include jaundice, central nervous system or ocular involvement, thrombocytopenia, hematuria, bleeding diatheses, and death. Patients may have multiple relapses before seeking medical attention and many relapse under the care of a medical provider due to lack of illness recognition [56]. Those infected with *B. miyamotoi* tended to have a longer hospital length of stay and a higher incidence of systemic symptoms. Erythema migrans (EM) was substantially more common in patients with *B. garinii* and *B. burgdorferi*, although a low percentage of patients infected with *B. miyamotoi* may have EM likely secondary to co-infection. Proteinuria and elevated transaminase levels were slightly more common among patients with *B. miyamotoi* but without clinical features [211].

Diagnosis is often made clinically in the appropriate endemic setting and epidemiologic exposure. Spirochetes are motile, helical bacteria less than 25 μm in length, with an inner and outer cell membrane and one or more flagella. While dark-field microscopy, Wright stain, or Giemsa stain of thin and thick blood smears can be performed during spirochetemia, the yield is often low. Wet mounts can also be employed, although the yield is also poor and, as with other methods of direct visualization, is operator dependent and may depend on the laboratory's level of experience. Xenodiagnosis, whereby mice are inoculated with infected blood to amplify the spirochetes to a detectable level, is reserved mainly for research purposes [57]. While specific media are available for cultivation of blood specimens, including Kelly's medium or Barbour–Stoenner–Kelly (BSK) medium, the overall yield is poor and may require up to 2 weeks of cultivation with regular examination under dark-field microscopy [8]. The yield of culture may be increased if collected prior to the administration of antibiotics [56]. Serologic confirmation through the indirect IFA or ELISA confirmed by Western blot is more commonly employed but is limited to relatively few diagnostic laboratories. A fourfold or greater rise in antibody titer between acute and convalescent sera, a single elevated convalescent serum, or a single acute IgM can be diagnostic although false positives between other spirochete species can occur. Serology is also limited by antigen variation in *Borrelia* spp. and the lack of well-defined, specific antibodies [56,57,87,225]. Polymerase chain reaction has been helpful to identify *Borrelia* to the species level but detects both viable and nonviable spirochetes and should be applied to the clinical context [87,225].

There is little evidence of acquired antimicrobial resistance among *Borrelia* spp. Tetracyclines and penicillins

remain the treatment of choice. The most commonly used treatments include doxycycline 100 mg twice daily and tetracycline 500 mg four times daily. Alternatively, erythromycin 500 mg four times daily or ceftriaxone 1–2 g daily can be used for children, pregnant women, or those intolerant to tetracyclines [57,225].

The Jarisch–Herxheimer reaction (JHR) is well described following the initiation of treatment for spirochete illnesses including relapsing fever. The JHR often occurs within 1–4 h after initiation of treatment due to rapid spirochete death and release of inflammatory mediators, resulting in a massive cytokine cascade. Symptoms may include tachycardia, fever, chills, diaphoresis, and severe cases can progress to cardiovascular collapse and death [57]. Close observation should be undertaken during the first hours after treatment initiation. The incidence rate in a study by Platnov et al. in those with TBRF due to *B. miyamotoi* was 15% [211].

Prevention of TBRF includes measures to decrease tick and rodent burdens in common dwellings. Proper construction of homes and cabins with well-sealed walls and roofs reduces the rodent habitat. Avoiding infested areas such as burrows and caves, wearing long pants and long-sleeved shirts, insect repellants, and permethrin-coated clothing are also mechanisms to reduce acquisition of tick bites.

21.8 *Rickettsia parkeri*

Species of *Rickettsia* are intracellular Gram-negative bacteria that are almost always associated with arthropods. Human disease caused by *Rickettsia* has long been recognized. Although the causative organism was not known, epidemic typhus was recognized as a distinct clinical entity as early as the 16th century. New species of *Rickettsia* are continuously being identified as causes of emerging infectious diseases in humans [219]. Since 2002, six species of *Rickettsia* have been newly recognized to cause disease in humans: *R. aeschlimannii* [217], *R. parkeri* [199], *R. heilongjiangensis* [179], *R. massillae* [270], *R. raoultii* [204], and *Rickettsia* 364D [239]. This discussion will focus on *R. parkeri* for which the most human cases have been reported from this group and therefore for which the most information is available.

R. parkeri was first described by R. R. Parker in 1939 as “maculatum disease.” He isolated an infectious agent from Gulf Coast ticks (*Amblyomma maculatum*) collected in Texas in 1937. He classified the organism as a “rickettsia-like infectious agent” based on the illness it caused in guinea pigs and on its cross-immunity with Rocky Mountain spotted fever (RMSF), and boutonneuse fever [203]. Further investigation by serologic testing demonstrated that the infectious agent first isolated by Parker was in fact spotted fever group rickettsia, with

it officially being named as *R. parkeri* in 1965 [143,200]. Subsequently, *R. parkeri* has been found in ticks across the southern United States in Arkansas [259], Florida [121,201,252], Georgia [36,252], Kentucky [121,252], Mississippi [60,201,252], North Carolina [266], Oklahoma [252], South Carolina [252], Tennessee [36], and Virginia [74,121,288], and in South America in Argentina [187], Brazil [194,243], and Uruguay [198,268]. In the United States, *A. maculatum* is the most common vector for *R. parkeri* [60,74,121,201,252,259,266,288], although there is one report of *R. parkeri* in *A. americanum* ticks in Tennessee and Georgia [36]. In South America, *R. parkeri* has most often been isolated in *A. triste* [187,198,243,268], with one report of *R. parkeri* in *A. nodosum* in Brazil [194].

For decades, *R. parkeri* was not thought to cause disease in humans. However, in 2004, the first case of *R. parkeri* causing human infection was reported in a patient in Virginia [199]. Since then, confirmed and probable cases of *R. parkeri* infection have been described in patients in Virginia [202,279], Alabama [202], Florida [202], Kentucky [202], Maryland [202], Mississippi [202], South Carolina [43,202], Texas [43], Argentina [229], and Uruguay [38]. The hallmark of the clinical syndrome is fever, eschar, and rash. A single eschar is usually present at the site of the tick bite. Rarely, multiple eschars have been reported. The rash is typically maculopapular or papulovesicular; petechiae have infrequently been reported. The rash may involve the palms and soles. Other associated symptoms include headache, myalgias, arthralgias and regional lymphadenopathy, which are variably present. Laboratory findings may include mild leukopenia, thrombocytopenia and mild elevated transaminases levels [38,43,199,202,229,279]. Compared to patients with RMSF, patients with *R. parkeri* infection are much less likely to have nausea, vomiting, or diarrhea or to require hospitalization. Patients with RMSF may experience coma, delirium, or seizure and the infection can be fatal. None of these outcomes have been reported in patients with *R. parkeri* infection [202].

Diagnosis in the reported cases of *R. parkeri* infection was made by the combination of the following criteria: exposure to an area with ticks known to harbor *R. parkeri*, a compatible clinical syndrome and positive serologies for spotted fever group (SFG) rickettsia (probable case) or positive PCR or cell culture from a skin biopsy specimen (confirmed case) [38,43,199,202,229,279].

There is cross-reactivity between the different human pathogens of the SFG rickettsia. *R. parkeri* antigens are known to cross-react with antigens from other SFG rickettsia, including *R. rickettsii* the agent of RMSF. It is possible that patients diagnosed with RMSF, especially mild cases, actually have been misdiagnosed due to this cross-reactivity and have another type of spotted fever such as *R. parkeri* infection. In fact, 15 cases originally

diagnosed as RMSF were reviewed and the specimens were retested with microimmunofluorescence, and when possible, Western blot. Titers for *R. parkeri* were higher than or equal to the titers for *R. rickettsii* in 11 cases. Four specimens reacted with a 120-kD protein of *R. parkeri*, suggesting these infections were due to this organism rather than *R. rickettsii* [218].

Doxycycline remains the treatment of choice for patients with SFG rickettsiosis. All of the cases of *R. parkeri* reviewed here were successfully treated with doxycycline except for one. That patient recovered without doxycycline [43,199,202,229,279].

21.9 *Mycobacterium lepromatosis*

Leprosy has a long history dating back to ancient times. The bacterium *M. leprae* was discovered by Armauer Hansen in 1873 and was the only known cause of leprosy [131]. However, it was unable to be cultured *in vitro*, a problem that exists until today [224]. Leprosy primarily is a disease of the skin and nerves. A well-established spectrum of disease exists. At one end of the spectrum is tuberculoid type leprosy (TT), characterized by a small number of skin lesions, few bacilli in lesions, and good T lymphocyte function. At the other end is lepromatous type leprosy (LL), where there are larger number of skin and nerve lesions, involvement of internal organs, high bacillary load, and ineffective T lymphocyte function towards the organism. There is geographic variation in the distribution of the different subtypes. TT predominates in Africa and India, while most cases are LL in Mexico and Central America. The two forms are equally distributed in Southeast Asia [131]. Despite the lack of the ability to cultivate *M. leprae* on artificial media, replication in the nine-banded armadillo, the footpads of immunodeficient mice, and the use of molecular techniques have enabled some characterization of the organism. More recently, genome sequencing studies have shown that many strains of *M. leprae* from Asia, Africa, and the Americas were remarkably clonal and differed only by single nucleotide polymorphisms or variable number of tandem repeats [182]. Excluding host immune factors, this genetic homogeneity does not explain the wide variation in clinical presentation.

A diffuse form of lepromatous leprosy (DLL), called leprosy with the Lucio phenomenon or diffuse leprosy of Lucio and Latapi, has been reported in patients from Mexico and the Caribbean [223,224]. Clinically, patients present with diffuse, non-nodular cutaneous infiltration that may develop into bluish or purple hemorrhagic plaques that ulcerate. Pathologically, there is a necrotizing vasculitis caused by endothelial invasion by *M. leprae*. The lesions may become generalized, leading to overwhelming secondary bacterial infection and death [223,232]. This

clinical picture is unique to DLL. The etiologic agent for this disease has been presumed to be due to *M. leprae*, although no specific studies have been conducted in this regard as *M. leprae* cannot be cultivated on artificial media. However, in 2008 Han et al. reported the use of multigene analysis on a mycobacterium obtained from two Mexican patients' tissue specimens who died of DLL. Their results demonstrated a new organism that differs from *M. leprae* and other mycobacteria and likely represents a new *Mycobacterium* species. The name they proposed was *M. lepromatosis* to distinguish it from the genetically homogeneous species *M. leprae* [96].

In their paper, Han et al. described two patients in Arizona of Mexican origin with clinical pictures consistent with DLL, who died in the course of their illness [96]. The cases occurred 5 years apart. Biopsies of skin and other organs showed necrotizing vasculitis and numerous acid-fast bacilli (AFB) that were unable to be cultured, supporting the diagnosis of DLL. Using PCR techniques, the 16S ribosomal RNA gene (unique to each species) was amplified and sequenced along with three other genes – the housekeeping genes *hsp65* and *rpoB* (a heat-shock protein and RNA polymerase β subunit, respectively) and *rpoT* (RNA polymerase σ factor) genes. The *Mycobacterium* was obtained from biopsy or autopsy tissue. The organisms from both patients matched fully and were closely related to, but distinct from, *M. leprae* with a 97.9% homology [96]. *Mycobacterium hemophilum* was the second-best match with 97.3% homology, followed by decreasing homology with other mycobacteria. According to the authors, this supported a species level difference as an approximately 3% sequence divergence in the signatory 16S rRNA gene as one of the main criteria for species definition [34,96]. In comparison, no variation in the 16S rRNA gene was noted for all *M. leprae* strains previously evaluated. In a subsequent paper, Han et al. further characterized the sequences of 20 genes and pseudogenes of the new organism from one of the two cases [97]. Their results again indicated that *M. leprae* and *M. lepromatosis* do have a species-level divergence (9.1%) and probably diverged about 10 million years ago from a common ancestor [97].

Since then, additional reports have been published describing cases of DLL with the identification of *M. lepromatosis* on sequence analysis of skin biopsy specimens. One report described an 86-year-old Mexican woman with an illness consistent with DLL with Lucio's phenomenon that occurred in 2005 [269]. Her skin smears were positive for AFB and she was treated with prednisone and multidrug therapy for multibacillary leprosy with improvement. She died at home 3 months later of unknown causes. Subsequent PCR testing on the archived biopsy specimen targeting *M. leprae* specific foci gave negative test results.

Further sequence analysis showed 100% identity with the *M. lepromatosis* sequences. Three more cases have been reported since, two from Singapore with DLL who died and another case from Ontario, Canada with a leprosy-like illness who died of an unrelated illness [119,293]. These cases point to the existence of the species beyond Mexico, in Asia and North America. The two cases from Singapore occurred in Chinese men in 1999 and they were found to have dual infection with *M. leprae* and *M. lepromatosis* when the archived skin biopsy samples were analyzed with primers for both species [293]. This was the first time dual infection was found. In other unpublished data by Han et al., 120 leprosy cases from Mexico were analyzed again. *Mycobacterium lepromatosis* was found to be the specific cause of all cases of DLL in that series and it caused even more cases of LL than DLL, exceeding *M. leprae* as the etiologic agent by a ratio of 3:1 [98]. This indicates *M. lepromatosis* as the main cause of leprosy in Mexico. More dual infections were also found (three cases). Ongoing studies of leprosy tissue samples from several countries including Brazil, Myanmar, and Uganda have also detected *M. lepromatosis*. Therefore this bacterium likely has a global importance.

Some have suggested that before concluding that *M. lepromatosis* is the causative agent for LL, DLL, or DLL with the Lucio's phenomenon, additional work needs to be done demonstrating that this does indeed cause leprosy [86]: concerns that this agent may actually be only a variant of *M. leprae* and not a new species have been expressed by some [86]. Sincere attempts to grow the organism in standard mycobacterial media and/or passage in mouse foot pads with subsequent demonstration of the bacteria's ability to infect nerves in the armadillo would help confirm that it is capable of causing a leprosy-like infection. However, these studies are difficult and are based on the assumption that *M. lepromatosis* will produce disease in the armadillo like *M. leprae*. Traditionally, leprosy has been diagnosed based on clinical manifestations and confirmed by skin biopsy showing AFB. When clinical and histopathological features were inconclusive, PCR has been used to help identify a few cases [224]; although not commercially available, PCR can be performed at a few research centers. This may help explain why this bacterium may have remained unknown previously. There have been no studies that have evaluated treatment for *M. lepromatosis* specifically. It is not known whether the drugs used to treat leprosy caused by *M. leprae* such as dapsone, rifampin, and clofazimine will be equally effective in treating *M. lepromatosis*, but it seems plausible to assume that multidrug therapy would be necessary. For the future, parallel testing for both species by a reliable PCR method would be needed to identify additional cases and establish the extent of disease due to *M. lepromatosis*. Until then, physicians

should be alert for *M. lepromatosis* as an emerging pathogen causing lepromatous leprosy, especially the diffuse variant in patients not only from Mexico but also from around the world.

21.10 Bocavirus

Human bocavirus (HBoV) was first described in 2005 by Tobias Allander in Sweden [3]. He analyzed two series of nasopharyngeal aspirates from children with respiratory infections using PCR cloning to try to identify the causative agents of their illness. A large percentage of isolates tested had the new virus detected. This virus most closely resembles the parvoviruses and has been placed in the family *Parvoviridae*, joining parvovirus B19 as one of only two human pathogens in the family. Since this report, many publications have described HBoV in association with acute respiratory infections in children [234].

HBoV has been found all over the world and does not have a specific geographical or seasonal distribution. In one study of 974 respiratory samples, HBoV was the third most commonly identified virus behind RSV and adenovirus [255]. What has been interesting is that HBoV is not usually present alone. In another study 72% of samples had another virus isolated in addition to the HBoV. Twenty of 45 asymptomatic children had HBoV shedding noted and continued up to 75 days [267]. Interestingly, co-infection with HBoV was associated with prolonged respiratory illness compared to those without HBoV. Based on these results it is difficult to assess whether HBoV is the primary pathogen or whether it is a co-pathogen or passenger.

Prevalence of HBoV in children has ranged between 1.5 and 19% in various studies. Most children have been infected by 2 years of age; however, older children up to 3 years of age have been noted to have acute infection as well. In a seroprevalence study by Endo et al. in Japan, over 90% of children 6–8 years of age had antibodies to HBoV, reflecting previous infection with the organism [62]. It is not clear how it is transmitted, however, high concentrations in respiratory secretions suggest aerosol and contact transmission to be the likely method.

The most common clinical findings in children with HBoV include fever, rhinitis, and cough. Other findings described include otitis, bronchitis, bronchiolitis, and pneumonia. Symptoms have ranged from 2 days to 3 weeks but most infections last about 1–2 weeks in duration. In one study in Germany, symptoms were severe enough for patients to have chest X rays and 10 of 11 of them had pulmonary infiltrates suggestive of pneumonia [271]. There has also been a strong association between HBoV infection and acute wheezing or asthma exacerbation. Chung et al. tried to assess what percentage of children admitted to their hospital had an acute viral infection. HBoV and RSV were

each positive in 13.8% of the patients that were checked and represented the highest prevalence in this population. Human metapneumovirus was found in 7.6% of them [32].

There are no methods to grow HBoV in culture, but it can be detected by PCR performed on nasopharyngeal aspirates and swabs: the PCR assays detect the NS1 or NP1 genes of the virus and are highly sensitive. In addition to PCR, antibody detection has been utilized in studies to detect the seroprevalence of the organism [163]. There are no studies in the literature at this time, which describe possible therapeutic options for the treatment of HBoV infection. At this point, supportive therapy is recommended.

These data suggest that HBoV can be a newly described pathogen causing acute respiratory illness in children and may be associated with acute reactive airway disease. Development of an animal model and rapid methods for isolation of this virus can lead to better understanding of this organism's role in human disease.

21.11 Human metapneumovirus

The *Paramyxoviridae* family is composed of two subfamilies consisting of *Paramyxovirinae* and *Pneumovirinae* that comprise a number of human and nonhuman pathogens. The *Pneumovirinae* are further divided into the *Pneumovirus* (respiratory syncytial virus or RSV) and the *Metapneumovirus* genera, whose sole member was until recently avian pneumovirus (APV) or turkey rhinotracheitis virus (TRTV). The classification of the two genera is based upon specific gene differences, as metapneumoviruses lack nonstructural proteins NS1 and NS2 and contain an alternate gene order than pneumoviruses [263].

Until 2001, metapneumoviruses were thought to be a nonmammalian infection but a recent discovery by van den Hoogen et al. in 2001 isolated a previously unknown pathogenic paramyxovirus that was genetically similar to the *Pneumovirinae* family and had a high level of homology with APV serotype C within the *Metapneumovirus* genus [125]. The pathogen, isolated from 27 nasopharyngeal specimens collected over the past 20 years from children less than 5 years of age in the Netherlands, was aptly termed human metapneumovirus (hMPV) [263]. Further animal experiments by van den Hoogen et al. have demonstrated that hMPV is not an avian virus but does cause disease in primates [263].

hMPV is an enveloped, single-stranded, non-segmented negative sense RNA genome composed of subgroup A and B. Within each subgroup exist two clades designated A1, A2, B1, and B2, which often circulate concurrently. On electron microscopy, hMPV are pleomorphic, spherical, and filamentous particles measuring 150–600 nm with short (15 nm), envelope glycoprotein projections and helical nucleocapsids. hMPV is known to make three viral

glycoproteins (major attachment [G], fusion [F], and small hydrophobic [SH]) inserted into the lipid envelope. The G and F proteins have roles in attachment and viral fusion to the host cell membrane, and variability in the G protein sequence suggests a role in serotype variation, which may distinguish hMPV subtypes. The helical nucleocapsid is composed of viral RNA and nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), polymerase protein (L), and regulatory transcriptional enhancer proteins (M2-1 and M2-2) [105,209,263].

Animal models including cotton rats, BALB/c mice, and cynomolgus macaques have demonstrated the pathophysiologic and histopathologic changes associated with hMPV infection. These included peribronchial lymphoplasmocytic infiltrates, edema of the bronchial submucosa, loss of ciliated epithelium, hypersecretory bronchial epithelium with sloughing and debris within bronchial lumen, and red cytoplasmic inclusions in respiratory epithelial cells [94,105,285]. hMPV appears to preferentially target ciliated epithelial cells of the human respiratory tract via attachment and fusion of surface glycoproteins [69].

Humans are the only known natural host for hMPV and infection is likely transmitted person to person by respiratory droplets or contaminated secretions, with an incubation of 4–6 days [69]. The infection has been reported worldwide and almost all children by age five are seropositive [263]. hMPV is seasonal, with late winter and early spring epidemics from December to April in the Northern Hemisphere and late spring and summer in Asia. hMPV occurs concurrently with RSV but often slightly later than RSV epidemics, and children tend to be slightly older [117,183,205,284,286]. Infection from one subgroup may not be protective from a virus from another subgroup, resulting in new infections throughout life, possibly due to incompletely protective immune responses and/or acquisition of new genotypes [17,63,92,284].

In children, hMPV accounts for 2–12% of lower respiratory tract infections and 5–15% of cases of upper respiratory tract infections with a peak age of 3–5 months. Children present with bronchiolitis, pneumonia, acute wheezing, and/or otitis and symptoms include cough, coryza, fever, rhinitis, pharyngitis, irritability, poor feeding, tympanic membrane abnormalities, and wheezing, with less commonly vomiting or diarrhea [8,10,11,13]. hMPV has also been found to be a stronger trigger for asthma exacerbation in children than RSV or influenza [209,221]. The hospitalization rate is highest among infants 0–5 months, followed by infants 6–11 months of age [283].

hMPV infection in adults is less well studied than in children but numerous accounts of acute respiratory illness have been documented, although asymptomatic shedding is also encountered frequently [16,66,247,273]. hMPV has been isolated in multiple adult cohorts with

highest rates among young adults that live with children and residents of long-term care facilities. Overall, fever was uncommon and young adults tended to experience more hoarseness compared to the elderly, who experienced more dyspnea and wheezing. Cough was a nearly universal symptom, often associated with nasal congestion and rhinorrhea. Hospitalized adults were more likely to have a chronic heart or lung condition and were often former or active smokers, with 25% having infiltrates on chest radiograph. Mean duration of illness ranged from 10 days in young adults to 16 days in high-risk adults. There is also a possible association between hMPV infection, asthma, and chronic obstructive pulmonary disease (COPD) exacerbations in adults. Studies on hospitalized patients with acute asthma or COPD exacerbations have detected hMPV in 6.9% and 12%, respectively [174,282].

hMPV has been documented to cause severe and prolonged disease in the immunocompromised, especially hematopoietic stem-cell transplant (HSCT) and lung transplant recipients. Many lung transplant patients will experience a decline in allograft function without progression to bronchiolitis obliterans syndrome (BOS), and mortality remains low. Lung function often recovers within 3–6 weeks but viral replication may be prolonged [108,147,238]. The incidence and burden of hospitalization from hMPV-LRTI is fivefold greater in HIV-infected compared to HIV-uninfected children [169].

hMPV was first isolated on tertiary monkey kidney (tMK) cells and requires supplementation with trypsin for replication [263]. Since discovery, hMPV has been shown to grow efficiently in rhesus monkey kidney (LLC-MK2), human laryngeal carcinoma (HEp-2), and human liver hepatocellular carcinoma (HepG2) cell lines, and slowly or poorly on African green monkey kidney (Vero), human lung adenocarcinoma (A549), and Madin-Darby canine kidney (MDCK) cell lines [26,105,125]. Cytopathic effect is indistinguishable from RSV, with rounding of infected cells and occasionally the formation of syncytia. While RSV replicates quickly, hMPV replicates slowly, with cytopathic effects seen 10–14 days post-inoculation [263].

EIA and IFA utilizing anti-hMPV antibodies to the F or N protein for direct antigen testing and shell vial cultures are available and recognize both hMPV subgroups [105,125]. The sensitivity and specificity of IFA compared to RT-PCR in Japanese children was found to be 73% and 97% [59]. EIA was found to have a sensitivity and specificity of 81% and 100%, respectively [139]. ELISAs to detect hMPV antibody in sera using recombinant N, F, and G proteins are available to evaluate for seroprevalence and seroconversion of hMPV serotypes and subgroups [105,139].

RT-PCR is the most common method used to detect hMPV from nasopharyngeal samples and BAL specimens and has been shown to have a higher sensitivity than

culture or IFA [59,85,167,251]. Reported detection rates are 43% by shell vial culture, 69% for DFA, and 92% by RT-PCR. Côté et al. compared the sensitivity of RT-PCR assays to amplify the N, M, F, P, and L genes, demonstrating superior sensitivity of primers for the N and L gene, although N-based primers may have lower sensitivity for serotype B [40,264].

There is no approved therapy for hMPV infection, and treatment is generally supportive. Ribavirin has been the most well studied agent and has been shown to inhibit hMPV replication and inflammation *in vitro* and in animal models [93,291]. The addition of glucocorticoids had no effect on decreasing hMPV replication but did reduce inflammation, suggesting a possible role in severe human disease when combined with ribavirin [93]. Ribavirin has been used in humans to treat hMPV infection, mostly in lung transplant patients [108,222,238]. A prospective analysis of hMPV and RSV LRTI in lung transplant patients treated with intravenous ribavirin (mean of 10 days) and methylprednisolone showed less mortality and BOS with hMPV, with similar rates of allograft dysfunction, time to recovery of lung function, and overall rate of lung function recovery [108]. Antibody preparations including intravenous immunoglobulin (IVIG) have been shown to contain relatively high titers of hMPV-neutralizing antibodies and inhibit hMPV replication, although use for hMPV in humans is limited [291]. A report of ribavirin in combination with IVIG has been noted to be clinically successful in two HSCT patients [238]. The monoclonal antibody palivizumab, used to prevent RSV infection in high-risk infants, has had no benefit with hMPV. It is unclear whether these outcomes represent the natural course of lung disease caused by hMPV or if treatment truly offers a clinical benefit, but the addition of hMPV testing in immunocompromised patients with respiratory tract infections may reduce the unnecessary use of antibiotics [265].

21.12 Severe fever with thrombocytopenia syndrome virus

Severe fever with thrombocytopenia syndrome (SFTS; also known as fever, thrombocytopenia, and leukopenia syndrome and Huaiyangshan virus) is an emerging infectious disease first recognized in central China as a result of surveillance of febrile illnesses in the country. In 2009, physicians in Hubei and Henan Provinces noted increasing cases of a syndrome of fever, gastrointestinal symptoms, leukopenia, and thrombocytopenia for which no clear etiology could be identified. A novel virus was identified in a patient with fever and thrombocytopenia of unknown cause after incubation of a blood sample from the patient with a DH82 cell line. The virus was subsequently identified in serum from 11 additional

patients with the same clinical syndrome. After genome sequencing and phylogenetic analysis, the virus was identified as a unique member of the *Phlebovirus* genus in the family *Bunyaviridae* and named severe fever with thrombocytopenia syndrome virus (SFTSV) [294].

Investigators then developed a RT-PCR assay to test for the virus in serum as well as assays for serum antibodies. During a 15-month period in 2009–2010, 241 hospitalized patients were identified with SFTS by clinical criteria (fever, thrombocytopenia, and no known cause). Of these patients, 171 had laboratory evidence of SFTSV (detection of the virus in cell culture, positive RT-PCR for SFTSV, seroconversion, or fourfold increase in antibody titers to SFTSV). In contrast, 200 matched healthy controls from the same geographic area had no evidence of SFTSV by RT-PCR or serologic testing. The mortality rate for these laboratory confirmed cases was 12% (21 deaths). Complete clinical information was available for 81 of the laboratory confirmed cases. All patients had fever. Gastrointestinal symptoms were common and included: anorexia (75%), nausea (69%), abdominal pain or tenderness (49%), vomiting (47%), and diarrhea (42%). The majority of patients had thrombocytopenia (95%), leukopenia (86%), elevated transaminases (ALT 83%, AST 94%), and proteinuria (84%) [294].

Another group of researchers [292] performed a similar study to Yu et al., investigating patients in Henan Province with the same clinical syndrome, which they called fever, thrombocytopenia, and leukopenia syndrome (FTLS). Out of 285 patients with clinical FTLS, RT-PCR and/or serologic testing confirmed 238 to have novel bunyavirus infection. All 130 control patients (either healthy volunteers or patients with respiratory infections) had no evidence of the virus by RT-PCR. Symptoms and laboratory findings of confirmed cases occurred with similar frequency to that described in patients with SFTS by Yu et al. The genetic sequence of the bunyavirus identified by Xu et al. is 99% identical to SFTSV.

Given that the majority of patients with SFTS are farmers from wooded areas who have recently worked in fields, an arthropod-borne reservoir of the virus was suspected. Yu et al. tested 5900 mosquitoes for SFTSV RNA, all of which were negative. However, 5% of ticks (*Haemaphysalis longicornis*) collected from domestic animals tested positive for SFTSV RNA, which was extremely closely related (though not identical) to SFTSV RNA isolated from case patients [294]. Since the initial description, several reports have identified clusters of SFTS, with evidence of person to person transmission via contact with blood of an index patient [5,6,82,157]. All index patients had hemorrhagic complications such as hematemesis, hematochezia, hemoptysis, or bleeding from intravenous (IV) line sites.

A quantitative rRT-PCR assay for SFTSV, with a sensitivity of 98.6% and specificity of 99.1%, has been

developed. The assay is able to detect elements from the L, M, and S segments of the SFTSV genome. RT-PCR was positive in some patients before antibodies were detected, indicating that this test could be useful for earlier diagnosis of the disease [253].

Currently there is no specific antiviral therapy available to treat SFTSV. Treatment is entirely supportive. The mortality rate is about 12% [294,296]. In a prospective study of 49 patients with SFTSV, multiple significant predictors of mortality were found including older age (median age of fatal cases 62 years old), prolonged blood coagulation times (median thrombin time 76 s, median activated partial thromboplastin time 55 s), elevated transaminases (median AST 558 U/L), and higher serum viral loads. Eight of ten patients with admission viral loads ≥ 105 copies/mL died, while all 39 patients with admission viral loads < 105 copies/mL survived. Viral load subsequently decreased over 3–4 weeks in correlation with clinical improvement, but in some patients it remained detectable at low levels for > 30 days. In this study, markers of the innate immune response (IL-6, IL-10, and IFN- γ) were also increased in case patients versus healthy controls, and in case patients the elevations were significantly higher in those who died versus those who survived [296].

21.13 Zika virus

The genus *Flavivirus* of the family *Flaviviridae* consists of mostly zoonotic or arthropod-transmitted viruses, of which yellow fever, dengue, Japanese encephalitis, and West Nile encephalitis viruses are well-known members [267]. Zika virus (ZIKV) was a lesser-known member within the same genus until a recent outbreak occurred in 2007, highlighting new areas with virus transmission and the potential for further expansion.

The virus was first isolated in 1947 from a febrile Rhesus monkey that had been stationed in the Zika forest near Entebbe, Uganda as part of research into the epidemiology of yellow fever [51]. In the subsequent year, ZIKV was isolated from a batch of wild-caught *Aedes africanus* mosquitoes from the same forest [168]. Human infection was suggested by the presence of antibodies against ZIKV in serological studies carried out in Uganda and Nigeria as early as the 1950s and in the 1960s and 1970s [50,65,168]. However, the first human ZIKV isolation, published in 1954, was from a 10-year-old African girl with fever and headache who had complete recovery from her illness [168]. Further human isolates were obtained from eastern and western Africa over the next two decades during routine arbovirus surveillance studies, but no epidemics occurred [65,244].

ZIKV is a single-stranded RNA virus with 10,794 nucleotides and is closely related to Spondweni virus [141]. The genome has a single open reading frame

flanked by 5' and 3' untranslated regions. It encodes a polyprotein that is cleaved into three structural proteins for the capsid, membrane, and envelope proteins, and seven nonstructural proteins. There are three ZIKV lineages: East African, West African and Asian [145].

Boorman and Porterfield conducted an experiment using artificial blood-membrane preparations in 1956 to study possible ZIKV vectors [18]. Their study suggested that the extrinsic incubation period in mosquitoes was approximately 10 days. They also showed that the virus was capable of multiplying in *Aedes aegypti* mosquitoes, the infection persisted for at least 10 weeks and those mosquitoes were capable of transmitting the virus to mice and monkeys [18]. Subsequent studies have isolated ZIKV from a variety of *Aedes* mosquito species in both Asia and Africa [102]. It is likely that ZIKV is maintained in a sylvatic cycle involving nonhuman primates and mosquitoes, with humans serving as incidental hosts [18,90]. But in areas lacking nonhuman primates, humans serve as the amplification hosts and possibly reservoir hosts.

The clinical manifestations following ZIKV infection are varied. Simpson in 1964 described his own mild illness from ZIKV, which began with frontal headache on day 1 followed by a diffuse maculopapular rash with fever on day 2 and both resolved over the next 2 days [244]. He was able to isolate the virus during his illness and demonstrate a rise in ZIKV antibody with convalescence. Filipe et al. published another description in 1973, where one of the authors developed a laboratory-acquired ZIKV illness shortly after yellow fever vaccination [71]. It was characterized by fever of 38°C with chills, retro-orbital pains, neck pains and arthralgias without a rash. The symptoms resolved by the end of the first week. Again, the virus was isolated from the patient's serum. However, the initial antibody response was against yellow fever (an anamnestic response) followed by a steady rise in antibodies against ZIKV, highlighting the importance of virus isolation to definitively establish a diagnosis in areas where other flaviviruses coexist.

In 1981, a case series by Olson et al. described seven patients from Indonesia with rising ZIKV antibodies detected during hospital-based surveillance for febrile illnesses [195]. The most common manifestations included high fever, malaise, abdominal pain, dizziness, and anorexia. None had headache or a rash, and only one each had arthralgia and conjunctivitis. In 2007, an outbreak of ZIKV occurred on Yap Island, Federated States of Micronesia, which is the first reported human outbreak [54]. There were 49 confirmed cases and 59 probable cases of ZIKV disease between April and July 2007. Rash, fever, arthralgia, arthritis, and conjunctivitis were the common symptoms. Other symptoms were myalgia, headache, retro-orbital pain, edema, and vomiting. The rash lasted for a median of 6 days while arthralgia lasted

for 3.5 days. There were no hospitalizations, hemorrhagic complications, or deaths. Females had a higher attack rate of disease, as did older persons, while the prevalence of IgM antibody to ZIKV was higher in men and evenly distributed across age groups. The outbreak was thought to have occurred from a recent introduction of ZIKV by an infected mosquito or viremic human with undetected infection.

A more recent report published in 2011 indicates a probable nonvector-borne transmission of ZIKV [76]. Two male American scientists contracted ZIKV while working on a mosquito-sampling project in Senegal in 2008. Both became ill after their return to the United States, with a maculopapular rash, fatigue, headache, arthralgia, and arthritis without fever. One of them also developed perineal pain, dysuria, and hematospermia. This patient's wife also developed similar symptoms 9 days after without any travel history. Serological tests indicated development of antibodies to ZIKV and yellow fever in acute and convalescent sera of the men and to ZIKV alone in the woman. This may indicate direct person-to-person, possibly sexual, transmission of the virus as other family members did not develop illness.

The diagnosis of ZIKV infection can be made by RNA PCR tests on acute-phase serum samples, virus isolation, and specific antibody tests showing rise in titer between acute and convalescent sera. The Arboviral Diagnostic and Reference Laboratory of the CDC (Atlanta, GA, USA) has developed an ELISA to detect IgM to ZIKV [145]. Cross-reactivity has been reported with dengue virus but also other flaviviruses, such as yellow fever and Japanese encephalitis. IgM has been detected as early as 3 days into the illness and up to day 8 [145]. The plaque reduction neutralization assay has better specificity over the immunoassay in primary infections but indeterminate results may be seen in secondary flavivirus infections (previous vaccination or exposure to another flavivirus) [54,145]. A one-step RT-PCR has also been developed that is rapid, sensitive, and specific but needs further validation using clinical samples [68]. In the Yap epidemic, viral RNA concentrations were between 900 and 729,000 copies/mL with most samples being collected ≤ 3 days after onset. One patient had detectable virus by PCR on day 11 after onset [145]. There is no specific treatment for ZIKV infection as is the case for most viral infections.

ZIKV is an emerging pathogen with spread outside Africa and Asia, causing illness that has been mild and self-limited. Clinical disease resembles other arboviruses such as dengue, and serologic cross-reactivity, especially to dengue viruses, may have led to underreporting of cases. The recent outbreak on an isolated island bears witness to the possibility of virus spread across larger areas with global travel. Virus isolation or detection using RT-PCR will help elucidate the pathophysiology and epidemiology of this virus.

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Automated and Manual Systems for Antimicrobial Susceptibility Testing of Bacteria

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22.1 Introduction

A key function of the clinical microbiology laboratory is to perform antimicrobial susceptibility testing (AST) on clinically significant bacterial isolates. Studies have shown that performing an AST method in a timely manner has the potential to improve patient outcomes by the rapid detection of resistance and by the subsequent selection of appropriately targeted antimicrobial agents [3,23,26]. Clinical microbiology laboratories in the United States, Canada, and Europe primarily use commercial broth microdilution testing methods for AST in an automated or semiautomated format [38] for improved test turnaround time. A few of the commercial broth microdilution panels also have the option to be set up and read manually. Since the broth microdilution panels have a fixed configuration of antimicrobial agents and are approved by the US Food and Drug Administration (FDA) for specific microorganism groups, the testing of additional antimicrobial agents or slower growing bacteria is often performed with supplemental agar-based manual AST methods, which include gradient diffusion and disk diffusion. All AST methods provide the qualitative interpretive category results of susceptible, intermediate, and resistant, and the broth microdilution and gradient diffusion methods also provide quantitative minimal inhibitory concentration (MIC) results.

Detailed procedural descriptions of broth microdilution, agar gradient diffusion, and agar disk diffusion methods are not presented in this chapter, and can be found in the *Manual of Clinical Microbiology*, 9th edn [41,58], in the Clinical and Laboratory Standards Institute (CLSI) documents for antimicrobial susceptibility testing [18–21], and in a review by Jorgensen and Ferraro [38]. In the United States both the CLSI and the FDA set antimicrobial MIC breakpoints for susceptible, intermediate, and resistant interpretive criteria, and in Europe the MIC breakpoints are set by

the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [25]. The criteria for establishing breakpoints differ slightly among, CLSI, FDA, and EUCAST and there may be minor MIC breakpoint dilution differences and minor inhibition zone diameter differences when comparing the three standards. Both CLSI and EUCAST documents for the interpretive criteria for MICs of relevant antimicrobial agents are reviewed and updated frequently, usually once per year. Commercial AST systems in the United States must use FDA breakpoints. There is a process in place by which the CLSI may periodically submit updated data to the FDA for review with the intention of aligning CLSI and FDA breakpoints for selected antimicrobial–organism combinations. If a regulatory authority such as the FDA changes breakpoints, then a commercial device manufacturer may be required to submit updated data to the FDA for review and approval, which would require 1–3 years for an interpretive breakpoint change to be implemented by a commercial AST system. In the United States either FDA or CLSI susceptibility interpretive breakpoints are acceptable to clinical laboratory accrediting bodies [21].

Commercial systems for AST can be divided into automated, semiautomated, and manual. An automated testing system consists of automated inoculation of MIC panels followed by computer-assisted incubation with reading, interpretation, and reporting functions that do not require manual intervention. A semiautomated system consists of manual or automated inoculation of panels and manual off-line incubation of panels. Each panel is then loaded into an automated reader, and computer-assisted reading and interpretive reporting of MICs is performed. Manual AST panels are inoculated and incubated manually, read visually by laboratory personnel, and the results are either recorded by hand or

manually entered into a computer for interpretation and reporting. Each automated, semiautomated, or manual broth microdilution system provides a selection of AST panels with a fixed configuration of antimicrobial agents. Upon request, several companies will prepare custom AST panels for clinical or investigational use.

It is recognized that susceptibility testing methods and panels are subject to periodic modifications and that new systems and panels are introduced into various global markets as an ongoing process. The purpose of this chapter is to review currently used commercial systems, and any omission of a specific method or published evaluation is unintentional. Older published evaluations are reviewed in the first edition of this text.

22.2 Evaluation of commercial AST performance

The evaluation of AST systems is based on guidelines established by the US Food and Drug Administration [71]. Error rates for commercial AST panels are determined by comparing AST results with the reference CLSI broth dilution method using at least 100 unselected clinical isolates of a single genus or species. The detection rate for very major errors (false susceptibility) should be $< 1.5\%$ of the test isolates, and major errors (false resistance) should be $< 3\%$ of the test isolates. Essential agreement with the reference method (± 1 dilution) should be $\geq 90\%$ and categorical agreement with the reference method (same susceptible (S), intermediate (I), resistant R category result) should be $\geq 90\%$ for each antimicrobial agent and organism combination.

22.3 Automated broth microdilution AST systems

The automated broth microdilution AST systems currently available in the United States include the Vitek 2 (bioMérieux Inc., Durham, NC), the MicroScan WalkAway *plus* (Beckman Coulter, Inc., Brea, CA), the Phoenix System (BD Diagnostics, Sparks, MD), and the Sensititre ARIS 2X (Thermo Scientific, Lenexa, KS). All four systems provide periodic software and panel upgrades, with most test panels showing good overall essential agreement with the CLSI reference broth dilution method. A listing of the four automated systems and their respective AST panels is presented in Table 22.1.

The automated and semiautomated systems discussed in this chapter have the capability of producing standardized or customized patient test reports generated by computer software packages referred to as data management systems. These software systems usually contain an epidemiology component which can archive results, thereby providing specialized reports such as summary

reports, infection prevention reports, organism trending reports, and hospital antibiograms, or formatted reports of cumulative susceptibility data. To optimize the availability and transcription accuracy of rapid patient reports, the integrated data from automated and semiautomated systems may be transferred through a computer interface to a laboratory information system (LIS). Specialized reports may then be generated by the LIS in addition to reports generated by the automated system's software.

An additional level of software enhancement in automated systems is referred to as "expert software," which examines and validates the antimicrobial susceptibility profile or phenotype of an individual isolate. These expert systems use specific rules or algorithms (preprogrammed or user defined) to flag unlikely resistance patterns and recommended changes. Expert software may also predict cross-resistance to other antimicrobial agents and can facilitate the addition of footnotes or comments to a patient's report regarding the resistance pattern. An additional advantage of some of the automated systems is a software package that enables interfacing with a pharmacy system so that the microbiology results can be matched with the patient's record of antimicrobial therapy. Thus, the early detection of *in vitro* resistance could potentially affect patient outcome if the pharmacy system was alerted to the need to modify therapy. The names of the expert systems available for the automated systems are listed in Table 22.1. A detailed review of evaluations of expert systems for the Vitek 2, MicroScan, and Phoenix systems has been published by Winstanley and Courvalin [76].

The relationship between an automated rapid susceptibility report and the clinical and economic impact on patient care has been the subject of several investigations [3,23,26,39,55,70]. While empiric therapy is commonly initiated in a significant number of hospitalized patients, a physician's modification to a more appropriate therapy in response to rapid susceptibility reports has been recorded for about 10–20% of cases [23,26]. Modification of therapy may result in a direct cost saving to the hospital [23,26] and lower mortality rates [23]. Thus, automated systems have the potential to offer improved patient care in addition to labor savings, standardization of testing, reproducibility, and data management.

Along with the advantages of automated systems, the purchaser should also be mindful of the limitations of each system. It has been common practice with commercial systems over the past two decades that following the publication and identification of a selective limitation with a pathogen and corresponding antimicrobial agent in an *in vitro* test panel, the manufacturer often voluntarily removes the panel from the market. An updated panel and software is then often reintroduced after the completion of additional clinical testing and FDA clearance of the new panel. In this chapter, each of the four current automated

Table 22.1 Comparison of automated AST systems

Item	Vitek 2 (bioMérieux, Inc.)	MicroScan WalkAway <i>plus</i> (Beckman Coulter, Inc.)	Phoenix (BD Diagnostics)	Sensititre ARIS 2X (Thermo Scientific)
Number of wells per panel	64	96	136	96
Number of drugs/GN panel	16–18 on GN panels/cards 19 on GN extension card, XN06 35 for 2 GN cards (GN69+XN06)	21–23 on Neg Combo panels 19–24 on Neg/Urine Combo 22–29 on Neg BP Combo panels 26 on Neg MIC panel 11 on ESBL plus panel 20	18–21 on NMIC/ ID panels 18–21 on NMIC panels 28 on Emerge NMIC-140 panel 20	21–24 on GN MIC panels 23 on NF MIC panel 9 on GNUR MIC panel 14 on ESBL panel 7
Number of Gram-negative panels	27	20	20	7
GN AST panel designations	GN66–GN93 Extension card: XN06	Neg Combo 31, 32, 44, 50 Neg/Urine Combo 33, 35, 45, 46, 51, 60–62 Neg BP Combo 30,34, 41, 44, 47 Neg MIC 38; ESBL plus	NMIC/ID 121–124, 126–130, 132–134, 139 NMIC 121, 123, 124, 126, 129, 133 Emerge NMIC-140 panel	GN2F–4F, GNX2F NF GNUR2F ESB1F
ESBL confirmation	Yes, on 18 panels	Yes, on 15 panels	Yes, on 20 panels	Yes, on 1 panel, ESB1F
GN urine panels (with nitrofurantoin)	Yes, on 24 panels	Yes, on 15 panels; 9 Neg/Urine Combo panels	Yes, on 20 panels	Yes, on 3 panels
GN panels for <i>Pseudomonas</i> spp. and nonfermenters (with pip/ tazo, ceftaz or ceftaz & carbapenem)	Yes, on 23 panels	Yes, on 19 panels	Yes, on 17 panels	Yes, on 6 panels
Lower GN CLSI breakpoints: for cephalosporins and for carbapenems (CLSI 2010)	Yes, on 27 panels Yes, on 27 panels	Yes, on 1 panel, ESBL plus Yes, on 8 panels	Yes, on 2 panels Yes, on 20 panels	Yes, on 6 panels Yes, on 6 panels
Number of Gram-positive panels	2	10	11	3
GP panel designations for <i>Staphylococcus</i> spp, <i>Enterococcus</i> spp, Group B Strep	GP75	Pos Combo 20, 21, 29, 33, 34 Pos BP Combo 20, 23 Pos MIC 26, 29	PMIC/ID 105–109 PMIC 106–109	GPN3F GPALL1F
<i>S. pneumoniae</i> AST panel	GP74	MICroSTREP plus	SMIC/ID-101, SMIC-101	STP6F
Data Management Software	Observe	LabPro Information Manager	BDXpert	Sensititre Windows (SWIN)
Expert System	Advanced Expert System (AES)	LabPro Alert _{EX} System	Advanced BDXpert	SWIN

GN, Gram-negative; GP, Gram-positive; BP, breakpoint; Neg, negative; Pos, positive; pip/tazo, piperacillin/tazobactam; ceftaz, ceftazidime; cefep, cefepime; CLSI, Clinical and Laboratory Standards Institute.

systems is discussed individually and evaluations (advantages and limitations) are presented as an overview of the current literature.

22.3.1 Vitek 2

The Vitek 2 instrument is a broth microdilution-based AST system (Figure 22.1), which uses 64-well plastic cards containing 17–20 antimicrobial agents (Figure 22.2). Combinations of primary Gram-negative susceptibility



Figure 22.1 Vitek 2 system. (Courtesy of bioMérieux.)
(See insert for color representation of the figure.)

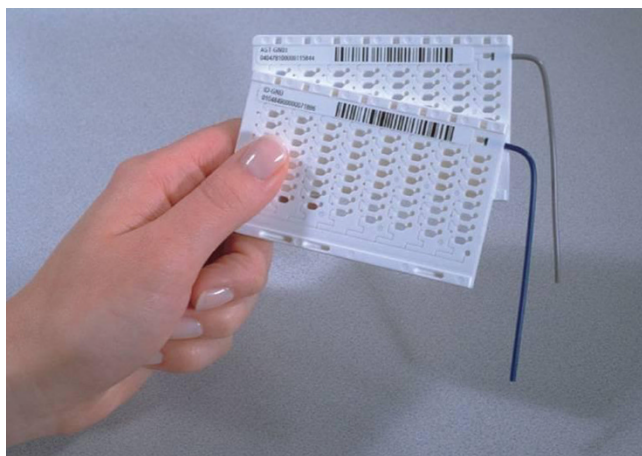


Figure 22.2 Vitek 2 Gram-negative ID and AST cards. (Courtesy of bioMérieux.)

cards can be combined with extension susceptibility cards resulting in the testing of 18 or 19 additional antimicrobial agents on the same isolate. The Vitek 2 measures changes in turbidity over time (growth curve), comparing a growth control well with wells containing various drug concentrations. Results are reported in 4–18 h as MIC and S-I-R category, depending on the growth rate and susceptibility parameters of the organism. The Vitek 2 instrument replaced the prior less automated Vitek 1 (Vitek Legacy) instrument over the period 1999–2010. Two Vitek 2 instruments are available with test card (ID and AST) capacities of 60 cards (Vitek 2) and 120 cards (Vitek 2 XL) with an expanded capability to connect two Vitek 2 modules to the same computer. In addition, three Vitek 2 Compact instruments are available with test card capacities of 15, 30, and 60 cards. Table 22.1 lists a variety of susceptibility cards, which are available for Gram-negative and Gram-positive organisms.

The Vitek 2 system contains computer software to also deduce the susceptibility results for selected Gram-positive bacteria (*Staphylococcus* spp., *Enterococcus* spp., *Streptococcus agalactiae*) for an additional four to ten antimicrobial agents, resulting in the reporting of 23–30 antimicrobial agents per card (Table 22.1). The Vitek 2 system incorporates test setup and sample verification with a Smart Carrier component. All AST inoculum dilutions are performed by the instrument, as well as card sealing and incubator loading functions. Optical reading of cards is performed every 15 min in the Vitek 2, with a multichannel fluorometer and photometer to record fluorescence, turbidity, and colorimetric signals. Susceptibility results are reported in 4–18 h, depending on the organism and susceptibility parameters. The data management system of the Vitek 2 is a PC Windows-based platform termed PC/Observa (Table 22.1). The software is FDA-cleared and is configured by the manufacturer

to FDA-defined MIC interpretive breakpoints, as are all automated AST systems. However, the Vitek software also gives the customer the option to configure MIC interpretive breakpoints according to the most recent CLSI AST performance standards [21]. The PC/Observa platform also provides a selection of quality control options, and has multi-instrument access capabilities. The detection of antimicrobial resistance is also facilitated in the Vitek 2 by the Advanced Expert System (AES). This software system validates MIC results by a set of *in vitro* testing rules based on 20,000 MIC distributions with over 2000 phenotypes, provides result interpretations and corrections, and adds footnotes (CLSI or laboratory defined) for communication to physicians. A biological correction is recommended by the Vitek 2 AES if it detects a single MIC inconsistency with the bacterial identification compared to the internal database. The Vitek 2 system for AST can be connected with the automated microbial identification system, Vitek MS, which uses mass spectrometry technology termed MALDI-ToF (matrix assisted laser desorption ionization time of flight) to provide culture isolate identification results in minutes. The AST and ID systems are connected via the laboratory informatics system, the Vitek Myla.

In an evaluation by Barry et al., MIC values from the Vitek 2 with AES was compared to a reference MIC agar dilution method against clinical isolates tested in five laboratories [4]. The essential agreement (MIC within one dilution of the reference method) for the Vitek 2 was 95.5% for 3074 Gram-negative rods, 96.7% for 1674 staphylococci, and 96.5% for 315 enterococci. In the same study, the Vitek 2 with AES appropriately detected a subset of characterized resistance mechanisms among the Gram-negative rods, staphylococci, and enterococci [4]. Blondel-Hill et al. evaluated the Vitek 2 with AES in comparison to a reference broth microdilution method against 300 Enterobacteriaceae and reported an overall correlation of susceptibility interpretations of 96.2% [8].

Performance characteristics for the Vitek 2 were compared with the Phoenix system (BD Diagnostics) in a study by Eigner et al. with an evaluation of 307 clinical isolates including Enterobacteriaceae, Gram-negative non-fermenters, *Staphylococcus* spp., and *Enterococcus* spp. [24]. The overall category agreement for both instruments was 97.0% compared to a reference broth microdilution method, and the minor error, major error (false positive), and very major error (false negative) rates for the Vitek 2 were 2.8, 0.2, and 1.7, respectively. The Vitek 2 with AES was evaluated for its ability to identify β -lactam phenotypes in a test panel of 196 isolates of Enterobacteriaceae and *P. aeruginosa*, and overall, the AES was able to ascertain the β -lactam phenotype for 93.4% of the isolates tested [62].

The Vitek 2 was evaluated along with five other commercial MIC systems for the accuracy of detecting

vancomycin MICs with a range of ≤ 1 –8 mg/mL among 129 *Staphylococcus aureus* isolates from the CDC strain collection [67]. The essential agreement (± 1 dilution) for the Vitek 2 GP61 card (currently replaced by the GP75 card, Table 22.1) was 100% and the overall category agreement was 90.7% [67]. Junkins et al. evaluated the implementation of cefoxitin on the Vitek 2 GP66 card to enhance the detection of methicillin-resistant *S. aureus* (MRSA) in comparison to two reference methods: oxacillin MIC reference broth microdilution and *mecA* gene detection [42]. For the 448 MRSA and 172 methicillin-susceptible *S. aureus* tested, the categorical agreement for the Vitek 2 was 99.7%. In a study of susceptibility testing of enterococci, the Vitek 2 was evaluated with 150 clinical isolates of enterococci, which included *vanA*, *vanB*, and *vanC* strains and six species of enterococci [29]. The essential agreement results for ampicillin, vancomycin, teicoplanin, and high-level gentamicin resistance were 93, 95, 97, and 97%, respectively. Kobayashi et al. evaluated the Vitek 2 against 32 strains of vancomycin-resistant enterococci (VRE), which were characterized by PCR as 23 *vanA* and 9 *vanB* [45]. In addition to the PCR analysis the Vitek 2 results were compared with two CLSI reference methods, agar dilution and broth microdilution. Among the seven antimicrobial agents tested, the Vitek 2 exhibited one very major error for teicoplanin, no major errors to any of the antimicrobials tested, and seven minor errors for teicoplanin. Overall, 54% of MIC test results were completed by the Vitek 2 within 7 h and all MIC values were determined within 13 h of incubation [45]. The Vitek 2 is capable of performing susceptibility testing of *Streptococcus pneumoniae* with a configured MIC card (GP74, Table 22.1) containing enriched growth medium. In a report by Jorgensen et al., 53 challenge strains of pneumococci with known resistance mechanisms and 818 clinical isolates were studied with the Vitek 2 compared with CLSI reference broth microdilution method in three separate laboratories [37]. The overall agreement of the Vitek 2 MIC values with the 10 antimicrobial agents studied was 96.3% with a mean time of results generated of 8.1 h.

22.3.2 MicroScan WalkAway plus System

The MicroScan WalkAway plus System (Figure 22.3) automated AST system is a broth microdilution method utilizing a standard 96-microwell panel (Figure 22.4). The WalkAway plus System is available in both 40- and 96-panel capacity models and was designed to replace the WalkAway-40 and the WalkAway 96 SI models. The instrument is capable of reading either conventional or rapid panels. The microwells contain serial dilutions of dehydrated antimicrobial agents. Three types of panel configurations for Gram-negative and three for Gram-positive bacteria are available: (i) Combo panels containing both antimicrobial dilutions and



Figure 22.3 MicroScan WalkAway plus system. (© 2015 Beckman Coulter, Inc., all rights reserved, used with permission.) (See insert for color representation of the figure.)

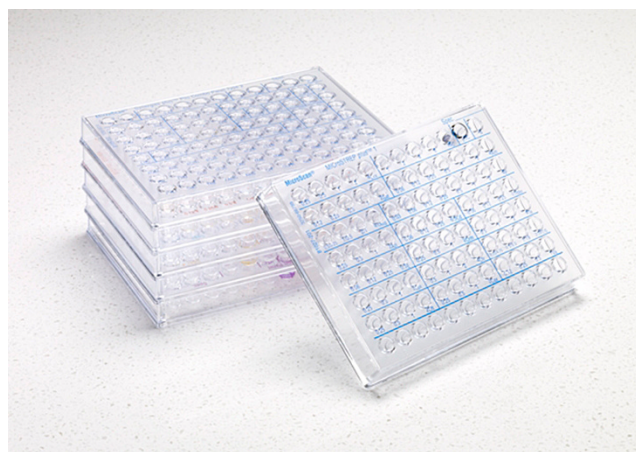


Figure 22.4 MicroScan Combo ID and AST panels. (© 2015 Beckman Coulter, Inc., all rights reserved, used with permission.) (See insert for color representation of the figure.)

identification (ID) substrates; (ii) Breakpoint Combo panels containing antimicrobials in a breakpoint dilutions and ID substrates; and (iii) MIC panels containing only antimicrobial dilutions. Breakpoint panels contain only two or three concentrations of each antimicrobial agent, and the resulting MIC corresponds to the interpretive category S, I, or R. In contrast, standard MIC panels contain a wide range of doubling dilution antimicrobial concentrations for the determination of the MIC. The MicroScan panel types are listed in Table 22.1. The panels are manually inoculated and rehydrated by using the RENOK inoculator and then placed into the incubator-reader component of the WalkAway. Results are obtained after 15 to 18 h by turbidimetric readings of overnight conventional panels and after 4.5–18 h readings of rapid panels. Combination Gram-negative ID and AST panels called MicroScan Synergies plus

Negative and rapid/S *plus* Negative panels were introduced in 2005, voluntarily recalled in 2013, and no longer available in the United States. The Synergies *plus* Negative 96-well panels were configured with 36 wells containing fluorogenic substrates for rapid organism identification (2.5 h) and 60 wells with antimicrobials in Mueller–Hinton broth for the turbidimetric determination of MICs utilizing a proprietary technique to optimize growth and allow readings from 4.5 to 18 h when needed. Since the rapid panels were no longer available at the time of writing this chapter, only the conventional panels are listed in Table 22.1. The WalkAway *plus* System includes the LabPro Information Manager system software for ID and AST data management. The optional LabPro Alert System software incorporates the detection of unusual resistance results and incorporates institution-specific infection prevention guidance into footnotes or comments for physician review. An expert software package was added in version 3 of the LabPro Alert_{EX} System [76].

An evaluation of the MicroScan WalkAway system was conducted by Rittenhouse et al., with results from a conventional MicroScan Gram-negative MIC panel compared to reference broth microdilution using 500 Gram-negative clinical isolates tested against nine antimicrobial agents [60]. Very major errors (false susceptibility) were detected at a rate of 3.0% (15 of 500), and no major errors (false resistance) were detected. Jorgensen et al. evaluated the MicroScan ESBL *plus* panel against 100 genetically characterized extended-spectrum β -lactamase (ESBL)-producing isolates consisting of 64 Enterobacteriaceae that produced CTX-M, 17 that produced SHV or TEM ESBL, and 19 that produced CTX-M and SHV ESBLs [40]. The MicroScan ESBL *plus* panel detected 98% of the isolates. A study by Bulik et al. compared MicroScan panels and reference broth microdilution in an evaluation of meropenem MICs from carbapenemase-producing *Klebsiella pneumoniae* with KPC genotypes [14]. A total of 46 *K. pneumoniae* isolates were tested, which were all modified Hodge test positive and *bla*_{KPC} positive. The MicroScan panels demonstrated a 95.6% essential agreement with the reference method, with 2.2% minor errors and no major or very major errors [14].

MicroScan Pos MIC conventional (overnight) panels were evaluated by Tenover et al. for the ability to detect linezolid susceptibility against a challenge panel of 50 enterococci and 50 staphylococci [69]. The challenge panel included 17 enterococci and 15 staphylococci that were nonsusceptible to linezolid. The MicroScan Pos MIC panels were read using the WalkAway with LabPro software and the category agreement with the CLSI broth microdilution reference method was 96.0%. In a study by Swenson et al., 129 *S. aureus* isolates were evaluated for the ability of the MicroScan Pos MIC 26 panel to detect vancomycin

susceptible ($n = 84$) and vancomycin intermediate ($n = 45$) strains from the CDC collection [67]. The MicroScan Pos MIC 26 panel demonstrated an overall category agreement of 92.2% identifying all 45 of the VISA strains and categorizing 10 of the 84 vancomycin-susceptible strains as VISA. MicroScan has a specific panel for the susceptibility testing of *Streptococcus pneumoniae* called the MICroSTREP *plus* panel (Table 22.1). In a report by Mittman et al., 311 clinical isolates of *S. pneumoniae* were tested using the MICroSTREP *plus* panel compared to a reference broth microdilution method [52]. The overall categorical agreement of the MicroScan MIC values with the 13 antimicrobial agents studied was 99.5% and the categorical agreement for penicillin was 98.7%.

22.3.3 Phoenix System

The BD Phoenix Automated Microbiology System utilizes combination panels for bacterial ID and AST. The combination MIC/ID panels contain a total of 136 wells, of which 51 are utilized for ID and up to 85 are utilized for AST (Table 22.1). A bacterial inoculum for ID and a second inoculum for AST are poured into receptacles of the respective ID and AST sections of the single combination panel. The panels are tilted and each dehydrated well is then self-inoculated. Susceptibility results are determined in the Phoenix system by utilizing a resazurin-based redox dye as well as kinetic measurements of turbidity to detect bacterial growth in the presence of an antimicrobial agent. Up to 100 panels can be placed into the Phoenix system for ID, AST, or combination of ID and AST. The panels are automatically incubated for up to 18 h, and the results are read and reported. The addition of a new Gram-negative susceptibility panel in 2014 called the Emerge NMIC-140 (Table 22.1) was designed for MIC susceptibility testing on the Phoenix system to be used in combination with the BD Bruker MALDI-ToF Biotyper system for the rapid identification of culture isolates using mass spectrometry. The two systems are connected through the BD EpiCenter software system for data management. The Phoenix system is shown in Figure 22.5, and the Phoenix Combo ID/AST panels are shown in Figure 22.6.

An evaluation of the Phoenix system was performed by Eigner et al. with an evaluation of 307 clinical isolates including 141 Enterobacteriaceae, 22 Gram-negative non-fermenters, 93 Staphylococcus spp., and 51 Enterococcus spp. [24]. The category agreement for the Phoenix system compared to a reference MIC broth microdilution method was 96.5% for Enterobacteriaceae, 84.8% for Gram-negative non-fermenters, 98.9% for Staphylococcus spp., and 98.0% for Enterococcus spp. The overall category agreement was 97.0% with 3.0% minor errors, 0.3% major errors (false positives), and 0.6% very major errors (false negatives). The evaluation was performed using NMIC/ID



Figure 22.5 Phoenix system. (Courtesy and © 2015 Becton, Dickinson and Company.) (See insert for color representation of the figure.)

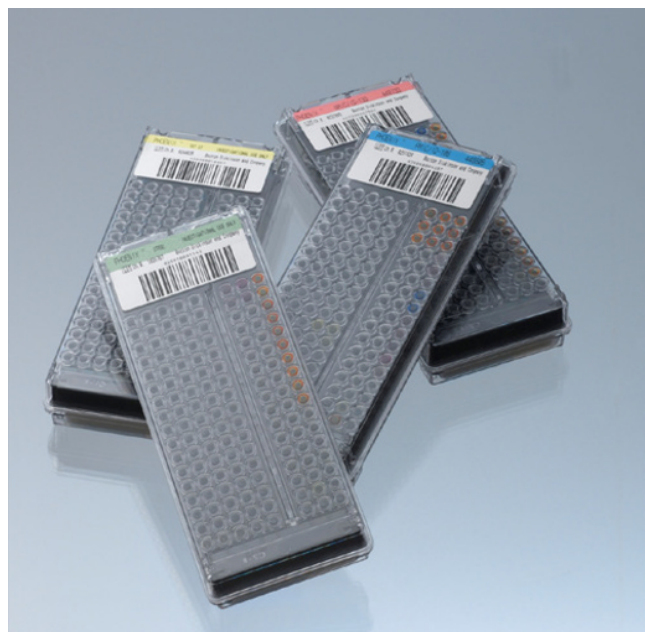


Figure 22.6 Phoenix ID/AST panels. (Courtesy and © Becton, Dickinson and Company.)

14 panels for Gram-negative rods and PMIC/ID 13 panels for Gram-positive cocci, which have since been replaced and updated on the BD Phoenix system (Table 22.1).

Junkins et al. evaluated the implementation of cefoxitin on the Phoenix PMIC/ID-102 panel to determine if the detection of methicillin-resistant *S. aureus* (MRSA) was enhanced in comparison to two reference methods: oxacillin MIC reference broth microdilution and *mecA* gene detection [42]. For the 448 MRSA and 172 methicillin-susceptible *S. aureus* tested, the categorical agreement for the Phoenix was 99.8%. The Phoenix PMIC/ID-100 panel was evaluated by Tenover et al. for the ability to detect linezolid susceptibility against a challenge panel of 50 enterococci and 50 staphylococci [69]. The challenge panel included 17 enterococci and 15 staphylococci that were nonsusceptible to linezolid. The overall categorical

agreement for the Phoenix panel compared to the CLSI broth microdilution reference method was 89.6% and the essential agreement ($\pm$ one MIC doubling dilution) was 95.8%. Buchan et al. evaluated the ability of the Phoenix system to detect inducible resistance to clindamycin among 524 clinical isolates of *Staphylococcus* spp. [11]. Compared to a double-disk diffusion reference method to detect inducible clindamycin resistance, the Phoenix showed a sensitivity of 100% and specificity of 99.6%.

Richter et al. conducted a multicenter evaluation of the Phoenix system for susceptibility testing of 13 agents against 2,013 streptococcal isolates, including 938 *S. pneumoniae*, 396 group B streptococci, 369 viridans group streptococci, 290 β -hemolytic *Streptococcus* groups A, C, and G, and 20 other streptococci [59]. In comparison to a broth microdilution reference method, the categorical agreement of the Phoenix panels ranged from 92 to 100% with one exception for viridians streptococci and penicillin, which was 87%. Mittman et al. evaluated 311 clinical isolates of *S. pneumoniae* using the Phoenix SMIC/ID-100 panel compared to a reference broth microdilution method [52]. The overall categorical agreement of the Phoenix panel with the 13 antimicrobial agents studied was 99.3% and the categorical agreement for penicillin was 95.5%.

22.3.4 Sensititre ARIS 2X

Sensititre ARIS (Automated Reading and Incubation System) 2X is an automated, bench-top incubation and reading instrument that accommodates up to 64 Sensititre 96-microwell panels for broth microdilution antimicrobial susceptibility testing and/or organism identification. Sensititre antimicrobial susceptibility testing panels for diagnostic use contain serial dilutions of dehydrated antimicrobial agents and are intended to provide a MIC ($\mu\text{g}/\text{mL}$) and MIC interpretation (susceptible, intermediate, resistant). Panel configurations are available for Gram-positive and Gram-negative bacteria and are listed in Table 22.1; some antimicrobial agents are only tested at MIC breakpoint concentrations. In addition to the commercial panels listed in Table 22.1, Thermo Scientific (previously distributed by Trek Diagnostics Systems) can manufacture custom MIC panels (dehydrated or frozen) for investigators performing surveillance studies and evaluations of new antimicrobials. Sensititre panel inoculation may be automated, using the Sensititre AIM (Automated Inoculation Delivery System), or performed manually using a Sensititre Electronic Pipette. The inoculated panels are then loaded manually into the Sensititre ARIS 2X. Antimicrobial susceptibility testing panels for diagnostic use employ a proprietary substrate (non-fluorescent) in the media used to suspend the bacterial isolate and perform panel inoculation. Hydrolysis of the substrate by bacterial



Figure 22.7 Thermo Scientific™ Sensititre™ ARIS™ 2X ID/AST System. (Photo courtesy of ThermoFisher Scientific, copying is prohibited.) (See insert for color representation of the figure.)

enzymes releases fluorescence which is used to monitor growth in each well and determine MIC endpoints; a computer algorithm converts the fluorescent signal to a MIC. The Sensititre ARIS 2X uses an internal bar code scanner to identify each panel type, then it assigns an appropriate incubation time, and when this assigned time has elapsed (16 to 24 h), the system transports the panel to the OptiRead Automated Fluorometric Plate Reading System within the ARIS 2X with no manual intervention. The Sensititre Windows (SWIN) software system offers automated expert analysis, semi-automated analysis, or manual options on a single software platform. The current Sensititre ARIS 2X instrument was released in 2004 and is shown in Figure 22.7 and the Sensititre panels are shown in Figure 22.8.

Peer-reviewed published studies comparing currently available Sensititre Gram-positive and Gram-negative bacterial AST panels on the Sensititre ARIS 2X instrument to reference methods for antimicrobial susceptibility testing are not available at the time of this writing. However, Chapin and Musnug evaluated AST results from the earlier Sensititre ARIS system for 231 Gram-negative isolates (*Enterobacteriaceae* and non-fermenters) and 95 Gram-positive isolates (*Staphylococcus* spp. and *Enterococcus* spp.) and compared AST results to MicroScan panels on the WalkAway 96 system with discrepancy analysis performed by a CLSI reference broth microdilution method [16]. The essential agreement for the 231 Gram-negative isolates was 95.8% between the ARIS and the WalkAway



Figure 22.8 Sensititre MIC panels. (Photo courtesy of ThermoFisher Scientific, copying is prohibited.)

system. Following discrepancy analysis the ARIS yielded 1.3% minor categorical errors, 0% major errors, and 0.4% very major errors. For the analysis of the 95 Gram-positive isolates, including 24 MRSA, the essential agreement was 93.5% with 0.9% minor categorical errors, 0.6% major errors, and 0.4% very major errors. Sensititre has a specific panel for the susceptibility testing of *S. pneumoniae* called the STP6F panel (Table 22.1). In a report by Zhang et al., 131 clinical isolates of *S. pneumoniae* were tested using the earlier STP5F panel with 14 antimicrobial agents compared to the reference CLSI broth microdilution method [78]. The panels were read by a Sensititre SensiTouch

reader. The essential agreement of the Sensititre MIC values ranged from 91.7% for trimethoprim/sulfamethoxazole to 100% for meropenem. The categorical agreement for antimicrobial agents with S, I, and R results ranged from 87.0% for ceftriaxone (nonmeningitis breakpoints) to 98.5% for penicillin (parenteral meningitis breakpoints) with minor errors of 9.2% for meropenem, 13.0% for ceftriaxone (nonmeningitis), and 17.7% for penicillin (parenteral nonmeningitis). No significant major errors and 0% very major errors were encountered in the study.

22.4 Semiautomated and manual broth microdilution AST systems

Manufacturers of microwell broth dilution MIC panels usually offer both semiautomated instruments and manual devices to facilitate the reading of the panels. The choice of a test system may be influenced by the laboratory budget and available personnel. However, the upgrading of a manual system to a semiautomated or automated system may also depend on the need to handle a larger volume of tests and/or the need to provide rapid computer-assisted reports. The basic manual system relies on the observer to read, record, and interpret MIC results. A computer-assisted manual system provides the observer with a touch screen, keypad, or light pen to enter and record results. The next level of instrumentation is a semiautomated system, which automatically reads, records, and interprets the MIC result from an AST panel that has been manually loaded into the reader. Both

manual and semiautomated systems can provide a variety of patient and laboratory reports, depending on the type of microcomputer and software that is chosen, and these computers often have the ability to be interfaced with an LIS. A listing of the four semiautomated systems for broth microdilution AST panels is presented in Table 22.2.

22.4.1 MicroScan AutoScan 4

In addition to the automated WalkAway *plus* system, MicroScan has a semiautomated instrument, the AutoScan 4. Use of the AutoScan 4 requires off-line incubation of the conventional 96-well broth microdilution MicroScan AST panels. The panels are either inoculated manually or inoculated with the MicroScan Renok instrument, which is designed for the hydration and inoculation of all types of MicroScan panels. After inoculation, the panels are manually loaded one at a time into the AutoScan 4 and read automatically. The MicroScan LabPro Information Manager can also be coupled with the AutoScan 4 for the computer-assisted application of AST interpretative criteria and the generation of patient and epidemiologic reports. The AutoScan 4 has been used typically in smaller laboratories with a lower testing volume or has been used for the testing of supplemental antimicrobial agents.

22.4.2 Sensititre OptiRead Fluorometric Plate Reading System

The Sensititre susceptibility system uses dehydrated 96-microwell panels which can be read by automated, semiautomated, and manual Sensititre instruments. In

Table 22.2 Semiautomated and manual AST systems

System/test type	Test System/reader	Manufacturer	Panel inoculation	Computer-assisted AST interpretive criteria
Semiautomated broth microdilution AST systems	MicroScan AutoScan 4	Beckman Coulter, Inc.	Manual pipettor, or MicroScan Renok	LabPro Information Manager
	Sensititre OptiRead Fluorometric Plate Reading System	Thermo Scientific	Sensititre Electronic Pipette or Sensititre AIM	Sensititre Windows Software (SWIN PC)
	Sensititre Vision Digital MIC Viewing System	Thermo Scientific	Sensititre Electronic Pipette or Sensititre AIM	Sensititre Windows Software (SWIN PC)
	BIOMIC V3 Microbiology System (96-well plate reader)	Giles Scientific	Inoculation dependent on panel manufacturer	BIOMIC V3 PC software
Manual agar-based gradient diffusion and disk diffusion AST Tests	Etest (gradient diffusion)	bioMérieux, Inc.	N/A	N/A
	M.I.C.E. Evaluator* (gradient diffusion)	Oxoid	N/A	N/A
	Disk Diffusion Tests	BD, Hardy, i2a, Oxoid, Remel	N/A	N/A
Semiautomated agar-based gradient diffusion and disk diffusion AST Readers	BIOMIC V3 Microbiology System (disk and gradient diffusion reader)	Giles Scientific	N/A	BIOMIC V3 PC software
	SIRSCAN 2000 (disk diffusion reader)	i2a (Intelligence Artificielle Applications)	N/A	SIRSCAN PC software

*Not marketed in the United States at the time of this writing.

addition to the automated ARIS 2X, which was discussed above, panels may be read by the semiautomated Sensititre OptiRead Fluorometric Plate Reading System, which was FDA-cleared in 2011, and replaced the previous Sensititre AutoReader instrument. When the OptiRead is utilized, a fluorogenic substrate is added to the bacterial inoculum. Panels are incubated off-line for 18 h and then loaded one at a time onto the Sensititre OptiRead instrument, where bacterial growth is detected by the presence of fluorescence. The fluorometric reading of each 96-well microtiter plate is completed in 13 s. The Sensititre data management software, SWIN (Table 22.1) can be coupled with the OptiRead instrument and patient reports can be generated with MIC results and interpretive categories, as well as epidemiology reports. Sensititre panel inoculation may be automated, using the Sensititre AIM, or performed manually using a Sensititre Electronic Pipette, as previously noted in section 22.3.4.

22.4.3 Sensititre Vizion Digital MIC Viewing System

A manual nonfluorometric method for growth detection for Sensititre 96-well AST plates is the Sensititre Vizion Digital MIC Viewing System, which is used in combination with a SWIN monitor/touch screen and software. The Vizion system was FDA-cleared in 2007 and replaced the previous Sensititre SensiTouch Reader. Following offline incubation for 18 h, a Sensititre MIC plate is placed on the Vizion reader and displayed on the SWIN PC screen with dilution templates. The MIC results are recorded by manually touching the screen or by using the mouse to record the MIC. Manual reading of Sensititre MIC plates can also be performed without computer-assisted software by the use of the Sensititre Manual Viewer, whereby the observer reads and records the MIC results. As with the OptiRead, MIC panels may be inoculated manually with the Sensititre Electronic Pipette, or automatically with the Sensititre AIM system. Sensititre JustOne strips for the supplemental testing of a single antimicrobial agent are also available from Thermo Scientific.

22.4.4 BIOMIC V3 Microbiology System for plate reading

The BIOMIC V3 Microbiology System (Giles Scientific, Santa Barbara, CA) utilizes color digital imaging to read and interpret 96-well AST plates. Plates are inoculated and incubated off-line and then placed on the BIOMIC V3, which reads turbidity, color, and ultraviolet wells. The instrument has the capability of reading Sensititre and MicroScan AST and ID plates, and custom 96-well AST plates. The system consists of a BIOMIC V3 reader cabinet with a high-resolution color digital camera and a PC with software for recording and interpreting test results and for data management. In a report by Fader et al., 3117 Gram-negative bacteria were

inoculated into a variety of AST/ID MicroScan panels and read initially on the MicroScan WalkAway and then by the BIOMIC V3 [27]. The overall category agreement for BIOMIC V3 MICs compared to MicroScan was 98.4% with 1.4% minor, 0.2% major, and 0% very major errors. In the same study, 5233 Gram-positive bacteria were evaluated and the overall category agreement was 98.7% for MicroScan with 0.9% minor, 0.3% major, and 0.1% very major errors. The BIOMIC V3 has the ability to read and report susceptibility results for disk diffusion zones of inhibition on agar, which was the original development purpose of the instrument, and can also read Etest gradient diffusion plates.

22.5 Manual and semiautomated agar antimicrobial gradient diffusion and disk diffusion susceptibility tests

As described in this chapter, the automated and semi-automated broth microdilution AST commercial systems that have been introduced over the past two to three decades provide diagnostic microbiology laboratories with more rapid MIC test results, reduced hands-on time, and computer-formatted reporting. During the same time period, manual agar-based methods, such as gradient diffusion and disk diffusion, have continued to be utilized as supplemental methods for the testing of antimicrobial agents that are not included on selected MIC panels, for the testing of fastidious bacteria, and also as the primary test method in some laboratories. The agar-based methods traditionally have been set up and read manually, but within the past two decades, semiautomated commercial readers have been introduced to facilitate the rapid reading and recording of both the gradient diffusion and disk diffusion methods. A listing of the manual and semiautomated agar-based test methods for gradient diffusion and disk diffusion AST tests is presented in Table 22.2.

22.5.1 Manual agar gradient diffusion method

The manual agar gradient diffusion method of susceptibility testing is performed by the epsilometer test, or Etest strips (bioMérieux, Inc., previously distributed by AB Biodisk), which is available in the United States and in global markets, and by the M.I.C. Evaluator (M.I.C.E.) strips (Oxoid, Basingstoke, UK), which are currently available outside of the United States. The initial gradient method was the Etest, which is a plastic strip with a predefined gradient of approximately 15 concentrations of antimicrobial agent on the bottom surface and a MIC interpretive scale on the upper surface. The Etest procedure requires the inoculation of the entire surface of a large, 150-mm-diameter agar plate with a standard suspension of the test organism ($1-2 \times 10^8$ CFU/mL) similar to that used in the disk diffusion procedure. Up to six Etest strips

may be placed in the radial fashion on the inoculated plate. The plates are incubated overnight, and an elliptical zone of growth inhibition due to the antibiotic gradient formed in the agar is visualized. The MIC is read as the point of intersection of the elliptical zone with the edge of the strip (Figure 22.9). Studies comparing the Etest to broth and agar dilution MIC methods using selected sets of Gram-negative and Gram-positive bacteria have been published [2,34]. In these studies, the MIC reported by Etest compared favorably to the reference methods within one twofold dilution interval. In an evaluation of vancomycin susceptibility results for enterococci, Tenover and colleagues reported no very major or major errors with the Etest method [68]. In a study by Swenson et al., 129 *S. aureus* isolates were evaluated for the ability of the Etest to detect vancomycin susceptible ($n = 84$) and vancomycin intermediate ($n = 45$) strains from the CDC collection with the Etest compared to the CLSI broth microdilution reference method [66]. The Etest yielded an essential agreement (within ± 1 dilution) of 100% and a category agreement of 90.7%, with the Etest categorizing 11 of 84 (13%) vancomycin-susceptible isolates as vancomycin-intermediate. Mittman et al. evaluated 311 clinical isolates of *S. pneumoniae* using the Etest compared to a reference broth microdilution method [52]. The overall categorical agreement of the Etest with the 13 antimicrobial agents studied was 99.5% and the categorical agreement for penicillin was 97.7%.

22.5.2 Manual disk diffusion test

The manual disk diffusion test is a standardized antimicrobial susceptibility method that was published by Bauer et al. in 1966 [5] and is described in the *Manual of Clinical Microbiology* by Jorgensen and Turnidge [41]. The paper disks containing standardized concentrations of antimicrobial agents are commercially available in the United States from BD Diagnostics (Becton Dickinson; Sparks,

MD), from Hardy Diagnostics (Santa Maria, CA), and from Oxoid Limited (UK; distributed by Remel/ThermoFisher Scientific, Lenexa, KS). Disks are also available from these manufacturers in Europe and also from i2a (Intelligence Artificielle Applications, Montpellier, France). The disk diffusion procedure is performed by inoculating the entire surface of a large, 150-mm-diameter Mueller–Hinton agar plate with a standard suspension of the test organism ($1\text{--}2 \times 10^8$ CFU/mL). Up to 12 commercial disks are placed on the inoculated agar surface and incubated at 35°C for 16–24 h. Following the incubation period, the zone diameter of growth inhibition for each antimicrobial agent disk is manually measured in millimeters and the qualitative interpretive criteria are recorded as S, I, or R, according to the CLSI published standards [20,21]. The disk diffusion method, which has been used for over four decades, does not require instrumentation and therefore has the lowest reagent cost of performing susceptibility testing. Other potential advantages of this method include the flexibility to test new antimicrobial agents and the ability to detect resistant subpopulations of organisms. With the use of various disk combinations the disk diffusion method also has the ability to demonstrate interesting phenotypic phenomenon, such as the inducibility of clindamycin resistance (D-Zone test), and the confirmation of ESBL production using a clavulanate-containing disk. Disadvantages of the disk diffusion method are that it provides only qualitative S, I, R results, and it may not reliably detect intermediate or resistant categories for select antimicrobial agent and pathogen combinations [41]. The disk diffusion method also requires more hands-on time than automated systems. However, there are commercially available semiautomated zone reader systems that can reduce the time for visually reading disks and also provide derived MIC results based on stored algorithms.

22.5.3 BIOMIC V3 Microbiology System for disk and Etest reading

The BIOMIC V3 System (Giles Scientific, Santa Barbara, CA) uses digital imaging to read, interpret, and record disk diffusion susceptibility agar plates. After a disk diffusion plate is placed in the digital reader and an image appears on the screen, which is reviewed for possible adjustment by the operator, quantitative zone diameters are calculated by digital image analysis and both qualitative (S-I-R) and quantitative (MIC) results are provided. Korgenski and Daly performed an evaluation of disk diffusion susceptibility plates for rapidly growing Gram-negative and Gram-positive organisms, comparing the earlier BIOMIC System with visually measured zones for 3339 organism–antimicrobial agent combinations [46]. The BIOMIC results demonstrated 0.1% very major (false-susceptible reads) and 0.2% major errors (false-resistant reads). Berke

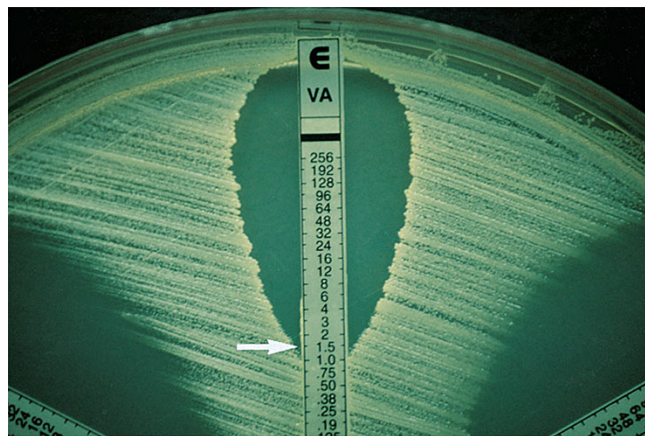


Figure 22.9 Etest. (Courtesy of bioMérieux.) (See insert for color representation of the figure.)

and Tierno compared the BIOMIC System with the Vitek System and reported overall MIC agreements between the two systems of 91.8% for Gram-positive bacteria and 91.7% for Gram-negative bacteria [7]. The BIOMIC V3 also has the capability to digitally read Etest zones and record MIC results with expert interpretation.

22.5.4 The SIRSCAN 2000 System for disk reading

The SIRSCAN 2000 System (i2a, Intelligence Artificielle Applications, Montpellier, France) is a semiautomated image analyzer that measures zone diameters of disk diffusion susceptibility plates. The SIRSCAN 2000 reports both interpretive category and MIC results and is coupled with a user-programmed expert system. Medeiros and Crellin compared readings of disk diffusion plates by the earlier SIRSCAN system with visual readings by experienced observers using a hand-held caliper [51]. A total of 368 clinical isolates, including 241 Gram-negative rods and 127 *Staphylococcus* spp., were tested. For all organisms and antibiotics tested, the SIRSCAN results yielded 0.3% very major errors. The SIRSCAN 2000 software stores data and has the capability of producing individual patient and epidemiology reports.

22.6 Phenotypic detection of antimicrobial resistance using chromogenic media

Chromogenic agars have been developed over the past two decades for the purpose of screening patient samples for colonization or infection due to organisms with specific antimicrobial-resistant phenotypes. The inventor of chromogenic culture media technology was Alain Rambach, and in 1993 the first commercial company to produce chromogenic media was incorporated, CHROMagar Microbiology (Paris, France). The chromogenic media

used in active surveillance are typically inoculated with swab specimens from anatomic areas such as nasal, axilla, groin, and rectal sites. Chromogenic agars are formulated such that the organism to be identified accumulates and then enzymatically hydrolyzes a carbohydrate substrate complex, in the presence or absence of one or more selective agents (e.g., antibiotics, NaCl), allowing chromogen (e.g., bromoindoxyl, chloroindoxyl) to accumulate within bacterial cells and to pigment colonies [57]. The pathogens with specific resistant determinants that may be detected using commercial agar screening methods are collectively termed MDRO (multidrug-resistant organisms) and include MRSA (methicillin-resistant *S. aureus*), VRE (vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*), ESBL (extended-spectrum β -lactamase-producing Enterobacteriaceae and other third-generation cephalosporin-resistant Gram-negative rods), and CRE (carbapenem-resistant Enterobacteriaceae and other Gram-negative rods). The manufacturers and names of the specific agars are listed in Table 22.3. The list may not be inclusive, as there may be additional manufacturers and additional chromogenic media either available or in development in countries outside of the United States.

22.6.1 MRSA detection by chromogenic media

To control the transmission of MRSA in hospitals, which is the most common cause of hospital-associated infections (HAIs), active surveillance cultures of patients' nares and other body sites and a comprehensive infection prevention program are recommended [15]. In fact, several states in the USA have passed laws requiring selective testing and reporting of MRSA rates by hospitals. A cost-effective approach for the phenotypic screening of MRSA is the use of chromogenic media. An evaluation of five chromogenic media for the detection of MRSA was conducted by Malhotra-Kumar et al. using 213 nasal and

Table 22.3 Chromogenic and selective agars manufactured to detect resistance phenotypes

Manufacturer	Resistance phenotype detected/agar name			
	Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA)	Vancomycin-resistant <i>Enterococcus</i> (VRE)	Extended-spectrum β -lactamase (ESBL)	Carbapenem-resistant Enterobacteriaceae (CRE)
CHROMagar Microbiology	CHROMagar MRSA	CHROMagar VRE	CHROMagar ESBL CHROMagar CTX	CHROMagar KPC
BD Diagnostics bioMérieux, Inc.	BBL CHROMagar MRSA II chromID MRSA	— chromID VRE	— chromID ESBL*	— chromID CARBA chromID OXA-48* SUPERCARBA*
Bio-Rad Laboratories	MRSAselect Medium	VREselect Medium	—	—
Hardy Diagnostics	HardyCHROM MRSA	HardyCHROM VRE	HardyCHROM ESBL	HardyCHROM CRE Agar
Oxoid Diagnostics	Brilliance MRSA Agar* Denim Blue Agar	Brilliance VRE Agar*	Brilliance ESBL Agar*	Brilliance CRE Agar*
Remel Diagnostics	Spectra MRSA	Spectra VRE	VACC Agar (selective)	—

*Not marketed in the United States at the time of this writing.

groin clinical swabs with results compared to reference culture-based and molecular PCR *mecA*-based methods [49]. Prior to inoculation onto the chromogenic media, swabs were individually incubated overnight in a selective enriched broth of brain heart infusion with 7% NaCl and 4 mg/mL oxacillin. Based on the reference methods, 87 MRSA strains were detected. When the chromogenic media were read after 24 h incubation, the sensitivity and specificity respectively were as follows: *Brilliance* MRSA agar (Oxoid) 89.9% and 86.9%; BBL CHROMagar MRSA (BD) 82.9% and 99.2%; chromID MRSA (bioMérieux) 82.8% and 96.3%; CHROMagar MRSA (CHROMagar) 81.9% and 99.1%; and MRSASelect (Bio-Rad) 80.7% and 97.2%. Denys et al. conducted a study of three chromogenic MRSA media using dual nasal surveillance swabs and compared the results to a standard nonselective trypticase soy agar with 5% sheep blood with MRSA confirmed by reference identification and susceptibility methods [22]. The sensitivity and specificity respectively, were as follows: MRSASelect (Bio-Rad) 89.0% and 93.4%; BBL CHROMagar MRSA II (BD) 87.7% and 98.6%; and Spectra MRSA (Remel) 83.6% and 92.1%. A list of manufacturers and names of chromogenic media for MRSA are included in Table 22.3.

22.6.2 VRE detection by chromogenic media

Since VRE is an important cause of HAIs, a current strategy for the early detection and management of VRE is the use of active surveillance to screen patients for the gastrointestinal colonization (rectal swabs) with VRE [53]. The development of chromogenic media for the phenotypic detection of VRE has the advantage of reducing detection time by 2–3 days compared to selective agars, and most media are able to differentiate *E. faecium* from *E. faecalis*. A review by Johnson et al. summarized published reports for five commercial chromogenic media which were compared to reference methods using selective media for the detection of VRE [36]. The sensitivity and specificity after 24 h of incubation for each media using a direct inoculation with rectal swabs were as follows: chromID VRE (bioMérieux) 80% and 100%; BBL CHROMagar VanRE (BD) 86.6% and 99.1%; Spectra VRE (Remel) 98.2% and 99.3%; *Brilliance* VRE (Oxoid) 98.6% and 99.8%; and VRESelect (Bio-Rad) 90.1% and 79.4%. The review showed that use of an enrichment broth prior to plating increased the sensitivity of the chromogenic media and that incubation beyond 24 h decreased specificity due to overgrowth of contaminating flora.

22.6.3 ESBL detection by chromogenic media

The proliferation of *Enterobacteriaceae* and other Gram-negative isolates producing β -lactam hydrolyzing enzymes, including extended-spectrum β -lactamases (ESBLs), have become a great concern over the past two decades, since

these organisms are associated with higher morbidity and mortality, and longer length of hospital stay [47]. As with other chromogenic media for the detection of MDROs, the rapid phenotypic detection of ESBL-producing bacteria would potentially facilitate earlier targeted selection of antimicrobial agents for appropriate patient management. An evaluation of two chromogenic media for the detection of ESBL-producing *Enterobacteriaceae* was conducted by Huang et al. using 528 clinical samples, which included stool, rectal swabs, respiratory tract samples, wound, and other samples, and the results were compared to reference culture-based and molecular PCR-based methods [35]. Of the 528 samples analyzed, 70 were confirmed as ESBL-producing isolates. The sensitivity and specificity after 24 h of incubation for chromID ESBL agar (bioMérieux) was 94.9% and 94.9%, and for *Brilliance* ESBL agar (Oxoid) was 94.9% and 95.1%. The same study also tested both chromogenic agars against *Enterobacteriaceae* strains with defined mechanisms of resistance to β -lactam agents. Overall, 98 of 98 ESBL-producing isolates, 30 of 34 AmpC cephalosporinase-producing isolates, and two of two carbapenemase-producing isolates were recovered on both the chromID ESBL and *Brilliance* ESBL agars [35]. In a study by Willems et al. the same two chromogenic media were evaluated using direct inoculation of 139 perineal and nasal swabs and 16 ESBL-producing *Enterobacteriaceae* were recovered [74]. The sensitivities and specificities after 24 h of incubation for chromID ESBL (bioMérieux) were 81.3% (13/16) and 80.7%, and for *Brilliance* ESBL (Oxoid) were 87.5% (14/16) and 82.1%. The lower specificities (false-positives) were mostly due to the growth of AmpC-producing *Enterobacteriaceae*, mainly *Enterobacter* spp., which is not a disadvantage if the laboratory is attempting to detect third-generation cephalosporin resistance due to any mechanism. A list of manufacturers and names of chromogenic media for ESBL detection are included in Table 22.3.

22.6.4 CRE detection by chromogenic media

Since carbapenems are often the last option for treating ESBL-producing *Enterobacteriaceae* and other multidrug-resistant Gram-negative pathogens, the worldwide occurrence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE) has become a major health concern. Due to the longer turnaround time associated with the use of enrichment broths with carbapenem disks subcultured to MacConkey (MAC) agar for the detection of CRE, the introduction of chromogenic media with direct inoculation of patient surveillance rectal swabs has presented a faster, simpler, more sensitive method for the phenotypic detection of CRE [61]. Wilkinson et al. evaluated four chromogenic media for their ability to support the growth of 130 well-defined CRE isolates (NDM, KPC, OXA, VIM, IMP) using a low inoculum of 100 CFU/

spot [75]. The sensitivity and specificity for detecting carbapenemases after 18 h of incubation for three of the media using a low inoculum were as follows: chromID Carba (bioMérieux) 91% and 89%; *Brilliance* CRE (Oxoid) 78% and 66%; and Colorex KPC made with CHROMagar KPC dehydrated media (CHROMagar) 56% and 77%. The study by Wilkinson et al. also included chromID ESBL agar (bioMérieux), which was designed to detect ESBLs, but when detecting CRE isolates showed a sensitivity of 96% and a specificity of 19%. The lower specificity may not be of concern for screening MDR Gram-negative isolates because the false-positive results (lower specificity) for CRE were due to the detection of ESBL- and AmpC-producing strains, which were included in the study. The same study also included two selective broths: trypticase soy broth (TSB) plus a 10 mg ertapenem disk followed by subculture to MAC with a sensitivity of 78% and specificity of 69%, and TSB plus a 10 mg meropenem disk followed by subculture to MAC with sensitivity of 47% and specificity of 79%. Girlich and colleagues tested 131 *Enterobacteriaceae* with reduced susceptibility to carbapenems against three chromogenic media [30]. The sensitivities were 96.5% for SUPERCARBA medium (bioMérieux), 76.3% for *Brilliance* CRE (Oxoid), and 43% for CHROMagar KPC (CHROMagar). The manufacturers and names of chromogenic media for CRE detection are listed in Table 22.3.

22.7 Genotypic detection of antimicrobial resistance determinants in positive blood cultures

The rapid genotypic detection of antimicrobial resistance determinants along with identification of selected pathogens from positive blood cultures presents a faster approach for the management of bacterial blood stream infections (BSIs). A quicker and more accurate antimicrobial management of BSIs is critical, since it is recognized that bacterial BSIs are a leading cause of morbidity and mortality worldwide, and that the rapid identification and treatment of these infections can reduce healthcare costs and improve patient outcomes [3,54].

In a study by Bauer et al., the clinical and economic outcomes of a rapid rPCR (real-time polymerase chain

reaction) assay were evaluated for the differentiation of methicillin-susceptible and methicillin-resistant *S. aureus* in positive blood cultures comparing a pre-PCR and post-PCR period [6]. In the post-PCR period, the mean time to switch from vancomycin to cefazolin or nafcillin in patients with MSSA was 1.7 days shorter, the mean length of stay was 6.2 days shorter, and the mean hospital costs were \$21,387 less [6]. The three rapid molecular systems that currently are FDA-cleared in the United States for use with positive blood cultures and the resistance determinants that are detected by each system are listed in Table 22.4.

22.7.1 Xpert MRSA/SA BC

A study by Wolk et al. reported a multicenter evaluation of the Xpert MRSA/SA blood culture (BC) assay run on the Cepheid GeneXpert PCR System to detect MRSA and *S. aureus* from positive blood cultures [77]. The GeneXpert System is a closed automated platform that performs onboard sample extraction, rPCR target amplification, and product detection within 1 h. The Xpert MRSA/SA BC assay detects the *mecA* gene along with the *attB* insertion site to identify the presence of the SCC_{mec} cassette and cassette variants with *mecA* deletions to reduce false-positive results. The multicenter study was conducted with the following positive BC broths from the Bactec (BD) instrument: Bactec Peds Plus/F, Plus Aerobic/F, Plus Anaerobic/F, Standard/10 Aerobic/F, and Lytic 10/Anaerobic/F. The study also included the following BC broths from the VersaTREK instrument (ThermoFisher Scientific): VersaTREK Redox 1 aerobic and Redox 2 anaerobic. The Xpert MRSA/SA BC assay for MRSA detection from positive BCs was compared to a standard reference culture method and CLSI disk diffusion reference method for oxacillin resistance, and performed with a sensitivity of 98.3% (57/58) and specificity of 99.4% (346/348).

22.7.2 FilmArray Blood Culture Identification panel

Altun and colleagues performed an evaluation of the FilmArray Blood Culture Identification (BCID) panel to detect the presence of MRSA and methicillin-resistant coagulase-negative *Staphylococcus* spp. (MR-CoNS)

Table 22.4 Rapid genotypic detection of resistance determinants in positive blood cultures

Assay name	Manufacturer	Instrument (time to results)	Resistance determinants
Xpert MRSA/SA BC	Cepheid	Cepheid GeneXpert System (1 h)	<i>mecA</i>
Film Array BCID	BioFire Diagnostics	BioFire Film Array (1 h)	<i>mecA</i> , <i>vanA/B</i> , KPC
Verigene BC-GP	Nanosphere, Inc.	Verigene Sample Processor Verigene Reader (2.5 h)	<i>mecA</i> , <i>vanA</i> , <i>vanB</i>
Verigene BC-GN	Nanosphere, Inc.	Verigene Sample Processor Verigene Reader (2.5 h)	CTX-M, KPC, NDM, VIM, IMP, OXA

from positive blood cultures [1]. The Film Array System is a closed automated system in a disposable pouch that performs onboard nucleic acid extraction and a nested multiplex rPCR target amplification, which includes a first-stage multiplex PCR reaction and up to 102 individual second-stage PCR reactions in separate microwells. The test concludes with a high-resolution melt-curve analysis, and the entire assay time is about 1 h. The BCID panel detects 24 target organisms in positive blood cultures in addition to four antimicrobial resistance genes: *mecA*, *vanA/B*, and the KPC gene. The study was conducted using 206 positive blood culture bottles, which included BacT/Alert FA Plus aerobic, BacT/Alert PF Plus pediatric, BacT/Alert FN Plus anaerobic, and Bactec Mycosis IC/F bottles. The study used a comparator standard reference culture method and susceptibility testing was performed by disk diffusion according to the EUCAST method. In three cases of discordant *mecA* results, conventional PCR was performed [1]. In the FilmArray analysis there were a total of 67 *mecA* results for *Staphylococcus* spp., and the BCID had a sensitivity of 94% (16/17) and a specificity of 96.0% (48/50). In the study, none of the 24 *Enterococcus* spp. detected by the FilmArray was positive for *vanA* or *vanB*, and there were no KPC-producing *Enterobacteriaceae*, which agreed with the reference method.

22.7.3 Verigene BC-GP and Verigene BC-GN

Buchan and colleagues reported a multicenter evaluation of the Nanosphere Verigene BC-GP (blood culture Gram-positive) nucleic acid assay to detect the *mecA* gene in *Staphylococcus* spp. and the *vanA* gene in *Enterococcus* spp. from positive blood cultures [12]. An aliquot of each positive blood culture is placed into a nucleic acid extraction tray and inserted into the Verigene Sample Processor. The extracted nucleic acid is then automatically transferred into a test cartridge and hybridized to complementary nucleic acid capture probes immobilized on a glass microarray slide. The nucleic acid target sequence is detected using a second hybridization with a gold nanoparticle-conjugated detection probe. The reading of the array is conducted in the Verigene Reader and the entire assay time is about 2.5 h. The Verigene BC-GP test detects 12 Gram-positive bacterial targets and three antimicrobial resistance genes, *mecA*, *vanA*, and *vanB*, from positive blood culture broths. The study of the Verigene BC-GP assay was conducted at five medical centers using BACTEC plus Aerobic/F (BD) and BacT/Alert FA FAN (bioMérieux) blood culture bottles. Identification of the *mecA* gene in 599 blood cultures containing *S. aureus* or *S. epidermidis* was 98.6% (343/348) sensitive and 94.3% (232/246) specific compared to the reference cefoxitin disk method [12]. In 81 blood cultures containing *E. faecium* or *E. faecalis* identification of the *vanA* gene was 100% sensitive and specific.

The Verigene BC-GN test detects nine Gram-negative bacterial targets and six antimicrobial resistance determinants, KPC, NDM, CTX-M, VIM, IMP, and OXA from positive blood culture broths. Mancini et al. evaluated the Verigene BC-GN test for the detection of Gram-negative pathogens and antimicrobial resistance determinants among 102 positive blood cultures and results were compared to disk diffusion, Vitek 2 broth microdilution, other standard phenotypic assays, and confirmation of resistance markers by PCR and sequencing [50]. Among the 94 isolates detected by the BC-GN, 28 isolates contained a resistance determinant: 14 were CTX-M, nine KPC, three VIM, and two OXA. The CTX-M markers detected in 12 *E. coli* and two *K. pneumoniae* were confirmed by PCR, as were the KPC carbapenemases detected in nine *K. pneumoniae* isolates. The BC-GN assay also detected VIM carbapenemases in two *P. aeruginosa* and one in *K. oxytoca*, and OXA carbapenemases in two *Acinetobacter* spp., which were all confirmed by PCR. The authors reported that eight positive blood cultures yielded genera of Enterobacteriaceae not found in the BC-GN panel and that three *P. aeruginosa* isolates showed a carbapenem-resistant phenotype without any resistance genes detected by BC-GN or PCR. Overall the study reported that the Verigene BC-GN assay detected resistance genes with a positive predictive value of 95.8% in Enterobacteriaceae and 100% in *P. aeruginosa*.

22.8 Next generation methods for antimicrobial susceptibility testing

Within the past two decades there have been advances in the use of rapid automated systems for growth-dependent bacterial identification and susceptibility testing. Also, there have been a few rapid commercial molecular test systems that have been impactful for earlier targeted patient therapy, but have been restricted to the detection of known antimicrobial resistance determinants from positive blood cultures. In addition, the past few years has seen the growth in rapid bacterial identification using proteomic analysis of positive culture material, namely the MALDI-ToF mass spectrometry (MS) system, which has become commercially available from bioMérieux and BD/Bruker. The MALDI-ToF/MS approach has gained interest and acceptance in clinical laboratories, and studies have shown that the time for reporting the identification of key pathogens from culture isolates has been reduced by approximately 24 h compared to growth-dependent methods [17,32,66]. The use of MALDI-ToF/MS for the detection of antimicrobial resistance has been reviewed, and studies have focused on the detection of the alteration of a single antimicrobial agent or a single class of agents utilizing MS analysis after the patient's isolate is incubated for 1–4 h in a broth containing the antimicrobial agent

[17,32]. Challenges exist with proteomic methods since antibiotics are usually small molecules and their detection can be complicated due to interaction with the matrix used for MALDI-ToF [32]. Applications and previous studies using MALDI-ToF/MS for the detection of antimicrobial resistance have focused on several approaches. The primary approach has been the direct detection of modifications of the antibiotic molecule itself causing a mass shift due to bacterial degradative enzymes, such as hydrolysis of a β -lactam ring [10,33,48,66]. Other approaches have been the detection of altered bacterial target proteins responsible for resistance, such as modified penicillin-binding proteins (PBPs) or methylation of ribosomal RNA [32,64]. A modification of MS has been studied for the detection of bacteria and for select antimicrobial resistance markers directly from tissue samples by the use of PCR-electrospray ionization (ESI) MS [9]. The current limited number of resistance markers that are detected include *mecA*, *van A/B*, and *bla*_{KPC}.

A limitation of current methods for the detection of antimicrobial resistance is the requirement for a relatively large inoculum of bacteria, which is true for broth microdilution systems, agar-based systems, molecular detection from positive blood cultures, and additional incubation times for MALDI-ToF/MS in antimicrobial broth. Several next generation methodologies for antimicrobial susceptibility testing have been reviewed by van Belkum and Dunne and they have included an extensive list of several new methods with detailed descriptions of a few of the near-future alternatives to traditional AST systems [72]. In addition to MS-based methods, the review discussed flow-cytometry-based methods, whereby the viability of bacteria are tracked after exposure to antimicrobial agents, and the time to detection of resistance is reduced to 1–2 h. The method is facilitated by the use of nucleic acid dyes that only permeate dead bacteria, and the proportion of dying cells after exposure to an antibiotic can be rapidly assessed [65,72].

Vibrating cantilevers containing bacterial cells is another new methodology that has been studied for the detection of antimicrobial resistance [31,44,72]. Cantilevers are structures that are anchored at one end, and these devices can be constructed with small fluidic pathways that allow bacterial passage. Following exposure to an antibiotic, the density of bacteria changes and this can be measured in cantilevers that vibrate continuously. An additional method included in the published review is isothermal microcalorimetry (IMC) [72,73]. The IMC device measures heat produced by the metabolic activity of growing bacterial cells exposed to doubling concentrations of antimicrobial agents. IMC correlates well with broth microdilution methods when net heat production over time is compared to a growth curve [73]. The IMC technology has been further developed as

chip calorimetry, and has been applied to detecting the effects of antimicrobial agents on the loss of activity and bacterial death in biofilms [13,72].

A novel approach to detect and quantify antimicrobial resistance has been the use of asynchronous magnetic bead rotation (AMBR) biosensors [43,72]. In this system, magnetic beads coated with bacterial antibodies are incubated with a bacterial broth suspension and then exposed to a revolving magnetic field to detect the change in the frequency of rotation of the beads with and without the addition of antimicrobial agents. The rotational period (RP) of the beads increases as bacteria multiply and attach to the antibody-coated beads, and when bacterial cell division is inhibited by a susceptible antimicrobial agent, the increase in RP over time is prevented. The structure and rotational rate of the beads and bead aggregates are measured by a biosensor by illuminating a hanging drop culture with a light-emitting diode (LED) or laser [43,72]. The hanging drop approach has the advantage of substantially reducing the test volume and reducing the time to result [63].

The use of digital imaging of small volume bacterial broth cultures in the presence or absence of antimicrobial agents presents another approach that has the potential to reduce the time to result of an AST. A recent study evaluated a real-time automated optical screening method based on time-lapse imaging of multiple bacteria–antimicrobial agent combinations [28]. The test method, called the oCelloScope detection system (Unisensor Ltd, Allerød, Denmark) measures bacterial growth kinetics using a contrast segmentation and extraction of surface area (SESA) algorithm, and the report showed that the detection of an antibiotic effect on *E. coli* was detected within 6 min [28]. The investigators also reported that the oCelloScope detection system could obtain AST results within 30 min from complex samples such as urine specimens.

The use of automated microscopy of immobilized live bacterial cells represents another recent rapid phenotypic method for AST, utilizing the concept of detecting growth changes in a small volume bacterial suspension [56]. The automated microscopy tests (Accelerate Diagnostics Inc., Tucson, AZ) were described using a small bacterial inoculum of 20–30 μ L placed into a disposable multichannel fluidic cassette. After a process of electrostatic immobilization of live bacterial cells, test solutions with and without antimicrobial agents were added to individual channels of the cassette, and microscopic dark-field images of progeny clones were analyzed as time-lapse images. The image analysis software was then able to generate growth probability scores based on the rate of a progenitor cell growing into a clone of daughter cells. The study by Price and colleagues examined 131 CDC-characterized isolates of *S. aureus* and reported that the method was able

to detect as few as ten growing clones over a 4 h period for MRSA, MSSA, and clindamycin-resistant *S. aureus*, including phenotypic expression of heteroresistance and inducible resistance [56]. The method of automated microscopy using accelerated population analysis profiles was also able to differentiate heterogeneous vancomycin intermediate *S. aureus* (hVISA) and VISA strains from vancomycin-susceptible *S. aureus*.

It is recognized that antimicrobial resistance is a complex process, which may involve multiple mechanisms, such as the altered uptake of drug due to porin loss or active efflux, the degradation of the antimicrobial agent due to hydrolytic enzymes, and an altered drug target such as modified PBP, or a methylated ribosomal RNA site. In the future, new test systems that are capable of performing rapid detection of the cumulative effects on bacterial cells due to multiple mechanisms of resistance (heteroresistance) will correlate more closely with the present phenotypic growth-dependent measurements of resistance, and these types of tests may have the opportunities to be the next generation systems for antimicrobial susceptibility testing. An additional challenge will be the development of extensive databases to support the next generation systems.

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Bioterrorism

James W. Snyder¹ and Michael A. Pentella²¹ Department of Pathology and Laboratory Medicine, University of Louisville Hospital and School of Medicine, Louisville, KY, USA² Massachusetts Department of Public Health, Boston, MA, USA**23.1 Introduction**

Ever since the anthrax attacks of 2001, known as Amerithrax, it has been recognized that diagnostic clinical laboratories are the first tier of an integrated response system of laboratories whose role is the early detection of biological agents, and rapid ruling out or referral of suspicious agents based on the use of standardized testing guidelines. Diagnostic laboratories are on the front line of the laboratory system that serves to protect the nation from the use of biological weapons. As a result of the emergency preparedness initiatives that have occurred nationally, there are strong linkages between clinical laboratories, public health laboratories, local government administration, and public health experts nationwide. This chapter is devoted to an overview of test systems in clinical microbiology that may be used to rule out microorganisms that might be suspected as agents of bioterrorism. Automated blood culture systems may be used to detect some biothreat agents and usually within a 5-day incubation protocol. The use of commercially available kits or automated test systems is not recommended for the identification of potential biothreat agents. This recommendation is based on the inability of some kits and automated systems to accurately identify these agents and because of the safety risk to the clinical laboratorian who may handle the potential biothreat agent without the benefit of adequate safety precautions. Diagnostic laboratories must partner with their nearest Laboratory Response Network (LRN) reference laboratory to complete the identification of the potential biothreat agent. In most instances, the LRN reference laboratory is the state public health laboratory.

23.2 History of bioterrorism

The Germ Theory of Disease, which explains how microorganisms invade the body and cause certain diseases, was unknown prior to the 19th century but use of biological weapons, in the form of microbial pathogens or toxins, dates back to the ancient Greeks and Romans. Prior to the 20th century, these weapons were used exclusively in military operations, commonly known as biowarfare, defined as the “intentional or threatened use of bacteria, fungi, or toxins from living organisms to produce death or disease in humans, animals, and plants,” and involves “intimidation of nations or people to accomplish political or social ends,” and targeted military personnel and supplies [7]. Although crude in nature, the weapons were used in three ways: (i) to poison or contaminate the enemy’s water and food supplies; (ii) expose the enemy to infectious agents and/or toxins using delivery systems such as catapults and arrows; or (iii) distributing biologically infected fomites (blankets, clothing previously used by or in contact with infected individuals) to transmit disease [8]. In 700 BC, the Assyrians intentionally poisoned water wells with rye ergot, a fungal mycotoxin; the Tartars, in the 1300s, catapulted plague-infected corpses over the walls of Caffa (Crimera), resulting in infected residents leaving the city, which is believed to be responsible for the origin of the “Black Death” epidemic in Europe [11]. In 1710, the Russians catapulted plague-containing cadavers into the city of Reval, Estonia, and in 1785 the Tunisian Moslem military forces catapulted plague-infected clothing into the city of La Calle [8]. A chronological summary of biological warfare events from the 18th century to the present is summarized in Table 23.1.

Table 23.1 Chronological examples of biological warfare events from the 18th century to present

Year/event	Biological warfare example
1763	French and Indian War: British provide Native Americans with blankets from smallpox patients
1797	Intentional flooding of the plains adjacent to Mantua, Italy by Napoleon to facilitate the spread of malaria
1863	The sale of clothing from yellow fever and smallpox patients to Union troops by Confederate military personnel
World War I	German and French Agents use agents of anthrax and glanders
World War II	Japanese use agents of anthrax, plague and other diseases Germans use anthrax on United States military personnel
1984	Spraying of <i>Salmonella</i> on salad bars in Dulles, Oregon by members of the Rajneesh Cult
2001	Letters laced with anthrax spores mailed to NBC News, American Media and office of Senator Tom Daschle

Beginning in the 19th century, the use of biowarfare became more sophisticated as a result of Koch's postulates, which led to a better understanding of infectious diseases, specific microbial pathogens, and transmission of disease [6]. Significant developments in microbiology, especially the culturing of microorganisms, led to organism identification and association of agents with specific diseases. In both World Wars, the more developed nations advanced the use of biological agents through experimentation, refinement, and the capability to target their enemies in both overt and covert operations. During the first half of the 20th century, the military was the primary state-sponsored agency to use biological agents as weapons on a worldwide basis. Although the United States discontinued research and development of biological agents for offensive purposes in 1972 in support of the Biological Toxin Weapons Convention, and focused on a defensive strategy such as the development of vaccines, antibiotics, and protective measures, other countries, particularly the former USSR, continued to support and conduct research for offensive purposes through the program known as Biopreparat [8]. The threat of biological and chemical agents was a major concern for the US and coalition forces during Operation Desert Shield following the invasion of Kuwait by Iraq in 1990, and again in 2003 with Operation Iraqi Freedom. Although intelligence reports indicated that the Iraqi regime had an active biological and chemical program, no physical evidence was discovered before or during this period of military operations.

With improvements and advances in the understanding in how to maximize the use of infectious agents or their byproducts combined with the availability of advanced equipment to produce and release agents to commit acts of bioterrorism, the period between 1980 and 1990 was

still regarded as “crude rather than advanced terrorism.” For example, although later repudiated, a primitive laboratory located in a Red Army Faction “safe house” in Paris was alleged to contain a bathtub in which was stored containers filled with botulinum toxin [8]. In 1984 in Dulles, Oregon, a cult, the Rajneesh, for the purpose of influencing the outcome of local elections, purposely contaminated salad bars with *Salmonella typhimurium*. The objective was to prevent local citizens from voting in the local election. Although no deaths were recorded, 750 individuals were infected with 40 requiring hospitalization [8,10]. On several occasions a Japanese cult, Aum Shinrikhyo, attempted to release and disperse anthrax, botulinum toxin, *Coxiella burnetti*, the agent of Q Fever, and Ebola virus. Several means of disbursement were utilized, including the use of an automobile exhaust system for botulinum toxin, anthrax via a sprayer system, and use of modified brief cases containing botulinum strategically placed in the Tokyo subway system [8,10]. In 1996, members of the laboratory staff at a Dallas, Texas medical center received an invitation via e-mail to come to their break room to partake of donuts and muffins. Hours later, 12 individuals developed severe diarrhea, eight of which tested positive for *Shigella dysenteriae* type 2. Following a detailed investigation, a laboratory employee was found to be responsible for the attack [6]. However, the most memorable event was the use of the US postal system to send letters containing anthrax spores to selected media outlets and governmental personnel. A total of 22 cases of anthrax were identified of which 11 were of the inhalational form, and 11 (seven confirmed, four suspected) of the cutaneous form [6]. Following many years of investigation, the Federal Bureau of Investigation (FBI) concluded that a scientist working at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) was responsible for what is today regarded as a biocrime.

23.3 Bioterrorism in the future

The threat and possibility of bioterrorism attacks in the form of biowarfare, biocrimes, or biothreats is greater today than ever before. Lessons learned from early historical records up to today clearly demonstrate the impact that biological agents can have on the military and civilian sectors. Although the progression of using biological agents as weapons has advanced from “crude” to advanced forms, the unique features of biological agents as weapons have remained constant [8]. The selection of what agent to be used is dependent on the financial, technical, and economic capabilities of the agency, state, organization, or individual, planning to execute an attack. The anthrax event in 2001 was, overall, successful and essentially served as a “wake up call” to the respective governmental, law enforcement, emergency response, and medical

communities. Although the morbidity and mortality was low, and the event was confined to the east coast, public fear and concern prevailed throughout the country. Just the observation of first responders, law enforcement, and medical personnel in full protective personnel equipment on television and in the print media has a psychological impact: people feel threatened and do not know what will happen next [8,10]! This fuels public distraction and anxiety. In addition to the development, sustainment, and implementation of preparation and response plans by the respective medical, public health, law enforcement, and governmental agencies targeting rapid response measures, maintenance of an active surveillance and intelligence program in addition to providing diagnostic laboratories with resources, including technology, training, and education, play a key role in the recognition, detection, and identification of a variety of biological agents that cause microbial-mediated diseases. Diagnostic laboratories, independent of their classification as private, public, or federal-based, are constantly in search of improved diagnostic tools designed to detect and reliably identify the agent as rapidly as possible. Having knowledge of the specific agent influences surveillance, infection control, patient therapeutics and management, epidemiology, and preventive measures [10].

23.4 Laboratory Response Network

Recognizing the need for, and the importance of, the diagnostic laboratory in responding to bioterrorism, the Laboratory Response Network (LRN) was created in 1999 through the collaborative efforts of the Centers for Disease Control and Prevention (CDC), the Federal Bureau of Investigation (FBI), and the Association of Public Health Laboratories (APHL). The primary mission of the network is to “ensure an effective laboratory response to bioterrorism” but more recently, in addition to bioterrorism, emerging infectious diseases [5]. Currently, the LRN is an integrated network of local, state, and federal public health, sentinel (e.g., hospital and commercially based), food testing, veterinary, environmental testing, and international laboratories providing diagnostic capability and capacity for biological, chemical, and radiological threat detection, emerging infectious diseases and other public health emergencies [5]. Laboratories are categorized as either a Sentinel Clinical Laboratory in the Public Health Laboratory System (city, county and state public health department), Sentinel Clinical Laboratory in the Laboratory Response Network (private sector; hospital and commercially based), Reference Laboratory (city, county or state public health department) or Federal (CDC, United States Army Medical Research Institute of Infectious Diseases [USAMRIID]). The primary function of the LRN sentinel laboratories is to recognize

Table 23.2 Major biothreat and emerging infectious disease agents

Biothreat (BT) agents (disease)	Toxin-mediated disease	Emerging pathogens*
<i>Bacillus anthracis</i> (anthrax)	<i>Clostridium botulinum</i> (botulism)	Novel influenza viruses
<i>Brucella</i> spp. (brucellosis)	<i>Staphylococcus aureus</i> (enterotoxin b)	Chikungunya
<i>Burkholderia mallei/pseudomallei</i> (glanders, melioidosis)	Ricin	Dengue fever
<i>Coxiella burnetii</i> (Q fever)		Japanese encephalitis virus
<i>Francisella tularensis</i> (tularemia)		<i>Cryptococcus gatti</i>
Variola virus (smallpox)		SARS virus/MERS virus
<i>Yersinia pestis</i> (plague)		

*See Chapter 21 for additional information.

potential biothreat agents using standardized testing protocols and submit suspicious isolates to the nearest LRN reference laboratory as soon as possible for confirmatory identification. The LRN reference laboratory then refers the identified agent(s) to the LRN federal laboratory for additional characterization (susceptibility testing, nucleic acid sequencing, and archiving) [9]. Of particular note is that, with the exception of LRN sentinel public health laboratories, LRN clinical laboratories are not allowed to accept environmental and animal specimens for evaluation and are required to refer them to the nearest LRN reference laboratory [9].

This formal, integrated, tiered laboratory structure links clinical laboratories with local, state, and federal public health laboratories to promote rapid, timely detection and recognition, and full characterization for a variety of biothreat agents (Table 23.2). In support of this, the LRN is continuously seeking new and innovative high quality assays for the rapid detection of biothreat agents. An LRN sentinel clinical laboratory is defined as a laboratory certified to perform high complexity testing under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) by the Centers for Medicare and Medicaid Services (CMS) for the applicable microbiology specialty. Laboratory in-house testing includes Gram stains and at least one of the following: lower respiratory tract, wound or blood cultures. The primary responsibility of sentinel clinical laboratories is to “rule out” or “refer” suspicious biothreat agents. Responsibilities include: (i) being familiar with reportable disease guidelines in its geographical jurisdiction and having policies and procedures in place to refer diagnostic specimens or isolates suspected to contain agents of public health significance to the local or state public health laboratory; (ii) ensuring that personnel have met the applicable federal regulations for packing and shipping of infectious substances; (iii) having policies and procedures for referral

of suspect biothreat agent specimens and/or isolates to the LRN reference laboratory that reflect the American Society for Microbiology (ASM) *Sentinel Level Clinical Laboratory Guidelines for Suspected Agents of Bioterrorism and Emerging Infectious Diseases* (www.asm.org)[1]; (iv) maintaining the capability to perform testing outlined in the aforementioned ASM Guidelines and demonstrating annual competency by participation in proficiency testing or exercises, such as the Laboratory Preparedness Exercise or state-developed challenge sets; (v) having a Class II or higher Certified Biological Safety Cabinet; (vi) complying with Biosafety Level II (BSL-2) practices as outlined in the current edition of the Biosafety in Microbiological and Biomedical Laboratories guidelines; (vii) complying with applicable Occupational Safety and Health Administration (OSHA) regulations for a respiratory protection program; (viii) complying with the rules and regulations of the Select Agent Program. Sentinel clinical laboratories are encouraged to refrain from using automated or kit-based identification systems when dealing with suspicious or known biothreat agents. The use of advanced technologies (nucleic acid amplification techniques, sequencing, etc.) is restricted to LRN Reference and Federal Laboratories who are certified to handle such agents under the Select Agent Program.

The conventional laboratory methods used by sentinel clinical laboratories are summarized in Table 23.3 and are available at the ASM webpage. Automated systems do not consistently correctly identify biothreat agents, for example, *Yersinia pestis* isolates are commonly misidentified as *Shigella* spp. Macroscopic growth characteristics are dependent on the biothreat agent considered. Some biothreat agents require increased carbon dioxide concentrations of 5–10%. These tests are informative to the potential of the

Table 23.3 Key sentinel laboratory methods

Essential testing	May be performed depending on biothreat agent suspected
Culture on appropriate media includes that necessary for the source of the specimen.	Oxidase (0.5% tetramethyl-p-phenylenediamine)
Other media that may be helpful for isolating or characterizing some bacterial BT agents include:	Catalase
Motility media	Urea (urea agar or rapid urea disks)
Cysteine-supplemented agar (Thayer Martin)	Indole
Lysis-centrifugation (Isolator) media	TSI/KIA
<i>Burkholderia cepacia</i> selective agar	Beta-lactamase test
Gram stain: to observe microscopic characteristics of biothreat agents	Catalase (3% hydrogen peroxide)
	Colistin or polymyxin B disk
	Motility
	Satellite test
	Arginine
	Growth at 42°C
	Nitrate

Table 23.4 Technology for detection of biothreat agents in the environment

Immunoassay
PCR
Immunoassay combined with PCR
Other: Optical Sensor, Raman Spectroscopy, Microarray, Mass Spectrometry*

* Matrix assisted laser desorption/ionization time of flight (MALDI-ToF) is replacing conventional methods in some laboratories. MALDI-ToF cannot be reliably used to identify biothreat agents and the process risks laboratory exposures to these pathogens during preparation of the sample.

isolation of a biothreat agent, and the performance of these tests, have a minimal risk to laboratory personnel working with the suspect agents. One commercial manufacturer offers a product to identify colonies of *Bacillus anthracis* using an immunoassay that contains a monoclonal antibody that binds to a surface protein found on *Bacillus anthracis*. Suspect colonies isolated from clinical specimens and cultured on sheep blood agar plates are tested using an immunochromatographic assay. The result of *B. anthracis* or not *B. anthracis* must be confirmed by a LRN reference laboratory.

23.5 Rapid methods

There are a wide variety of rapid methods (see Table 23.4) in the market place designed to detect biothreat agents in environmental samples [2,3,4]. Generally, the tests are not to be used for human medical diagnostic testing and none are FDA approved for clinical testing. While the results of these tests have good performance characteristics, they cannot be used alone to base response decisions.

23.6 Conclusion

Since automated systems and kits are not recommended for use with suspect biothreat agents, it is important that all technical staff receive training on the recognition of these suspect agents early in the identification process before the manipulation of these cultures results in exposures of individuals working in the laboratory. There should be policies and procedures in place instructing technical staff on the correct testing to be performed. It is also important that laboratories maintain conventional test capabilities so that when the laboratory encounters a suspect agent there will not be a delay in the identification process.

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Clinical Microbiology: Looking Ahead

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24.1 Introduction

In the first edition of this text, Wolk and Persing asked the question, “What will the future hold in terms of laboratory diagnosis of infectious disease?” [264]. In 2002, they predicted that clinical microbiologists would face ample opportunities to change the face of their practice, and predictions for change included the following:

- an increased need for rapid diagnostic response to emerging pathogens and microbes introduced via new medical procedures, or medical practices;
- the expansion of the breadth of pathogens we will encounter and diseases they will cause;
- options for screening for drug resistant mutations in addition to phenotypic testing;
- use of gene expression patterns as they relate to pathogenicity;
- monitoring of the immunological response to infection via biomarkers and gene expression profiling;
- assessment of human genetic variations as they relate to host susceptibility and drug metabolism;
- introduction of high throughput automated technologies;
- the democratization of molecular technology to adapt to a variety of different clinical laboratory settings;
- the use of sequence identification as a stand-alone or adjunctive detection method;
- the rise of automated nucleic acid extraction platforms;
- the expansion of real-time polymerase chain reaction (PCR) assays and instruments;
- the transition from single target to multiplex target testing;
- parallel testing with random access instruments;
- introduction of the applications of mass spectrometry and protein analysis;
- application of peptide nucleic acid probes;
- introduction of flow cytometry applications;

- increased need for standardization in quality control practices;
- increased need for bioinformatics;
- additional efforts to identify cost benefit;
- increased emphasis on interdisciplinary healthcare and industry teams;

The preceding list of 20 items, predicted in 2002, creates a clear and singular description of clinical microbiology diagnostic practice today. Since the first edition of this book, diagnostic capabilities in clinical microbiology have grown exponentially, in part due to the impact of new molecular and proteomic tools to detect human pathogens.

In the first edition of this book, the routine integration of real-time PCR and robotic instrumentation for PCR, reverse transcription (RT) PCR and nucleic acid extraction was predicted [264]. Most of the technology previously reviewed is now either commonplace or has come and already gone with the rush of new technology in clinical microbiology. The technologies cited in the first edition have evolved by several generations in many cases, with new software and engineered improvements. Real-time PCR, molecular beacon probes, DNA sequencing, and robotic nucleic acid (NA) extraction, have become routine, and best practices for these methods have made their way into guidelines from the Clinical and Laboratory Standards Institute (CLSI) and College of American Pathologists (CAP) checklists. Solid microarrays have not yet become entrenched, but their liquid version counterpart, Luminex-based multiplex assays, and multiplex assays with integrated detection systems that followed (Nanosphere, GenMark, and BioFire) have revolutionized the way we perform virology alongside bacteriology as routine components of diagnostic testing for respiratory pathogens.

New technology will continue to rapidly influence the diagnostic sciences. This chapter will focus on recently introduced or novel methods and advances that have changed or are poised to change the landscape of clinical microbiology. Trends in identification of human pathogens, commonly encountered in diagnostic settings, will also be discussed. Of note, this chapter focuses primarily on technology that is US Food and Drug Administration (FDA) reviewed, approved, or being targeted toward FDA clinical trials; no trademarks or similar notations will be used in this chapter, please consult with the manufacturers to obtain this information.

In this review, the reader will learn how new technological advances provide us tools with which we can attempt to improve patient care and disease management. In addition, we will detail examples of how clinical microbiologists can document both benefits and limitations of new technology and related clinical practices. The benefits of technology generally include the ability to provide fast results, increased sensitivity and specificity, greater versatility, and more accurate results that are not necessarily dependent on isolation of pathogens in culture. An additional benefit is the ability to obtain multiple answers from one test process; for example, the result may document the presence of multiple pathogens or identify the pathogen and common antimicrobial resistance markers. Their limitations generally include: (i) higher costs; (ii) inability to detect emerging genetic or proteomic sequences that may not be targeted by current primer or probe combinations; (iii) inability to detect sequence variants not yet placed into genomic databases; and (iv) the inability to access cultivated microbes for susceptibility testing or genotyping.

Over the past 12 years, clinical microbiology strategies transitioned from a primary “culture all pathogens” approach to a combination of culture and “amplify multiple genetic targets” approach. The excitement surrounding

rapid development and diversity of new technology is tempered by current healthcare expectations to describe clinical and financial utility of laboratory testing prior to, or quickly after implementation. As first noted in 2002, Wolk stated; “We have opportunities to shape the future of our clinical practice. Progress must be tempered with common sense so that optimal patient benefit is achieved and documented [264].” Although the evidence-based laboratory intervention literature is currently sparse, there is hope that the merits of recent improvements will be documented to increase the cost-benefit of such laboratory testing [234,235].

24.2 Connectivity between extraction and amplification platforms

24.2.1 Requirements for upstream nucleic acid extraction

In general, purified NA is necessary as the primary template for most amplification technologies and may be performed as a preliminary step for diagnostic assays. Starting from a clinical sample, NA extraction can be performed by either manual or automated methods (Table 24.1). Determining which method to use is a critical step in successful NA purification and use in downstream amplification assays. The extraction methods that may work for one pathogen and specimen type may not necessarily be effective for other specimen types and pathogens. In addition, structural characteristics of microorganisms and specimen inhibiting substances can affect NA extraction.

Manual methods are time-consuming and are generally less reproducible than automated methods, and they are moving towards extinction in clinical laboratories where automation is overtaking manual methods. With today's assays moving from single-target assays to those with multiple target amplification, the extraction requirements

Table 24.1 Common automated nucleic acid extraction systems

Kit/platform	Manufacturer	Technology	Number of samples/ total time	Specimen type(s)
m2000sp*	Abbott	Magnetic silica particles	24–96/2–2.5 h	Serum, plasma, vaginal, rectal, nasopharyngeal swabs, stool
NucliSENS EasyMAG	bioMérieux	Proprietary “Boom” technology with magnetic silica	24/batch	Most sample types (plasma, serum, whole blood, stool, respiratory samples, etc.) 50–1000 µL
BioRobot EZ1	Qiagen	Magnetic silica particles	6/20 min	Dermal, genital, nasal swabs
QIAcube			24/1.5 h	
QIAagility			96/1.5 h	
QIAsymphony*			96/2 h	
MagNA Pure LC	Roche Applied Science	Magnetic silica particles	32/90 min	Serum, whole blood, plasma, stool, sterile body fluids, respiratory tract specimens, dermal, genital swabs
MagNA Pure Compact			8/30 min	
COBAS Ampliprep*			96/2 h	Blood stain cards, buccal swabs, whole blood, tissue
Maxwell	Promega	Paramagnetic particles	16/45 min	

* Automated instrumentation with extraction module and assay preparation. Not available as a stand-alone extractor.

are more important than ever before, requiring optimized upstream NA extractions that produce high yield and high purity extract [19].

Automated linked or integrated extraction systems are becoming more common and practical for use in clinical laboratories. Linked automated extraction systems incorporate a modular approach to extraction, amplification, and target detection, typically two or three different instruments are linked by software that recognizes and tracks samples via barcodes as they move, either robotically or manually through the molecular testing process. Linked extractors are primarily intended for use with only certain downstream detection systems, typically sold by the same manufacturer or a corporate partner of the manufacturer. Thus, while adding ease and additional throughput to molecular laboratory processes, a linked system typically locks the user into purchasing only the testing reagents that are designed for use with that one system, limiting flexibility in reagent selection. In contrast, integrated systems are those in which the processing cartridge incorporates all phases of testing, namely, extraction, amplification, and detection. Advantages of these systems include the speed of results and common Clinical Laboratory Improvement Amendments (CLIA) classification of moderate complexity. Limitations include higher costs and smaller throughput than the larger linked systems, which commonly base testing on 48–96 tests per run.

Linked and integrated systems provide several benefits over manual extraction methods, which are losing favor in many busy multitasking molecular laboratories feeling the effects of workforce shortages and limited resources. Moreover, automated NA extraction systems tend to yield more reproducible NA recovery and reduce the risk for contamination of other samples and the work environment [94]. The robotic components of linked or integrated automated extraction instruments ensure greater workload efficiency with very minimal manipulation or attention [117,258]. Many offer inventory control and full process documentation to support best practices in clinical laboratories.

One example of a new concept, the linked automated extraction platform, is the Qiagen QIASymphony SP system (Qiagen, Germantown, MD). The QIASymphony (Figure 24.1a) is designed to couple with the QIASymphony AS (assay setup) module (Figure 24.1b) for automated PCR assay preparation and the Qiagen Rotor-Gene Q real-time PCR instrument for target amplification (Figure 24.1c). Integrated extraction systems, such as the QIASymphony, typically provide prefilled reagent cartridges, bar-coding of samples and reagents, and touch-screen operation. Many robotic NA extraction systems allow loading of 1–96 samples per run, and some can perform different purification procedures within the same run of 96 samples.

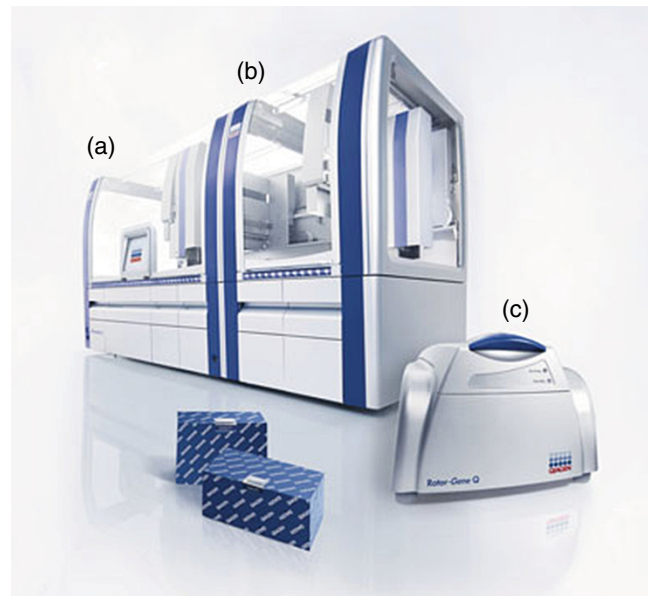


Figure 24.1 (a) Qiagen's QIASymphony SP instrument and integrated extraction system, is designed to work in conjunction with (b) the QIASymphony AS (assay setup) module, and (c) the Qiagen Rotor-Gene Q, real-time PCR instrument. (© Qiagen, all rights reserved.)



Figure 24.2 (a) The Roche COBAS system is comprised of the COBAS AmpliPrep instrument and (b) the COBAS TaqMan instrument. The two components are depicted here in “docked” mode, but can also be undocked and placed in separate laboratory areas. (Courtesy of Roche Diagnostics Corporation.)

Other examples of linked extraction platforms include the Roche COBAS AmpliPrep/COBAS TaqMan System (Roche Molecular, Indianapolis, IN), and the Abbott m2000sp (Abbott, Des Plaines, IL). The COBAS AmpliPrep platform (Figure 24.2a) isolates RNA or DNA with silica-coated glass magnetic beads and is linked with the COBAS TaqMan Analyzer (Figure 24.2b) or smaller COBAS TaqMan 48 Analyzer for real-time PCR and amplicon detection. Process integration occurs via the Roche AMPLILINK software. The system has a throughput of 168 samples in an 8-h shift and can load up to four different tests at once.

The Abbott m2000sp (Abbott, Des Plaines, IL) system (Figure 24.3a) is used for sample preparation and is



Figure 24.3 (a) Abbott's m2000 sp system for sample preparation is linked with (b) m2000rt for real-time amplification and detection to form a multicomponent system for qualitative and quantitative PCR and RT-PCR. (Courtesy of Abbott.)

integrated with their real-time PCR downstream applications performed on the Abbott m2000rt: RealTime PCR platform (Figure 24.3b). Throughput options range from 24 to 96 samples and the system has an advantage of a partially open mode, which can allow the users to alter the protocols for various sample types and input volumes. In addition, the m2000sp can be used with reagents from a corporate affiliation with Intelligent MDx (Cambridge, MA). Please refer to other chapters in this text for additional information.

Other emerging linked technologies include the Sentosa system (Vela Diagnostics, Fairfield, NJ), in which an Eppendorf robotic pipettor was adapted to become the Sentosa SX101, and combined with a Qiagen Rotor-GeneQ real-time cyler and options for an Ion Torrent Sequencer, adapted and branded as the Sentosa SQ301 (all sold and supported for research use only (RUO) by Vela Diagnostics). Vela currently offers a CE-IVD test, Sentosa SA Direct MRSA/SA PCR, for qualitative detection of *Staphylococcus aureus* (SA) and methicillin-resistant *S. aureus* (MRSA) from nasal swabs. They also offer RUO PCR-based surveillance testing for the identification of the mosquito-borne Chikungunya virus.

Despite the advantages of automated and integrated systems, there are circumstances when manual methods may be preferred or used in combination with automated methods. For example, manual methods may offer higher DNA yield or purity, or may be useful when microbial cell walls or inhibitors in specimen matrices become problematic. Manual extraction kits are usually less expensive, but do require several manipulations, which may offset the savings in reagent costs. Lastly, automated linked and

integrated extraction instrumentation can require the use of large footprints of space, a valuable commodity in many laboratories.

24.3 Polymerase chain reaction and RT-PCR: detection and characterization

24.3.1 Real-time PCR

According to predictions, real-time PCR is arguably the molecular method with the most profound impact on the clinical laboratory in the past two decades. Since its emergence in clinical laboratories in the late 1990s, real-time PCR integrated into diagnostic practice relatively quickly, serving as a rapid method to identify or quantify many common pathogens [25,59,257,260]. PCR methods continue to evolve and redefine the optimal diagnostic strategy in clinical microbiology laboratories.

With the development of even simpler and more rapid methods, multi-target chemistries, smaller instrument footprints, integrated extraction, and amplification with minimal manipulation [59,60], real-time PCR methods are steadily supporting our ability to improve patient care. Such benefits currently decrease the time to result, the hands-on time demand and requirements for qualified molecular technologists to perform the testing. It is not surprising that molecular pathogen identification is strikingly more common than it was a decade ago.

There are now several assays and instruments available, which are classified as "moderate complexity" by CLIA, making them amenable to testing by most laboratory staff, for 24/7 testing, which can be used in small community hospital laboratories and large reference laboratories alike

[25,59]. With the advent of completely integrated systems, the ability to apply such technologies to direct point-of-care testing and bedside identification is promising.

Currently, two main types of diagnostic real-time PCR assays are used for the detection of pathogens: (i) multi-step real-time PCR assays that require off-platform specimen processing, extraction of NA from the specimen, PCR assay set-up, and amplification/detection; and (ii) integrated assays that incorporate the three steps into the subsequent amplification/detection step. Examples of the latter type, in order of appearance to the US market, include Cepheid's GeneXpert system, Becton Dickinson's BD Max system, BioFire Technology's FilmArray and Focus Diagnostic's Simplexa Assays on the Integrated Cyclers. The benefits of the integrated real-time PCR assays are decreased hands-on time, no manipulation of the extracted nucleic acids, which decreases the risk of contaminating the work area, and in most cases decreased reaction time and overall turnaround time.

Since there are hundreds of real-time PCR examples, all cannot be reviewed here and we refer readers to recent extensive reviews of methods [78,257,260]. Likewise, it is not possible to review the entire breadth and depth of laboratory-developed tests (LDTs). We will review examples of common or emerging technology with impact to clinical microbiology laboratories.

24.3.2 Recent innovations for reverse-transcription and real-time PCR assays

24.3.2.1 Simplexa assays

Focus Diagnostic (Cypress, CA) markets the eight-well Simplexa Direct Amplification Disc assays (Figure 24.4a) that are fully integrated and do not require any upstream NA extraction steps, thus decreasing turn-around time and enabling performance of molecular testing in places where engineered molecular laboratory space is not available. Simplexa Direct assays include influenza A/B and respiratory syncytial virus, herpes simplex viruses 1 and 2, and Group A *Streptococcus*. Simplexa assays are performed on the four-channel 3M Integrated Cyclers (Figure 24.4b), which allows processing of multiple pathogen targets simultaneously. The growing test menu of molecular assays includes detection of *Clostridium difficile*, *Bordetella pertussis/parapertussis*, *Chlamydia* (*Chlamydophila*) *pneumoniae*, Group A *Streptococcus*, *Mycoplasma pneumoniae*, and *Borrelia* spp. directly from patient specimens [57,127,248].

For assays in which NA extraction is required prior to sample loading, amplification and detection of up to 96 samples can occur on one Simplexa disc, with results available in about an hour after loading the instrument. The assays and instrument support software programs for DNA and RNA, qualitative and quantitative PCR



Figure 24.4 (a) The Focus Diagnostics eight-well Simplexa Direct Amplification Disc combines with (b) the 3M Integrated Cycler for direct amplification and detection without the need for nucleic acid extraction. (Courtesy of Focus Diagnostics)

reactions, and a small instrument footprint. Assays require small sample volumes and multiple assays can be performed on the same disc. The menu of PCR and RT-PCR Simplexa assays is extensive.

24.3.2.2 IntelligentMDx assays

IntelligentMDx (Cambridge, MA) uses a unique proprietary bioinformatics process to provide analysis and verification of genetic sequences, thus producing assays with the potential to quickly adapt to genetic change. The IntelligentMDx bioinformatics process, NGENix, was specifically developed to address complex and evolving infectious targets. The NGENix system and processes may enable a better understanding of NA interactions and enhance the development of assays for any molecular target. Accelerated production of assays occurs by preselection of metrics, algorithms, and rules necessary to build robust, reproducible molecular assays for the detection of pathogens. The Intelligent MDx process is the first to use a systems approach and multifactorial computational capability that enables *in silico* assay design for primers, probes and amplification conditions. Built into this process, is the integration of input from expert end-users to preset the clinical indications and specifications for an assay. In theory, the assay should be robust and easily adapted to emerging genetic sequences as pathogens evolve.

Real-time PCR assays are designed with similar reaction conditions so that they can be multiplexed, performed in parallel, or performed separately, allowing clinical laboratories to optimize throughput and workflow.

There are three current IntelligentMDx assays commercially available in the United States. The *C. difficile* assay for the Abbott *m2000* is a PCR assay for the qualitative detection of conserved regions within toxin A and toxin B genes of the *C. difficile* genome in human symptomatic patients. The assay detects toxigenic *C. difficile*, including NAP1/027 hypervirulent strain and toxin B-variant type strains. In addition, the VanR assay detects *vanA* and *vanB* vancomycin resistance genes directly from human perirectal or rectal swabs or stool samples from patients at risk for vancomycin-resistant *Enterococcus* (VRE) colonization. More recently, the Flu A/B and RSV assay was approved for testing nasopharyngeal swabs and includes detection of the emerging H3N2 variant strains. All IntelligentMDx assays are intended for use on the Abbott *m2000* RealTime system, a high throughput system ranging from 22 to 96 samples per batch (Figure 24.3a,b).

24.3.3 Digital PCR

Digital PCR is noted to have potential for highly accurate and reproducible quantitation of viruses and other genetic targets [16,50,83,88,184,241]. Droplet Digital PCR (ddPCR) from Bio-Rad (Hercules, CA) is a technique based on partitioning of many PCR reactions in water-oil emulsion droplet technology. Bio-Rad's QX100 system fractionates each sample into 20,000 individual nanoliter-sized droplets (Figure 24.5), and PCR amplification of the template molecules occurs in each individual droplet.

PCR-positive and PCR-negative droplets from every sample are then counted to provide absolute target quantification in digital form. The ddPCR technology uses reagents and workflows similar to those used for most standard TaqMan probe-based real-time PCR assays. The massive sample partitioning is a key aspect of the ddPCR technique. Digital PCR has been successfully used for quantitation of HIV and cytomegalovirus, as well as qualitative analysis of pathogens such as *S. aureus* and *Mycobacterium tuberculosis* [88,92,116,185].

24.3.4 Integrated PCR assays

24.3.4.1 GeneXpert assays

The Cepheid GeneXpert (Cepheid; Sunnyvale, CA) is a completely integrated single-use cartridge real-time PCR instrument for direct detection of pathogens from clinical specimens. Specimen processing and decontamination occurs along with DNA extraction, amplification, and detection of the genetic target all within the same cartridge. A result is rapid, within 2 h, for all assays and requires minimal hands-on time. The system is composed of the GeneXpert instrument, with throughput designed to range from 1 to 96 samples simultaneously (Figure 24.6a and 24.6b, respectively) and a computer containing the required assay software to interpret the results. The various cartridges all incorporate *Bacillus globigii* spores, which serve as internal controls for extraction and amplification, making the test technically effortless with little manipulation by the operator. Currently, Cepheid offers assay cartridges for several important pathogens (references are listed for the first published method comparisons of the FDA-approved product found using search



Figure 24.5 Bio-Rad's QX100 Droplet Digital PCR system (ddPCR) is a novel way to accomplish quantitative PCR. (Courtesy of Bio-Rad Laboratories, Inc.) (See insert for color representation of the figure.)

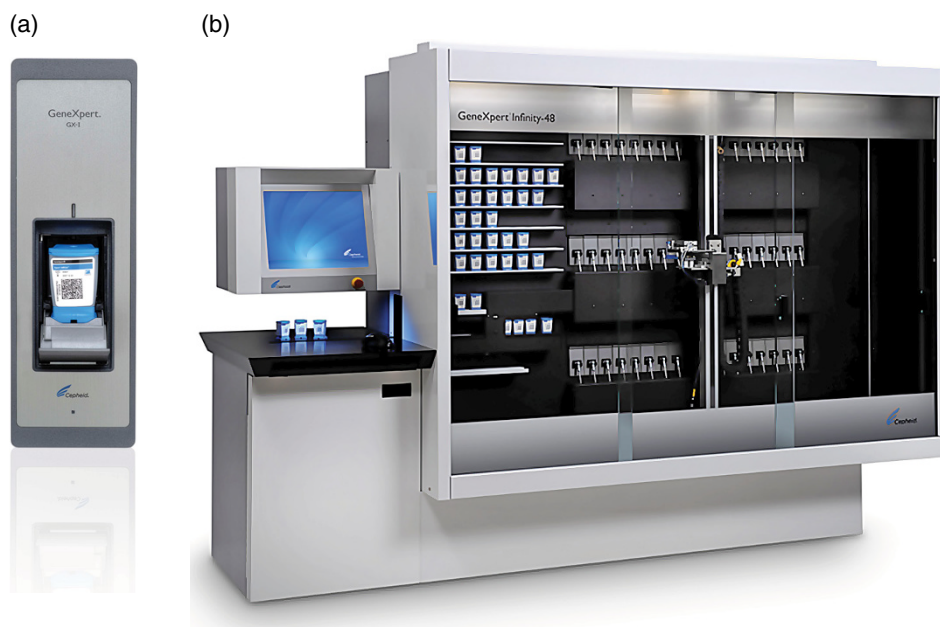


Figure 24.6 Cepheid GeneXpert systems range in throughput, from (a) one-cartridge to (b) a 96-cartridge instrument. (Courtesy of Cepheid) (See insert for color representation of the figure.)

terms Xpert or GeneXpert and the pathogen and/or the publications from multisite trials), including *S. aureus* [178,263], MRSA [202,262], *C. difficile* [102,172], VRE [21], Group B *Streptococcus* [74], enterovirus [120,167], *Chlamydia trachomatis*/*Neisseria gonorrhoeae* [76], and *M. tuberculosis* [18,90]; the latter assay has been associated with a recent eruption of publications, with more than 156 publications in 3.5 years.

The GeneXpert instrument is a random access instrument with rapid testing capabilities. Each individual reaction module can detect up to six genetic targets in a single cycle. Another advantage for pathogen detection is its multiplex capabilities, detecting the pathogen as well as some antibiotic resistance genes. Strain typing capabilities are also available, as in the case of presumptive identification of *C. difficile* NAP1/B1 strains [8,172]; yet there is no consensus for the utility of reporting the presumptive strain type. Methicillin resistance of *S. aureus* [202,215,236,261–263], vancomycin resistance of enterococci [21,46,75,144,272], and rifampin resistance of *M. tuberculosis* are discriminated by the system [84,95,104,152,158,159,273], but as with any laboratory method, vigilance is required because antibiotic resistance genes can mutate quickly and exceptions can occur [17,26,37,229].

24.3.4.2 Focus Simplexa assays

Because NA extraction is not required with the latest assays from Focus (Cypress, CA), the Simplexa Direct assays also bear mentioning in the integrated PCR section (Figure 24.4a,b). Due to the lack of an extraction step, the assay costs are relatively low and ease of use is

high; however, in some cases there may be some loss in sensitivity when compared to other molecular methods for assays that include extraction steps for influenza, RSV, and *C. difficile* [43,96,125,126,166,226,266]. Please see other chapters in this text for additional information.

24.3.4.3 The FilmArray

The FilmArray system (BioFire; Salt Lake City, UT) is a novel and completely integrated real-time multiplex PCR instrument (Figure 24.7a) that incorporates multiple single-plex PCR reactions in a disposable pouch (Figure 24.7b), [58,189]. Originally launched for the detection of respiratory viruses [132,186,189,196], with the capability to identify bacterial pathogens, the latest addition to their FDA-cleared menu includes a gastrointestinal (GI) panel for bacteria, viruses, and parasites, and the blood culture identification (BCID) panel for the detection of relevant bacteria and yeasts, as well as antimicrobial resistance bacterial genes. The BCID panel multiplexes 27 different genetic targets while the GI panel multiplexes 22. All NA's are extracted and purified directly from the unprocessed sample. Since the FilmArray is both an integrated and a multiplex PCR/RT-PCR assay, the platform will be further described in the multiplex section below.

24.3.5 Multiplex PCR/RT-PCR

24.3.5.1 FilmArray

The FilmArray performs a nested multiplex PCR assay. During the nested PCR the FilmArray performs a single, large volume, multiplexed reaction with primers specific

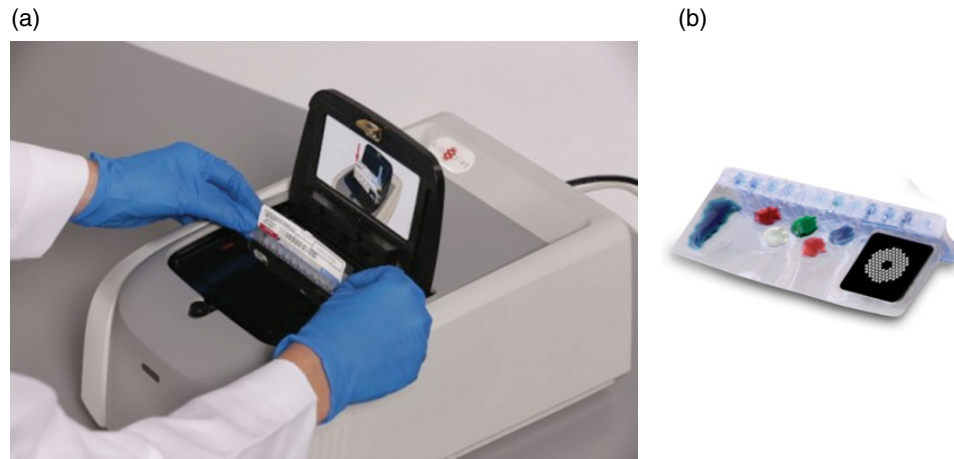


Figure 24.7 (a) BioFire Diagnostics' FilmArray instrument and (b) freeze-dried reagent pouches. (Courtesy of BioFire Diagnostics, Inc.) (See insert for color representation of the figure.)

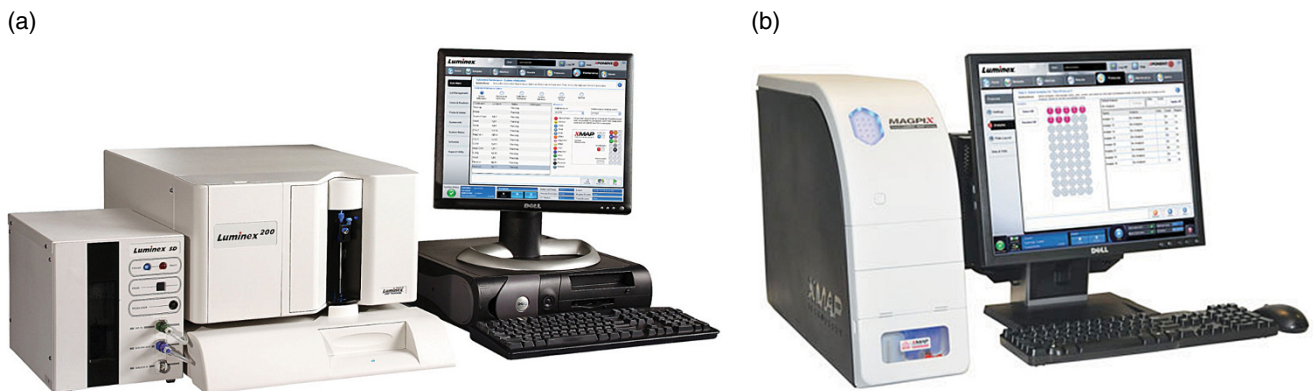


Figure 24.8 (a) The Luminex 100/200 system and (b) the Luminex MAGPIX instrument are used for detection of targets downstream to multiplex PCR. (Images provided by Luminex Corporation.) (See insert for color representation of the figure.)

to several targets. Following the multiplex PCR reaction, an individual single-plex second-stage PCR reaction further amplifies the products that amplified during the first round of multiplex PCR. The FilmArray software analyzes and interprets the end-point melting curve of the amplicons. New panels in development include a meningitis/encephalitis panel and a lower respiratory tract pathogen panel.

24.3.5.2 Bead-based assays

Although Luminex's bead-based technology (Luminex Corp, Austin, TX) has been in use in clinical virology since the mid-2000s, release of the Luminex Gastrointestinal Pathogen panel (GPP) in 2012 combined viral, parasitic, and bacterial targets and was the first appearance of an FDA-cleared bead-based assay to include bacterial targets. Luminex xTAG technology assays are performed on the Luminex 100/200 instruments (Figure 24.8a) or the MAGPIX (Figure 24.8b), the latest version of the technology. The Luminex assays are composed of a multiplexed PCR reaction that amplifies the regions

of interest in the target pathogen genes followed by treatment with exonucleases to remove excess nucleotides and primers. Subsequently, a primer extension step that is specific for the pathogen target that is being analyzed is included in the PCR reaction. The 5' end of the primers is attached to a universal tag sequence. The 5' universal tag sequence is hybridized to the complementary anti-tag sequence coupled to a particular xMAP bead set. After performing a wash step, detection is initiated. The hybridized beads are read by aspirating the assay samples, one after another, into the reader. The hybridized beads are carried by a microfluidic system in a stream of fluid through the measurement cuvette, where they are individually irradiated by dual reporter lasers. A red laser identifies each bead (or pathogen) by its color-coding, while a green laser detects the hybridization signal associated with each bead (indicating the presence or absence of a particular pathogen). Instrumentation reads the color-coded beads that attach to specific NA sequences, and results are analyzed by specific data analysis software [52,53].

Luminex xMAP technology is supported by the principles of flow cytometry and the major advantage of the technology is centered on the bead color-coding, which enables up to 100 possible separate interactions that can be analyzed simultaneously [52,105]. The entire procedure can be completed in approximately 5 h, making it a quick method to identify multiple genetic targets. Other significant advantages over traditional methods include: the versatility of one system to analyze several target types, the accuracy, the speed, and the standardization observed because of the reproducibility of high-volume production of xMAP microspheres within a single lot. The new Luminex instrument, the MAGPIX, is a compact instrument being launched to support bacterial panel testing.

Luminex xTAG panels are available, as the xTAG Gastrointestinal Pathogen Panel (xTAG GPP), to detect *Salmonella*, *Shigella*, *Campylobacter*, enterotoxigenic *Escherichia coli* (ETEC), *E. coli* O157, Shiga-like toxin producing *E. coli* (STEC), and *C. difficile* toxin A/B, in addition to viruses and parasites associated with gastrointestinal disease. The current FDA-cleared GPP includes 11 targets in all. Additional targets, *Vibrio cholerae* and *Yersinia enterocolitica* are available but not in the United States at the time of this writing. The Respiratory Virus panel, which includes eight viruses and subtypes, is being updated to include bacterial targets. The multiplex aspect of the xMAP panels allows the identification of infections due to multiple pathogens, with rapid diagnostic results for diagnosis and patient management [13,150,164,180,255].

24.3.6 Second generation multiplexes

After Luminex and BioFire (formerly Idaho Technologies, now bioMérieux) entered the market with multiplex assays with more than ten targets, the second round of assays emerged, including GenMark Diagnostics (Carlsbad, CA) and Nanosphere (Northbrook, IL) instrument platforms.

24.3.6.1 GenMark eSensor

The eSensor technology (XT-8) (GenMark; Carlsbad, CA) promotes high assay specificity and sensitivity owing to competitive DNA hybridization and electrochemical detection of the post-PCR target sequence. The technology is not based on fluorescence or optical detection as are the first generation multiplex assays, thus the protocols do not require as many of the time-consuming washing and preparation steps required by earlier multiplexing technologies. The XT-8 systems (Figure 24.9a) can be used in tandem with up to three modules per computer for a throughput of 1–24 samples in random access mode.

The eSensor cartridge (Figure 24.9b) is preprogrammed with a specific test protocol, lot number, and expiration date on an internal memory chip. In the cartridge, the preamplified target DNA is mixed with the signal probe solution, and then analyzed via electrochemical detection.

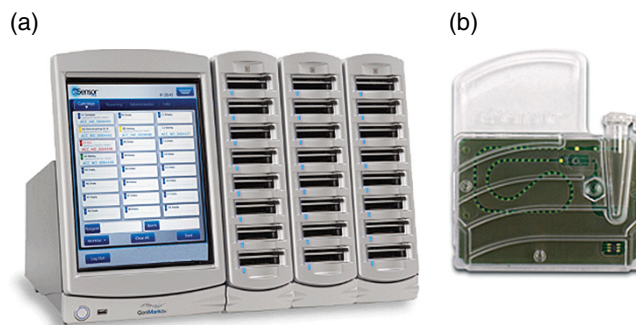


Figure 24.9 (a) The GenMark XT-8 system for detection of multiplex PCR products and (b) the test cartridge. (Images provided courtesy of GenMark Diagnostics.) (See insert for color representation of the figure.)

The first microbiological offering for eSensor technology was the Respiratory Viral Panel (RVP), an assay for respiratory virus detection and subtyping, which is a very sensitive RVP test. Based on independent studies these assays achieve up to 99.2% agreement with real-time PCR [187,188,204] and exhibit enhanced detection of coinfections, which can increase length of stay and may support changes in cohorting of the sickest patients [138]. The GenMark pipeline includes the HCV Genotyping Test (HCV 1a, 1b, 2a/c, 2b, 3, 4, 5, 6a/b) and the HCVg *Direct* test, with detection from serum or plasma [173,208]. New adaptation of the system toward one requiring less than the 60 min of hands-on time will make the GenMark system more easily adaptable to routine laboratory settings and is expected to be a fully integrated cartridge system with sample-to-answer workflows.

24.3.6.2 Nanosphere Verigene

Nanosphere's Verigene (Northbrook, IL) system uses a gold nanoparticle probe technology, typically 13–20 nm in diameter that carries a defined number of target-specific oligonucleotides or antibodies utilized in two methodologies, array and amplification, depending on the specific assay. The nanoparticles support increased sensitivity compared to fluorophores. Light scattered from one nanoparticle is reported to be equivalent to the light emitted from one half million fluorophores.

Automated NA purification and amplification is performed on the Verigene Processor SP (Figure 24.10a). Inside the Verigene Test Cartridges NA targets are hybridized to capture oligonucleotides on a microarray containing specific oligonucleotides and gold nanoparticle probes. Finally, signal amplification of probes occurs via a silver staining process with automated qualitative analysis performed on the Verigene Reader (Figure 24.10b). The first tier of assays available for the Verigene platform includes respiratory viruses, which are offered in amplification assays [3,107], and products for identification of select

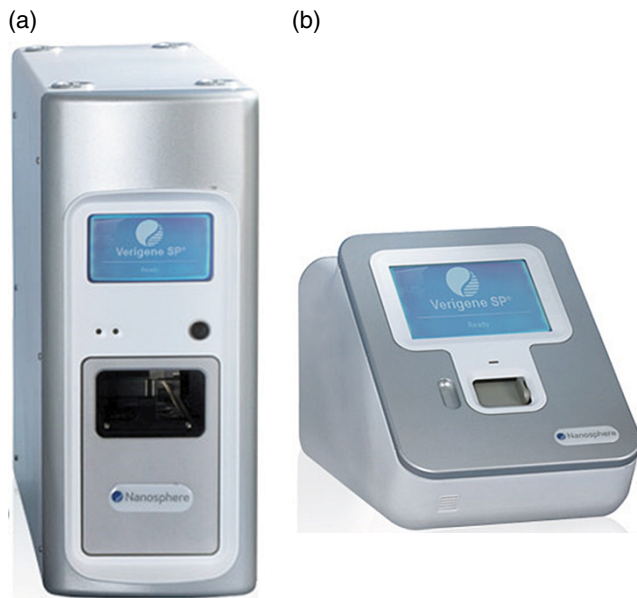


Figure 24.10 Nanosphere's Verigene SP system performs array-based detection of nonamplified or pre-amplified genetic targets by using (a) the Verigene SP processor and (b) the Verigene SP reader. (Courtesy of Nanosphere, Inc.)

Gram-positive and Gram-negative pathogens from positive blood cultures, which are probe-based without target amplification [2,213].

24.3.6.3 Curetis Unyvero

Another novel RUO application, the Unyvero system (Curetis AG, Germany) targets lower respiratory tract (LRT) bacterial pathogens using a highly multiplexed panel including multiple bacterial species and antibiotic resistance genes [106].

24.4 Other amplification methods

24.4.1 Transcription mediated amplification

Transcription mediated amplification (TMA) is the synthesis of a DNA strand complementary to a target NA, usually RNA [123]. The newly synthesized cDNA becomes the template for the subsequent *in vitro* transcription reactions. An excess of RNA is created and is used as the substrate for another round of transcription. Much like PCR, this process continues at an exponential rate and several million copies of the target can be created. The amplified target is then detected using various methods. Hologic/Gen-Probe (San Diego, CA) launched the PANTHER (Figure 24.11), the smaller version of the TIGRIS, a fully automated random-access TMA instrument for high-volume testing. Like the TIGRIS, which combines detection and decontamination into one instrument, the PANTHER also utilizes Gen-Probe's target capture technology for sample processing, TMA amplification, and hybridization protection assay (HPA)



Figure 24.11 Hologic's PANTHER instrument tests ThinPrep liquid cytology specimens via Aptima HPV mRNA-based assay is a nucleic acid amplified test that detects 14 high-risk strains of human papillomavirus (HPV) associated with cervical cancer and precancerous lesions. (Courtesy of Hologic, Inc.)

[78]. The PANTHER provides the ability to test for four targets from one specimen using Gen-Probe's APTIMA assays for *Neisseria gonorrhoeae/Chlamydia trachomatis* (GC/CT), human papilloma virus (HPV) [131], and *Trichomonas vaginalis*, providing a multi-targeted STD testing option. Currently the assays are limited to end-point assays; however, the PANTHER has assays currently in development with real-time TMA capabilities to compete with quantitative PCR applications, specifically viral loads [245].

24.4.2 Loop-mediated isothermal amplification of DNA

Loop-mediated isothermal amplification of DNA (LAMP) is a novel method that is quickly impacting molecular detection of pathogens [170]. This method rapidly amplifies DNA under isothermal conditions with high specificity and efficiency. During the LAMP process, four to six different primers, specifically designed to recognize six or eight distinct regions on the target sequence, participate in amplification and detection of the target sequence, which can be completed in a single step [162,247].

The *illumigene* LAMP (Figure 24.12) amplification assays (Meridian Bioscience; Cincinnati, OH) use specific primers that continuously facilitate isothermal amplification producing the byproduct, magnesium pyrophosphate, in the reaction. The accumulation of magnesium pyrophosphate



Figure 24.12 Loop-mediated isothermal amplification of DNA (LAMP assay) can be performed on Meridian's Illumigene instrument. (Courtesy of Meridian Bioscience, Inc.) (See insert for color representation of the figure.)

results in a precipitated solution. The resulting turbidity from the precipitate can be detected by the instrument, which functions as both an incubator and reader [156]. When the pathogen, specifically the gene target of interest, is not present, precipitate does not form and the amplification reaction is negative. When the gene target is present, the resulting amplification produces a turbid solution and a positive result. The *illumigene C. difficile* assay detects the pathogenicity locus (PaLoc) of toxigenic *C. difficile* by targeting a region of *tcdA*, with results available in about 1 h [113,114,124,168,169]. The *illumigene* test menu has expanded to include Group A streptococci, *B. pertussis* and *Mycoplasma pneumoniae* [5,124,197].

The New Alere i instrument (Alere; Waltham, MA) uses a semimanual process and visual touchscreen-driven process for isothermal amplification of influenza A and B genetic targets. The Alere i produces results in 15 min from samples such as nasal swabs and swabs in viral transport media. The instrument is designed with a small footprint for use in small throughput point-of-care settings. Isothermal amplification can be performed without the use of thermal cyclers in a wide variety of laboratory conditions; therefore, the isothermal nucleic acid amplification technology (INAAT) market is growing. Please consult current literature and company web sites for further information.

24.5 Probe technology

24.5.1 Peptide nucleic acid–fluorescent *in situ* hybridization

Peptide NA (PNA) probes are fluorescent probes attached to PNA molecules that have a noncharged polyamide (peptide) backbone as opposed to the negatively charged sugar-phosphate backbone of DNA and RNA. PNA probes have the same nucleotides (adenine-A, guanine-G, cytosine-C, and thymine-T) and follow the same base-pairing rules allowing them to bind normally to complementary sequences of NA. There

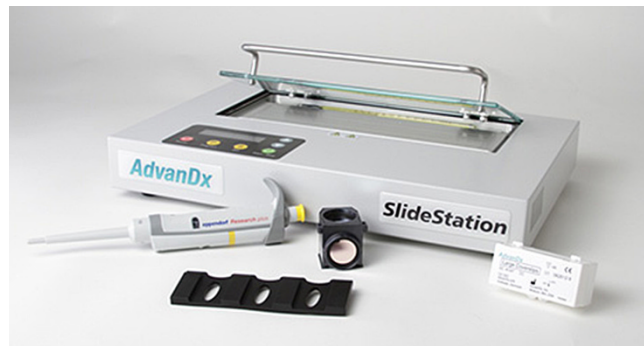


Figure 24.13 AdvanDx QuickFISH System offers results in 20 min of positive blood culture with 5 min of hands-on time. (Courtesy of AdvanDX)

are several advantages to the structure of PNA probes over NA probes, namely: (i) PNA probes bind more strongly during the hybridization reaction than a DNA probe – PNA chemistry hybridizes more readily to the target of interest due to very small dye-labeled probes, approximately 12–20 nucleotides in size; (ii) The neutral charge of the PNA backbone reduces natural repulsion with negatively-charged backbones (i.e., DNA), therefore they bind tightly; (iii) greater specificity as a result of more precise binding to the target and exquisite base-discrimination; and (iv) PNA probes provide greater sensitivity – they add very low background with better visualization of the fluorescent signal.

AdvanDx, Inc. (Woburn, MA) commercially markets FDA-cleared PNA FISH (fluorescent *in situ* hybridization) kits for clinical diagnostic use and identification of *S. aureus*, *Enterococcus* spp., *Mycobacterium* spp., *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, among other targets, including *Candida* spp. [32,69–71,93,154,155,239]. More cost-effective than PCR techniques, PNA FISH does not require extensive knowledge or experience in molecular techniques, since the workflow is much like routine immunofluorescence staining procedures.

The chemistry of PNA FISH probes make this technology just as sensitive but more specific than Gram staining and direct fluorescence staining [239]. Because the PNA FISH kits are relatively easy to use, they are now widely accepted as a method for rapid identification of bloodstream pathogens. The rapid identification of bloodstream pathogens via PNA FISH may be the most widely studied of all rapid methods in terms of outcomes [27,69–71]. Originally the PNA FISH turnaround time was about 1.5 h; however, the next generation of PNA FISH, called QuickFISH (Figure 24.13), eliminates the wash step, thus reducing the assay time from 90 to only 20 min after the blood culture flags positive [30,44,45,145]. Limitations of PNA FISH are the inability to test multiple pathogens and the absence of complete antibiotic susceptibility information [68].

24.6 Mass spectrometry

24.6.1 Matrix assisted laser desorption ionization time-of-flight spectrometry

Matrix assisted laser desorption ionization time-of-flight (MALDI-ToF) methods leverage the high sensitivity of mass spectrometry (MS) with linkage to large microbial databases, to provide users with species-specific spectra that can be used to reproducibly identify microorganisms. Analysis of whole cells was first proposed in 1975 to study the biomarker profile of various bacterial species following pyrolysis-MS for low molecular weight products [6]. However, it was not until 1996 that the first MALDI-ToF/MS experiment was successful for identifying bacteria directly from whole colonies based on protein biomarkers [40,97]. A great number of developments have been made over the last decade on whole-organism MALDI-ToF/MS. The protein biomarkers that are measured in MS of microorganisms are highly expressed proteins responsible for housekeeping functions, such as ribosomal components, chaperones, and transcription/translation factors [38].

Relying on identification of protein profiles (generally measuring mass to charge ratios (m/z) between 2 and 20 kDa), MALDI-ToF/MS derives its information from profiles of highly conserved bacterial proteins. For MALDI-ToF, the protein profiles are generated from direct ionization of an intact colony or a bacterial protein extract, after manual extraction. Identification occurs after

correlating an isolate's spectral signature to a database of spectra collected from reference strains (Figure 24.14).

In its current form, MALDI-ToF/MS requires cultivated microbes and a small quantity of matrix (typically less than 1 $\mu\text{g}/\text{sample}$). The matrix and target spotting plates/slides are the primary consumables and are relatively inexpensive in disposable or reusable formats. Ongoing costs include the equipment maintenance agreements. MALDI-ToF/MS is widely used, for its high accuracy, low consumable cost, and speed of analysis. A typical process consists of growth of bacteria or yeast, colony selection and placement on a target, addition of matrix, and analysis with MALDI-ToF/MS. Mass spectrometry identification has diverse possibilities, as it is a fast and sensitive method to measure proteins, lipids, and other targets that does not require prior knowledge about the organism. Several reviews of MALDI-ToF/MS technology are available [15,38,49,101,128,190,214].

24.6.1.1 Bruker MALDI Biotyper

Commercial MS systems exist that can integrate with traditional antimicrobial susceptibility systems. Among them, the MALDI Biotyper from Bruker Daltonics (Figure 24.15a) is among the most widely tested and has proven accuracy in the identification of bacteria [135]. The system uses a 48-spot MALDI target plate (Figure 24.15b) and is partnered with the Becton Dickinson Phoenix

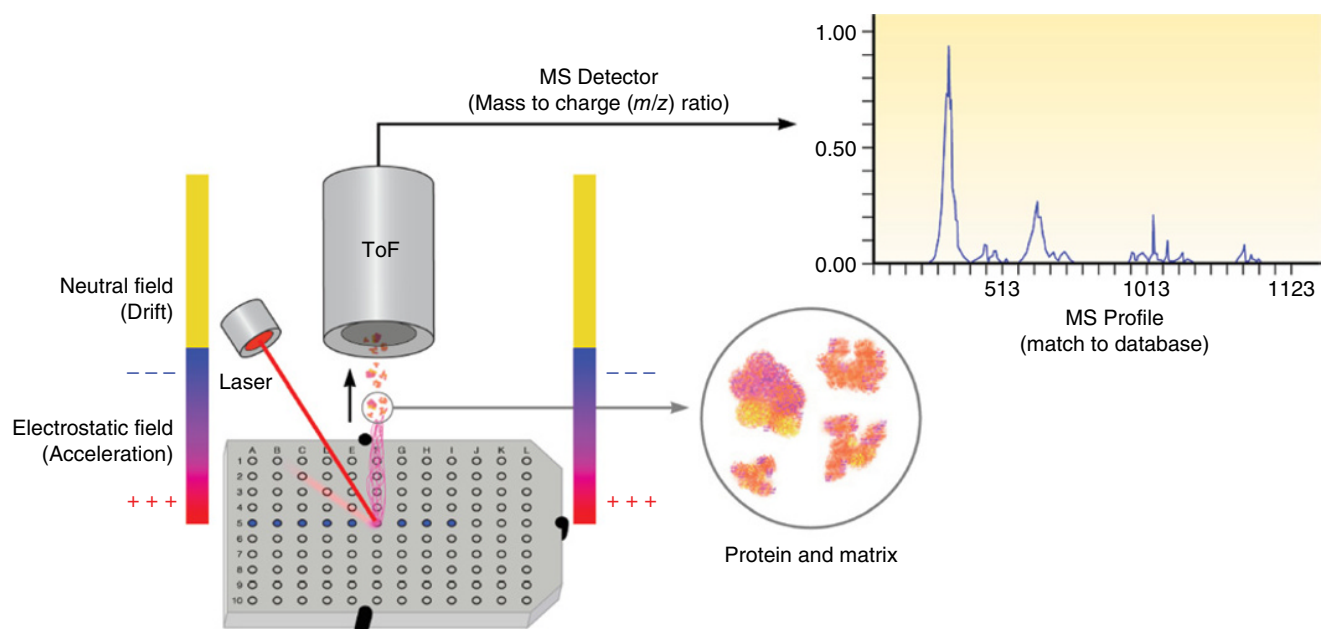


Figure 24.14 Schematic for MS analysis of ionized microbiological isolates and clinical material. Once appropriately processed, samples are added to the MALDI plate, overlaid with matrix and dried, then the sample is bombarded by the laser. This bombardment results in the sublimation and ionization of both the sample and matrix. These generated ions are separated based on their mass-to-charge ratio via a ToF tube, and a spectral representation of these ions is generated and analyzed by the MS software, generating an MS profile. (Copyright © American Society for Microbiology [Clinical Microbiology Reviews, Volume 26, Number 3, 2013, pp. 547–603] doi: 10.1128/CMR.00072-12.) (See insert for color representation of the figure.)



Figure 24.15 (a) The Bruker MALDI Biotyper CA System includes a bench-top microflex MALDI-ToF mass spectrometer, software, reagents, a library of microorganism reference spectra, and (b) a 48-spot MALDI target plate. (Courtesy of Bruker Daltonics.)

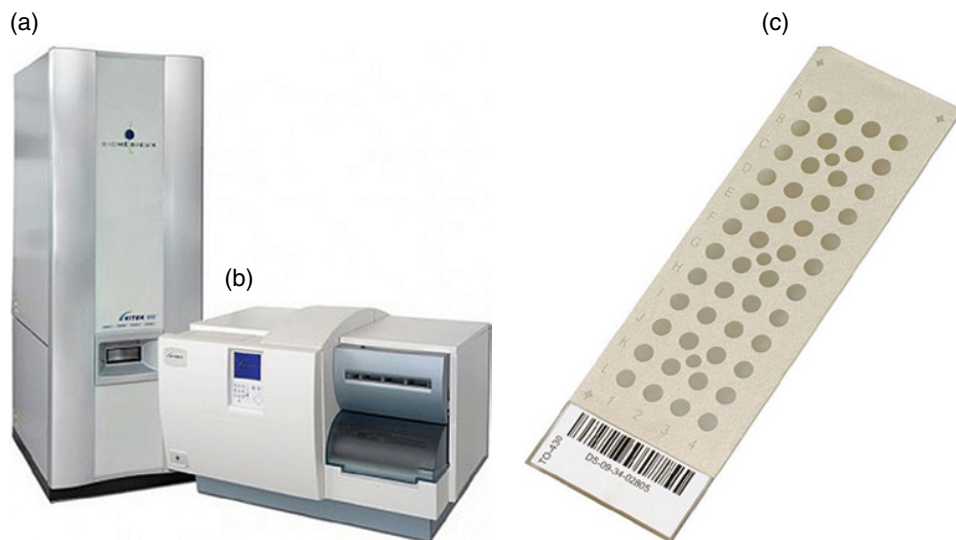


Figure 24.16 (a) The bioMérieux Vitek MS MALDI-ToF technology, depicted here with (b) the Vitek 2 system for susceptibility testing, couples with reagents, a comprehensive database of clinically relevant species, and (c) a disposable array. (Courtesy of bioMérieux.)

System or the Siemens MicroScan, which supply antimicrobial susceptibility results that pair with bacterial identification. Evaluations of the system show high accuracy for rare or fastidious bacteria, yeasts, and fungi, which represent a challenge to clinical laboratories using phenotypic identification evaluation due to their limited biochemical reactivity [1,141,142,207].

A few reports show promise for the identification of microorganisms using MALDI-ToF/MS without subculture [66,160,194,217,240], but the methods were limited by the need for a large number of cells. These adaptations showed successful identification for only approximately 80% of blood cultures. A promising new technology, the

MALDI Sepsityper system (Bruker Daltonics), aims to identify bacteria and yeast directly from positive blood-culture bottles [118,145,217,271].

24.6.1.2 bioMérieux Vitek MS

Another MALDI-ToF system, the bioMérieux Vitek MS (Figure 24.16a), exhibits similar capabilities; no statistically significant difference was observed between the two platforms for organisms clinically encountered [35]. A multicenter clinical trial group published several reports to document the high degree of accuracy supported by Vitek MS version 2.0 [23,73,147,256]. The accuracy of Vitek MS was high when compared with the reference method

(DNA sequencing) and provided an accurate, species-level identification for 96% of 226 isolates; an additional 1% were accurately identified to the genus level [23,137,205]. Similarly, of 651 isolates analyzed, 91.2% exhibited the correct anaerobic species identification based on 16S rRNA gene sequencing: at the genus level, the agreement rate increased to 92.5% [73]. For yeast, compared to sequence analysis of the D2 region of the 26S rRNA gene, 823 isolates (96.6%) were correctly identified to the genus level and 819 isolates (96.1%) were correctly identified to the species level [256]. The Vitek MS uses middleware name “Myla” to connect to the Vitek 2 (Figure 24.16b) and uses a 48-well disposable array (Figure 24.16c).

24.6.2 Limitations of MS-based technologies

While fundamentally equivalent in performance, the Vitek MS differs from the Bruker Biotyper in its instrumentation scoring algorithm and its databases. Both instruments are supported with an FDA-approved database and both are partnered with automated systems for antimicrobial susceptibility testing, but both have limitations in that the databases need to be expanded to include rare human pathogens. Neither system currently performs antimicrobial susceptibility testing (AST); neither have high accuracy from direct specimens, with the exception of the Bruker Sepsityper. With Sepsityper, positive blood cultures are purified and subjected to rapid identification using MALDI-ToF/MS [134].

The need for growth of organisms with isolated colonies is still required in order to obtain an accurate result because the technique's ability to resolve polymicrobial mixtures is lacking. Additionally, a high number of bacterial cells are required for identification, such that a whole intact colony is typically used for analysis, limiting the ability to rapidly identify microorganisms directly from biological fluids where the bacterial count is expected to be relatively low. Research is currently being performed to mitigate some of these requirements. Until accurate determinations of resistance factors can be made, parallel culture-based recovery of positive cultures will most certainly be required for the foreseeable future for antimicrobial susceptibility testing [217].

The major barrier to implementing MS-based technologies to the clinical laboratory is the cost associated with the capital purchase of a dedicated mass spectrometer [259]. Thus, as is true for most newly emerging technologies, these methods may be limited to larger reference laboratories until the cost benefit is fully evaluated or until smaller more affordable bench-top options are developed. Nevertheless, the potential for high analytical sensitivity, accuracy, and broad-based applicability of MS-based platforms have the potential to change the practice of clinical microbiology in the future and offers the promise of

off-setting some portion of the complexity and workload in clinical microbiology.

24.7 DNA sequencing

24.7.1 Capillary sequencing

Sequence-based identification, strain typing, and comprehensive isolate fingerprinting can be utilized for the tracking and control of pathogenic organisms; therefore molecular methods like sequencing have been a widely used tool for epidemiological fingerprinting of isolates important to public health. In 1995, the first two complete DNA sequences of the bacterial genomes *Haemophilus influenzae* and *Mycoplasma genitalium* were revealed by scientists at The Institute for Genomic Research (TIGR), followed in 1996 by the first complete genome sequencing of an Archaea, *Methanococcus jannaschii*. Since then automated sequencing using fluorescent chain-terminating nucleotides during elongation of a DNA template (referred to as Sanger sequencing) has made it possible to rapidly sequence the genomes of many pathogens and environmental microorganisms both in research and diagnostic settings. A major advantage of sequencing is it allows for the comparison of genomes identifying differences between virulent and avirulent medically important pathogens. The comparison data generated at the genomic level of a pathogenic species can provide information about host or tissue specificity and antimicrobial resistance patterns. The clinical applications of sequencing extend far and wide, including but not limited to, identification of cultured and nonculturable isolates, pathogenicity and drug resistance, strain typing in outbreak situations, and emergence of new drug-resistant strains.

24.7.2 Short read pyrosequencing

Pyrosequencing is a DNA sequencing method that detects released pyrophosphate as DNA polymerase acts on a target sequence. Pyrosequencing assays have been developed for several applications including genotyping, single nucleotide polymorphism (SNP) detection, and microorganism identification. Pyrosequencing is real-time sequencing and thus can be used for not only microbial species identification but for the discrimination of point mutations that confer antimicrobial resistance. There are several major advantages to pyrosequencing (PPI): it is high throughput, sensitive, rapid, simple, customizable, and highly specific [109,110,195,274].

The principle of the pyrosequencing reaction relies upon annealing of a target primer to a single stranded template. After binding, DNA polymerase acts to elongate the sequence, much like PCR. As the polymerase adds deoxynucleotides, in a predefined pattern, the primers extend and release inorganic pyrophosphate as part of



Figure 24.17 QIAGEN's PyroMark Q24 pyrosequencer.
(© Qiagen, all rights reserved.)

the synthesis. Inorganic pyrophosphate is converted into ATP by ATP-sulfurylase. The accumulating ATP is utilized by firefly luciferase to produce light. The pattern of light signals, correlating to the added nucleotide, is a true representation of the sequence that is being analyzed. The amount of light generated by the reaction is directly proportional to the PPi produced or the number of nucleotides that were incorporated into the newly generated sequence. Any nucleotides that are not incorporated into the new sequence are enzymatically degraded. The sequence generated by pyrosequencing is quantitative in nature. Select pyrosequencing methods utilize a solid-phase template preparation of streptavidin beads that interact with biotinylated 5' ends of single-stranded DNA as opposed to the enzyme-based template preparation.

The reaction is rapid, less than 2 h to result, and DNA extracted from multiple microbial species in a single specimen can be sequenced in the same assay batch. Still, disadvantages to the technology consist of the method being relatively hands-on, labor intensive, and open to potential contamination events. In addition, the sequencing reaction is only able to produce sequence for short targets, in some cases < 100 nucleotides. Qiagen PyroMark (Qiagen Inc.; Valencia, CA), a commercial system (Figure 24.17), is available in three system formats, Q24, Q96, and Q96MD, while Roche markets the GS Junior and GS Flex pyrosequencers.

24.8 Emerging technology

24.8.1 Automated microscopy: ID/AST

A novel combination of automated microscopy paired with electrophoresis of microbes, and FISH genetic probes, the Accelerate instrument (Figure 24.18a; Accelerate Diagnostics, Tucson, AZ) utilizes both genomic and phenotypic detection technologies to significantly decrease

time to result while sustaining high sensitivity and specificity. This process will occur on a circular cartridge (Figure 24.18b). Although not yet FDA cleared as of this writing, a recent report details use of the ID/AST prototype to correctly classify *Klebsiella pneumoniae* carbapenemase (KPC) positive or -negative strains [29]. In another report, susceptibility of *S. aureus* was characterized by assessing immobilized live bacterial cells and differentiating them as susceptible or nonsusceptible using as few as 10 growing clones after 4 h of growth, compared to overnight growth required for traditional culture-based methods [193]. In this report, 77/77 MRSA phenotypes and 54/54 MSSA phenotypes were correctly identified. In addition, 53/56 clindamycin-resistant and 75/75 clindamycin-susceptible strains were detected and heterogeneous and inducible resistance was characterized [193]. The instrument can also perform microbial identification; the prototype was successfully used to identify *Pseudomonas* and *Staphylococcus* directly from bronchoalveolar lavages (BAL) [151].

24.8.2 Next generation sequencing and high throughput sequencing

Traditionally, sequencing methods were based on slab gel electrophoresis that separated DNA fragments produced from *in vitro* Sanger sequencing reactions. These methods involved laborious molecular cloning and PCR techniques. Several instruments have recently been introduced that utilize molecular cloning techniques (minus any bacterial vectors), PCR amplification, and capillary electrophoresis to produce sequencing data. Next generation sequencing (NGS) takes amplified templates isolated by molecular cloning techniques within the instrumentation and discriminates the incorporation of nucleotides (adenosine, thymine, cytosine, or guanine) on two-dimensional arrays. Digital imaging monitors the nucleotides as they are incorporated on a cycle-by-cycle basis. NGS is high-throughput and the DNA sequencers can synthesize >100 megabases of DNA sequences per assay, drastically shortening the time required to sequence an entire bacterial genome. Though the principles are similar, the currently available systems use sequence synthesis chemistries that are distinct to each system.

24.8.2.1 Roche sequence analysis

The Roche GS Junior bench-top system incorporates the power of Roche 454 sequencing systems. Applications include, but are not limited to, pathogen detection, direct amplicon sequencing, *de novo* microbial genome sequencing, and metagenomic characterization of samples. In these systems, beads are loaded onto a nanoscale well device with a single capture bead per well and all the required enzyme reagents. A camera detects chemiluminescent light signals as complementary nucleotides, which flow sequentially across the wells and are incorporated into the template strand attached to the beads.

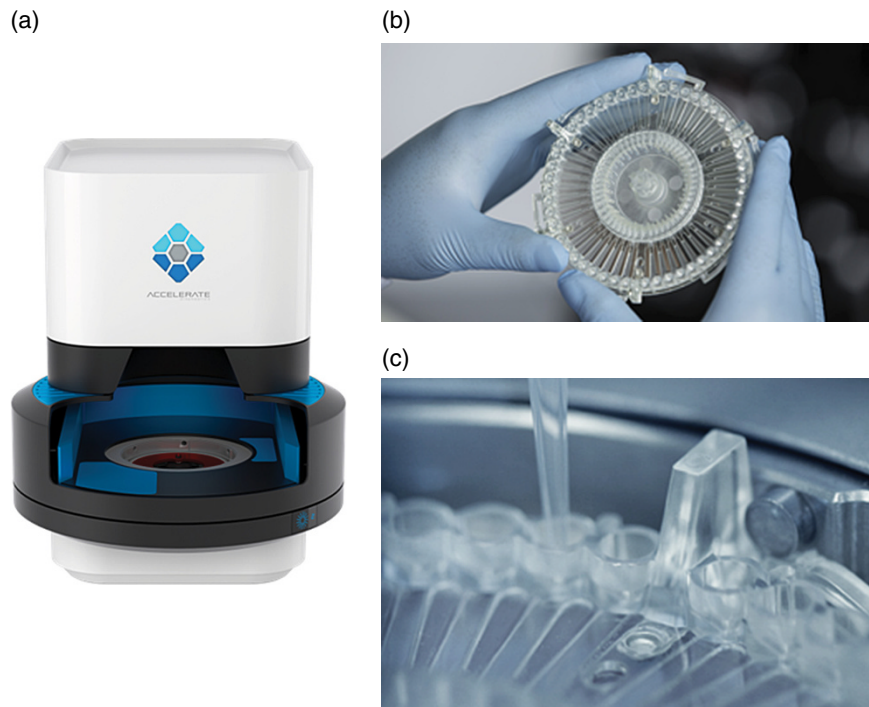


Figure 24.18 (a) The Accelerate ID/AST System uses (b) a 48-channel round cassette as well as (c) a pre-filled reagent cartridge for rapid, multiplex microbial identification and antimicrobial susceptibility testing. (Courtesy of Accelerate Diagnostics.)

24.8.2.2 Illumina MiSeq

The Illumina MiSeq (Illumina Inc., San Diego, CA) system uses fluorophore-labeled nucleotides with terminating 3' ends that are added to the end of a primer one at a time. The addition of the fluorophore-labeled nucleotides results in laser excitation of the fluorophore and detection by the imaging system; subsequently the 3' end terminators and fluorophore are cleaved, allowing for the addition of the next nucleotide. The nucleotides equally compete for incorporation, resulting in superior strain specificity. MiSeq permits approximately 150 bp (base pair) paired sequence reads and results can be achieved in less than 8 h. The Illumina HiSeq2500 expands on the capabilities of the MiSeq. Any genome can be sequenced using this technology with the ability to uncover genetic variants and antimicrobial resistance mechanisms.

24.8.2.3 ABI SOLiD

The ABI SOLiD (Applied Biosystems, Foster City, CA) utilizes a similar reaction chemistry of labeled nucleotides with blocked 3' termini that are enzymatically attached to the end of a primer; however, the central dinucleotide is flanked with universal and degenerated nucleotides. This provides added specificity to the sequencing chemistry. The nucleotides are fluorophores, labeled and added in blocks of eight nucleotides or octamers that specify the sequence for two nucleotides. Once added and the fluorophore is detected, the entire octamer is removed. The subsequent ligation reactions use smaller

and smaller primers until ultimately a 35 bp sequence is read. The ABI SOLiD sequencers can sequence multiple whole bacterial genomes in less than 2 weeks as opposed to months.

24.8.2.4 Ion Torrent

The Ion Torrent Personal Genome Machine (Life Technologies, Grand Island, NY) is based on semiconductor technology as opposed to light chemistry to acquire genomic sequences (Figure 24.19a). Chemical signals are translated into digital signals, which are then detected by the proprietary ion sensor (Figure 24.19b). The chemical signals are generated as nucleotides become added to the NA strand (Figure 24.19c). When a complementary nucleotide is incorporated, the release of hydrogen ions results in a pH change that is ultimately read as a voltage change. The chip is flooded sequentially with nucleotides and if the nucleotide is not a match, no change in voltage occurs. If two or more nucleotides are complementary to the strand the voltage change is multiplied proportionally to the number of correct nucleotides. Recording sequence synthesis by the ion sensor is analogous to a small pH meter and is extremely rapid, since lasers, scanning, and cameras are not necessary for detection, with sequence acquisition in about 2 h.

The MinION (USB single-use device) and GridION (Oxford Nanopore Technologies, Oxford, UK) use a novel technology called “nanopore sensing,” comprising biosensors that measure the disruption in current as an analyte

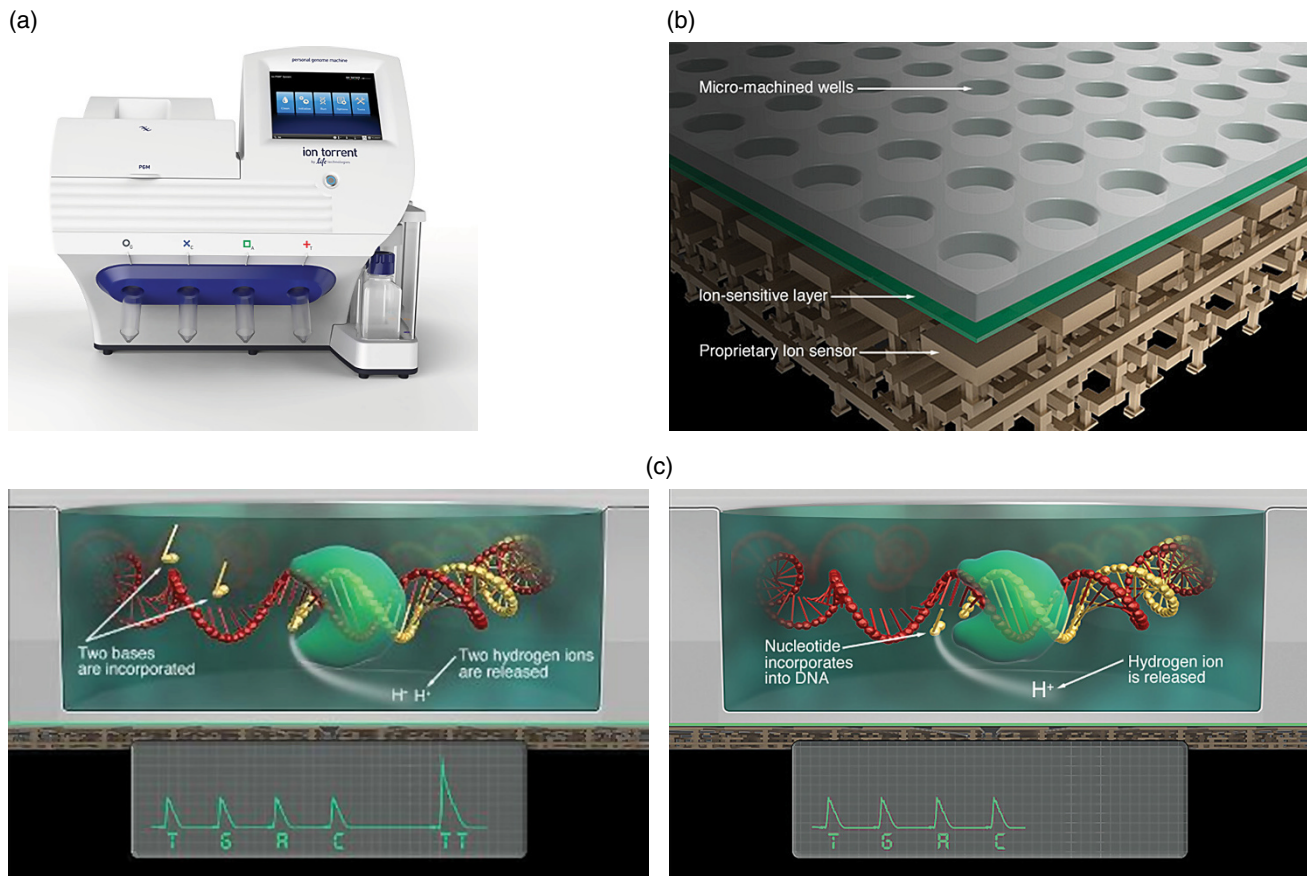


Figure 24.19 (a) The Ion Torrent Personal Genome Machine (Ion PGM™ System) Sequencer from Life Technologies utilizes (b) an ion sensor to identify the type and number of nucleotides in a sequence, as depicted in (c). (Photo courtesy of ThermoFisher Scientific, copying is prohibited.) (See insert for color representation of the figure.)

passes through a nanopore or near its aperture while determining the identity of the base, in the case of a DNA strand. The MinION (Figure 24.20) plugs directly into a laptop or desktop computer through a USB port, and is a self-contained device to deliver real-time data. The system is designed to be compatible with blood or serum and can not only distinguish between the four DNA bases, but also modified bases, proteins, and small molecules [219]. The MiniION and GridION, with 512 and 2000 individual nanopores, respectively, report read lengths of 5–100 kb (kilobases) at a rate of 150 Mb per hour for up to 6 h [136].

24.8.2.5 Optical mapping

Optical mapping (OpGen, Inc., Gaithersburg, MD) offers whole-genome mapping based on restriction fragment patterns, or map code, of whole-genomes (bacteria, yeast, fungi) without prior knowledge of DNA sequence information. High-molecular-weight DNA is extracted and applied to an optical chip via microfluidics and capillary action. Once the DNA is bound to the chip, a restriction endonuclease is utilized to fragment the DNA. A fluorescent dye labels the DNA fragments, which are then visualized via a fluorescence microscope. The size



Figure 24.20 The MinION (Oxford Nanopore Technologies) plugs directly into a laptop or desktop computer through a USB port, and is a self-contained device to deliver real-time data. (Courtesy of Oxford Nanopore Technologies.)

and order of the fragments are analyzed via image analysis software that measures the fluorescence intensity of each fragment to determine its size in kilobases. The optical data is then converted into digital data to create the optical map of the whole-genome (Figure 24.21).

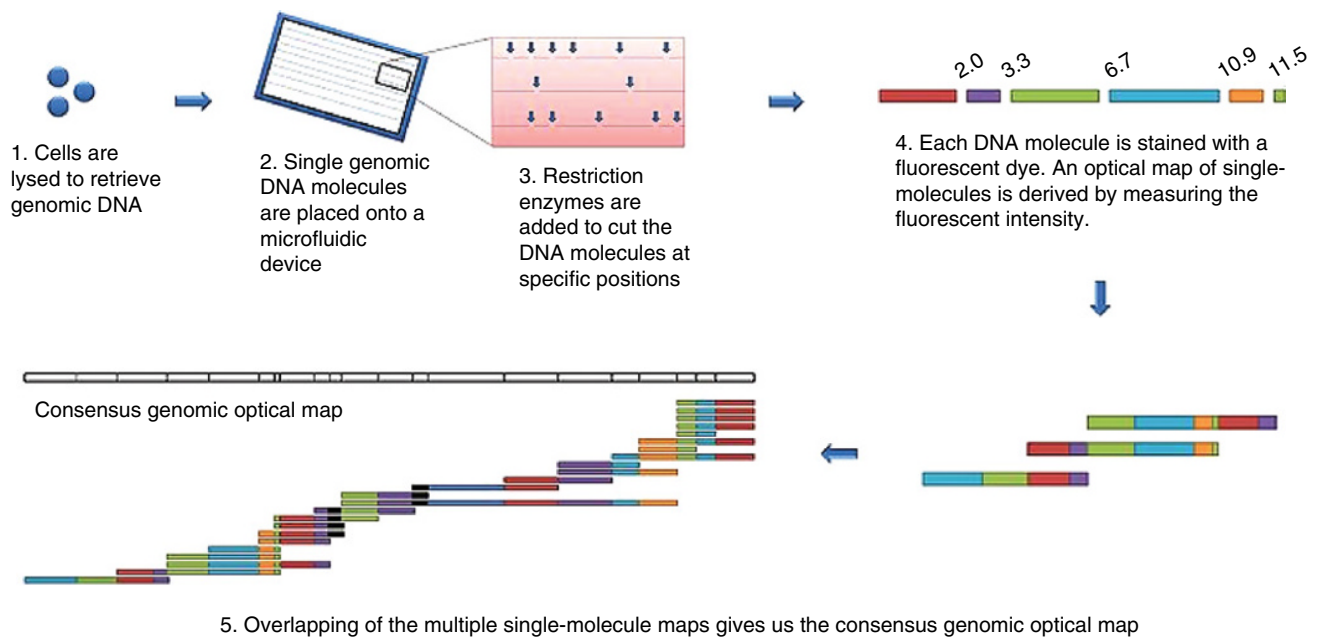


Figure 24.21 Overview of procedural steps for optical mapping. (Source: Chan and Wang, Used with permission, <https://upload.wikimedia.org/wikipedia/commons/e/e1/opticalmapping.jpg>; used under CC-BY 3.0, <https://creativecommons.org/licenses/by/3.0/deed.en>) (See insert for color representation of the figure.)

The applications of whole-genome mapping include sequence assembly, comparative genomics, and strain typing. Depending on the resources available, *de novo* sequence assembly can be time-consuming and costly [163]. Optical mapping provides an *in silico* template for DNA sequence assembly, which can enhance the assembly process by directing the orientation and alignment of contigs (aka continuous sequences of DNA that are assembled from overlapping cloned DNA fragments) and aid in the elimination of sequence gaps [175]; thus creating a high-resolution map more efficiently [199].

Together, optical mapping and whole-genome sequencing are powerful tools that allow variation of genomes to be assessed, also known as comparative genomics. Unique genetic elements (polymorphisms, gene rearrangements, insertions and deletions), as small as 2 kb, can be detected using optical mapping [121]. Additionally, because optical mapping is the compilation of several hundred restriction fragments, it provides higher resolution data than traditional strain-typing methods such as PFGE (pulse field gel electrophoresis), ribotyping and *spa* typing for MRSA. Thus, closely related species can be more easily differentiated due to the large number of genetic markers available for analysis. Optical mapping has been utilized to identify isolates associated with outbreaks [86,121], differences in antimicrobial resistance [34,130], phylogenetic [82,224] and epidemiological studies [39].

24.8.2.6 Single-molecule sequencing

Single-molecule sequencing (SMA) was first demonstrated in 2003 [24] and has become an advantageous sequencing method due to its simplicity, requiring few or no enzymes to prepare the DNA. Preliminary cloning and amplification steps are not required to read the DNA and the method can also read RNA. In addition, some systems allow for direct identification of nucleotide modifications.

Pacific Biosciences (Menlo Park, CA) market the Single Molecule Real Time (SMRT) sequencing technology (Figure 24.22a), which is a single-molecule, real-time sequencing system for extra-long read lengths (5–8 kbp on average) and total throughput of about 3 Gbp per day, with the added benefit of the ability to detect base modifications, such as methylations. Finally, the Helicoscope Single Molecule Sequencer (Helicos Biosciences, Cambridge, MA), the first commercial single-molecule sequencer (Figure 24.22b) technology is based on extension of single DNA molecules using predefined primers and individually labeled fluorescent nucleotides that contain dye terminators preventing incorporation of multiple nucleotides per extension cycle [22]. This method has been used to successfully sequence bacterial genomes [238] and the M13 phage viral genome [85].

Although NGS still requires highly trained operators, possibilities exist for the use of NGS for the direct detection and characterization of microorganisms present in clinical specimens and cultures [12,81,87,119,198]. The development of bench-top sequencing systems and portable systems make NGS systems more affordable options for

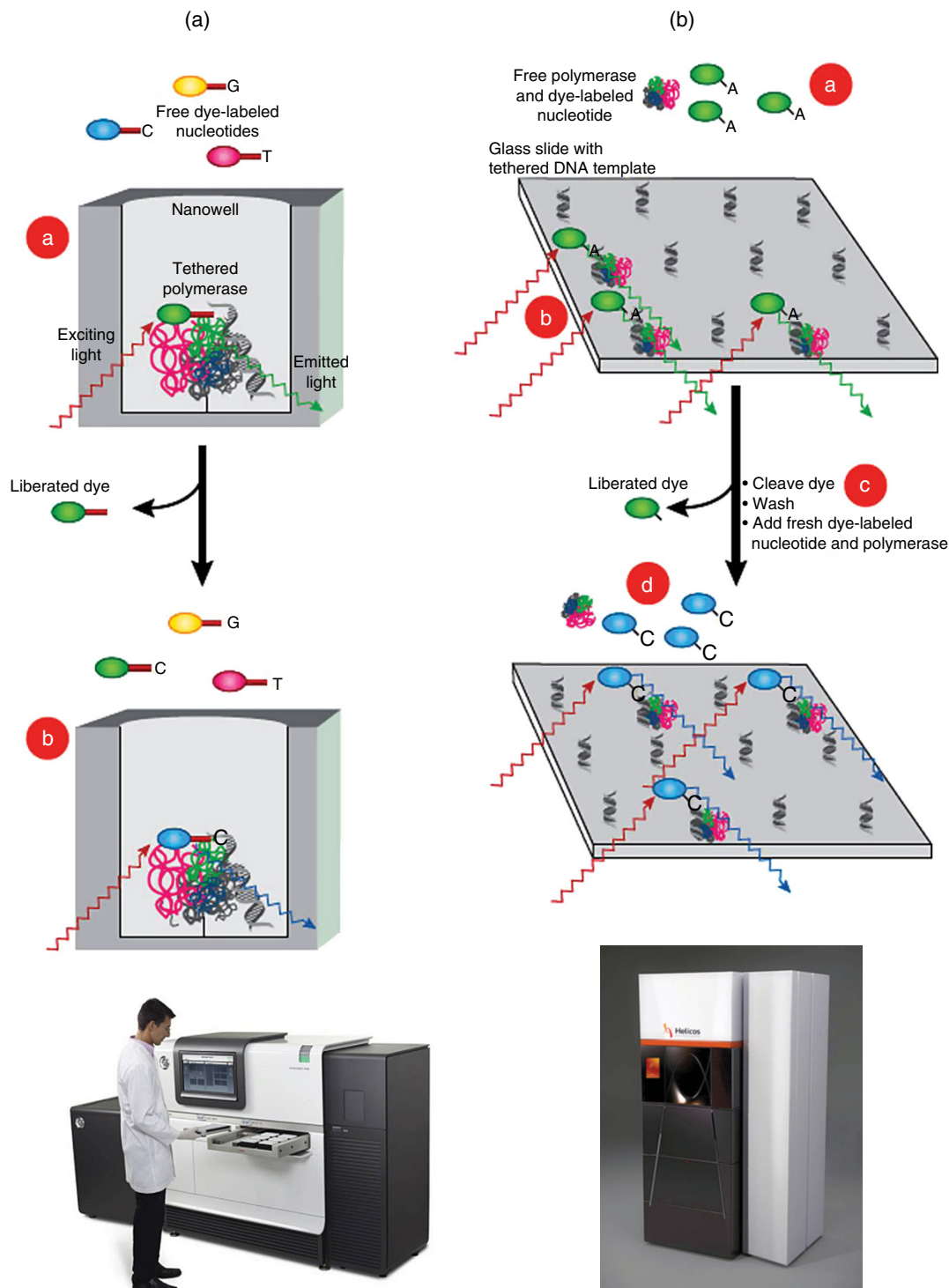


Figure 24.22 Single-molecule sequencing schemes: (a) Pacific Biosciences SMRT sequencer and (b) Heliscope Bioscience's HeliScope Genetic Analysis System represent novel research technologies. No applications are currently available for clinical use. (Osherovich 2010, reproduced with permission of Nature Publishing Group.) (See insert for color representation of the figure.)

detection, identification, genetic characterization [7,9,12], and genotyping for outbreak investigations [206,253]. These include MRSA [218], including vancomycin-intermediate *S. aureus* (VISA) strains and heteroresistance hVISA strains [148,200], *C. difficile* [61,62], *Acinetobacter baumannii* [267],

carbapenem-resistant *K. pneumoniae* strains [233] and *N. gonorrhoeae* [79]. Additionally, microbial community assessment via NGS is a research tool that could bring new awareness to infections associated with biofilms and biomes, as it has for the human microbiota [9,63,67,225,232,270].

24.8.2.7 PCR mass spectrometry: Abbott IBIS

An emerging new technology, PCR electrospray ionization (ESI) MS, by Abbott Molecular (DesPlaines, IL) has both the potential to identify nearly all known human pathogens directly from clinical specimens and to identify genetic evidence of undiscovered pathogens [243] and/or genetic changes to diagnostic targets. Originally developed for biodefense and public health safety, the first use of PCR/ESI-MS was reported in 2005, and the technology was improved in 2008 [55,56,209–212]. In comparison to MALDI-ToF, PCR-ESI/MS shows no statistically significant differences in performance, has greater capabilities and is substantially more expensive [111].

The T5000 Biosensor System was the PCR-ESI/MS prototype instrument [56]. The system couples equipment that desalts PCR products and sends them to a ToF analyzer, which delivers results to characterize mass accuracy of sufficient resolution to discern the base compositions of the amplicons produced in multiplex PCR. The refined current version of the instrument is now called the PLEX-ID (Figure 24.23). The system can perform identification (approximately 6–8 h, depending on NA type) and characterization of bacterial, viral, fungal, and other infectious organisms as well as analysis of human DNA, achieving a wide breadth of pathogen identification. As the PLEX-ID is relatively new, several published reports describe research performed on its prototype, the Ibis



Figure 24.23 The Abbott PLEX-ID system for PCR/ESI-MS is depicted here with a see through cabinet to unveil internal components. (Courtesy of Abbott.)

T5000 [55,56,111,209,212]. The PLEX-ID system is in its clinical infancy yet is a technology that could shift the way microbiologists think about the diagnosis of infections. It appears accurate and flexible, and has potential for implementation in large reference laboratories and in public health surveillance.

24.8.2.8 Magnetic resonance:

T2 Biosystems T2Dxt

T2 Biosystems (Lexington, MA) offers the T2Dxt instrument, a miniature magnetic resonance (MR) based platform that measures water molecules reacting in the presence of magnetic fields (Figure 24.24). The T2MR technology does not require sample preparation because the signal is not disrupted by background in the sample. For target-specific testing, nanoparticles with magnetic properties that enhance the magnetic resonance signals are used. The performance characteristics of this product have not yet been established in a clinical setting, yet the technology offers significant promise [165]. According to the manufacturer, T2Candida detects five species of *Candida* in whole blood specimens in approximately 3 h with a limit of detection as low as 1 CFU/mL.

24.8.2.9 Raman spectroscopy

Raman spectroscopy is not yet clinically available but has the potential to translate from research use into clinical laboratories as a point-of-care technology that is a fast and noninvasive way to detect pathogens by using whole-organism fingerprints, and which requires only a small volume of sample [48,139,153,275]. Raman spectroscopy uses the interaction of light with molecules present in the sample. It measures vibrational states of a molecule's functional groups. When incident light or photons (usually originating from a highly focused laser), interact with



Figure 24.24 T2Dx Instrument from T2 Biosystems uses magnetic resonance for identification of microbes. (Courtesy of T2 Biosystems, Inc.)

a molecule, they can induce a transition that puts the molecules in an excited vibrational state while rendering the photon with a loss of energy. The photon's energy loss results in a frequency shift in the incident light, which causes a color change that is measured by the instrument. Most of a molecule's scattered photons possess the same energy as the incident light, but a small fraction of the photons are scattered at different frequencies, an event called Raman scattering, from which the technique derives its name. The difference in scattered frequencies (the Raman shift) is measured in units of cm^{-1} , called wavenumbers. Usually, the frequency of the Raman-scattered photon is less than the original frequency of light (the incident frequency, or Stokes scattering), but differences can be measured in either direction from the incident frequency.

Surface enhanced Raman spectroscopy (SERS) (Figure 24.25) is shown to provide accurate results for

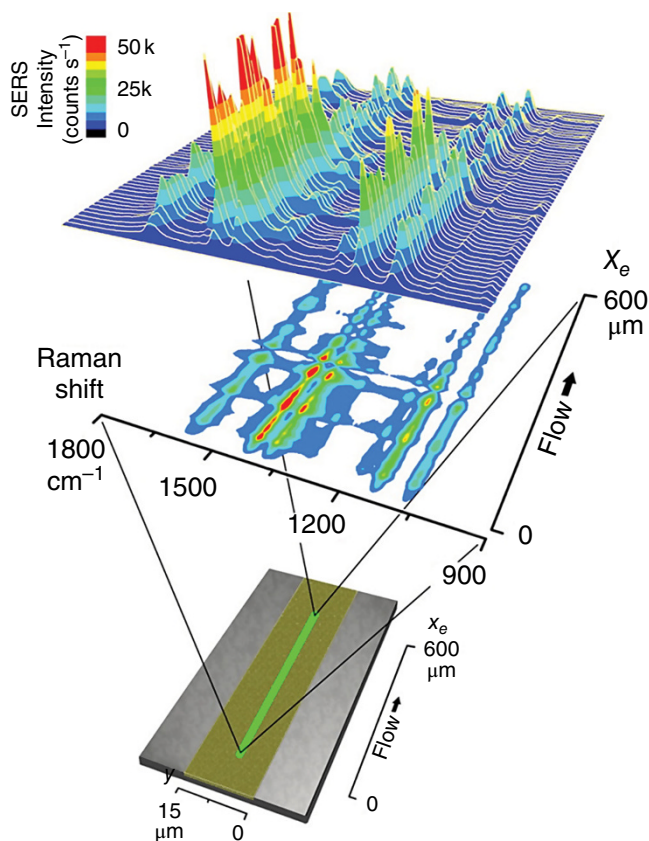


Figure 24.25 For surface enhanced Raman spectroscopy (SERS) a typical series of SERS spectra, obtained by the stepwise measurement of Raman scattering along the microchannel in 10- μm increments, is shown as a function of position down the streamwise direction of the microchannel. (Free-surface microfluidic control of surface-enhanced Raman spectroscopy for the optimized detection of airborne molecules, PNAS, Vol. 140, No.48, pp. 18898–18901, Nov. 7, 2007. Copyright (2007) National Academy of Sciences, USA.) (See insert for color representation of the figure.)

identification of bacteria, viruses, fungi, and mycoplasma [51,64,91,181,191,222,269] and can identify pathogens in human body fluids such as urine and blood [64,191]. It is not yet commonly used in clinical laboratories due to traditional challenges of Raman light scattering. These include inherently weak signals that can require long acquisition times, relatively high limits of detection in specimen matrix, background signals arising from fluorescence, sample interference, relatively high cost, and the complexity of the spectra. These require high-level data analysis and extensive database comparisons for reliable and reproducible interpretation.

Recently, combined Raman spectroscopy and dielectrophoresis were used for the classification of pathogens recovered from the urinary tract [222]. Dielectrophoresis technology allows for direct manipulation of bacteria in suspensions with nonuniform spatial electrical fields. A quadrupole electrode is used to capture bacteria directly from fluids. Viability staining shows that bacteria survive this procedure and the dielectrophoretic enrichment of bacteria allows for obtaining high quality Raman spectra in dilute suspensions with an integration time of only 1 s. In a pilot study, *E. coli* and *Enterococcus faecalis* were successfully identified in a urine matrix [222].

24.8.3 Emerging practices in total laboratory automation

As we face a critical shortage of skilled laboratory workers and increased test volumes with universal healthcare, an aging population, and decreasing reimbursement, automation in microbiology can play a key role in maintaining quality and productivity. Ranging from pre-analytical specimen processors to total laboratory automation (TLA) with digital imaging of agar plates that allow for remote specimen examination, the laboratory of today may look little like the laboratory of tomorrow.

Inoculation instrumentation like the Inocula (Figure 24.26a; Becton Dickinson, Sparks, MD) and the Walk-Away Specimen Processor (WASP), (Figure 24.27; Copan Diagnostics, Quebec, Canada), and the PREVI Isola (bioMérieux, St. Louis, MI) are coupled with digital plate reading systems like the BD Kiestra TLA (Figure 24.26b) and smart incubators (Becton Dickinson), or with the WASPLab (Copan Diagnostics), respectively. The TLA systems are promoting interest from clinical microbiology laboratories across the globe in efforts to streamline and standardize workflow and increase productivity. Vendors are developing ancillary instruments to inoculate the MS template and tubes for susceptibility testing [160].

Inoculation instruments automate the plating of liquid specimens regardless of the specimen container type or size. Reading of the primary specimen barcode and labeling of the plated media with patient identifiers allows

for traceability of all specimens, and allows coupling to downstream instrumentation, digital imaging, and digital plate reading. Costs of laboratory automation vary, but most require a substantial investment even for the purchase of modular components [171]. The financial and

clinical impact of total automation on clinical laboratory workflow and patient outcomes has yet to be fully defined.

24.9 Other strategies and concepts that will impact clinical microbiology

There are many emerging diagnostic and treatment concepts that are sure to impact the practice of clinical microbiology. From our perspectives, the initiatives that are most likely to have the most impact on clinical microbiology practice include characterization of the human microbiome [72,103,108,129,146,220,223] and biofilms associated with human disease [28,31,89,176,201,227,230,249]. In a recent report from the American Academy of Microbiology, the genetic microbiome is defined as “the entire collection of genes found in all of the microbes associated with a particular host”. The microbiome initiatives will challenge clinical microbiologists and researchers to identify not only pathogens, but also identify imbalances in normal human flora (human microbiome) that predispose people to infection or other diseases [4]. In addition, there are many technological advances that add to our ability to identify new pathogenic associations for uncultivable or fastidious pathogens [33,80,112,115,122,203,231,244] and facilitate new awareness of the impact of globalization on the differential diagnosis and treatment algorithms, with viral threats being particularly worrisome [112,157,179,182,250,254,265,268]. Therefore, we will no longer have the luxury of testing for only common pathogens and common antimicrobial resistance markers in real-time; there may be certain global pathogens and resistance mechanisms that require

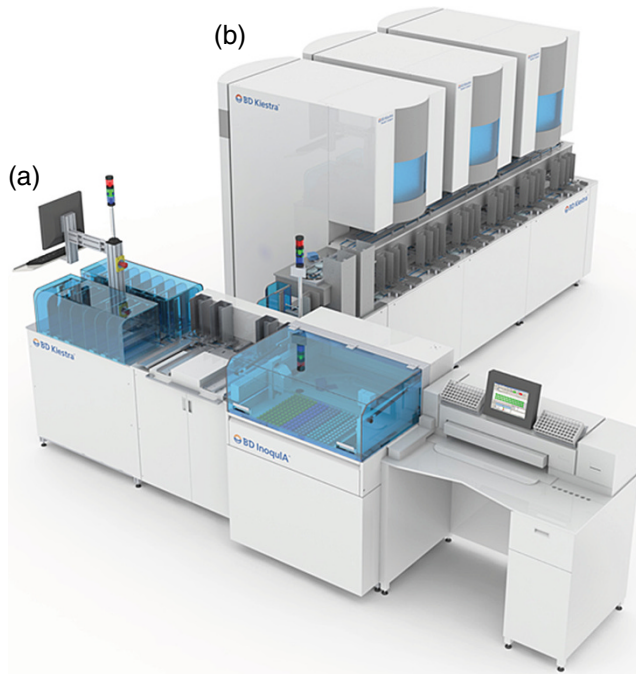


Figure 24.26 (a) Becton Dickinson's Inoqula with (b) smart incubators attached. (Courtesy and © Becton, Dickinson and Company.) (See insert for color representation of the figure.)



Figure 24.27 COPAN's Walk-Away Specimen Processor (WASP) is part of the WASPLab system for automated microbiology. (Courtesy of Copan Diagnostics.) (See insert for color representation of the figure.)

rapid testing for surveillance or outbreak situations, as well as for diagnostic and prognostic purposes. Furthermore, the creative thinking associated with new nonantimicrobial treatment strategies will challenge us to assess the relationship of probiotics with pathogens [42,161,177,216,237,250]. The impact of testing for hospital-associated infections will continue to grow and current initiatives like the Surviving Sepsis Campaign [10,47,221] will change the way microbiology laboratories operate, forcing the global acceptance of 24/7/365 microbiology laboratories in all modern hospitals, regardless of size. Finally, nonclinical issues, such as finances, reimbursement, regulations, and data dissemination to the public will continue to impact our practices on a daily basis.

24.10 Some new responsibilities for the coming years

24.10.1 Cost-benefit: the future impact of systematic reviews

With great technology comes great responsibility. Historically, laboratories store and analyze large amounts of data; however there are many instances in which the utility of the data is not well explored. Compilation and analysis of laboratory data linked to clinical data is critical to clinical research and to the emerging field we named “interventional diagnostics”. Interventional diagnostics embraces the examination and analysis of the impact of diagnostic test intervention to patient care. Data to describe post-analytic effects of actionable interventions based on relevant laboratory tests is the key to the future of laboratory science and the growing awareness of the importance of laboratory medicine to nearly all aspects of healthcare practice. For new technology platforms to integrate successfully the laboratory budget can no longer be considered on its own; benefits must be documented at the level of the healthcare organization itself.

Clinical laboratory scientists have been leaders in quality assurance practice for decades, but have not always shared results in the peer-reviewed literature to further improve patient outcomes. The laboratory intervention approach to research aligns with evidence-based medicine (EBM), a practice that refers to the application of existing data to answer questions and solve problems through systematic synthesis and appraisal of data. In the EBM process, standardized data acquisition and analysis is used so that the data derived from the process will add to the evidence-based body of knowledge. The underlying premise of an evidence-based approach is that healthcare decisions are guided by the synthesis and appraisal of evidence from well-designed studies balanced with clinical expertise and individual patient preferences. The US Agency for Healthcare Research and Quality (AHRQ) is one leader in this initiative, dubbed

“Patient-Centered Outcomes Research” (<http://www.ahrq.gov/index.html>) along with the American Society for Microbiology (ASM)/Centers for Disease Control’s (CDC) Laboratory Medicine Best Practices (LMBP) initiatives (<http://wwwn.cdc.gov/futurelabmedicine/>) that adapt EBM concepts to clinical laboratory practice and quality improvement (QI) studies. Current LMBP systematic reviews can be found at <http://wwwn.cdc.gov/futurelabmedicine/> and are described in general terms in a recent publication [228].

Challenges to practicing EBM in laboratories include the lack of research time allocated to the clinical laboratory, lack of experimental design and biostatistical training in medical laboratory science (MLS) programs and MLS fellowships, lack of institutional support for mentoring and implementing an evidence-based approach, the lack of evidence from well-designed diagnostic studies, and misconceptions about practicing and contributing to EBM [36,228].

The premise of evidence-based practice recommendations relies on two processes, “systematic reviews” and “meta-analysis” [228]. A systematic review collates and analyzes the findings from well-designed studies and pools the results from individual studies to reduce bias and maximize the interpretation of the conclusions [246]. Various terms are used for systematic reviews, including “comparative effectiveness” and “patient-centered outcomes research,” but the common objective is to use available data to make optimal decisions for diagnostic laboratory practice and patient care. Systematic review methods consist of key steps to address a problem about the quality of a practice [36,228].

Within LMBP, systematic reviews can provide information to support decision making for health outcomes achieved from the use of a diagnostic test and additional outcomes that influence cost and quality of patient care (e.g., decreased mortality, inpatient length of stay, earlier treatment). Considering that evidence generated in the laboratory is typically derived from observational studies rather than randomized controlled trials, the LMBP A-6 Methods are applied to conduct reviews that assess the effectiveness of practices in improving the quality of laboratory medicine service delivery. Via the LMBP systematic review process, unpublished quality improvement (QI) data, which typically are not disseminated through the peer-reviewed literature process, are incorporated with data from the peer-reviewed literature. Often, the QI data is hampered by a lack of the following elements of sound research design: (i) explicit focused purpose of the study; (ii) defined sample size; (iii) defined practice of interest; (iv) collection of baseline data or data on the usual (or current) practice (comparator); and (v) defined outcome measures [36,228].

Key evidence-based practice principles to improve QI projects include the following:

- 1 frame quality problems as clinical questions using what is called the PICO (Population, Intervention, Comparison, and Outcome) format;
- 2 conduct literature searches;
- 3 apply elements of research study design to QI project design;
- 4 capture and disseminate project results.

A systematic review is designed to answer a clearly stated question. The important steps in a systematic review process include:

- 1 pose an explicit statement of one or more clinical questions;
- 2 develop a search strategy for published primary studies;
- 3 review titles and abstracts generated by the search;
- 4 select and review full papers and extract relevant data;
- 5 analyze data including use of meta-analytic tools;
- 6 summarize findings;
- 7 identify further research needed.

Types of PICO Questions and Measures include [100,192,242]:

- Intervention: What practice, policy, or therapy leads to the best outcome?
- Diagnostic: Which test is more accurate and precise in diagnosing a condition?
- Prognosis: What is the likelihood of a particular outcome for a population, with specific changes observed in the results of specific laboratory questions?

For the analysis in the ASM/CDC LMBP initiative, a review team is compiled that is composed of experts in the fields such as clinical microbiology, biostatistics, epidemiology, laboratory sciences, infectious diseases, healthcare management, and other related specialties. This team works to perform and document literature searches, using PubMed (www.pubmed.gov), Cochrane Library (www.cochrane.org), and other free databases available to search the current peer reviewed literature to identify publications, called primary studies.

The team reviews primary studies, unpublished QI projects, and other literature according to a critical appraisal checklist for evaluating the quality of published primary studies. This LMBP Study Quality Appraisal Checklist can also guide further planning and documentation of QI projects, standards, or guidelines for optimal laboratory practice [36,149] and provide templates for preparing a publication about the findings of a project.

In addition to the ASM-LMBP initiatives, other assessments and guidelines for diagnostic accuracy studies are available. The STARD initiative (STAndards for the Reporting of Diagnostic accuracy studies) provides guidelines to improve the accuracy and completeness of diagnostic accuracy studies [20,174]. Adherence to STARD criteria facilitates the assessment of key

manuscript properties, such as the potential for study bias, and evaluation of the generalizability of the data and conclusions. The STARD statement consists of a checklist of 25 items and recommends the use and publication of a flow diagram to describe the design of the study and the flow of patients [20,174].

24.10.2 Antibiotic stewardship: why not laboratory stewardship?

Antimicrobial stewardship teams were first suggested in the 1990s and great strides were made due to the integrated membership of workgroups and focus on one common theme, i.e., conservation efforts and strategic use of antimicrobial therapy with proper and rapid targeting/de-escalation. While many techniques focused on providing rapid identification, few have focused on performing rapid AST or identifying genetic surrogates of antimicrobial resistance [14,68–71,133]. To expand the impact now available from AdvanDx's PNA FISH, BioFire's FilmArray BCID, Nanosphere's Verigene panels, and other systems, full AST methods that provide accurate and rapid results to clinicians are sought, and may change the face of clinical microbiology and its impact on patient care and stewardship efforts. The emerging trend to use real-time PCR for determining antimicrobial resistance of organisms leaves us facing risks of not being able to identify all means of resistance. By analyzing only the genes known to confer resistance [59,143,144,262,263], as opposed to merely discerning the phenotypic determinants, we face the difficult decision of improving care for some, but not for others. Back-up plans to continue to identify unusual resistance mechanisms must be carefully designed to mitigate potential harms.

The capacity to generate timelier, clinically actionable results, based on antimicrobial resistance genes is a key item on the wish list of many clinicians and microbiologists. The development of bacterial panels for direct detection of pathogens, and in some cases their resistance mechanisms, from blood cultures is likely to impact antimicrobial stewardship practices for certain pathogens [68]. A number of exciting alternatives appeared in the literature focusing on whole-genome sequences (WGS) derived from NGS [54,251]. Genetic antibiograms may also be the norm in the future. For the highly clonal *S. aureus* [46], a recent study showed that overall sensitivity and specificity of a ten-digit antibiogram based on a staphylococcal WGS were 97 and 99% respectively [77].

In addition to WGS, the use of MALDI-ToF/MS strategies may further assist to identify antimicrobial resistance by providing evidence of antimicrobial inactivation or modification of antibiotics such as β -lactams by β -lactamases [99] and correlating changes in the protein spectrum of a microorganism exposed to an antimicrobial agent with or without resistance [140].

Progress is also being made using LC-MS/MS to detect antimicrobial resistance [183]. For additional advances in AST, please refer to other recent reviews [252].

24.10.3 Microbiological biorepositories

A microbial sample biorepository receives, stores, processes, and disseminates biospecimens and associated data to provide resources to advance translational biomedical research, navigation through the FDA process for *in vitro* devices, verification and validation of diagnostic methods, and personalized medicine [11,41,65]. Method development and biomedical research require a wide variety of sample types and microbial diversity; therefore, clinical microbiology will be expected to expand its role as guardians of clinical samples related to infectious disease diagnostic testing and research. Navigating the regulatory, ethical, and legal issues involved in the preservation and use of human biospecimens is complex, but storage of biological samples, along with associated and validated data, is of paramount importance and well worth the effort to advance the health sciences [11,41]. The merger of electronic medical records and laboratory data can support proper labeling and annotation of samples for improved research utility [41].

Future needs for biorepositories include implementation of best practices for sample collection, storage, transport, distribution, and data handling [41]. In order to fully realize the opportunities that biorepositories offer to medicine, laboratories must be able to guarantee high-quality biospecimens, associated information, and a system able to respond to needs for method verification, and quality control, or to fulfill the requests of scientific colleagues. As the field matures, educating a new generation of biospecimen/biobanking scientists will be an important need.

24.11 Summary

Although molecular and protein-based diagnostic testing is becoming commonplace, and offers the prospect of major improvements in speed and accuracy of clinical microbiology methods, in the short term there remains a strong need to document the utility of the new methods. The trend toward miniaturization, multiplexing, sample-to-answer instrumentation, and broad-based testing is primed to continue and should greatly impact the practice of clinical microbiology in the future. Technology is becoming easier to use, smaller and more versatile than predecessors, driving the adoption and utilization of new innovative tests. Through prudent and evidence-based use of new technology, we can improve both the care and the economics of patient care. Laboratory tests that streamline diagnostic laboratory processes can significantly impact our ability to advance clinical practice. To best leverage new technology, clinical microbiology networks, like the

ASM/LMBP initiatives, which propose meta-analysis of literature to support evidence-based guidelines, are poised to enable sooner translation of new technology as well as much faster assessment of the cost/benefit of new technology.

Culture methods are not dead yet, and we will continue our culture-based testing for instances when identifying genotypes and mutated strains escape detection by genetically targeted technology. While there are many advantages of rapid molecular and proteomic methods, laboratories are certain to keep traditional cultures, as well as phenotypic and biochemical characterization methods available for some time.

We live in a time of both abundance and scarcity: abundance of technology, yet restriction on capital and human resources. There is a critical need to undertake initiatives to manage the large amounts of medical and financial informatics required to assess the utility and cost/benefit of new technology. Initiatives like the human microbiome projects will contribute vast amounts of information relevant to health care and will challenge clinical microbiologists to think differently in terms of our views of infectious disease and the balance with “microbial health”. In the next decade, we will certainly be challenged to provide new testing algorithms, techniques, and specimen storage and annotation capacity to help unveil the secrets of the microbiome, globalized infections, and antimicrobial resistance, among other challenges. Future workplace needs include clinical training requirements for biostatistics, medical informatics, bioinformatics, and techniques for sample biorepositories.

Collaboration is a prerequisite skill for laboratorians, pharmacists, clinicians, other healthcare staff, and our patients. Initiatives, such as the American Board of Internal Medicine’s (ABIM) “Choosing Wisely” program (<http://www.choosingwisely.org/>), the Robert Wood Johnson Foundation’s “Open Notes” initiative (<http://www.rwjf.org/en/grants/grantees/OpenNotes.html>), and the Institute for Healthcare Improvement’s (IHI) “Triple Aim Initiative” (<http://www.ihl.org/Engage/Initiatives/TripleAim/>), are examples of the importance of collaboration that fosters the stewardship of healthcare resources, the meaningful use of healthcare and laboratory data, and clinical decision support to improve health while reducing costs. These are just a few examples of programs that overlap and interface with clinical microbiology. In the next decade, it will be imperative that laboratorians seize the opportunity to get involved in important initiatives such as these. The IHI’s “5 million lives” campaign reminds us that, “Some is not a number. Soon is not a time.” We must act now. We must be able to compile metrics to help quantify improvements, deliver diagnostic interventions that help save lives, reduce morbidity, and support the patient’s health and satisfaction.

Rapid and targeted antibiotic administration is a practice for which laboratories can provide actionable results and microbiology laboratories are expected to maintain or expand microbiology testing to operate 24/7/365. There are published examples of how laboratory data is used to help drive clinical decisions and antibiotic de-escalation, thus decreasing mortality, decreasing costs, and supporting antimicrobial stewardship [69,70,98], but we must continue our efforts and expand the breadth and depth of our laboratory's scope. In order for laboratories to assess the effectiveness of new technology and document improvements in health outcomes, we must think and train differently, with added emphasis on strategy, experimental design, biostatistical analysis, cost-benefit, and evidence-based interventions. Here, we coin the terms "diagnostic interventionist" and "laboratory stewardship specialist" to describe possibilities for new interdisciplinary subspecialties in laboratory medicine. The future of clinical microbiology stands on the strong foundation of quality systems but will require these new directions as we move forward. It may seem as though we are in too deep or over our heads, as we race to do more, do better, be faster – all with fewer resources; to that end, we leave you with a quote from T.S. Eliot, who said, "If you aren't in over your head, how do you know how tall you are?" Clinical microbiologists of this generation can walk tall knowing we have adapted and changed with the ebb and flow of today's health care, and we will continue to do so in the future. As was true in 2002 when Wolk stated, "In our lifetime, technology will only be as good as the collective efforts, ethics, and judgment of the scientists who design and provide diagnostic services. Working alone, we may not be able to achieve the goals for effective integration of new technology; working together, it seems we can." Today, that sentiment still rings true. Clinical microbiology remains a team-based venture with partners in health care, administration, education, industry, and in the communities in which we work and live.

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Introduction

Since the introduction of clinical (diagnostic) microbiology as an accepted discipline in laboratory medicine (and clinical pathology) in the early to mid-20th century, the development of tests, protocols, media and methods has been largely isolated by country, or at least by region (i.e., North America, Central America, South America, Europe, Australia, Africa, Asia, etc.). The US federal agency that regulates *in vitro* diagnostic medical devices (IVDMDs) is the Food and Drug Administration (FDA). In the European Union (EU) the CE marking (Conformité Européenne, European Conformity; or formerly Communauté Européenne, European Community) on a product is a manufacturer's declaration that the product complies with the essential requirements of the relevant European health, safety, and environmental protection legislations.

Many countries have developed their own societies of microbiology, and infectious diseases; and each country (or region, such as the European Union) has developed their own regulatory standards and federal agencies for the acceptance or approval of commercially available diagnostic products in the area of clinical (diagnostic) microbiology.

In the mid- to late part of the 20th century, collaboration and interchange of information and products (both commercially available and in-house) for the diagnosis of infectious diseases started to be expanded between and among countries and regions of the world. Now, in the early part of the 21st century there seems to be a rapid expansion, one might say an explosion of cooperation and interchange of products, information, techniques and protocols around the globe. Although it is still somewhat difficult for specialists to attend international meetings on a regular basis, a number of international

specialty meetings have developed over the recent years; and the Internet and scholarly scientific publications have acted as a backbone to transmit important new products, methods, and protocols for the rapid diagnosis of infectious diseases and their treatment with antibiotics and chemotherapeutic agents.

We are truly becoming a global marketplace for commercially available products in the area of Clinical Microbiology. One needs only look at the number and breadth of global biotechnology companies that market their products around the world. And each of these companies must interact with the regulatory agencies of each country or region.

In this second edition of the *Manual of Commercial Methods in Clinical Microbiology, International Edition*, we have attempted to take the first global snapshot of the international scope of commercially available diagnostic products, and the regulatory environment around the globe for these products. We have included a number of countries, and international experts in clinical (diagnostic) microbiology, laboratory medicine, and infectious diseases to help us understand the nature and breadth of clinical microbiology testing and product regulation in their country.

Since this truly is a moving target, we are sure that there are some products, tests, instruments and methods that may have been missed in these summaries, and we apologize for any missing information. We encourage all readers to contact the publisher, editor, and authors regarding additional information that will improve the presentation for future editions. The countries were selected based upon the author's willingness to participate and the Editor's attempt to include as many countries and regions as practically feasible. We realize that this

is the first ever attempt to include a truly international component to a reference text on commercially available clinical microbiology products and their regulatory requirements. We hope that this serves as an incentive for others to either establish or expand the global marketplace for products and standards in clinical microbiology. Web sites and vendor contact information are current at the time of writing for each chapter and the reader should

confirm all web sites and vendor information at the time he/she is searching for current information.

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Clinical Microbiology *In Vitro* Diagnostic Medical Devices in Argentina: Regulatory Requirements and Product Information

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25.1 History

Medical devices, drugs and food are a matter of serious concern due to their close connection with the health of the population. In Argentina, the production and commercialization of all products used in human medicine have been regulated by the National Medicines Act 16463/64 [1] since 1964. The Instituto Nacional de Normatización de Drogas y Medicamentos, later known as Instituto Nacional de Farmacología y Bromatología (INFyB), also started in 1964.

Clinical microbiology is a very dynamic field with frequent changes. Therefore, it must be regulated and supervised by a health authority that guarantees that rules and procedures will be duly met. Thereby, medical devices, drugs and food available to the population will meet the international standards of quality, efficacy, and safety.

The regulation of new drugs and medical devices for their commercialization in Argentina has been modified over the years. In 1992, the Medicines Act was amended, which required the creation of a specific agency with technical and administrative capacities to guarantee that the amended act would be in force. Thus, the National Administration of Drugs, Foods and Medical Devices (Administración Nacional de Medicamentos, Alimentos y Tecnología Médica, ANMAT) was established [2].

25.1.1 ANMAT: development and objectives

ANMAT was started as a health agency with a regulatory mission (Law Decree 1490/92) [3], which was defined as follows:

- To authorize and register the manufacture and marketing of medicinal, diagnostic and cosmetic products, as well as their updates and modifications.
- To monitor the premises engaged in the manufacture, import, fractionation and/or marketing of the

above-mentioned products, in addition to setting the regulations and technical specifications they must comply with.

- To control the composition, quality, efficacy, and harmlessness of the above-mentioned products.
- To authorize, register, control, and monitor the quality and healthiness of foodstuff including dietary supplements and any material in contact with foodstuff. All this is carried out as a joint task together with the federal health jurisdictions.
- To participate in the creation and update of regulations.
- To authorize medical devices as well as the materials and equipment to be used in human medicine. To formulate and enforce regulations assuring the quality of medical devices manufacture, import, and storage processes.
- To watch for undesired effects, inefficiency, and quality failure of the products it regulates. For this purpose, information networks were developed to gather any notifications made by professionals, institutions, or users by means of the Pharmacovigilance, Technovigilance and Food Vigilance National Systems.
- To communicate and disseminate any health-related significant information among professionals and public in general, through periodical and nonperiodical publications.
- To promote the “Good Practices for Clinical Pharmacology Studies” in any clinical investigation on new drugs in compliance with international ethics regulations and with a specific inspection program.
- To authorize the advertising of over-the-counter drugs, dietary supplements, and the medical devices facilitating their consumption and use, as well as guiding consumers in doing so.

With the support of qualified professionals and technicians, ANMAT can achieve the aforementioned objectives.

The following entities depend on the ANMAT organization:

- INAME (Instituto Nacional de Medicamentos) National Institute of Drugs: supervises those entities engaged in the production, import, or commercialization of drugs and cosmetic products. This institute further controls the related activities, processes, and technologies to provide certified quality products.
- INAL (Instituto Nacional de Alimentos) National Institute of Food: this entity is engaged in the registration and control of food, goods used for food production, household products, and materials coming in contact with food. These tasks require an interaction with the provinces, and technical assistance to address health supervision.
- National Directorate Of Medical Devices (Dirección Nacional de Productos Médicos): this division supervises the quality of medical devices as well as the activities, processes, and technologies performed for their manufacture, fractionation, import, export, and storage.

25.1.2 ANMAT: Reference Regional Drug Regulatory Authority

As a World Health Organization (WHO) initiative to promote the accreditation of drug regulation national authorities, ANMAT was evaluated by the Pan American Health Organization (PAHO). PAHO fosters this WHO initiative in America through examination processes carried out by experts in those countries willing to participate. After comprehensive consultation and approval procedures, all of the American countries agreed on the implementation of an assessment tool to analyze both the regulatory requirements the national authority must meet, and the indicators evidencing the authority compliance. In December 2009 ANMAT was the first Regional Regulatory Authority to obtain the highest qualification (Level IV) by PAHO/WHO.

The audit focused on ten modules containing the standards set by PAHO:

- 1 National Regulatory Agency (NRA): its organization and legal foundations.
- 2 National Regulatory System: organizational structure, institutional development, and quality management.
- 3 Marketing Authorization: outlines, guidelines, procedures, evaluation mechanisms, registers, and information availability.
- 4 Market vigilance: import and export controls, recall procedures.
- 5 Pharmacovigilance: outlines and guidelines, organization and structure, and evaluation procedures.
- 6 Regulatory oversight of clinical trials: guidelines, ethical framework, organization and structure, evaluation procedures.

- 7 Licensing of manufacturers, importers, and retailers
- 8 Regulating inspections and monitoring activities: guidelines, outlines, planning, and registry.
- 9 National quality control laboratory: organization, structure, quality management, human resources, equipment, and safety structure.
- 10 NRA Lot Release.

This evaluation process and resulting recognition of ANMAT as a PAHO Reference Drug Regulatory Authority was a powerful incentive for the whole agency. In light of the above mentioned, the professional, technical, and administrative staff are deeply committed to the objective in the knowledge that the achievement of this result would mean a significant improvement and advancement in the area of diagnostic medical devices. The working teams displayed an excellent technical and professional performance, which was reflected in the final qualification obtained.

In 2011, at the *VI Pan American Conference on Drug Regulatory Harmonization* (CPANDRH) [4] organized by the Pan American Network for Drug Regulatory Harmonization (PANDRH) [5] in the city of Brasilia, ANMAT was acknowledged by PAHO as a Reference Regional Drug Regulatory Authority for a 3-year period.

25.1.3 MERCOSUR

In 1991, the Treaty of Asunción was signed between Argentina, Brazil, Paraguay, and Uruguay to establish the Southern Common Market (MERCOSUR) [6]. Later, Bolivia (1997) and Venezuela (2004) joined as associate countries. The MERCOSUR ministers of health have worked hard on the process of harmonizing several topics, such as health in border areas, environment, drugs policy, the Millennium Development Goals, disasters, primary health care, epidemiological surveillance (e.g., malaria and dengue), sexual and reproductive health, HIV/AIDS, and review of the International Sanitary Code. Since 1995, MERCOSUR Ministers of Health have met annually.

A special working group (Working Subgroup No. 11/Health – SGT N°11) is also entirely engaged in health issues and is focused on the following: harmonization of laws and directives; technical cooperation; actions taken among the parties in the fields of health care, goods, services, raw material, health products, professional practice, epidemiological surveillance and sanitary control. The aim is to promote and protect health and life, overcome the obstacles related to regional commerce, and foster overall health care [7].

Argentina guarantees that the laws issued by MERCOSUR bodies shall be fulfilled. Thus, Argentina incorporates the MERCOSUR Provisions into its national legislation.

25.2 Regulation of clinical microbiology *in vitro* diagnostic medical devices in Argentina

In Argentina, the regulation of reagents, diagnostic products, and any other product for human medicine started in 1964. With the advent of globalization emerged the need to adapt and update the regulatory system. Thus, since 1994 the registration of all entities engaged in the manufacture, import, export, or sale of *in vitro* diagnostic (IVD) medical devices became mandatory. In addition, all products commercialized in Argentina should be previously registered. The ANMAT Resolution N° 2674/99 has the requirements for IVD registration [8]. As a result of the technical advances in IVD, the regulations concerning registration of these products have been amended.

Regulations about inspections of companies engaged in IVD manufacture or import were also passed pursuant to ANMAT Resolution N° 3266/13 [9]. Regulations concerning the qualification of entities engaged in these activities [10] and requirements about IVD distribution and commercialization (ANMAT Resolution N° 6052/913) were also passed. Recommendations on Good Manufacturing and Control Practices, and Inspections Guidelines for IVD manufacturers, importers, and exporters were harmonized with the MERCOSUR group.

The reagents to be used in molecular biology techniques are regulated under Decree N° 3619/97. These products are antibodies, specific receptor proteins, ligands, nucleic acid sequences, and other similar molecular biology products or reagents. According to this regulation, companies must submit a form and catalogs of those reagents to be commercialized. In addition, those companies that manufacture or sell these products must be licensed by ANMAT.

25.2.1 IVD definition

The IVD medical devices are those reagents, instruments, and systems that allow performing a qualitative, quantitative, or semiquantitative determination in specimens obtained from a human body. Therefore, an IVD should not be ingested, injected, or given to humans. They have been designed and developed to assess health or disease, supervise treatments, detect or follow a medical condition, diagnose, prevent, carry out blood donor screening, and determine the safety and compatibility with potential recipients. This includes reagents, calibrators, control materials, specimen receptacles, software, and related instruments or apparatus or other devices. The international reference materials (e.g., WHO) and materials used for external quality assessment schemes are excluded.

Specimen containers are considered to be IVD medical devices. These receptacles, whether vacuum-type or not, are specifically made for the containment and preservation of specimens derived from the human body for IVD examination purposes.

Table 25.1 IVD classification: requirements and examples

Class A: professional use only	Declaration of conformity Dossier	Hormones, cardiac markers, clinical chemistry, hematology Neonatal screening Down syndrome Tumor markers Therapeutic drug monitoring HLA system Antibiotic susceptibility tests
Class B: professional use only	Declaration of conformity	Rubella, Epstein–Barr virus, dengue
Class C: professional use only	Dossier Dossier and product samples	CMV, <i>Chlamydia</i> . HIV 1/2, HBV, HTLV 1/2, syphilis, HCV, Chagas, brucellosis, ABO system HCG, glycemia
Class D: self-testing	Dossier and product samples	

All IVDs require ANMAT's authorization before their commercialization. Companies engaged in the manufacturing, distribution or marketing should also meet the requirements and obtain authorization from ANMAT.

Products for general laboratory use (such as glassware, pipettes, tubes and similar products) are not IVDs.

25.2.2 IVD medical device classification

The IVD medical devices are classified into four regulatory categories for their registration (Table 25.1). This classification is based on the intended use and indications for use as specified by the manufacturer:

- Class A: to diagnose noninfectious, nontransmissible diseases;
- Class B: to diagnose infectious diseases, except for those in Class C;
- Class C: to diagnose sexually transmitted infectious diseases or infectious diseases transmitted by blood or blood products, and to identify blood groups;
- Class D: self-testing devices/products.

The level of regulatory control increases with each class.

25.2.3 General requirements for the registration of IVDs

The IVD requirements for their registration are described in Resolution N° 2674/99. They were harmonized by MERCOSUR and compiled in a document entitled "Interzone Registry of *In Vitro* Diagnostic Products" (MERCOSUR GMC / RES. N° 79/96).

25.2.4 General documents required

- 1 Authorization to work as an IVD manufacturer.
Certificate of Good Manufacturing Practice (GMP) compliance.

- 2 Registration of the technical director (Licensed Professional).
 - 3 Product characteristics or “dossier”.
 - 4 Agreement or certificate of representation between the local firm and the manufacturer (for imported products only).
 - 5 A “free sale” certificate stating that the product is commercialized in a country of high health surveillance (such as USA, UK, Germany, France, Austria, Belgium, Denmark, Sweden, Netherlands, Spain, Italy, Australia, Japan, Finland, Israel, Canada). The “free sale” certificate must be issued by the Health Authority of these countries and must include the Apostille of The Hague Accords (for imported products only).
 - 4 Safety characteristics: description of precautions, special care, and specifications about the risks of handling and disposal.
 - 5 Performance evaluation:
 - (a) Sensitivity.
 - (b) Specificity.
 - (c) Potency, accuracy, and precision.
 - (d) Stability studies: storage conditions.
 - (e) Reference values.
- Information should include the reference method or reference materials. This evaluation should be based on adequate scientific references and a clinical or analytical study.
- 6 Basic flowchart of the production process, containing a brief description of each phase or stage leading to the finished product.
- Analysis of all the documents outlined under “general documents” and “product characteristics” are performed for all classes. In addition, Classes C and D should submit samples of the product to be analyzed by ANMAT or other recognized entities. Some IVDs used in Microbiology are manufactured in Argentina (Table 25.2).

25.2.5 Product characteristics or “dossier”

- 1 Trade name.
- 2 Product description based on its intended use.
- 3 Chemical and physical composition of each component in the kit.

Table 25.2 *In Vitro* diagnostic (IVD) medical devices manufactured in Argentina

Test/product		Intended use
HIV	HIV 1+2 Rapid Test Cassette	Rapid test for the detection of anti-HIV-1, anti-HIV-1 group O and anti-HIV-2 antibodies in serum, plasma, and whole blood
	HIV 1+2 ELISA 3rd generation	3rd generation enzyme-linked immunosorbent assay (ELISA) for the detection of anti-HIV-1 and anti-HIV-2 antibodies in serum or plasma
	DIA HIV1+2	Dot immunoassay to detect anti-HIV-1, anti-HIV-1 group O and anti-HIV-2 antibodies
	HIV Ag/Ac ELISA 4th generation	Immunoenzymatic assay (ELISA) for the simultaneous detection of p24 HIV-1 antigen and anti-HIV-1 and anti-HIV-2 antibodies in serum or plasma
	HIV PCR	Polymerase chain reaction assay for the qualitative detection of human immunodeficiency virus type 1 (HIV-1) nucleic acids from human plasma
HTLV	HTLV I+II ELISA recombinant	Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to HTLV I and HTLVII
Streptococci β-hemolytic	ASO Calibrator Turbitest AA	Latex agglutination test for determination of antistreptolysin O in serum. Increase in ASO titer indicates recent infection produced by group A β-hemolytic streptococci, such as tonsillitis, scarlet fever, puerperal sepsis, erysipelas, etc.
	ASO latex ASO latex direct ASO latex Turbitest O-Streptolysin	Hemolytic method for determination of anti-streptolysin O (ASO) titer
Chagas disease (also known as American trypanosomiasis)	Rapid Tests for <i>Trypanosoma cruzi</i> Ab	Immunochromatographic test for qualitative detection of antibodies anti- <i>Trypanosoma cruzi</i> in serum, plasma and whole blood
	ELISA recombinant 4th generation	For screening and serologic diagnosis of <i>T. cruzi</i> infection
	ELISA recombinant 3rd generation	3rd generation method for detection of antibodies to <i>Trypanosoma cruzi</i>
	ELISA dried blood	Enzyme immunoassay recombinant for serologic diagnosis from dried blood spot samples
	ELISA cell lisate	Enzyme immunoassay for the qualitative detection of antibodies anti- <i>T. cruzi</i> in human serum or plasma samples
Toxoplasmosis	HAI Screening A-V	Indirect hemagglutination assay for the detection of antibodies to <i>Trypanosoma cruzi</i>
	HAI test	Indirect hemagglutination assay for the detection of antibodies anti- <i>Toxoplasma gondii</i>
	Latex test	Latex agglutination test for the detection of antibodies anti- <i>Toxoplasma gondii</i>
Syphilis	Venereal disease research laboratory (VDRL) test	Stabilized antigenic suspension to perform modified VDRL (unheated serum reagin [USR]) test for detection of syphilis
	Rapid plasma reagin (RPR) slide test	Rapid test for RPR for serologic detection of syphilis
	Syphilis ELISA recombinant	Enzyme immunoassay for the qualitative detection of antibodies anti- <i>Treponema pallidum</i>
	Syphilis ELISA recombinant v.4.0	Enzyme immunoassay for the qualitative detection of antibodies anti- <i>Treponema pallidum</i> .

Table 25.2 (Continued)

Test/product	Intended use
Hepatitis C virus	HCV ELISA 3rd generation Hepatitis C (anti-HCV) ELISA HCV PCR assay 3rd generation ELISA method for detection of hepatitis C virus Enzyme immunoassay for detection of antibodies to hepatitis C virus Reverse transcription-polymerase chain reaction (RT-PCR) assay for the quantitation of hepatitis C viral ribonucleic acid (HCV RNA) in human serum and plasma from HCV-infected individuals
Hepatitis B Virus	HBV PCR assay HBsAg ELISA RT-PCR assay for the quantitation of hepatitis B viral ribonucleic acid (HBV RNA) in human serum and plasma from HBV-infected individuals Enzyme immunoassay for detection of surface antigen of the Hepatitis B virus (HBsAg) for diagnosis of Hepatitis B infection.
Mononucleosis	Hepatitis B (anti-HBc) ELISA EBV test (Epstein-Barr virus) Slide test for diagnosis of infectious mononucleosis.
HPV	HPV PCR assay Qualitative PCR assay for amplification and detection of human papillomavirus (HPV) DNA in cervical smears or tissue biopsies
Dehydrated culture media*	Alkaline Peptone Water A broth medium for the enrichment of <i>Vibrio</i> species from food, water and clinical samples Glucose Azide Broth For the detection of enterococci in water Brain Heart Infusion Broth A highly nutritious infusion medium recommended for the cultivation of <i>Streptococcus</i> , <i>Neisseria</i> and other fastidious organisms Brain Heart Infusion Agar A solid medium which contains the highly nutritious infusions recommended for the cultivation of fastidious organisms DRCM Broth Medium for cultivation and enumeration of sulphite-reducing anaerobes by the most probable number technique (MPN). It is widely used in the analysis of water and food Ec Medium Selective medium for the enrichment of <i>Escherichia coli</i> O157H7 from food. Ec Modified Medium Mossel Broth An enrichment medium for <i>Enterobacteriaceae</i> Lactose Broth A liquid medium for use in the performance or confirmation of the Presumptive Test for coliforms Buffered Listeria Enrichment Broth An enrichment medium for <i>Listeria</i> spp. This medium complies with the modifications made by FDA/BAM MacConkey Broth* A differential medium containing neutral red for the detection of coliform organism MacConkey Agar* A differential medium for the isolation of coliforms and intestinal pathogens in water, dairy products and biological specimens. MacConkey Sorbitol Agar A selective and differential medium for the detection of <i>Escherichia coli</i> O157 MRS Broth Medium for growth of Lactobacilli and other lactic acid bacteria from clinical samples and food MRS Agar A non-selective general purpose medium Nutrient Broth A non-selective general purpose medium Nutrient Agar Nutrient culture medium and nonselective for microorganisms. When incorporated into the "Friis Medium" it is used for growth of <i>Mycoplasma</i> species. PPLO Broth A selective medium Selenite Broth A selective medium Selenite Cystine Broth A selective medium Tetrathionate Broth Base A selective medium Thioglycollate Fluid Media A selective medium Thioglycollate with Indicator A selective medium Todd Hewitt Broth A selective medium Trypticase Soy Broth* A nonselective general purpose medium Trypticase Soy Agar* A nonselective general purpose medium Tryptose Phosphate Broth A nonselective general purpose medium Brilliant Green Bile (2%) Broth This medium is used to detect or confirm the presence of members of the coli-aerogenes group; the brilliant green content suppresses anaerobic lactose fermenters, such as <i>Clostridium perfringens</i> , and the medium is recommended for the 44°C confirmatory test for <i>Escherichia coli</i> . Brilliant Green Agar A selective medium for the isolation of <i>Salmonella</i> , other than <i>Salmonella typhi</i> Antibiotic Medium 1 A medium recommended for the seed layer in the preparation of plates for the microbiological assay of antibiotics Antibiotic Medium 3 Assay broth used in the serial dilution assay of penicillin and other antibiotics Müller Hinton agar A selective and diagnostic medium for the isolation and enumeration of <i>Bacillus cereus</i> Müller Hinton broth <i>Bacillus cereus</i> Selective Medium – PEMBA <i>Bacillus cereus</i> Selective Agar (Mossel) Baird Parker Base-Agar

(Continued)

Table 25.2 (Continued)

Test/product	Intended use
<i>Burkholderia cepacia</i> Agar Base	A medium for the selective isolation of <i>Burkholderia cepacia</i> from the respiratory secretions of patients with cystic fibrosis and for routine testing of non-sterile inorganic salt solutions containing preservative
Clostridia Differential Agar	Medium used for the cultivation and enumeration of clostridia in food and other materials of sanitary importance
Reinforced Clostridia Agar	
Bile Esculin Agar	A differential medium for the isolation and presumptive identification of enterococci/Group D streptococci
Bile Esculin Azide Agar	A selective medium
Brucella Agar Base	A selective medium
Cetrimide Agar Base*	A selective medium for the isolation of <i>Pseudomonas</i> species
CLED Medium (Cystine-Lactose-Electrolyte Deficient media)	Recommended for diagnostic urinary bacteriology. The medium supports the growth of all urinary potential pathogens giving good colonial differentiation and clear diagnostic characteristics
GC Agar Base	A selective medium for the isolation of <i>Neisseria</i> species
Hc Agar Base	
Clostridia Medium Duty*	A selective medium
Columbia Agar Base*	
EMB Levine Agar	A selective medium
Lowenstein-Jensen Medium Base	A selective medium
Hektoen Enteric Agar	A differential, selective medium for the isolation of <i>Shigella</i> and <i>Salmonella</i> species from enteric pathological specimens
Mold And Yeast Medium	It is used to cultivate mold and yeast
M-Green Yeast And Mold Broth	
Mt.Ge Broth	
Salt Manitol Agar*	A selective medium for the isolation of <i>Staphylococcus aureus</i>
Oxford Agar Base Modified	
Agar Base Palcam	
Potato Glucose Agar*	
Proteose Peptone No. 3 Agar	
<i>Pseudomonas</i> Isolation Agar	A selective medium for <i>Pseudomonas</i> spp.
R2a Agar	
Plate Count Agar	
Sabouraud Dextrose Broth*	It is used to cultivate dermatophytes and other types of fungi
Sabouraud Dextrose Agar	It is used to cultivate dermatophytes and other types of fungi
Sabouraud Dextrose Agar Antibiotics*	It is used to cultivate dermatophytes and other types of fungi
Salmonella Shigella Agar	A differential selective medium for the isolation of <i>Salmonella</i> and some <i>Shigella</i> species from clinical specimens and foods
Blood Agar Base	A non-selective general purpose medium which may be enriched with blood or serum.
Blood Agar Base (with Azide)	
TCBS Medium	A selective isolation medium for pathogenic vibrios
Violet Red Bile Agar	A selective isolation medium
Violet Red Bile Glucose Agar	A selective isolation medium
Vogel Johnson Agar	A selective isolation medium
Acetate Differential Agar	Culture media for identification tests
Tryptone Water	
Sugar Basal Medium (streptococci)	
BAM with Urea Medium	
Christensen Medium (Urea Agar Base)	
DNase Agar	
Enterococci Hypertonic Medium	
Phenylalanine Agar	
Giolitti Cantoni Medium	
Glucose Nitrate Medium	
Kligler Iron Agar	
Lysine Iron Agar	
MIO Medium	
Möeller Decarboxylase Medium Base	

Dehydrated
culture media
for
identification
tests

Table 25.2 (Continued)

Test/product	Intended use
	Motility Agar
	MR-VP Medium
	Hugh and Leifson Basal Medium
	Pseudomonas Agar F
	Pseudomonas Agar P
	SIM Medium
	Simmons Citrate Agar
	TSI Agar
	Urease Test Medium
Transport medium	Stuart Medium
	Stuart Agar
	Amies Medium
	Amies with or without Charcoal Agar
	Cary-Blair Gel

DIA: dot immunoassay; ASO: antistreptolysin O.

* Culture media formulations in compliance with the requirements of Harmonization of the European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP) and United States of America Pharmacopoeia (USP).

25.2.6 Abridged procedure

The “abridged procedure” is a shortened procedure that is used to register products that have low risk (Classes A and B). These products are to be used in laboratories by professionals only.

The manufacturer attests that its IVD complies fully with all applicable essential principles for safety and performance as documented in the “Declaration of Conformity” according to ANMAT resolution N° 2275/06 [11].

The products belonging to Classes A and B may follow the abridged procedure except for: neonatal screening, Down Syndrome, tumor markers, monitoring of therapeutic drugs, HLA System, and antibiotic susceptibility tests. These products should follow the regulatory procedure as declared by Resolution N° 2674/99.

25.2.7 National IVDs

Most IVDs are imported reagents, although there are Argentinian companies that manufacture IVDs and export them overseas. Some of these national IVDs are:

- Dehydrated culture media
- Culture media (ready-to-use) in plates, flasks or tubes.
- Transport medium
- Disks for antibiotics
- Immunoenzymatic assay (ELISA) for Chagas disease
- Immunoenzymatic assay (ELISA) HBV (HBsAg, anti-HBc)
- ELISA HCV
- ELISA HIV (4th generation)
- HTLV 1/2 ELISA
- Mononucleosis
- Dot ImmunoAssay HIV 1/2

- Immunochromatographic Rapid Tests for *Trypanosoma cruzi* Antibody (Chagas disease)
- HIV Immunochromatographic Rapid Tests
- VDRL test
- RPR slide test
- Syphilis ELISA (recombinant)
- HAI (Hemagglutination Inhibition) or latex test for toxoplasmosis
- HIV PCR assay
- HBV PCR assay
- HCV PCR assay
- HPV PCR assay

Dehydrated culture media formulations comply with the requirements of international Pharmacopoeia. Please note that other tests, devices and instruments manufactured in the United States, the EU and other countries may be used in healthcare settings in Argentina. Please refer to other chapters in this text for additional information. Also refer to ANMAT databases and other references and sites for specific vendors and contact information.

References

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- 2 ANMAT (home page). <http://www.anmat.gov.ar/>
- 3 Decreto 1490/92: Creación de ANMAT. http://www.anmat.gov.ar/webanmat/Legislacion/NormasGenerales/Decreto_1490-1992.pdf
- 4 VI Pan American Conference on Drug Regulatory Harmonization (CPANDRH) http://new.paho.org/hq/index.php?option=com_content&view=article&id=5101%3Avi-conferencia-panamericana-para-la-armonizaciun-de-la-reglamentaciun-farmacuoutica-cparf&catid=1156%3Ahss-pan-american-network-for-drug-re&Itemid=1685&lang=en

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Clinical Microbiology *In Vitro* Diagnostics in Australia: Regulatory Requirements and Product Information

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Australia uses the international definition of *in vitro* diagnostic devices (IVDs) (Directive 98/79/EC) [1] and Australian diagnostic microbiology laboratories use a mix of imported and in-house IVDs. While IVDs licensed overseas often have the advantage of prior rigorous testing (e.g., in North American and/or European jurisdictions) and published track records of clinical performance, regional variation and minor epidemiological differences can occasionally prove problematic. Australia manufactures relatively few IVDs and the majority of IVDs in use are produced overseas. However, in-house devices, i.e., devices “developed or modified by providers for use in their own laboratories”, are common.

Several organizations govern the development and implementation of diagnostic services in Australia, including imported and in-house IVDs:

- 1 TGA – Therapeutic Goods Administration
- 2 NPAAC – National Pathology Accreditation Advisory Council
- 3 NATA – National Association of Testing Authorities
- 4 RCPA QAP – Royal College of Pathologists of Australasia Quality Assurance Programs.

26.1 The Therapeutic Goods Administration

The Therapeutic Goods Administration is part of the Australian Government Department of Health, and oversees the regulatory control process on all therapeutic goods, including any medical devices manufactured, sold, and used in Australia [2]. The TGA authority over medical devices derives from the “Therapeutic Goods Act 1989”. With Food Standards Australia and New Zealand, providing for oversight of foodstuffs [3], the TGA is the Australian counterpart of the American Food and Drug

Administration. The main roles of the TGA are to provide standards and conformity assessment procedures, post-marketing activities, compliance testing and licensing, and to allow for adverse event reporting and recalls, as well as provision of access to unregistered IVDs in cases of special needs. The TGA specifies both performance and safety guidelines. Foreign IVD manufacturers must have an Australian sponsor sharing legal responsibilities and participating in registration of the device. Prior to market release, all IVDs must be registered on the Australian Register of Therapeutic Goods (ARTG) under standard Global Medical Device Nomenclature (GMDN). The TGA recovers operational costs through charges and fees to manufacturers and sponsors, as well as penalties for a range of offences from illegal importation, exportation, manufacture or supply of medical devices, to making false declarations (Therapeutic Goods Act 1989).

The TGA defines IVDs as “pathology tests and related instrumentation used to carry out testing on human samples, where the results are intended to assist in clinical diagnosis or in making decisions concerning clinical management”. All IVDs for use in diagnostic laboratories, at the point of care, and in the home come under TGA jurisdiction. The TGA’s regulation of IVDs is separate from that of other medical devices and carries its own specific classification system originally in line with the Global Harmonization Task Force (GHTF) (now replaced by the International Medical device Regulation Forum – IMDRF) [4]. IVDs classified and regulated by the TGA legislation must be explicitly intended for therapeutic use. Products without therapeutic purpose (e.g., tests for parentage and kinship testing; alcohol and illicit drugs tests) and those used solely in veterinary medicine are exempt from TGA regulation. As medical devices, all IVDs must comply for their entire lifespan with a series of

Essential Principles set out by the TGA for quality, safety and performance [5], and the manufacturer is responsible for ensuring compliance and must provide evidence of performance and safety of the product to the TGA. Prior to 2010, IVD regulation was limited with no form of pre-market examination. Since 2010, however, efforts have been in place to update the regulatory framework in order to standardize Australian legislation with international guidelines [6]. All established and yet-to-be developed IVDs, both commercial and in-house, from tests for serious infectious diseases, tests for screening blood, tissues and organs, through to home pregnancy tests and glucose monitors, are expected to be regulated under this new framework from July 2014.

26.1.1 IVD classification

The TGA's current legislative framework ensures both pre-market assessment and post-market control for all IVDs, with level of scrutiny based on the risk associated with each specific device. Both commercial and in-house IVDs are classified by the TGA based on the risk they pose and on their intended use [7]. Classification rules generally aligned with the GHTF guidelines and IVDs fall into four categories as follows:

Classification	Level of risk	Example
Class 1 IVD	No public health risk or low personal risk	Gram's staining solutions; general culture media
Class 2 IVD	Low public health risk or moderate personal risk	Viral tissue culture system; specific DFA assays
Class 3 IVD	Moderate public health risk or high personal risk	HIV genotyping; blood/CSF* testing
Class 4 IVD	High public health risk	blood supply screening; HIV diagnosis

* Any test where an erroneous result could lead to death or major injury, e.g., detection of malaria parasites in blood, cryptococcal antigen in CSF, etc.

The legislation requires each IVD to be classified separately when used in conjunction with other IVDs. In systems and procedure packs containing one or more IVDs along with non-IVD devices (calibration, quality control materials, etc.), the IVD with the highest class will determine the class of the overall pack.

26.1.1.1 Class 4

IVDs used for the detection of transmissible agents that present a high threat to the health of the Australian population belong to Class 4. Specifically, Class 4 includes IVDs used to detect:

- 1 transmissible agents in blood, blood components, blood products, cells, tissues or organs or any derivatives of these products of human or animal origin, in order to assess their suitability for transfusion or transplantation;

- 2 a transmissible agent that causes serious disease with a high risk of propagation in the Australian population.

Front-line or screening assays, confirmatory and supplemental assays, IVDs that detect structural components or surrogate markers of transmissible agents that cause serious disease, belong to this highest risk class. Intended use is also significant. For example, a syphilis assay will be a Class 4 IVD if used to screen blood and tissue donations, but Class 3 if used for diagnostic purposes only.

Tests to establish the safety of blood and blood components for use, such as exotoxin activity assays (pyrogenicity tests) when specifically used to detect bacterial contamination of blood, and IVDs used to diagnose clinical infections that cause serious disease with a high risk of transmission from person to person in the Australian population, are Class 4 IVDs, including tests for detection of severe acute respiratory syndrome-associated coronavirus (SARS-CoV), highly virulent pandemic influenza, viral hemorrhagic fevers such as Ebola virus, all assays to diagnose HIV-1 and 2 infection, and hepatitis C and B viruses.

26.1.1.2 Class 3

These IVDs are designed to identify a high individual risk, being tests aimed to provide a direct diagnosis, whereby an erroneous result would lead to an immediate threat to life, such as:

- 1 testing for notifiable diseases, and/or sexually transmitted diseases, with particular emphasis on the detection of gonorrhoea (*Neisseria gonorrhoeae*), chlamydia (*Chlamydia trachomatis*), syphilis (*Treponema pallidum*), donovanosis (*Klebsiella (Calymmatobacterium) granulomatis*), genital herpes (herpes simplex virus 2), lymphogranuloma venereum (*C. trachomatis* serovars L-1, L-2, L-3), genital warts (human papillomavirus) and trichomoniasis (*Trichomonas vaginalis*), are Class 3 IVDs (excluded are tests for HIV, HCV, and hepatitis B virus regarded as serious diseases and graded as Class 4);
- 2 detection of an infectious agent with a risk of limited propagation in cerebrospinal fluid or blood;
- 3 detection of an infectious agent where an erroneous result is likely to cause death or severe disability to an individual or fetus being tested (e.g., MRSA);
- 4 pre-natal screening of women in order to determine their immune status towards transmissible agents;
- 5 detection of biological markers to assess the immunological compatibility of blood, blood components, tissues or organs for transfusion or transplantation, if not classified higher, like transfusion antibodies not included in Class 4 (e.g. C^w or V from the Rhesus system, Cellano (k) from the Kell blood group system, any markers from MNS and Cartwright blood group systems);

- 6 determining infectious disease or immune status, when a therapy decision is to immediately follow diagnosis and when the device is not used for mere monitoring purposes (Class 2 IVD);
- 7 selecting patients for (i) selective therapy and management, (ii) disease staging, (iii) diagnosis of cancer;
- 8 human genetic testing – all devices are Class 3 IVDs (Philadelphia chromosome, Huntington's disease or cystic fibrosis IVDs), as are IVDs screening for congenital disorders (pre- and post-natal tests for trisomy, Klinefelter's syndrome, etc.) and any kits or individually supplied reagents intended to be used for the *in situ* hybridization of targeted gene polymorphisms;
- 9 monitoring levels of medicines, substances or biological components;
- 10 managing patients suffering from a life-threatening infectious disease;
- 11 self-testing IVDs, where the diagnosis has to be validated by a health professional and safe treatment supervised (blood glucose self-monitoring tests).

26.1.1.3 Class 2

Class 2 comprises devices that pose a low public health risk or moderate personal risk. The results from IVDs included in Class 2 are not meant to be used alone for a definitive correct diagnosis, and the likelihood that an erroneous test result will have a significant negative impact on patient outcome is negligible.

Class 2 IVDs are those used for/as:

- 1 monitoring a condition (e.g., total PSA detection) or initial screening for a condition (e.g., fecal occult blood screening test) where the result requires further investigation prior therapy;
- 2 self-testing devices not used to diagnose serious ailments or used to test preliminary conditions (i.e., pregnancy kits or urine self-test strips);
- 3 nonassay-specific quality control material (controls, calibrators, and standards not assigned for use with a specific assay);
- 4 detecting infectious agents that are not easily propagated in a population;
- 5 any other medical device not specified elsewhere in the legislation;
- 6 staining solutions intended for a specific diagnostic purpose.

The manufacturer's specified intended use for a reagent, stain, or kit becomes a very important discriminating factor in classification of low-risk IVDs. Although general reagents not manufactured or supplied specifically for *in vitro* diagnostics are not considered IVDs, separately supplied reagents labeled and intended for use with a specified diagnostic test or set of tests, or used specifically to determine a specific analyte/parameter are IVDs, classified according to the class of the assay/analyte/parameter

they are intended to be used with. For example, separately supplied reagents with an intended specified use with a specific microbial identification assay that determines the biochemical identification of clinical isolates, are Class 2 IVDs (e.g. labeled 3% hydrogen peroxide for use in a catalase test; tetramethyl-*p*-phenylenediamine (TMPD) for use in an oxidase test).

26.1.1.4 Class 1

Class 1 comprises devices, assays or reagents that pose low or no personal risk and no public health threat. Any IVD that has multidisciplinary use or is suitable for multiple diagnostic applications is considered a Class 1 IVD.

Class 1 IVDs are:

- 1 reagents and instruments intended specifically by the manufacturer to be suitable for *in vitro* diagnostic procedures across multiple disciplines and multipurpose;
- 2 specimen receptacles (e.g., vacuum-type except those intended for use in self-testing);
- 3 microbiological culture media (e.g., ready-to-use plain or containing selecting agents).

Staining solutions provided with instructions linking the stain to a particular staining process are classified on the basis of the overall intended purpose of the stain. Class 1 includes as general purpose reagents all single staining solutions supplied separately and without instructions, such as unspecified Gram iodine solution used in multiple disciplines such as clinical microbiology, histology, and cytology.

Manual, automated or semi-automated instruments (e.g. enzyme immunoassay analyzer, thermal cycler for performing nucleic acid amplification in a clinical specimen) are Class 1 IVDs. Nonassay specific reagents for use on a specific instrument are considered to be Class 1 IVDs. Microscope counting chambers suitable for use across multiple disciplines or in several different diagnostic applications (hemocytometer) specifically intended for the microscopic examination of body fluids are Class 1 IVDs.

Reagents (powders, agar bases), standard solutions (buffers, saline solution), general laboratory equipment (waterbaths, centrifuges, pipettes, laboratory tubes, plain ground-glass microscope slides) are not considered IVDs unless specifically labeled as intended for *in vitro* diagnostic use by the manufacturer. Quality control materials, instruments, and software specifically assigned for use with a specific assay are to be classified as the parent test based on the common intended purpose.

26.1.2 Alternative approach to licensing by the TGA if already licensed overseas

In order to ensure that an IVD and its manufacturer comply with Australian standards, conformity assessments are performed by the TGA. For all IVDs, except all

Class 1 IVDs and Class 2 and 3 in-house IVDs, the TGA requires one of the following as conformity assessment evidence:

- TGA Conformity Assessment Certificate – mandatory for some manufacturers and for IVDs in some risk classes (Australian manufacturers; Class 4 IVDs);
- EC certificates issued by an EU Notified Body under the EU *In vitro* Diagnostic Medical Device Directive (IVDD) 98/79/EC (mandatory for Class 2 and 3 IVDs);
- a compliance certificate for ISO 13485 (medical devices, quality management systems, requirements for regulatory purposes) issued by a recognized body including: those designated under the IVDD 98/79/EC; Canadian Medical Devices Conformity Assessment System (CMDCAS) recognised Registrar; a certification body accredited by a competent member of the International Accreditation Forum (IAF);
- for Class 3 IVDs supported by an ISO 13485 certificate, a Class III or IV Medical Device Active Licence Listing (MDALL) issued by Health Canada under the Canadian Medical Devices Regulations.

Australian conformity assessment requirements, although aligned with those of the EU and Canada, are unique, therefore the manufacturer and sponsor may be required to obtain additional evidence or undergo additional review upon applying for registration in the ARTG (particularly relevant for Class 3 IVDs developed overseas). Because the US classification system and the Australian regulations do not align, the TGA does not accept as evidence certificates from the US Food and Drug Administration (FDA). Manufacturers may also be required to present evidence relating to manufacturing process or device design. Manufacturers and sponsors must report adverse events or problems within a specified timeframe and post-marketing TGA surveillance continues with compliance testing, technical review, and certification review (certificates are valid for 5 years), as well as ad hoc review triggered by reports from health-care professionals and consumers.

26.2 National Pathology Accreditation Advisory Council

The National Pathology Accreditation Advisory Council (NPAAC) was established in 1979 as the body responsible for the accreditation of pathology laboratories in Australia, and determines the accreditation criteria for Australian pathology services [8].

The compulsory accreditation system related to Medicare Australia benefits for pathology was introduced in 1986. Quality standards and guidelines and other specific accreditation requirements are defined in the Health Insurance (Accredited Pathology Laboratories – Approval) Principles 2002. The NPAAC

develops accreditation policies and standards of practice, coordinates legislative and administrative functions for pathology services, implements educational programs on pathology laboratory practice and advises government on specific accreditation matters. The administrative aspects of pathology laboratory accreditation are carried out by The Department of Human Services (Medicare Australia). The National Association of Testing Authorities (NATA) conducts audits against accreditation standards and guidelines, and NATA reports are reviewed by Medicare Australia for determining access to the Medicare Benefits Scheme. Medicare rebates for eligible pathology tests are only available to facilities holding NATA accreditation in the specific area.

Medicare Australia rebates are only payable for approved pathology services performed in an accredited pathology laboratory owned by an approved pathology authority and provided by or on behalf of an approved pathology practitioner (e.g., a clinical microbiologist certified by the RCPA). Among the required accreditation criteria is performance in national quality assurance programs provided by the RCPA (see below) and for practitioners, appropriate qualifications under the auspices of the RCPA. The RCPA certifies medical professionals in a standard post-graduate medical microbiology training scheme (5 year fellowship by examination following completion of medical training and general registration as a medical practitioner, with requirement for yearly professional continuous development) and this is the usual qualification requirement for an approved pathology practitioner.

26.3 National Association of Testing Authorities

The NATA provides accreditation of laboratories, inspection bodies, calibration services, producers of certified reference materials, and proficiency testing scheme suppliers throughout Australia with the goal of improving “the business efficiency and competitiveness of Australian industry in international and national markets” [9]. Established in 1947, NATA was the world’s first comprehensive laboratory accreditation body and is still one of the largest. It is a nonprofit, mostly self-funded independent organization counting members and representatives from industry, government, and professional bodies. The NATA has memoranda with the Australian Government as well as mutual recognition agreements with United States and European bodies, acknowledging its prominent role in ensuring technical excellence in medical practices. The NATA represents Australia in the International Laboratory Accreditation Cooperation, the Asia Pacific Laboratory Accreditation Cooperation and the OECD Working Group [10]. NATA members are found both in Australia (>3000 facilities) and worldwide.

The Commonwealth Government relies on the NATA to meet its testing needs, encouraging State Governments and other agencies to use its facilities.

NATA accreditation of Australian laboratories provides the assurance of technical competence to perform specific testing, inspection, calibration, or other responsibilities. A yearly fee between AUD\$1000 and 6000, depending on type, must be paid to obtain accreditation, but a laboratory may have its accreditation withdrawn at any time or may be granted conditional status based upon redress of identified shortfalls in performance, documentation, training, or structural elements including physical facilities. It is a requirement of the Commonwealth Government that all its pathology laboratories pass NATA inspection in order to continue to provide diagnostic services. Accreditation is available for a variety of public health testing facilities (diagnostic imaging, biological testing, medical testing, etc.). The medical testing accreditation program is administered by the NATA and the RCPA (see below) and is open to facilities performing diagnostics in various fields of human pathology. Actual laboratory performance is assessed by the NATA to meet the NPAAC requirements as well as Australian and international standards for best practice. The national system of assessment comprises site visits to inspect quality assurance systems, laboratory training, and performance parameters. NATA inspectors are drawn from the professional body of diagnostic microbiologists (medical and nonmedical).

Other institutions involved in ensuring compliance of Australian facilities to standards of quality are Standards Australia [11], the National Measurement Institute (NMI) [12] and the Joint Accreditation System of Australia and New Zealand (JAS-ANZ) [13].

26.4 RCPA quality assurance programs

The training, licensing, and oversight of development of Australian pathologists, including competence to perform and supervise diagnostic testing, are carried out by the RCPA [14]. Quality Assurance Programs (QAP) for pathology laboratories are among the services offered by the RCPA to both Australian/New Zealand and overseas facilities. The NATA has a current Memorandum of Understanding with the RCPA for providing suitable QAP for accreditation of pathology laboratories. Participation in an appropriate NATA/RCPA scheme is a mandatory condition of accreditation for any Australian pathology laboratory since 1985. Separate programs for each pathology discipline (cytopathology, microbiology, serology, transfusion, etc.) are offered and QAP cover education and training of pathology staff, workshops, and seminars. The QAP offer support and consultation and provide quality assurance materials used for laboratory

training and procedural validation (test materials include detectable errors at all stages from sample labeling to assay performance).

The Microbiology QAP test the reliability of diagnostic methods against accreditation standards, and include specialized subprograms for bacteriology, virology, mycology, parasitology, mycobacteriology, molecular diagnostics, serology, urine dipstick chemistry, STAT microscopy, *Pneumococcal* serotyping, *Clostridium difficile* laboratory detection, VRE screening, and microbiological surveillance culture for endoscopes and bronchoscopes. The QAP are available to all laboratories in Australia, New Zealand and other countries and count approximately 500 participants worldwide, and specifically assess: (i) isolation and identification of pathogens in clinical samples; (ii) culture media; (iii) antibiotic susceptibility testing methods; (iv) microscopic examination of films of clinical specimens; (v) parasites and fungi identification; (vi) nucleic acid amplification methods. Participating laboratories select from the range of surveys and modules available. Program subscribers are supplied with clinical notes and directed to perform several surveys per year using their routine methodology and the sample organisms provided by the QAP. Within a set timeframe (e.g., 2 weeks for the urine dipstick chemistry and STAT, 3 weeks for the bacteriology, parasitology, and molecular diagnostics items, and 5 weeks for the mycology items), findings are compiled in a report and returned to QAP for scoring. All participants are given thorough reports and assessments for each survey, specific to the test material and modules used, as well as an end-of-year final report. Individual results and summaries of the data obtained from all responding laboratories are returned to each facility with ad hoc comments on performance, methods used, and sources of errors.

26.5 Manufacturers and suppliers of IVDs

In Australian pathology and clinical diagnostic laboratories, nucleic acid amplification testing (NAAT) has largely replaced other identification techniques [15–17]. The NAAT methods offer considerable advantages over traditional screening techniques, with higher sensitivity and considerably shorter time lags between detection and identification of infection and delivery of diagnosis to the patient. The NAAT technologies are often linked to fully integrated and automated screening platforms, provided by a number of different manufacturers (Table 26.1) [17], which allow the rapid processing of large numbers of samples simultaneously and efficiently. The NAAT assays are, for example, commonly employed in the detection of bloodborne viral infections (e.g., HIV, HCV, Epstein–Barr

Table 26.1 List of main manufacturers and suppliers of IVDs to Australian laboratories.*

IVD description		Manufacturers and Suppliers	
		Australian or in-house	Imported
General reagents (culture media identification discs, stains, reagents and chemicals, antibiotic discs and powder)		Australian Scientific Enterprise Edwards Group	Becton Dickinson (BBL-Difco) BioRad Don Whitley Scientific Hardy Diagnostics Hologic Merck Millipore
Microbiology instruments			ThermoFisher Scientific (Oxoid; Remel; life technologies; VersaTREK)
Specimen collection (vials, tubes, media)			Sigma-Aldrich
Cell Culture			
Anaerobic equipment			
Diagnostic bacteriology and mycobacteriology	Automated systems for blood culture, bacterial identification and antimicrobial susceptibility tests	In-house AusDiagnostics (Gram Positive 12 MT-PCR; Gram Negative) resistance MT-PCR; STD 9; vanR enterococcus)	Becton Dickinson (BACTEC System) bioMérieux SA (BAC T ALERT System; Vitek MS) Bruker (MALDI-Biotyper)
	Nucleic acid amplification tests for bacterial identification, quantitation and antibiotic resistance detection (<i>C. difficile</i> , enteric flora, streptococci, <i>H. pylori</i> , MRSA, mycobacteria, <i>N. gonorrhoeae</i> , <i>C. trachomatis</i>)	Genetic Signatures (GS EasyScreen)	Abbott Diagnostics Becton Dickinson (BD MAX System; BD GenOhm; Cepheid (GeneXpert System) Gen-Probe (APTIMA Combo 2; AccuProbe tests) Hain Lifescience (Geno Type Mycobacteria) ProbeTec ET and BD Viper System Sequenom (MLST)
Diagnostic virology	Immunodetection reagents/instruments point-of-care tests/ (influenza virus; RSV; HIV; enteric viruses; hepatitis B virus; human herpes virus; EBV)	In-house AusDiagnostics (respiratory virus screen)	Becton Dickinson Alere (Binax; Clearview) Chembio Diagnostic System Gaifar GmhB InTec Products Merck Millipore (Light Diagnostics) QUIDEL (Sofia Analyzer) Remel-ThermoFisher Scientific
	Nucleic acid amplification tests (coronavirus, CMV, adenovirus, EBV, enterovirus, herpes virus, HIV, influenza virus, measles, RSV, arboviruses, flaviviruses)		Abbott Diagnostics bioMérieux SA (NucliSens System) Cepheid (GeneXpert System) DiaSorin Nanogen (MGB Alert probes) Qiagen (Artus HBV assay) Roche Diagnostics (CAP/CTM v1.0; COBAS and Amplicor systems) Bayer-Siemens (Versant assays; TruGene HIV-1 assay)
Mycology	Automated systems for blood culture, bacterial identification and antimicrobial susceptibility testing Nucleic acid amplification tests (<i>Aspergillus</i> , <i>P. jirovecii</i> , <i>Candida</i>)	In-house AusDiagnostics	Becton Dickinson (BACTEC System; BD Affirm VPIII) bioMérieux SA (BacT/ALERT System) Becton Dickinson (BD Affirm VPIII) bioMérieux SA (NucliSens easyMag) Myconostica Roche
Parasitology	Immunoassays/ reagents/slides Nucleic acid amplification tests (<i>Cryptosporidium</i> , <i>Plasmodium</i> , <i>Giardia</i>)	In-house	Hardy Diagnostics (PARA-TECT) ThermoFisher Scientific (PROTOCOL vials; Remel products)
Serology*	Nucleic acid amplification tests (hepatitis; syphilis)	In-house	Becton Dickinson Roche Diagnostics
Bacterial subtyping and characterization	Rep-PCR technology	In-house	bioMérieux SA (DiversiLab)
	Automated systems for bacterial identification and antimicrobial susceptibility tests		Sequenom
	High-throughput nucleic acid sequencing		Illumina Life Technologies Roche

Table 26.1 (Continued)

IVD description		Manufacturers and Suppliers	
		Australian or in-house	Imported
Support for nucleic acid amplification and sequencing	Automated systems for nucleic acid extraction	Diagnostic Technology (Lab Turbo)	Abbott Diagnostics (m2000sp) bioMérieux SA (NucliSENS easyMag) Invitrogen (Iprep) Promega (Maxwel 1 16) Qiagen Roche Diagnostics (MagNA Pure) ThermoFisher Scientific (Kingfisher; Applied Biosystems technologies) Eppendorf Qiagen
	Thermal cyclers	Fisher Biotec	BioRad
	Gel visualization systems Real Time quantitative PCR (automated platforms, cyclers, probes, dyes, primers)	Fisher Biotec AusDiagnostics	Abbott Diagnostics Applied Biosystems-ThermoFisher Scientific (ABI Prism) Cepheid (SmartCycler) Life technologies-ThermoFisher Scientific (TaqMan and SYBR Green assays) Qiagen (TaqMan and SYBR Green assays; Rotor-Gene; Artus PCR kits) Roche Applied Science (LightCycler) Roche Molecular Systems (Amplicor and COBAS systems)

* This list is not inclusive of all manufactures and suppliers dealing in the Australian market. Other tests, instruments and methods may also be available from vendors and suppliers not listed here (please refer to vendor web sites and other chapters in this text for additional information).

virus, etc.) and for the determination of microbial loads (e.g., HCV, CMV). Both conventional PCR and RT-PCR-based methods are routinely used IVDs. Most “moving targets” nucleic acid based tests as well as more routine PCR-based assays are in-house IVDs in Australia, typically in reference laboratories in need to meet clinical diagnostic output and cost demands (e.g., respiratory virus testing, antibiotic resistance gene testing, etc.). Private pathology laboratories, less resourced for in-house test development, optimization and validation, will more readily elect commercially available and certified IVDs.

In-house IVDs are subject to regulation by the TGA and the NPAAC [18,19]. The change in requirements by the TGA in 2014 means that pathology providers have to either register an in-house assay (compliance requirements being quite expensive) or switch to commercial assays for which the cost of certification is borne by a large-scale manufacturer. In fact, with few exceptions, commercial IVDs in use in Australian pathology laboratories are manufactured overseas, although the small size of the Australian market may make compliance requirements unattractive for some overseas manufacturers. However, commercial assays that choose not to register with the TGA and pay the Australian registration fee can still be licenced as in-house assays by an end-user. In this case, the licence is user-specific and the pertaining TGA approval cannot be generalized for use of the aforementioned assay in another laboratory. IVD Australia is a

not-for profit company formed in 2009, representing Australian sponsors and manufacturers of IVDs, with the sole aim of promoting the safe and effective use of high-quality IVDs within the Australian community and of advancing the interests of its members with both local and overseas governments and institutions [20]. Several of the main manufacturing and distributing companies sharing the Australian market are registered as members with IVD Australia [21].

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Clinical Microbiology *In Vitro* Diagnostic Devices in Canada: Regulatory Requirements and Product Information

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In Canada, medical devices, including commercial *in vitro* diagnostic devices, are regulated by Health Canada, a department of the federal government, pursuant to the Food and Drugs Act [1] and the Medical Devices Regulations [2]. Within Health Canada, the Medical Devices Bureau, a division of the Therapeutic Products Directorate, serves as the national authority for monitoring and evaluating the safety, effectiveness, and quality of diagnostic and therapeutic medical devices before being authorized for sale in Canada. The Therapeutic Products Directorate ensures, to the extent possible, the safety, effectiveness, and quality of medical devices in Canada by a combination of premarket review, post-approval surveillance and documentation of quality systems in the manufacturing process. Under the Medical Devices Regulations all devices must meet safety and effectiveness requirements and must have a compliant label before they can be imported into, advertised, and/or sold in Canada.

Canada manufactures relatively few *in vitro* diagnostic devices. The majority of *in vitro* diagnostic devices used in the country are imported from manufacturers in the United States and Europe. Under the North American Free Trade Agreement (NAFTA), certain products, including most medical devices, that originate in Canada, Mexico, or the United States enjoy low or zero tariff rates when traded between these countries. In order to receive this preferential treatment, products that qualify must have a NAFTA Certificate of Origin.

In Canada, medical devices are assigned to one of four classes by the Therapeutic Products Directorate based on the risk associated with their use (Canadian Risk-Based Classification System). Class I (e.g., a thermometer, prepared bacterial culture media) medical devices are of lowest risk and Class IV (e.g., pacemakers, HIV diagnostics) are the highest risk. Prior to selling a device in Canada,

manufacturers of Class II, III and IV devices must obtain a Medical Device License. Class I devices do not require a Medical Device License but are monitored by Health Canada through Medical Device Establishment Licenses which allow importers, distributors, and manufacturers of Class I devices, who do not sell their products through a licensed importer or distributor, to operate in Canada. In general, Class II devices require that the manufacturer only attest to having safety and efficacy data on file; no premarket review is required. Following submission of a Medical Device License Application to Health Canada the length of review varies depending on the class of the device; Class III and IV license applications have a target review time of 75 days and 90 days respectively, and Class II license applications have a 15 calendar day target.

Canadian regulations require that medical devices be manufactured, or designed and manufactured, under a registered quality management system that meets the criteria of the Canadian National Standard CAN/CSA ISO 13485:03 *Medical devices – Quality Management Systems – Requirements for Regulatory Purposes*. The Canadian national standard version is modeled after the International Organization for Standardization (ISO) standard ISO 13485:2003 *Medical Devices – Quality Management Systems – Requirements for Regulatory Purposes*. Third party auditing organizations (also called registrars) are formally recognized by the Minister of Health under Section 32.1 of the Medical Devices Regulations for the purpose of issuing quality management system certificates to manufacturers of Class II, Class III, or Class IV medical devices for regulatory purposes. Quality management system regulations do not apply to Class I medical devices. The official list of Health Canada-recognized third parties (registrars) is maintained on the Health Canada website [3].

The Therapeutic Products Directorate also plays a role in monitoring medical devices after they are licensed to ensure their continued safety and effectiveness. If a medical device is found to no longer be safe and effective, its license can be suspended or the manufacturer may be requested to recall or refit the medical device. Manufacturers are required to ensure that medical devices meet safety and effectiveness requirements and to maintain data on these requirements. Manufacturers are likewise obligated to identify the risks inherent in the device, eliminate or reduce possible risk, and provide for appropriate protection from those risks. As an ongoing commitment to openness and transparency, Health Canada publishes summaries of its safety reviews on its website [4].

Under the Medical Devices Regulations, manufacturers, importers, and distributors of medical devices are required to maintain distribution records for each device sold in Canada [4]. The Medical Devices Regulations require that the distribution records contain sufficient

information to permit a complete and rapid withdrawal of the device from the market. The distribution records requirements do not apply to retailers and healthcare facilities that distribute medical devices for use within that facility. Distribution records are to be kept for at least two years from the shipment of the device or for its projected useful life, whichever period is longer.

Manufacturers, importers, and distributors are also obliged to maintain records of reported problems related to the performance and safety of the device as well as actions taken in response to these incidents including recalls. A preliminary and final report to the Ministry of Health is required from the manufacturer and importer regarding any incident (occurring within or outside of Canada) regarding a device that is sold in Canada that is related to the failure of the device or the death or “serious deterioration of the state of health of a patient.”

Table 27.1 summarizes companies that are based, in whole or in part, in Canada that manufacture a commercial

Table 27.1 Companies that are based, in whole or in part, in Canada that manufacture a commercial diagnostic test, device, or reagent relevant to clinical microbiology laboratory testing

Company	Location	Diagnostic test, device or reagent manufactured
Becton Dickinson Diagnostics	Quebec City, Quebec	BD GeneOhm Cdiff BD GeneOhm A ACP BD GeneOhm MRSA ACP Lysis Kit BD GeneOhm Staph SR BD GeneOhm Strep B BD GeneOhm VanR BD MAX Cdiff BD MAX CRE BD MAX Enteric BD MAX GBS BD MAX MRSA BD MAX OSRs (Open Systems Reagents)
Dalynn Biologicals	Calgary, Alberta	Manufacture-prepared culture media, spot-test reagents, disks, prerduced anaerobically sterilized transport media, supplements, and enrichments for media
Pro-Lab Diagnostics	Richmond Hill, Ontario	Prolex Streptococcal Grouping Latex Kits Prolex Blue Staph Latex Kits Prolex Blue <i>E. coli</i> Latex Test Reagent Kits Vision <i>Salmonella</i> Slide Agglutination Antisera Vision <i>Shigella</i> Slide Agglutination Antisera Prolisa Enzyme Immunoassay System Selective Microbiological Media Supplements Mucolyse Sputum Digestant <i>Legionella</i> Direct Fluorescent Antibody Kits and Reagents Prolex <i>Legionella</i> Latex Kit and Reagents Pro-Stains Microbiology Stains Prospot PYR Test Kit
Quelab Laboratories Inc.	Montreal, Quebec	Manufacture dehydrated culture media, prepared culture media, supplements, enrichments, stains, and reagents
Spectral Diagnostics Inc.	Etobicoke, Ontario	Endotoxin Activity Assay (EAA)
ThermoFisher Scientific (Oxoid Company)	Ottawa, Ontario	Manufacture-prepared culture media
Vista Technology, Inc.	Edmonton, Alberta	Automated petri plate streaker (Isoplater 180)

diagnostic test, device, reagent or instrument, relevant to clinical microbiology laboratory testing. This list was compiled by reviewing the Government of Canada website, under the section heading Industry Canada (<http://www.ic.gc.ca/app/ccc/sld/cmpny.do?>), which lists companies that import, distribute, and manufacture *in vitro* diagnostic devices and laboratory equipment and supplies for sale in Canada. The reader should refer to vendor web sites and other chapters in this text for additional information since other tests, devices reagents, and instruments may be available.

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Clinical Microbiology *In Vitro* Diagnostics in China: Regulatory Requirements and Product Information

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28.1 The regulatory requirements for clinical microbiology *in vitro* diagnostics products in China

28.1.1 Product classification and registration procedure

In China, the products for the laboratory diagnosis in clinical microbiology or infectious diseases belong to the category of *in vitro* diagnostics (IVD) and are regulated under “The Method for Registration and Administration of Medical Devices” issued by the State Food and Drug Administration (SFDA) of China according to “The Regulations for Supervision and Administration of Medical Device” [1]. The regulations are designated to ensure the safety and effectiveness of medical devices.

In the Method for Registration and Administration of Medical Devices, the IVD products are classified into three classes for each of the domestic and imported products, and each class is managed differently (Table 28.1) [2]. Nevertheless, the product classification is not completely unchangeable and flexibility exists among the classes, which is generally related to the general medical circumstances that are present at the time nationally (i.e., national medical priorities). In China, administrative services are conducted by the governments at different levels, i.e., the top level is the central government, the second level includes the governments of provinces, autonomous regions and municipalities directly under the central government, and the third level consists of the governments of the district; the latter are authorized by the governments at the second level. At the three levels, the SFDA and its branches are responsible for registration and administration of medical devices, and the authorizations are different depending upon the governmental

levels. Each class of products and the description of the regulation are described as follows [2,3]:

- Domestic Class I: those medical devices, the safety and effectiveness of which is guaranteed by a regular management procedure, shall be inspected, approved, and granted a registration certificate by the SFDA authority of the municipal government; examples of such devices are microbial culture media that are not used for microbial identification and susceptibility testing, i.e., nutrition and transportation media, and sample processing reagents, such as hemolytic agents, diluting liquid, and staining solutions.
- Domestic Class II: those medical devices, the safety and effectiveness of which is guaranteed, shall be inspected, approved, and granted a registration certificate by the SFDA authority of the provincial government; the products of Class II are reagents except those that have been clearly divided into Class III and Class I. Examples of such devices are the products used in detections of proteins, carbohydrates, hormones, enzymes, vitamins, and inorganic ions.
- Domestic Class III: those medical devices, implanted into the human body and used to support life, and products or reagents used for the detection of pathogenic microorganisms (including antigens, antibodies, and nucleic acids), blood type and tissue matching, human genes, narcotics, psychotropic and medically toxic drugs, therapeutic drug targets, tumor markers and allergens, shall be inspected, approved and granted a registration certificate by the SFDA of the central government.

All of the imported medical devices, including those from Taiwan, Hong Kong, and Macao, shall be inspected, approved, and granted a registration certificate essentially according to the approach for Class III products above.

Table 28.1 Materials required for registration application to the SFDA for IVD Products

Number	Material requirement	Domestic Class I	Domestic Class II	Domestic Class III	Imported Class I	Imported Class II	Imported Class III
1	Registration application form	✓	✓	✓	✓	✓	✓
2	Company Production license		✓	✓			
	Copy of business license	✓	✓	✓	✓	✓	✓
	Sales proof				✓	✓	✓
3	Product technical report		✓	✓		△	△
4	Risk analysis report		✓	✓		△	△
5	Product standard introduction	✓	✓	✓	✓	✓	✓
6	Product self-testing report	✓	✓	✓	△	△	△
7	Testing report for registration		✓	✓		✓/B	✓/B
8	Clinical testing data		✓	✓	✓	✓	✓
9	Medical device introduction	✓	✓	✓	✓	✓	✓
10	Production quality systematic inspection	✓/A	✓	✓	✓/A	✓	✓/C
11	Self-assurance statement	✓	✓	✓	✓	✓	✓
12	Quality assurance document				✓	✓	✓

✓, Required; A, company introduction about current production condition and quality management documentation (including testing methods); B, particular policies exists for high-end instruments; C, for the imported Class III products, extra requirements are included; △, for the imported products not yet approved by the original country or district, supplementary materials would be required.

The certificate of medical device registration shall be printed by the SFDA and the content filled in by the department within the SFDA responsible for the inspection. Upon its issue, the medical device certificate is valid for 4 years. In Figure 28.1 [4], a basic procedure for applying, inspecting, and approving medical device registration is briefly described and in Table 28.1 [4], the materials used in the process of registration application are listed.

28.1.2 Requirements and responsibilities for product certification [2]

- 1 The use of certificate: the certificate for medical device registration is attached with the registration record and they shall be used together.
- 2 Responsibility: the manufacturers that apply for the medical device registration shall take relevant legal responsibility and hold the certificate after approval.
- 3 Applicant: individuals who conduct the registration application should be authorized by the manufacturer, possess professional knowledge, and are familiar with the laws for medical device registration and administration as well as technical requirements.
- 4 Application for imported product registration: when the imported medical device registration is applied for, the overseas manufacturer shall authorize an assignee in China to handle the case and the latter should take relevant legal responsibilities. Meanwhile overseas manufacturers shall also select a qualified organization or authorize their own agency in China to facilitate the registered products in service.

- 5 Standard or reference for the products: the medical devices to be applied for registration shall have comparable and appropriate standards, which could be a national, industrial, or enrolled product standard, however, the latter should not be inferior to the former.
- 6 Qualification for the applicants: individuals who apply for Class II and Class III products shall be identified to possess the relevant manufacturing condition or quality system required by the SFDA.

28.2 IVD product review: devices and instruments

In Table 28.2, the products, devices, instruments, and reagents used for the laboratory diagnosis in clinical microbiology are reviewed with respect to the product name, registration authorities, and product identification numbers [5–14], producers/distributors, and contact information. For the products that are available from multiple companies, three are randomly selected as representative. Attempts were made to organize the products according to their clinical application. Most instruments used in China are imported from outside of China; and most of the reagents and media used in the China are manufactured within China. Many other products, devices, and tests manufactured in the United States, the European Union, and elsewhere may be available in China. Please refer to other chapters in this text and specific vendors and their web sites for additional information.

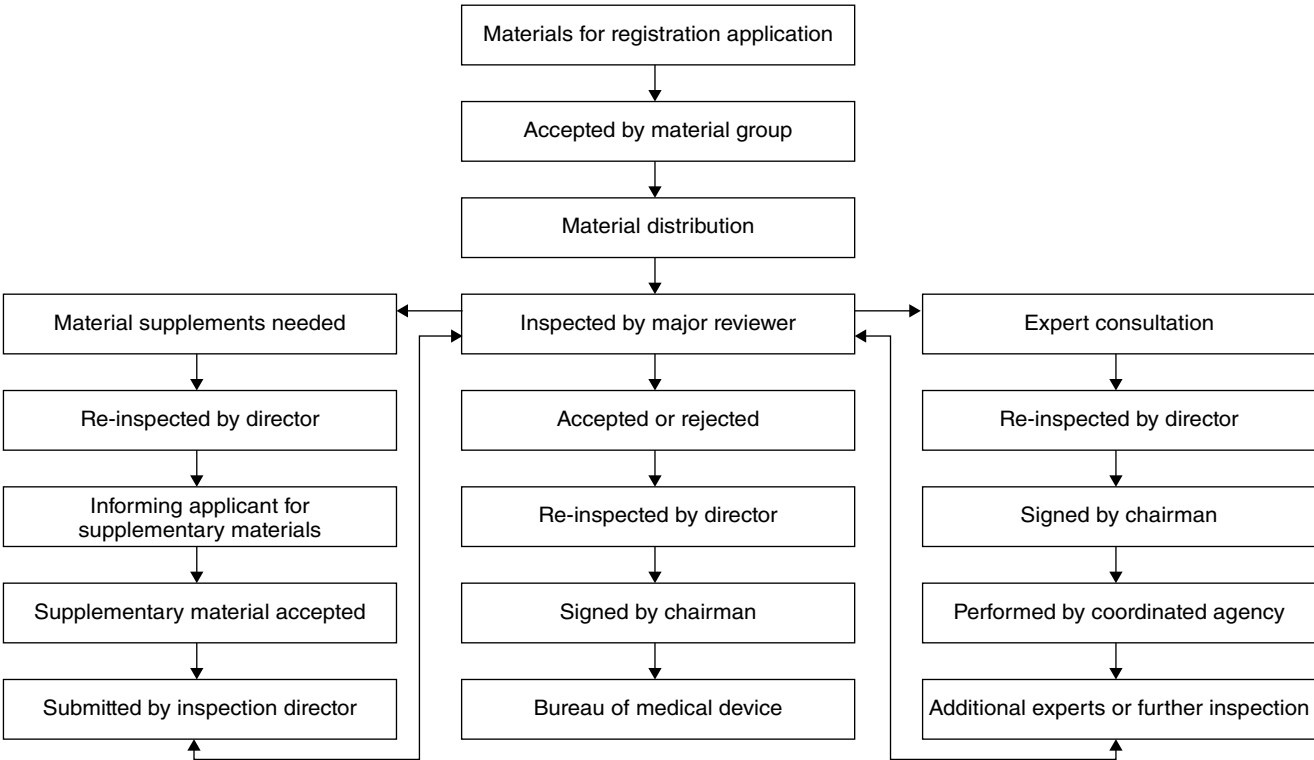


Figure 28.1 The basic procedure for registration application to the SFDA for IVD Products. As described above, two major reviewing steps must be completed: first, to examine all materials required for the application, second, to evaluate all products; this is conducted by the SFDA staff and relevant experts. In the SFDA at each of governmental level, the director or chief for the department has the authority to organize a committee of experts to review the application. The final approval for the product registration is signed by the SFDA chairman at each of the governmental levels.

Table 28.2 IVD product information for clinical microbiology testing in China: instruments and reagents*

Microorganism	Product	Type	Approval number		Company information†
			SFDA [5]	Local SFDA	
Bacteriology	Anaerobic chamber	I		(a) H-2008-2410523 [12] (b) Y-2012-2410089 [8]	(a), 1; (b), 2
	Automated blood culture system	I		(a) Y-2011-2400500 [8] (b) J-2012-2400316 [6] (c) Y-2012-2400155 [8] And others	(a), 3; (b), 4; (c), 5
	Automated rapid microbial mass spectrometry detection system (mass spectrometer)	I	2012-2402639		(a), 6 refer to other chapters in this text for additional vendors and information
	Bacterial culture system	I		(a) W-2010-2400116 [5] (b) W-2008-2400006 [5] (c) L-2011-2400103 [11] And others	(a), 7; (b), 8; (c), 9
	Bacterial identification/susceptibility analyzer	I	(a) 2009-3400236	(b) L-2010-2400250 [11] (c) W-2009-2400059 [5] And others	(a), 10; (b), 9; (c), 8
	Chemiluminescence immune analyzer (CLIA)	I		(a) J-2010-2401027 [6] (b) J-2010-2401030 [6] (c) Y-2011-2400260 [9] And others	(a), 11; (b), 12; (c), 13
	Chemiluminescence immune analyzer (automated)	I	(a) 2012-3401558 (b) 2012-3401267 (c) 2010-3400314	And others	(a), 14; (b), 15; (c), 14

Table 28.2 (Continued)

Microorganism	Product	Type	Approval number		Company information [†]
			SFDA [5]	Local SFDA	
	Chemiluminescence immune analyzer (semi-automated)	I		(a) J-2010-2400408 [6] (b) J-2012-2400023 [6]	(a), 16; (b), 17
	Chemiluminescence immune analyzer (upconverting phosphor technology,UPT)	I		J-2011-2400432 [6]	18
	ELISA reader	I		(a) S-2009-2400585 [10] (a) S-2010-2400431 [10] (b) S-2010-2400706 [10]	(a), 19; (b), 19; (c), 19
	ELISA reader (automated)			Y-2010-2400700 [8]	20
	<i>Helicobacter pylori</i> (Hp) tester	I		(a) Y-2010-2400370 [8] (b) Y-2010-2400157 [8] (c) Y-2010-2400156 [8]	(a), 21; (b), 21; (c), 21
	Immunity analyzer (IA)	I	(a) 2011-3401359 (b) 2009-2401783	(c) Y-2011-2400775 [8]	(a), 22; (b), 23; (c), 24
	Fluorescence immunity analyzer (FIA)	I	(a) 2012-2404084 (b) 2011-2402642	(c) J-2012-2400221 [6] And others	(a), 25; (b), 26; (c), 27
	Time-resolved fluorescence immunity analyzer (TRIFA)	I		(a) Y-2011-2400273 [8] (b) Y-2011-2400324 [8] (c) Y-2012-2400241 [8]	(a), 28; (b), 29; (c), 28
	Immunity analyzer (γ radiative)	I		J-2011-2330982 [6]	30
	Immunity analyzer (automatic infectious)	I		Y-2009-2400351 [8]	24
	Microplate Reader	I	(a)2010-2400660	(b) Y-2011-2400086 [8] (c) H-2009-2401121 [12] And others	(a), 31; (b), 32; (c), 33
	Anaerobic culture medium	R		Y-2010-2400014 [9]	34
	Anaerobic bacteria identification card	R		J-2010-2400036 [13]	35
	Bacterial identification/susceptibility analysis plate	R		(a) W-2012-2400051 [5] (b) W-2009-2400060 [5]	(a), 7; (b), 8
	Bacterial identification/susceptibility analysis kit came with analyzer	R		L-2010-2400251 [11]	9
	Blood culture medium	R		(a) H-2012-1400224 [12] (b) J-2009-1400022 [13] (c) Y-2009-2400017 [9] And others	(a), 6; (b), 38; (c), 34
	Chocolate Blood Agar Plate	R		(a) J-2012-1400227 [6] (b) Y-2010-2400068 [9] (c) H-2012-2400781 [12] And others	(a), 39; (b), 13; (c), 6
	<i>Clostridium tetani</i> antibody (IgG) quantitative detection kit (ELISA)	R	2011-3400138		40
	Diphtheria antibody (IgG) quantitative detection kit (ELISA)	R	2011-3400137		40
	<i>Helicobacter pylori</i> antibody detection kit (ELISA)	R	2012-3400365		41
	<i>Helicobacter pylori</i> antigen assay kit (ELISA)	R	(a) 2012-3400935 (b) 2009-3401036		(a), 42; (b), 43
	<i>Helicobacter pylori</i> detection kit (urease method)	R		(a) M-2012-2400039 [7] (b) H-2010-2400272 [12] (c) M-2011-2400096 [7] And others	(a), 44; (b), 45; (c), 46
	<i>Helicobacter pylori</i> diagnostic kit (pH indicator method)	R		(a) M-2010-2400044 [7] (b) Z-2010-2400367 [14]	(a), 44; (b), 47
	<i>Helicobacter pylori</i> IgG antibody detection kit (ELISA)	R	2012-3400936		42
	<i>Helicobacter pylori</i> nucleic acid detection kit (RT-PCR)	R	2011-3400106		48
	<i>Helicobacter pylori</i> (HP) rapid urease test card (biochemical method)	R		Y-2010-2400079 [8]	49
	<i>Helicobacter pylori</i> rapid assay kit (turbidimetry)	R		Y-2010-2400729 [8]	24

(Continued)

Table 28.2 (Continued)

Microorganism	Product	Type	Approval number		Company information†
			SFDA [5]	Local SFDA	
	Legionella (Lp) antigen detection kit (ELISA)	R		J-2010-2400039 [13]	35
	MRSA resistant gene detection kit (RT-PCR)	R	(a) 2010-3400274 (b) 2011-3400251		(a), 50; (b), 51
	<i>Neisseria gonorrhoeae</i> (NG) - ureaplasma urealyticum (UU) - <i>Chlamydia trachomatis</i> (CT) detection kit (ELISA)	R	2009-3400186		52
	Salmonella and <i>Shigella</i> detection kit (RT-PCR)	R	2010-3401081		53
	Antistreptococcal hemolysin "O" IgG/IgM antibody detection kit (ELISA)	R		J-2012-2400174 [6]	54
	Group B streptococci nucleic acid detection kit(RT-PCR)	R	2011-3400250		51
	<i>Treponema pallidum</i> antibody detection kit (CLIA)	R	(a) 2012-3400963 (b) 2012-3401331 (c) 2012-3401667 And others		(a), 55; (b), 56; (c), 57
Mycobacteriology	<i>Mycobacterium tuberculosis</i> antimicrobial susceptibility test medium	R		(a) Y-2011-2400304 [9] (b) Z-2010-2400208 [14]	(a), 58; (b), 59;
	<i>Mycobacterium tuberculosis</i> drug resistant gene detection kit (DNA microarray method)	R	2010-3400383		60
	<i>Mycobacterium tuberculosis</i> IgG antibody diagnostic kit (ELISA)	R	(a) 2012-3401559 (b) 2011-3400657 (c) 2011-3401273 And others		(a), 41; (b), 13; (c), 61
	<i>Mycobacterium tuberculosis</i> nucleic acid diagnostic kit (RT-PCR)	R	(a) 2012-3401427 (b) 2012-3400660 (c) 2011-3400260 And others		(a), 62; (b), 63; (c), 64
	<i>Mycobacterium tuberculosis</i> nucleic acid and resistance gene detection kit	R	2011-3401573		65
	Quantitative diagnostic kit for <i>Mycobacterium tuberculosis</i> IFN- γ release assay	R	2012-3400557		55
	<i>Mycobacterium tuberculosis</i> rifampin resistant mutation detection kit (PCR-reverse dot blotting)	R	2011-3401177		66
	<i>Mycobacterium tuberculosis</i> staining solution	R		J-2011-1400965 [6]	67
	<i>Mycobacterium tuberculosis</i> specific antigen diagnostic kit (ELISA)	R	2011-3400656		13
Mycoplasmas	<i>Mycoplasma pneumoniae</i> IgG antibody detection kit (ELISA)	R	2012-3400230		68
	<i>Mycoplasma pneumoniae</i> IgM antibody detection kit (ELISA)	R	(a) 2011-3400235 (b) 2012-3400920 (c) 2012-3404707		(a), 69; (b), 70; (c), 71
	<i>Mycoplasma pneumoniae</i> nucleic acid quantitative detection kit (RT-PCR)	R	(a) 2012-3400105 (b) 2011-3400808 (c) 2012-3400008		(a), 48; (b), 62; (c), 42
Chlamydia	<i>Chlamydia pneumoniae</i> IgG antibody detection kit (ELISA)	R	(a) 2012-3400541 (b) 2012-3400943		(a), 72; (b), 56
	<i>Chlamydia pneumoniae</i> IgM antibody detection kit (ELISA)	R	(a) 2012-3400919 (b) 2011-3401272 (c) 2012-3400542		(a), 70; (b), 56; (c), 72
	<i>Chlamydia trachomatis</i> nucleic acid detection kit (RT-PCR)	R	(a) 2012-3401193 (b) 2012-3400901 (c) 2011-3400537 And others		(a), 48; (b), 73; (c), 42

Table 28.2 (Continued)

Microorganism	Product	Type	Approval number		Company information [†]
			SFDA [5]	Local SFDA	
Virology	Bunyaviridae (in fever with thrombocytopenia syndrome) DNA detection kit (RT-PCR)	R	2011-3401666		48
	Coxsackie virus A16 IgM antibody detection kit (ELISA)	R	2011-3400427		55
	Coxsackie virus A16 nucleic acid detection kit (RT-PCR)	R	(a) 2012-3401684 (b) 2009-3400549 (c) 2012-3400224 And others		(a), 74; (b), 48; (c), 72
	Coxsackie virus A16, enterovirus 71 and universal enterovirus nucleic acid detection kit (RT-PCR)	R	(a) 2012-3400079 (b) 2010-3401300		(a), 33; (b), 72
	Coxsackie virus A16 and enterovirus 71 nucleic acid detection kit (RT-PCR)	R	(a) 2011-3401031 (b) 2012-3401035		(a), 75; (b), 76
	Coxsackie virus (group B) IgG antibody detection kit (ELISA)	R	2012-3400570		41
	Coxsackie virus (group B) IgM antibody detection kit (ELISA)	R	2012-3400604		41
	CMV (Cytomegalovirus) IgG antibody detection kit (CLIA)	R	(a) 2011-3401510 (b) 2010-3400006 (c) 2010-3400635 And others		(a), 12; (b), 17; (c), 78
	CMV (Cytomegalovirus) IgG antibody detection kit (ELISA)	R	(a) 2012-3403299 (b) 2013-3400011 (c) 2010-3403463 And others		(a), 79; (b), 77; (c), 80
	CMV (Cytomegalovirus) IgM antibody detection kit (CLIA)	R	(a) 2010-3400636 (b) 2009-3400167 (c) 2009-3400539 And others		(a), 78; (b), 79; (c), 81
	CMV (Cytomegalovirus) IgM antibody detection kit (ELISA)	R	(a) 2011-3400684 (b) 2012-3400927 (c) 2011-3400238 And others		(a), 55; (b), 82; (c), 69
	CMV (Cytomegalovirus) nucleic acid quantitative detection kit (RT-PCR)	R	(a) 2011-3400390 (b) 2012-3400007 (c) 2012-3400950 And others		(a), 48; (b), 42; (c), 33
	EB virus EA IgA antibody diagnostic kit (ELISA)	R	2010-3400636		83
	EB virus EA IgG antibody diagnostic kit (ELISA)	R	(a) 2011-3400640 (b) 2012-3400571 (c) 2010-3402926		(a), 13; (b), 55; (c), 83
	EB virus IgA antibody diagnostic kit (ELISA)	R	2009-3400720		84
	EB virus NA1 IgA antibody diagnostic kit (ELISA)	R	(a) 2012-3400206 (b) 2011-3401499		(a), 85; (b), 85
	EB virus NA1 IgG antibody diagnostic kit (ELISA)	R	(a) 2012-3400573 (b) 2011-3401217		(a), 41; (b), 83
	EB virus nucleic acid quantitative detection kit (RT-PCR)	R	2009-3400253		48
	EB virus Rta IgG antibody diagnostic kit (ELISA)	R	2009-3400893		70
	EB virus (VCA) IgA antibody diagnostic kit (ELISA)	R	(a) 2012-3400446 (b) 2011-3400814 (c) 2011-3400252 And others		(a), 86; (b), 87; (c), 88
	EB virus VCA IgG antibody diagnostic kit (ELISA)	R	(a) 2012-3400572 (b) 2010-3400151		(a), 41; (b), 83

(Continued)

Table 28.2 (Continued)

Microorganism	Product	Type	Approval number		Company information [†]
			SFDA [5]	Local SFDA	
	EB virus VCA IgM antibody detection kit (ELISA)	R	(a) 2012-3400569 (b) 2009-3402690	(c)Y-2012-2400079 [8]	(a), 41; (b), 83; (c), 85
	EB virus Zta IgA antibody diagnostic kit (ELISA)	R	2012-3400691		85
	Enterovirus 71 antibody IgG detection kit (ELISA)	R	2010-3400412		41
	Enterovirus 71 IgM antibody detection kit (ELISA)	R	(a) 2010-3400411 (b) 2010-3400526		(a), 41; (b), 55
	Enterovirus 71 nucleic acid detection kit (RT-PCR)	R	(a) 2009-3400551 (b) 2012-3401686 (c) 2010-3400272 And others		(a), 48; (b), 74; (c), 50
	Enterovirus CoxA16 nucleic acid detection kit (RT-PCR)	R	2012-3400417		63
	Enterovirus nucleic acid (universal) detection kit (RT-PCR)	R	(a) 2009-3400550 (b) 2012-3401206 (c) 2012-3400418 And others		(a), 48; (b), 76; (c), 63
	Epidemic hemorrhagic fever virus IgM antibody detection kit (ELISA)	R	2012-3400894		89
	Hantavirus IgM antibody detection kit (ELISA)	R	2011-3400277		55
	HAV (Hepatitis A virus) IgG antibody diagnostic kit (ELISA)	R	(a) 2011-3401291 (b) 2012-3401204		(a), 90; (b), 91
	HAV (Hepatitis A virus) IgM antibody diagnostic kit (CLIA)	R	(a) 2009-3400896 (b) 2012-3401332		(a), 17; (b), 56
	HAV (Hepatitis A virus) IgM antibody diagnostic kit (ELISA)	R	(a) 2012-3401180 (b) 2012-3400451 (c) 2012-3400804 And others		(a), 55; (b), 33; (c), 92
	HBcAb diagnostic kit (CLIA)	R	(a) 2011-3400832 (b) 2012-3400914 (c) 2012-3401723 And others		(a), 93; (b), 14; (c), 94
	HBcAb diagnostic kit (ELISA)	R	(a) 2012-3400741 (b) 2011-3400939 (c) 2012-3401092 And others		(a), 33; (b), 55; (c), 42
	HBcAb diagnostic kit (TRIFA)	R	(a) 2010-3400972 (b) 2010-3400197		(a), 95; (b), 28
	HBeAb diagnostic kit (ELISA)	R	(a) 2009-3400577 (b) 2012-3400739 (c) 2012-3401187 And others		(a), 85; (b), 33; (c), 13
	HBeAb diagnostic kit (TRIFA)	R	(a) 2010-3400968 (b) 2010-3400198		(a), 95; (b), 28
	HBeAg diagnostic kit (ELISA)	R	(a) 2012-3400738 (b) 2012-3401746 (c) 2010-3400462 And others		(a), 33; (b), 41; (c), 42
	HBeAg diagnostic kit (TRIFA)	R	(a) 2010-3400967 (b) 2012-3401024 (c) 2010-3400199		(a), 95; (b), 29; (c), 28
	HBsAb diagnostic kit (CLIA)	R	(a) 2011-3400828 (b) 2011-3401348 (c) 2012-3400005 And others		(a), 93; (b), 17; (c), 89
	HBsAb diagnostic kit (ELISA)	R	(a) 2012-3400738 (b) 2012-3401412 (c) 2012-3401182 And others		(a), 33; (b), 56; (c), 55

Table 28.2 (Continued)

Microorganism	Product	Type	Approval number		Company information [†]
			SFDA [5]	Local SFDA	
	HBsAb diagnostic kit (TRIFA)	R	(a) 2010-3400022 (b) 2011-3400245		(a), 96; (b), 28
	HBsAg diagnostic kit (CLIA)	R	(a) 2011-3400831 (b) 2011-3401621 (c) 2011-3400931 And others		(a), 93; (b), 89; (c), 97
	HBsAg diagnostic kit (TRIFA)	R	(a) 2010-3400023 (b) 2011-3400244		(a), 28; (b), 96
	HBV nucleic acid detection kit (RT-PCR)	R	(a) 2012-3401194 (b) 2011-3400145 (c) 2012-3401205 And others		(a), 48; (b), 98; (c), 74
	HBV PreS1-Ab diagnostic kit (ELISA)	R	2012-3400447		99
	HBV PreS1-Ag diagnostic kit (CLIA)	R	(a) 2010-3401082 (b) 2009-3401054 (c) 2009-3400898		(a), 57; (b), 100; (c), 17
	HBV PreS1-Ag diagnostic kit (ELISA)	R	(a) 2012-3401745 (b) 2012-3400415 (c) 2012-3400328 And others		(a), 41; (b), 90; (c), 101
	HCV (Hepatitis C virus) antibody detection kit (CLIA)	R	(a) 2012-3400702 (b) 2012-3400357 (c) 2012-3400403 And others		(a), 55; (b), 100; (c), 102
	HCV (Hepatitis C virus) core antigen detection kit (ELISA)	R	(a) 2012-3401179 (b) 2011-3401278		(a), 103; (b), 104
	HCV (Hepatitis C virus) core antigen/antibody detection kit (ELISA)	R	2012-3404425		105
	HCV (Hepatitis C virus) nucleic acid Quantitative Detection Kit (RT-PCR)	R	(a) 2012-3400209 (b) 2011-3400144 (c) 2011-3400109 And others		(a), 48; (b), 98; (c), 73
	HDV (Hepatitis D virus) antigen detection kit (ELISA)	R	2010-3400126		41
	HDV (Hepatitis D virus) IgG antibody diagnostic kit (ELISA)	R	2012-3400893		55
	HDV (Hepatitis D virus) IgM antibody diagnostic kit (ELISA)	R	(a) 2010-3400128 (b) 2012-3400895		(a), 41; (b), 55
	HEV (Hepatitis E virus) IgG antibody diagnostic kit (CLIA)	R	2012-3401113		56
	HEV (Hepatitis E virus) IgG antibody diagnostic kit (ELISA)	R	(a) 2010-3403403 (b) 2010-3400003 (c) 2012-3401611 And others		(a), 106; (b), 81; (c), 107
	HEV (Hepatitis E virus) IgG /IgG antibody diagnostic kit (ELISA)	R	2010-3400003		81
	HEV(Hepatitis E virus) IgM antibody diagnostic kit(CLIA)	R	2012-3401112		56
	HEV(Hepatitis E virus) IgM antibody diagnostic kit(ELISA)	R	(a) 2012-3400744 (b) 2012-3401299 (c) 2012-3401739 And others		(a), 33; (b), 56; (c), 41
	HEV(Hepatitis E virus) RNA detection kit (FQ-PCR)	R	2009-3400559		108
	HIV (Human immunodeficiency virus) antibody detection kit (CLIA)	R	(a) 2012-3400902 (b) 2012-3401501 (c) 2012-3401727 And others		(a), 56; (b), 17; (c), 57
	Human immunodeficiency virus antigen / antibody detection kit (CLIA)	R	(a) 2011-3401612 (b) 2010-3400629		(a), 55; (b), 100

(Continued)

Table 28.2 (Continued)

Microorganism	Product	Type	Approval number		Company information†
			SFDA [5]	Local SFDA	
	HIV-I (Human immunodeficiency virus) nucleic acids detection kit (PCR-chip electrophoresis)	R	2008-3401471		109
	HIV-I (Human immunodeficiency virus) nucleic acids quantitative detection kit (Nest-PCR)	R	2012-3401095		48
	HIV-I (Human immunodeficiency virus) nucleic acid quantitative detection kit (RT-PCR)	R	2012-3400077		48
	HIV-I (Human immunodeficiency virus) urine antibody detection kit (ELISA)	R	2012-3400441		110
	HIV-III (Human immunodeficiency virus) antibody detection kit (CLIA)		2011-3402742		111
	HPV (Human papillomavirus) nucleic acid detection kit (RT-PCR)	R	(a) 2009-3401039 (b) 2012-3404427		(a), 73; (b), 112
	HPV-6, HPV-11 nucleic acid detection kit (RT-PCR)	R	(a) 2011-3400033 (b) 2012-3400695 (c) 2011-3400535		(a), 50; (b), 51; (c), 42
	HPV-16, HPV-18 nucleic acid detection kit (RT-PCR)	R	(a) 2011-3400113 (b) 2011-3400121		(a), 33; (b), 50
	HPV-16, HPV-18, HPV-6B and HPV-11 nucleic acid detection kit (PCR-fluorescence polarization)	R	2012-3401606		113
	HR-HPV (High-risk HPV) nucleic acid detection kit (flexible multi-analyte profiling, xMAP)	R	2009-3400020		114
	HR-HPV (High-risk HPV, 8 types) nucleic acid quantitative detection kit (RT-PCR)	R	2012-3400104		48
	HR-HPV (High-risk HPV, 13 types) nucleic acid assay kit (RT-PCR)	R	(a) 2011-3400139 (b) 2011-3400935 (c) 2011-3401502		(a), 50; (b), 115; (c), 116
	HR-HPV (High-risk HPV, 14 types) nucleic acid detection kit (Hybrid Capture-chemiluminescence, HCCL)	R	2012-3401210		73
	HR-HPV (High-risk HPV, 14 types) nucleic acid detection kit (RT-PCR)	R	(a) 2012-3401448 (b) 2012-3401687		(a), 116; (b), 117
	LR-HPV (Low-risk HPV) nucleic acid detection kit (RT-PCR)	R	2012-3400467		115
	HSV-(Herpes Simplex virus) IgG antibody detection kit (CLIA)	R	(a) 2010-3400637 (b) 2010-3400007 (c) 2011-3401511		(a), 78; (b), 17; (c), 12
	HSV-(Herpes Simplex virus) IgG antibody detection kit (ELISA)	R	(a) 2012-3403300 (b) 2013-3400012 (c) 2012-3400556 And others		(a), 79; (b), 77; (c), 118
	HSV-(Herpes Simplex virus) IgM antibody detection kit (CLIA)	R	(a) 2010-3400641 (b) 2010-3400177 (c) 2011-3401508		(a), 41; (b), 77; (c), 82
	HSV-(Herpes Simplex virus) IgM antibody detection kit (ELISA)	R	(a) 2012-3403314 (b) 2013-3400010 (c) 2012-3400922 And others		(a), 79; (b), 17; (c), 12
	HSV-(Herpes Simplex virus) nucleic acid quantitative detection kit (RT-PCR)	R	(a) 2012-3400653 (b) 2012-3401683		(a), 51; (b), 74
	HSV-(Herpes Simplex virus) IgA antibody detection kit (ELISA)	R	2012-3403312		79
	HSV-(Herpes Simplex virus) IgG antibody detection kit		2010-3401496		105
	HSV-(Herpes Simplex virus) IgG antibody detection kit (ELISA)	R	(a) 2011-3400093 (b) 2012-3403369 (c) 2011-3400658		(a), 119; (b), 120; (c), 81

Table 28.2 (Continued)

Microorganism	Product	Type	Approval number		Company information [†]
			SFDA [5]	Local SFDA	
	HSV-(Herpes Simplex virus) IgM antibody detection kit (CLIA)	R	2010-3400003		12
	HSV-(Herpes Simplex virus) IgM antibody detection kit (ELISA)	R	(a) 2010-3401267 (b) 2010-3400686 (c) 2012-3403848		(a), 119; (b), 42; (c), 121
	HSV-(Herpes Simplex virus) nucleic acid quantitative detection kit (RT-PCR)	R	2012-3401685		74
	HSV-(Herpes Simplex virus) IgG antibody detection kit (CLIA)	R	(a) 2010-3400642 (b) 2010-3400008 (c) 2011-3401506		(a), 82; (b), 17; (c), 12
	HSV-(Herpes Simplex virus) IgG antibody detection kit (ELISA)	R	(a) 2011-3400094 (b) 2013-3400009 (c) 2012-3403301 And others		(a), 119; (b), 77; (c), 79
	HSV-(Herpes Simplex virus) IgM antibody detection kit (CLIA)	R	(a) 2010-3400638 (b) 2010-3400176 (c) 2010-3400004		(a), 82; (b), 17; (c), 12
	HSV-(Herpes Simplex virus) IgM antibody detection kit (ELISA)	R	(a) 2012-3400364 (b) 2012-3400199 (c) 2012-3403313 And others		(a), 41; (b), 90; (c), 79
	HSV-(Herpes Simplex virus) nucleic acid quantitative detection kit (RT-PCR)	R	(a) 2012-3400750 (b) 2011-3400248 (c) 2012-3401033 And others		(a), 48; (b), 98; (c), 74
	Influenza A virus antigen detection kit (ELISA)	R	(a) 2010-3400974 (b) 2009-3400605 (c) 2010-3400739		(a), 55; (b), 122; (c), 33
	Influenza A virus nucleic acid diagnostic kit (RT-PCR)	R	(a) 2009-3400603 (b) 2011-3400097 (c) 2011-3400098		(a), 122; (b), 63; (c), 63
	Influenza A/B virus nucleic acid diagnostic kit (RT-PCR)	R	2012-3401296		76
	Influenza B virus antigen diagnostic kit (ELISA)	R	2009-3400606		122
	Influenza B virus nucleic acid diagnostic kit (RT-PCR)	R	2009-3400604		122
	Measles virus IgG antibody detection kit (ELISA)	R	(a) 2012-3401744 (b) 2011-3401187 (c) 2012-3401453 And others		(a), 41; (b), 91; (c), 40
	Measles virus IgM antibody detection kit (ELISA)	R	(a) 2009-3400903 (b) 2012-3401742 (c) 2008-3401413		(a), 41; (b), 41; (c), 119
	Novel coronavirus N-protein detection kit (ELISA)	R	2009-3400948		119
	Rabies virus IgG antibodies quantitative detection kit (ELISA)	R	(a) 2010-3400942 (b) 2012-3401452		(a), 13; (b), 40
	Respiratory syncytial virus nucleic acid detection kit (RT-PCR)	R	(a) 2009-3400722 (b) 2012-3400214		(a), 48; (b), 122
	Rotavirus diagnostic kit (ELISA)	R	(a) 2009-3400672 (b) 2011-3400240		(a), 123; (b), 69
	Rubella virus IgG antibody diagnostic kit (CLIA)	R	(a) 2010-3400640 (b) 2010-3400010 (c) 2011-3401509		(a), 78; (b), 17; (c), 12
	Rubella virus IgG antibody diagnostic kit (ELISA)	R	(a) 2012-3404434 (b) 2013-3400013 (c) 2010-3400687 And others		(a), 120; (b), 77; (c), 42
	Rubella virus IgM antibody diagnostic kit (CLIA)	R	(a) 2010-3400002 (b) 2010-3400639 (c) 2010-3400009		(a), 12; (b), 78; (c), 17

(Continued)

Table 28.2 (Continued)

Microorganism	Product	Type	Approval number		Company information [†]
			SFDA [5]	Local SFDA	
Parasitology	Rubella virus IgM antibody diagnostic kit (ELISA)	R	(a) 2012-3401180 (b) 2012-3400451 (c) 2012-3400804 And others		(a), 55; (b), 33; (c), 92
	SARS coronavirus IgG antibody detection kit (ELISA)	R	2009-3400883		63
	SARS coronavirus IgM antibody detection kit (ELISA)	R	2009-3400882		63
	Schistosoma circulating antigen, antibody assay kit (ELISA)	R	(a) 2009-3400456 (b) 2009-3400458		(a), 93; (b), 14
	Schistosoma japonicum IgG antibody diagnostic kit (ELISA)	R	2013-3400004		86
	Hydatid IgG antibody diagnostic kit (ELISA)	R	2011-3400241		119
	Toxoplasma IgG antibody diagnostic kit (CLIA)	R	(a) 2011-3401507 (b) 2010-3400740		(a), 12; (b), 78
	Toxoplasma IgG antibody diagnostic kit (ELISA)	R	(a) 2012-3400216 (b) 2012-3401211 (c) 2013-3400008 And others		(a), 89; (b), 118; (c), 77
	Toxoplasma IgM antibody diagnostic kit (CLIA)	R	(a) 2010-3400634 (b) 2010-3400509		(a), 78; (b), 12
	Toxoplasma IgM antibody diagnostic kit (ELISA)	R	(a) 2012-3400479 (b) 2012-3400928 (c) 2012-3400934 And others		(a), 41; (b), 82; (c), 118

I, instrument; R, Reagent.

* This table includes IVD products registered by the SFDA at each governmental level in China collected from references 4–14 at the time of this writing; for the items represented by three and more manufacturers or vendors, only three products are randomly listed independent of the product performance. This listing is not meant to endorse any specific vendor or product, only to demonstrate a snapshot of product availability in China. Please refer to other chapters in this text and other vendors and their web sites for additional product availability.

^{†1} Shanghai Yuejin Medical Device Factory. 6166 Yang Gaonan road, Kangqiao town, Shanghai, China.

² Guangzhou HiTech Photoelectricity Biotechnology Co., Ltd. Science Park, High & New Technology Development District, Guangzhou, 510665, China.

³ Zhuhai MeiHua Bio-medical technology Co., Ltd. No. 10 KejiYi Road, Tangjiawan Town, Zhuhai, China.

⁴ Beijing BoShengTai BioTech Co., Ltd. Zhongguancun Science & Technology Industrial Park, Tongzhou District, Beijing, China.

⁵ Zhuhai DiEr BioEngineering Co., Ltd. No. 19 Jinhaian Avenue, Sanzao Town, Zhuhai, China.

⁶ bioMérieux Diagnostics (Shanghai) Co., Ltd. Chemin de l'Orme 69280 MARCY L'ETOILE.

⁷ Hefei HengXing Technological Co., Ltd. No.11 Tianda Road, High-Tech Area, Hefei, China.

⁸ Hefei WeiXing Biotechnology Co., Ltd. No. 8 Tianda Road, High-Tech Area, Hefei, China.

⁹ Shandong Xinke Biological Technology Co., Ltd. Phoenix Industrial Park, Liaocheng, Shandong, China.

¹⁰ Hunan Changsha TianDiRen BioTech Co., Ltd. LuGu International Industrial Park, Tongzipo Road (West), Changsha, China.

¹¹ Beijing HaMaMatsu Photo Co., Ltd. No. 188 Nansihuan Road (West), Fengtai District, Beijing, China.

¹² Beijing TigSun Diagnostic Co., Ltd. No. 115 Fucheng Road, Haidian District, Beijing, China.

¹³ Autobio Diagnostics co., Ltd. No.87 Jingbei Yi Road National Eco & Tech Development Area, Zhengzhou, China.

¹⁴ Beijing Bio-Ekon Biotechnology Co., Ltd. Fl 03, Bldg 1, No. 1 Haiying Road, Fengtai District, Beijing 100070, China.

¹⁵ Beijing Leadman BioChemistry Co., Ltd. No.5 Hongda Road (South), Beijing Economic and Technological Development, District, Beijing, China.

¹⁶ Beijing DiaCha Bioengineering Co., Ltd. No.18 Keyuan Road, Daxing Returned Student Venture Park, Beijing, China.

¹⁷ Beijing Yuande Biomedical Engineering Co., Ltd. No.24 Yongchang Road (North), Beijing Economic and Technological, Development Zone, 100176, China.

¹⁸ Beijing HotGen Biotechnology Co., Ltd. Room301, NO. 27 Keyuan Road, Daxing District Beijing 102600 China.

¹⁹ Nanjing Huadong Electronics Group Co., Ltd. No.19 Hengtong Avenue, Economic Development Zone, Nanjing, China.

²⁰ Shenzhen Aikang Electronics Co., Ltd. Building B, Zhangkeng Ind. Park, Minkang Road, Shenzhen 518131, Guangdong, China.

²¹ Shenzhen Zhonghe Headway Bio-Sci & Tech Co., Ltd. High-Tech Industrial Park South District, Shennan Road, Shenzhen, China.

²² Japan FujiReBio Co., Ltd. Nihonbashi, the central area of Tokyo, Japan.

²³ JOKOH Co., Ltd. Kikugawa, Shizuoka, Japan.

²⁴ ShenZhen YHLO Biotech Co., Ltd. No. 5 Building, Lishan Industrial Area, Xinghai Road, Nanshan District, Shenzhen, China.

²⁵ NaEnTai INC. JiuLao District, Seoul, Korea.

²⁶ Wallac Oy Mustionkatu 6, FI-20750 Turku, Finland.

²⁷ Beijing BoHui Inovation Technology Co., Ltd. No. 25 Beiwucun Road, Haidian District, Beijing, China.

²⁸ Guangzhou Fenghua BioEngineering Co., Ltd. No. 6 Yinyi Street, Zhicheng Road, Economic and Technological Development. Area, Guangzhou, China.

- ²⁹ Guangzhou DaRui Antibody Engineering and Technology Co., Ltd. No.19 Xiangshan Road, Science Park, High & New, Technology Development District, Guangzhou, 510665, China.
- ³⁰ Beijing Heihai High Technological Co., Ltd. 7th Area, Hepingli, Chaoyang District, Beijing, China.
- ³¹ Biochrom Co., Ltd. 22 Cambridge science Park, Cambridge, CB4 0FJ England.
- ³² Shenzhen Mindray Bio-Medical Electronics Co., Ltd. Mindray Building, Nanshan High-Tech Industrial Park, Shenzhen, China.
- ³³ Shanghai Kehua Bio-engineering Co., Ltd. No. 1189 Qinzhou Road (North), Shanghai 200233, China.
- ³⁴ Beiruite Bio-technology (zhengzhou) Co., Ltd. No. 96 Ruida Road., Hi-tech Developing Area, Zhengzhou, China.
- ³⁵ Tianjin Reagent Biotech Co., Ltd. No 6 Erwei Road, Huayuan industrial District, Tianjin, 300384, China.
- ³⁶ Shanghai KeMaJia BioTech Co., Ltd. No. 500 Jinian Road, Shanghai, China.
- ³⁷ Shanghai Upper Biomedcial Co., Ltd. No. 526 Ruiqing Road, Zhangjiang Morden Medical Device Park, Shanghai, China.
- ³⁸ Tianjin JinZhang Technological Co., Ltd. No.95 Xihu Road, Nankai District, Tianjin, China.
- ³⁹ ThermoFisher (Shanghai) Instrumental Co., Ltd. No. 27 Jinqiao Road, Pudong District, Shanghai, China.
- ⁴⁰ Zhengzhou ETE Biotechnology Co., Ltd. No. 97 Yulan Street, Hi-tech Development Zone, Zhengzhou, 450001, China.
- ⁴¹ Beijing Beier Bioengineering Co., Ltd. Lucheng Industrial Zone of Daxing District, Beijing, 102612, China.
- ⁴² Acon Biotechnology (Hangzhou) Co., Ltd. No.398 Tianmushan Road, Hangzhou.
- ⁴³ Hebei XieHe Pharmaceutical Co., Ltd. Yanjiao Economic & Technological Development Area, Sanhe, Hebei Province, China.
- ⁴⁴ Fujian SanQiang BioChemistry Co., Ltd. No. 2 Changan Road, Sanyuan District, Sanming, China.
- ⁴⁵ Shanghai HuiTai Medical Technology Co., Ltd. No. 145 Shangdong Road(Middle), Shanghai, China.
- ⁴⁶ Sanming AnXin Biological Technology Co., Ltd. Lvyancun, Meilie District, Sanming, 365000, China.
- ⁴⁷ Ningbo Medical System Biotech Co., Ltd. No.299 Qiming Road (South), Yinzhou District, Ningbo, China.
- ⁴⁸ Da An Gene Co., Ltd. of Sun Yat-sen University. No.19 Xiangshan Road, Science Park, High & New Technology Development, District, Guangzhou, Guangdong, 510665, China.
- ⁴⁹ Shenzhen Sciarry Biotechnology Co., Ltd. No. 3181 Shahe Road (West), Nanshan District, Shenzhen, Guangdong, China.
- ⁵⁰ Shanghai ZJ bio-Tech Co., Ltd. No. 528 Ruiqing Road, Zhangjiang High-tech Industrial East District, Shanghai, China.
- ⁵¹ Fujian Triplex Bioscientific Co., Ltd. No.55 Qianpu Industrial Area, Xiamen, China.
- ⁵² Shanghai ShenYou Bio Technology Co., Ltd. 17 Faraday Road, Shanghai, 201203, China.
- ⁵³ Shenzhen MABSKY Technology Co., Ltd. No.19 Langshan Road, High & New Technology Industrial Park, Nanshan, District, Shenzhen, 518057, China.
- ⁵⁴ Beijing LiMi BioTechnology Co., Ltd. No.4 Fufeng Road, Fengtai District, Beijing.
- ⁵⁵ Beijing Wantai Biological Pharmacy Enterprise Co., Ltd. No. 31 Kexueyuan Road, Changping District, Beijing 102206, China.
- ⁵⁶ Zhuhai Livzon Diagnostics Inc. No.1 Pingdong road, Nanping Science and Technology Industrial Park, Zhuhai, China.
- ⁵⁷ Weihai WeiGao BioTech Co., Ltd. No. 312 Shichang Avenue, Weihai, China.
- ⁵⁸ Henan Cellnovo Biotechnology Co., Ltd. No.1 Cuizhu Street, Zhengzhou high-tech development zone, Zhengzhou, China.
- ⁵⁹ Hangzhou WeiSheng Biotech Co., Ltd. No. 478 Jianduan Road, Tonglu County, Hangzhou, China.
- ⁶⁰ Capital Bio Co., Ltd. No.18 Life Science Parkway, Changping District, Beijing 102206, China.
- ⁶¹ Beijing Modern Gaoda Biotechnology Co., Ltd. No.7A Yongchang North Road, Economic and Technological Development Area, Beijing, China.
- ⁶² Shanghai FoSun Long March Medical Science Co., Ltd. No. 830 Chengyin Road, Baoshan District, Shanghai, China.
- ⁶³ Beijing BGI-GBI Biotech Co., Ltd. Area B, Konggang Industrial District, Beijing, China.
- ⁶⁴ Xiamen Amply Biotech Co., Ltd. No. 10 Yangguang Road, Xiamen, China.
- ⁶⁵ Hain Lifescience GmbH Hardwiesenstraße 1 72147 Nehren, Germany.
- ⁶⁶ Yaneng BioTech (ShenZhen) Co., Ltd. 2-309, Bioincubator, Gaoxin Zhong Yi Road, Hi-tech Park, Shenzhen, 518057, China.
- ⁶⁷ Beijing YiLi Fine Chemical Co., Ltd. West of Changdian Village, Jinzhan Town, Chaoyang District, Beijing, 100018, China.
- ⁶⁸ R-Biopharm AG Landwehrstrasse. 54 64293 Darmstadt, Germany.
- ⁶⁹ Shenzhen AnQun Biotech Co., Ltd. 6-8D Tianan Industrial Area, Shenzhen, China.
- ⁷⁰ Tarcine BioMed (China) Inc. No. 29 Life Science Park Road, Changping District, Beijing, 102206, China.
- ⁷¹ NovaTec Immundiagnostica GmbH Waldstraße 23 A6, D-63128 Dietzenbach, Germany.
- ⁷² Jiangsu Mole BioScience Co., Ltd. No. 1 Yaocheng Road, Taizhou, 225300, China.
- ⁷³ QIAGEN Bioengineering(ShenZhen) Co., Ltd. R3-B High & New Technology Development Area, Nanshan District, Shenzhen, China.
- ⁷⁴ Wuhan Biotech Gene Engineering Co., Ltd. The 5th floor of Hongye Mansion, Science and Technology Park of Wuhan, University, Wuhan East Lake Development Zone, Wuhan, China.
- ⁷⁵ Beijing AiPuYi Biotech Co., Ltd. No.1 Disheng Road (East), Beijing Eco & Tech Development Area, Beijing, China.
- ⁷⁶ Jiangsu BioPerfectus Technologies Co., Ltd. No.1 Yaocheng Avenue, Taizhou, China.
- ⁷⁷ Zhuhai Hua Ao Biotech Co., Ltd. No.213 Anjizhong Road, Jinwan District, Zhuhai, China.
- ⁷⁸ BioSino Bio-technology and Science Co., Ltd. No. 27 Chaoqian Road, Changping District Beijing, 102200, China.
- ⁷⁹ Institut Virion\Serion GmbH Friedrich-Bergius-Ring 19, 97076 Würzburg, Deutschland.
- ⁸⁰ Diesse Diagnostica Senese SPA. Milano (MI) Via Solari, 19CAP 20144.
- ⁸¹ Dia. Pro Diagnostic Bioprobes, S.r.l. Via Columella, 31 20128 Milano, Italy.
- ⁸² Beijing Jianan Biotech Co., Ltd. No.374 Lijiafen, Fengtai District, Beijing, China.
- ⁸³ Euroimmun Medizinische Labordiagnostika AG. Seekamp 31, D-23560, Lubeck, Germany.
- ⁸⁴ Shenzhen KangShengBao Bio-Technology Co., Ltd. Fuan Industrial Park Phase 1, Dayang Development Zone Sub-Zone, Fu yong Street, Baoan District, Shenzhen 518103, China.
- ⁸⁵ Zhongshan Bio-Tech Co., Ltd. No.1, Shengwugu Avenue, National Health Base, Torch Development Area, Zhongshan, 528473, China.
- ⁸⁶ Shenzhen Huakang Biomedical Engineering Co., Ltd. No.4 Taifeng Industrial Park, Shenzhen, China.
- ⁸⁷ Shenzhen HuiAn BioTech Co., Ltd. No. 25 Dengliang Road, Nanshan District, Shenzhen, China.
- ⁸⁸ Beijing Bioneovan Co., Ltd. No. 18 Keyuan Road Daxing Industry Zone, Beijing, China.
- ⁸⁹ Weifang kanghua Biotech Co., Ltd. No. 699 Yuehe Road, Weifang, China.
- ⁹⁰ Weifang 3V BioEngineering Co., Ltd. No. 1198, Zhiyuan Road, Weifang, 261061, China.

- ⁹¹ Jiangsu HuaGuan BioTech Co., Ltd. No.1 Yaocheng Avenue, Hailing District, Taizhou, China.
- ⁹² Shanghai Rongsheng Biotech Co., Ltd. No. 888 Xiangyang Road, Minhang District, Shanghai, China.
- ⁹³ Sichuan Maker Science And Technology Co., Ltd. No.16 Baichuan Road., Hi-Tech Industrial Development Zone, Chengdu, 611731, China.
- ⁹⁴ Beijing North Institute of Biological Technology. Panjiamao A20, Fengtai District, Beijing 100076, China.
- ⁹⁵ Beifang Biotech Research Institute. No. 7 Kexing Road, Fengtai District, Beijing, 100076, China.
- ⁹⁶ Suzhou Sym-Bio Life Science Co., Ltd. No. 115, Taiping Road(North), Taicang, China.
- ⁹⁷ Xianmen BoSon Biotech Co., Ltd. No. 90-94 Tianfeng Road, Jimei North Industrial Park, Xiamen, Fujian 361021, China.
- ⁹⁸ Hunan Sansure Biotech Co., Ltd. No. 198 Tongzipo Road (west), Hi-Tech Development Zone, Yuelu District, Changsha, China.
- ⁹⁹ Shanghai alpha biotechnology Co., Ltd. No. 269 Zhuyuan Road, Minhang District, Shanghai, China.
- ¹⁰⁰ Beijing KeMei Biotech Co., Ltd. No.7 Fengxian Road(Middle), Haidian District, Beijing, China.
- ¹⁰¹ National Bio-Founder Biotech Co., Ltd. No.1 China Medical City Avenue, Taizhou, Jiangsu,225316, China.
- ¹⁰² Zhengzhou Biocell Biotechnology Co., Ltd. No.28 First Street, Zhengzhou National Economic & Technological Development Area, Zhengzhou, China.
- ¹⁰³ Hunan KangRun pharmaceuticals Co., Ltd. No. 380 Baling Road (East), Yueyang, Hunan, China.
- ¹⁰⁴ Shandong LaiBo BioTech Co., Ltd. No. 750 Xinyu Road, Jinan Hi-Tech Area, Jinan, China.
- ¹⁰⁵ DiaSorin South Africa (Pty) Ltd. 22 Kyalami Boulevard, Kyalami Business Park, Kyalami 1684, South Africa.
- ¹⁰⁶ MP Biomedicals Asia Pacific Pte Ltd. 85 Science Park Drive, #04-01, The Cavendish, Singapore Science Park.
- ¹⁰⁷ Beijing KeWei Clinical Diagnostic Reagents Co., Ltd. No.19 Gucheng Street (West), Shijing District, Beijing, China.
- ¹⁰⁸ Beijing kinghawk pharmaceutical Co., Ltd. No. 7 Yuncheng Street, Beijing Economic-Technological Development Area, Beijing,100176,China.
- ¹⁰⁹ Shanghai Haoyuan Biotech Co., Ltd. No. 2933 Huqingping Road, Qingpu District, Shanghai, 215234, China.
- ¹¹⁰ Beijing JunHe pharmaceuticals Co., Ltd. No.4 Xinlong Road, Tongzhou District, Beijing, China.
- ¹¹¹ Ortho Clinical Diagnostics. 50-100 Holmers Farm Way-High Wycombe Bucks HP12 4DP GBR.
- ¹¹² AB Analytica. Srl ViaSvizzera, 16-35127, Padova, Italy.
- ¹¹³ Shanxi YangGuang BioEngineering Co., Ltd. No.66 Xibu Avenue, Hi-Tech Park, Xian, China.
- ¹¹⁴ Shanghai Tellgen Life Science Co. Ltd. No. 646 Songtao Road, Zhangjiang Hi-Tech Park of Pudong District, Shanghai 201203, China.
- ¹¹⁵ Genetel Pharmaceuticals (Shenzhen) Co., Ltd. 2-310 Bio-Incubator, Gaoxinzhong 1st Avenue, Hi-Tech Industrial Park, Nanshan District,Shenzhen, 518057, China.
- ¹¹⁶ Chaozhou HybriBio BioChemistry Co., Ltd. Economic Development Experimental Area, Chaozhou, China.
- ¹¹⁷ Beijing KangMei TianHong BioTech Co., Ltd. No.4 Yongan Road, Shilong Industrial Area, Beijing, China.
- ¹¹⁸ Hangzhou Watson Biotech Co., Ltd. No.88 Jiangling Road, Hangzhou, China.
- ¹¹⁹ Zhuhai S.E.Z HaiTai Biological Pharmaceuticals Co., Ltd. No 18 Chuangxinsi Road, Tangjiawan, Zhuhai, 519085, China.
- ¹²⁰ Adaltis S.r.l. Via Luigi Einaudi 7-Guidonia Montecelio 00012 Roma, Italy.
- ¹²¹ Adaltis Italia S.p.A. Via Cristoni, 12 - Casalecchio di Reno - 40033 Bologna, Italy.
- ¹²² Guangzhou HuaYin Medical Technology Co., Ltd. No 80 Lanyue Road, Science City, Guangzhou, China.
- ¹²³ Lanzhou institute of Biological Co., Ltd. No. 888 Yanchang Road, Lanzhou, 730046, China.

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- 1 The Method for Registration and Administration of Medical Devices (SFDA No.16.). <http://www.sda.gov.cn/WS01/CL0053/25844.html>
- 2 The Regulations for Supervision and Administration of Medical Device (Decree No. 276 of the State Council of P.R.C. Department of State No. 276). <http://www.sda.gov.cn/WS01/CL0063/16570.html>
- 3 Announcement on Classification Management of *in vitro* Diagnostic Reagents. <http://www.sda.gov.cn/WS01/CL0053/25844.html>
- 4 SFDA Medical Device Registration. <http://wenku.baidu.com/view/6f63928071fe910ef12df80b.html>
- 5 Medical Device: Equipments and Reagents. <http://www.sfda.gov.cn/WS01/CL0001/>
- 6 Beijing SFDA: Equipments and Reagents. <http://www.bjda.gov.cn/publish/main/index.html>
- 7 Fujian SFDA: Equipments and Reagents. <http://www.fjfd.gov.cn/>
- 8 Guangdong SFDA: Equipments and Reagents. <http://www.gdda.gov.cn/publicfiles/business/htmlfiles/gdffa/>
- 9 Henan SFDA: Equipments and Reagents. <http://www.hda.gov.cn/CL0001/>
- 10 Jiangsu SFDA: Equipments and Reagents. <http://www.jsfda.gov.cn/>
- 11 Shandong SFDA: Equipments and Reagents. <http://www.sdfda.gov.cn/>
- 12 Shanghai SFDA: Equipments and Reagents. <http://www.shfda.gov.cn/gb/node2/node3/index.html>
- 13 Tianjin SFDA: Equipments and Reagents. <http://www.tda.gov.cn/>
- 14 Zhejiang SFDA: Equipments and Reagents. <http://www.zjfd.gov.cn/>

Clinical Microbiology *In Vitro* Diagnostic Medical Devices in France: Regulatory Requirements and Product Information

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29.1 Regulatory requirements

The ANSM (Agence Nationale de Sécurité du Médicament et des produits de Santé's) mission is to ensure the protection of health and safety to health products including those for *in vitro* diagnostic use. The European Directive 98/79/EC regulates *in vitro* diagnostic medical devices (IVDMDs) and allows the harmonization within the European Union contributing to the process of commercialization for these devices.

29.2 European Directive 98/79/EC

Since June 7, 2000, the European Directive 98/79/EC of October 27, 1998 on IVDMD's [1] came into effect and allows the free movement of such devices throughout the European Community. Prior to commercialization, the manufacturer must obtain the Conformité Européenne (CE) marking on the device, ensuring compliance with the essential requirements of the Directive. The scope of the devices covered by Directive 98/79/EC is defined in Article I of the Directive and integrates these features to an end medical diagnosis from a human sample. Thus, the scope of products covered by this Directive covers all medical devices related to *in vitro* diagnosis, including reagents, laboratory automation, sample tubes, and accessories specifically designed for *in vitro* diagnostic use. It also covers the self-tests and devices that may be used in the care or medical practice to carry out an analysis by a health professional.

The principle of this Directive is based on the obligation of manufacturers to comply with the essential requirements of design, manufacture, and performance in order to obtain the CE mark for their devices. However, the procedure depends on the CE device. Thus, for the

majority of IVDMDs, compliance with these requirements allows the manufacturer to obtain the CE marking under his sole responsibility. For others, including self-tests and devices listed in Appendix II (please refer to Chapter 31 for selected examples) of the Directive (mainly for transfusion or maternal–fetal infection), the manufacturer cannot certify their conformity by itself. They must obtain a certificate of conformity issued by a notified body chosen in a Member State of the European Union. It is up to the competent authorities in each country to identify and control these devices.

In France, the competent authority (ANSM) will notify the LNE (Laboratoire National de Metrologie et d'Essais)/G-MED (Groupement pour l'évaluation des dispositifs médicaux) as the certification body in the medical field. In the certification process, the manufacturer may rely on existing standards, especially the harmonized European standards. When a product is designed, manufactured, and evaluated according to one or more harmonized standards, it is compliant with the essential requirements set out in the Directive. For the products listed in Appendix II A (please refer to Chapter 31 for some specific examples), there are specific performance evaluations called common technical specification standards; manufacturers are required to follow these, unlike harmonized standards, which are not mandatory. It should be noted that manufacturers that do not have their registered office in the European Union are required to appoint an agent with a registered office in an EU country. This office can then represent the manufacturer and address any issues from authorities and those in the European Community instead of the manufacturer directly. These are obligations and requirements of Directive 98/79/EC.

Table 29.1 Test, devices and instruments for clinical microbiology diagnostic testing in France*,†

Manufacturer/supplier	Bacteriology (aerobic/anaerobic)/mycobacteriology	Virology	Mycology	Parasitology	Mycoplasma, Chlamydia, rickettsia and miscellaneous
Abbott 12 Rue De La Couture BP 50203 94518 Rungis Cedex Tel: + 33 1 45 60 25 00	<i>Treponema pallidum</i> (TPPA – <i>Treponema pallidum</i> particle agglutination) – Architect	Abbott Molecular <i>m2000spViral</i> load: Hepatitis B Hepatitis C HIV AxSYM Immunoassay Analyzer: HIV HBsAg, HBsAb, HBcAg, HBcAb, HBeAg, HCV HAV HIV Ag/Ab HTLV CMV G+M			
AES Chemunex Rue M Bastie-Ker Lann-Cs 17219 Bruz Cedex 35172 Tel: + 33 223501212 aes@aeslaboratoire.com			Culture media: Sabouraud Sabouraud chloramphenicol gentamicin Antifungal susceptibility: RPMI agar		
Alere SAS 21 rue Albert Calmette Batiment B4 78350 Jouy-en-Josas, France Tel: + 33 139468318		Alere Determine HIV-1/2/ Dengue serology: Detection of dengue NS1Ag IgG/M			
All Diag 2 Rue Ettore Bugatti BP 6 Eckolsheim 67038 Tel: + 33 388788088					Rapid test chromatographic immunoassay in blood: <i>Plasmodium falciparum</i> , <i>P. ovale</i> , <i>P. malariae</i>
Applied Medical France 114 Bis Rue Michel Ange Paris 75016 Tel: +33 6 84 85 51 21	Applied Biosystems 3130 Genetic Analyzer				
Becton Dickinson France 11 Rue Aristide Berges Le Pont De Claix 38800 Tel: +33 0476683780 bd.service.clients. vacmed@europe.bd.com	BACTEC: Blood culture aerobic and anaerobic bottles BACTEC MGIT 960 System: Mycobacteria Mass spectrometry identification BD Phoenix: Identification and antibiotic susceptibility testing	Rapid test chromatographic immunoassay in nasal swab: Influenza and respiratory syncytial virus	Mass spectrometry identification		

<p>Bioavance 1 Av. Marne Et Gondoire Bussy St Martin 77600 Tel: +33 164616666</p> <p>Bio-Evolution 55, boulevard du Général Gallieni94 360 Bry-sur- Marne FRANCE Tel: +33 1 60 03 81 50 Email : contact@bio- evolution.fr</p>	<p>Syphilis serology: RPR latex kit</p>	<p>HTLV 1 and 2 Western blot</p>	<p>VIDAS/Serology: Rubella Ig G/M /avidity CMV IgG CMV IgM CMV IgG avidity Hepatitis A IgM Hepatitis B: HBsAg, HBcAb, HBcAb-IgM</p>	<p>Fungi identification Vitek card YST Culture media: <i>Albicans</i> ID Sabouraud</p>	<p>VIDAS/Serology <i>Toxoplasma gondii</i> G/M/ avidity</p>	<p>Specific media/Culture: A7 Mycoplasma agar</p>
<p>bioMérieux France Chemin De L' Orme Marcy L Etoile 69280 Tel. :+33 4 78 87 20 00 http://www.biomerieux.com</p>	<p>UF500 UF 1000: Cytology of urine sample BacT/ALERT 3D: Culture media for blood PREVI Color Gram: Gram staining</p> <p>PREVI Isola: Automated inoculation system for culture media</p> <p>Conventional culture media: Columbia agar + 5% sheep blood Columbia CNA agar + 5% sheep blood Chocolate agar + PolyViteX (PVX) Specific media: Campylosel agar BCSA agar (selective isolation of <i>Burkholderia cepacia</i>) Yersinia CIN agar BCP agar Mac Conkey agar Hektoen agar Mannitol Salt 2 agar (selective isolation of staphylococci) Muller Hinton agar: Muller Hinton agar blood VITEK 2: Identification and antibiotic susceptibility testing Mass spectrometry identification E TEST: Antibiotic susceptibility testing</p>	<p>VIDAS/Serology: HIV P24 II HIV Ag/AC Measles IgG+M</p>	<p>Vitek card: Antifungal susceptibility testing ATB FUNGUS 3 A OR AST-YS01 E test: Antifungal susceptibility testing NucliSens easyMAG extractor</p> <p>Mass spectrometry identification</p>			

(Continued)

Table 29.1 (Continued)

Manufacturer/supplier	Bacteriology (aerobic/anaerobic)/ mycobacteriology	Virology	Mycology	Parasitology	Mycoplasma, Chlamydia, rickettsia and miscellaneous
Bio-Rad 3 Bd Raymond Poincare Marnes La Coquette 92430 Tel: + 33 147956000	Serology: <i>Brucella melitensis</i> Rose Bengale and Wright seroagglutination Serology: <i>Salmonella</i> sp. Molecular biology/CFX Real-time PCR Detection System: <i>Streptococcus pneumoniae</i> <i>Neisseria meningitis</i>	Serology: HIV1/2 western blot Molecular biology/CFX Real-Time PCR Detection System: HSV VZV HHV6 Parvovirus B19 JC-BK polyomavirus Enterovirus Chikungunya virus Dengue Measles Mumps Toscana West Nile Lymphocytic choriomeningitis Influenza Parainfluenza Parechovirus Human Respiratory Syncytial virus Hantavirus Norovirus Rotavirus Japanese encephalitis virus Yellow fever ELISA/Evolzyzer (Tecan)/Serology Parvovirus B19 HHV6 IgG Immunofluorescence/Serology HHV6 IgM	Culture media: Sabouraud Chloramphenicol Gentamicin Agar Antifungal susceptibility FUNGITEST Kit	Molecular biology/CFX Real-Time PCR Detection System <i>Pneumocystis</i> <i>Plasmodium</i>	Molecular biology/CFX Real- Time PCR Detection System: <i>Coxiella burnetii</i> <i>Bartonella henselae</i> , <i>Bartonella quintana</i> <i>Rickettsia conorii</i> , <i>R. typhi</i> , <i>R. africae</i> , <i>R. slovaca</i> , <i>R. mongolitimonae</i> <i>R. felis</i> , <i>R. prowazekii</i> and others <i>Tropheryma whipplei</i> <i>Chlamydia trachomatis</i> <i>Neisseria gonorrhoeae</i> <i>Mycoplasma genitalium</i> <i>Mycoplasma pneumoniae</i> <i>Bordetella pertussis</i>
Biotrin 14 Rue Gorge De Loup Lyon 69009 Tel: 0472530461					
Bruker Daltonique Eurl Bp. 10002-34 Rue L Industrie Wissembourg Cedex 67166 Tel: + 33 3 88 06 60 40	Mass spectrometry identification: <i>Microflex LT</i>		Mass spectrometry identification: <i>Microflex LT</i>		

Cepheid Europe Vira Soleih Maurens Scopont 81470 Tel: +33 563825300	GeneXpert: MRSA <i>Streptococcus agalactiae</i> detection in vaginal swab <i>Clostridium difficile</i> in stool <i>Mycobacterium tuberculosis</i> rifampicin resistance	GeneXpert: Influenza A/B Enterovirus	Molecular biology/GeneXpert: <i>Chlamydia trachomatis</i> <i>Neisseria gonorrhoeae</i>
Diasorin S.p.A. 11 Rue Georges Besse BT4 Antony Cedex 92182 Tel: + 33 15590400 gilles.augrandjean@ diasorin.it	LIAISON/ELISA <i>Borrelia burgdorferi</i> IgG <i>Borrelia burgdorferi</i> IgM	LIAISON/ELISA EBV IgM VCA IgG EBNA IgG Parvovirus B19 IgG Parvovirus B19 IgM VZV IgG VZV IgM	LIAISON, ETH-MAX/ELISA <i>Mycoplasma pneumoniae</i> IgM/IgG/IgA <i>Chlamydia pneumoniae</i> IgM/IgG/IgA <i>Chlamydia trachomatis</i> IgM/IgG/IgA
Eurobio 7 Av De Scandinavie Les Ulis 91953 Tel: +33 169079477	<i>Helicobacter pylori</i> serology G/M; Rapid test: <i>Legionella pneumophila</i> Urinary antigen	Hepatitis E IgG/M	
Eurogentec France SASU 152 Av Patton-BP 80111 Angers Cedex 01 49001 Tel: + 33 241733373	Molecular biology mix: <i>Streptococcus pneumoniae</i> <i>Neisseria meningitis</i> <i>Bordetella pertussis</i>	Molecular biology mix: <i>Pneumocystis</i> <i>Plasmodium</i>	Molecular biology mix <i>Mycoplasma pneumoniae</i> <i>Coxiella burnetti</i>
Groupe Servibio 85 Rue Henri Barbusse Meudon 92190 Tel: +33 146264281	Rapid test immunochromatographic test for antigen detection: <i>Streptococcus pyogenes</i>		
InGen 17 Avenue du Parc 91380 Chilly-Mazarin France Tel: +33 16909793	Serology: <i>Borrelia burgdorferi</i> Western blot Syphilis Western blot Rapid test: Tetanus immunization status	Serology: HIV 1/2 Western blot Rapid test chromatographic immunoassay in nasal swab: Respiratory syncytial virus	
Iris Diagnostics France Bat D - Paris Nord II33 Rue Des Chardonn erets Bp 88052 Tremblay en France 95933 Roissy Cdg Cedex Tel : +33 1 82 71 30 10	iQ Series q: Cytology of urine sample		

(Continued)

Table 29.1 (Continued)

Manufacturer/supplier	Bacteriology (aerobic/anaerobic)/ mycobacteriology	Virology	Mycology	Parasitology	Mycoplasma, Chlamydia, rickettsia and miscellaneous
I2A BP 90002-PC Mediterranee Perols Cedex 34477 Tel: +33 467504800 i2a@i2a.info	SIR SCAN 2000/ Diffusion antibiotic testing				
J2L ELITech 16 Zone Industrielle Labarthe Inard 31800 Tel: +33 561885900	ASLO/DORNASE Aerospray Gram staining		Antifungal susceptibility: FUNGIFAST AFG		
Medac 23 Rue Pierre Gilles De Gennes Lyon 69007 Tel: +33 437661470		ELISA/Evolzyer (Tecan) Serology CMV IgM			ELISA: <i>Chlamydia trachomatis</i> IgG/A <i>Mycoplasma pneumoniae</i> Ig M/G/A
Meridian 34 Rue de Ponthieu 75008 Paris Tel: +33 1 42 56 04 40 Email: info.fr@ meridianbioscience.eu	Immunofluorescence/Serology <i>Legionella pneumophila</i> Rapid test: stool immunoassay <i>Helicobacter pylori</i> antigen		<i>Cryptococcus neoformans</i> : Antigen latex agglutination system in serum, cerebrospinal fluid		
MP Biomedicals Rue Geiler Kayserberg BP50067 Illkirch Cedex Tel: +33 388672267		Rapid test: Hepatitis E IgM			
Oxoid 6 Route de Paisy B.P. 13 Dardilly Cedex 69570 Tel: +33 472523370	Culture media				
Orgentec P.Pissaloup 4 Rue E. Branly Trappes 78190 Tel: + 33 130688000	ELISA: <i>Leptospira</i> IgG/M <i>Yersinia</i> IgG <i>Campylobacter jejuni</i> IgG				

<p>Qiagen SA ZA 3 Av Canada LP 809 Courtaboeuf Cedex 91974 Tel: +33 160920920</p> <p>Rbiopharm 17 Av C.Gaule Parc De Crecy St Didier Au Mont D OR69370 Tel: +33 478643200 d.info@rbiopharm.fr</p> <p>Roche Diagnostics 2 Avenue Du Vercors BP 59 Meylan Cedex 38242 Tel: +33 4 76 76 30 00</p>	<p>Molecular diagnosis/Easy one DNA extraction kit</p>	<p>Molecular diagnosis/Easy one RNA-DNA extraction kit</p> <p>Rapid test*/ immunochromatographic test for antigen detection Adenovirus and rotavirus Norovirus</p>	<p>LC 480 Candida albicans, Candida glabrata, Candida krusei, Candida parapsilosis, C. tropicalis Aspergillus sp Dermatophytosis Other fungi</p>	<p>LC 480 Cryptococcus neoformans Taenia solium Giardia Histoplasmosis Plasmodium falciparum, P. ovale, P. malariae Toxoplasma gondii Pneumocystis jirovecii Dirofilaria</p>	<p>Cobas 4800 Chlamydia trachomatis Neisseria gonorrhoeae</p>	<p>Molecular diagnosis/Easy one DNA extraction kit</p>
<p>Siemens SAS and Healthcare Diagnostics 9 boulevard Finot 93527 Saint-Denis Cedex 2 Tel: +33 149223100</p>	<p>Microscan Walk-Away/AutoSCAN4 Identification and antibiotic susceptibility testing</p>	<p>Evolyzer (Tecan): Mumps IgG Mumps IgM Measles IgG Measles IgM Parvovirus B19 IgG/M HSV-1/2 IgG HSV-1 IgG HSV-2 IgG HSV-1/2 IgM VZV IgG VZV IgM</p>				
<p>Theradiag Actipole 25 Bd Beaubourg Marne-La-Vallée Cedex 2 77423 Tel: + 33 1 64 62 10 12</p>					<p>Immunofluorescence/Serology Chlamydia pneumoniae Chlamydia psittaci</p>	

* This list represents a snapshot of some of the products available from the vendors included in this table. Other tests, devices, and instruments are also available; please refer to other chapters in this book and company literature and web sites for additional information.

† Commonly used tests, instruments, and devices that are manufactured in the United States, the EU, and other countries are also used in hospitals and laboratories in France.

29.3 Directive 98/79/EC in France

The European Directive was enacted into French law by Order No. 2001-198 of March 1, 2001. The decrees implemented the European text and specify the rules for the commercialization of devices, and enforceability of manufacturer requirements with offices in France. The manufacturers based in other European countries are subject to the rules in their own countries resulting from the enactment and implementation of the directive into their national law. Under the decree of February 25, 2005 of the Code of Public Health, any operator in France that is a manufacturer, agent, distributor, importer, or subcontractor, has an obligation to declare their activity. French manufacturers and agents must register as responsible for the commercialization. Manufacturers, agents, and distributors outside France, that is to say, any manufacturer that brings IVDMDs into France, must declare their device to the ANSM.

29.4 The implications of the regulatory level authorities

According to the European Directive, manufacturers assume the responsibility for the marketing of their devices. They ensure quality by using procedures that they implement in their design, in their manufacture, and the validation of their performance. The role of monitoring and controlling their product comes afterwards. Thus, besides its role in receiving information relating to the commercialization of products that are CE marked and according to the needs related to public health requirements, the ANSM may establish ad hoc requirements. For example, it may ask the manufacturer or any other subsidiary that has been documented to carry the CE marking, for any set of devices, to monitor the performance

of the new device entering in the market, to provide additional quality control records or to provide proof of compliance to the requirements listed in the Directive. The manufacturer must also take into account international recommendations when they exist. Moreover, the ANSM organizes a national system of reported incidents called "reactovigilance". The regulations require that industry, healthcare providers, and other users report any failure or deterioration of any IVDMD that may be fatal to human health. This system is enhanced by the further coordination of the regulatory process required at the European level. The ANSM also participates in various European groups, working on the development of texts, standards, criteria quality, and harmonization of working procedures between countries.

29.5 Assessment procedures

Please refer to Chapter 31 for information related to Assessment Procedures and other information related to the European Union requirements for IVDMDs.

Table 29.1 contains information related to tests, devices, products, and instruments available for clinical microbiology diagnostic testing as of this writing. Other products from the United States, the EU, and from other countries may also be available for use in France. Please refer to other chapters in this text, and other vendors and their web sites for additional products and current information.

Reference

- 1 Directive 98/79/CE : JO L 331 1998/12/7; Successive amendments and corrections to Directive 98/79/EC have been incorporated into the basic text. Règlement (CE) n° 1882/2003: JO L 284 2003/10/31; Règlement (CE) n° 596/2009: JO L 188. 2009/7/17

Clinical Microbiology *In Vitro* Diagnostic Medical Devices in India: Regulatory Requirements and Product Information

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India, with a population of more than 1.2 billion, currently has a healthcare sector of approximately US\$50 billion, which is expected to rise at the rate of about 2% per year as per the Fitch Rating agency report released in January 2011. Currently there are 336 teaching hospitals in India with diagnostic laboratory facilities and over 12,000 private hospitals of various sizes and more than 100,000 private clinical laboratories. Test volumes serviced by Indian laboratories vary from a few hundred tests a day for regional and hospital laboratories to a few thousand per day for larger laboratories. India's diagnostic laboratory sector is forecasted to grow at about 25% over the next 3 years and estimated to be about US\$2 billion in the near future.

India, like the rest of the world, has from the early 1990s witnessed a flooding of the market with commercial microbiological kits, particularly in the field of immunodiagnosics. At the beginning these were primarily imported kits from the United States or Europe, but gradually indigenous kits as well as those from various Asian countries like Korea, Malaysia, and China have made their presence felt. These later kits in many instances have performed equally well as the Western manufactured ones and are substantially economical.

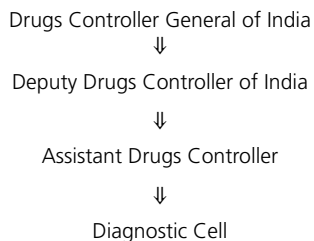
Analogous to the US Food and Drug Administration (FDA), the apex Government institution in India is the Central Drugs Standards Control Organization (CDSCO). This office is part of the Directorate General of Health and Family Welfare, Government of India. The CDSCO also has offices at the capitals of various states. The office of the CDSCO is the central licensing authority for all drugs, medical devices, and diagnostic kits. Licensing is a two-part procedure for select imported items (such as pharmaceutical products and medicines, and medical devices such as catheters and heart valves): the company interested in marketing a particular product

manufactured abroad first has to obtain the license to import that item to India. Once the license is obtained, testing of the product by a Central Government approved laboratory needs to be completed and if it passes the test, then the second license to market the drug or product is issued. The microbiology products in this select category, requiring a two-part licensing procedure, include the immunodiagnostic kits and nucleic acid testing systems for HIV, HCV, and HBV. For all other imported items related to diagnostic microbiology products, a single licensing procedure to import the particular test or reagent is required. For various items manufactured in India, the manufacturer has the items tested by Central Government approved laboratories, followed by application to the licensing authority along with the test reports so as to market the product in the country.

The CDSCO has the following responsibilities:

- 1 It implements policy and guidelines for quality assurance of diagnostic kits.
- 2 It establishes the standards for assuring quality, safety, and efficacy of the kits.
- 3 It ensures good manufacturing practice on the part of the manufacturers. To this end inspectors carry out visits to laboratories of the manufacturers from time to time without giving prior notice.
- 4 It issues the license for importation of diagnostic kits and maintains a registry of licensed manufacturers. It also issues the license to the kit manufactured in India after receiving a satisfactory report from the reference laboratory.
- 5 It carries out post-marketing surveillance to evaluate and monitor commercial kits that have been released into the market. It instructs the state laboratories to send representative kits being used to the reference laboratory for testing.
- 6 It can recall the kits from the market in the event of any deviation in the kit quality after marketing.

The organogram of the test license section (used for products imported and for those manufactured in India) is as follows [2]:



The manufacturer first has to apply for the license of the product, addressed to the Drugs Controller General of India office. This application goes through the successive offices of the Deputy Drugs Controller, followed by the Assistant Drugs Controller and finally to the diagnostic cell or wing. All applications for licensing of products will be ultimately directed to this section (the diagnostic cell or wing), which then gives instructions to the state-level drugs controller office to send the test kits to an approved laboratory for evaluation. Once a satisfactory report is obtained from the state-level office, the diagnostic cell forwards the report to the Drugs Controller General, who is the final signatory for grant of license; and the manufacturer receives a license to market the product after completing the relevant documentary procedures. The test-licensing procedure is governed by the Drugs and Cosmetics Act of 1940 and rules of 1945, as amended from time to time. The validity of a manufacturer's license is for a period of 3 years.

The apex testing laboratory in India is the National Institute of Biologicals (NIB) situated in the National Capital region of Delhi at Noida. It was established as an autonomous organization under the Ministry of Health and Family Welfare, Government of India registered in 1992. The overall objective of the institute is to ensure the quality of the biological products (which include diagnostic kits) and also to strengthen the regulation of biologicals in India. The institute assures and reviews the quality of biological products available through domestic manufacturer or imports. The operations are carried out in a state-of-the-art facility of the institute and in close collaboration with the Government of India's regulatory authority, i.e., office of CDSCO. The NIB plays the following roles in India:

- 1 Examination of *in vitro* diagnostic devices submitted for CDSCO approval. The examination is carried out consistent with international standards. It has enrolled and participated in External Quality Assurance programmes with the National Serology Reference laboratory (NRL) Australia, which is a National Association of Testing Authorities (NATA) accredited proficiency testing provider complying with

International Laboratory Accreditation Cooperation (ILAC) – G13:08/2007 and also a WHO Collaborating Centre for Diagnostic and Laboratory support for HIV and other bloodborne infections. At the national level, NIB has participated in the accreditation process by National Accreditation Board for Laboratory (NABL, India) as per ISO 17025.

- 2 It looks after the consistency of quality in the use of devices by total quality management. This is carried out by much testing of the products sent from various state laboratories as per the instructions of CDSCO.
- 3 It organizes training courses and develops training material in all aspects of quality management for all laboratories. It also conducts training for personnel from various hospitals and state reference laboratories.
- 4 It also provides the materials for External Quality Assurance (EQA) and supplies panels for testing not only to the manufacturing units, but also the state reference laboratories. The EQA is aimed primarily at kits that are used for infectious disease screening in transfusion practices. The institute maintains a panel of 100 positive and 300 negative sera each for HIV, HBV, HCV, and syphilis serology. These panels are intended for testing both ELISA and rapid immunochromatographic tests for HIV, HBV, and HCV, in addition to Western blot tests for HIV and HCV and neutralization testing for HBV. For syphilis, the panel is intended for rapid plasma reagin and *Treponema pallidum* haemagglutination assay kits.

To obtain an approximate idea about the number of kits in use in India, the NIB annual report of 2011–2012 has the data for HIV, HBV, and HCV kits. In 1 year it had evaluated 274 kits in this category (115 HIV, 75 HBV, and 84 HCV) from 13 countries including India. Of the 115 HIV kits, 33 were from India; followed by Korea, France, and Singapore. For HBV, almost equal numbers of kits (ranging from 11 to 16) were from India, France, China, and Korea; while for HCV the vast majority (38 out of 84) were from Korea, followed by Canada, China, and India. On evaluation, 86% of the HIV, 96% of the HBV, and 84% of the HCV kits met the specifications established for sensitivity and specificity [1].

The bulk of Indian microbiological diagnostic services, like other parts of the world, consist of routine bacteriological and serological tests. To keep up with the demand, there has been a mushrooming of indigenous manufacturers dealing with these products. For example, approximately two decades back, Hi Media was the single Indian manufacturer of dehydrated media for bacteriology and mycology, along with related stains, reagents, and biochemicals. But in recent years, the trend is changing, with other companies entering this segment of the market. Licensing of these products (which includes all bacteriological media, stains, disks, and reagents as

well as immunodiagnostic kits) requires testing of their quality by one of the CDSCO approved laboratories and obtaining their approval. Once the approval is obtained, the companies receive the license from the CDSCO to market these products, and the license needs renewal every 3 years. These products are not under any regulatory network in India once the license to market the products is obtained, and it is the end user who remains the best judge regarding the quality of the product. The imported culture media from the multinationals like Becton Dickinson and Company (BBL-Difco), Remel, Oxoid, and other companies are primarily used for research purposes since other options for products manufactured in India are becoming more readily available.

Many centers are using imported bacterial and yeast identification systems and antimicrobial sensitivity panels. Manual and semiautomated products in this field like API, BBL Crystal, Remel, and others, or automated systems like bioMérieux Vitek, Microscan, TREK, and the Becton Dickinson Phoenix are being used by a few government institutions, and by a large number of private hospitals and laboratories. For the automated systems for blood culture, identification, and sensitivity testing, India is totally dependent on the world market leaders in this segment, like Becton Dickinson, bioMérieux, Dade Microscan, TREK, and others, which manufacture outside of India. Please refer to other chapters of this book for additional information.

The antigen/antibody detection for infectious diseases is the other major segment of diagnostic services. The CDSCO plays an important role in licensing the products in this field for HIV, HBV, and HCV. As per the gazette notification number GSR 601(E) issued in 2002 by the Ministry of Health and Family Welfare, Government of India, the *in vitro* diagnostic devices for the above three viruses have been designated as drugs per the Drugs and Cosmetic Acts, 1940. Additionally, as per an order issued in January 2013, these medical devices require a registration certificate and import license for import into India. However, the devices that do not require the provisions of Drugs and Cosmetic Acts, 1940 may be allowed into the country without a “no objection certificate” or NOC. In an order issued in November 2012 by the Drugs Controller General of India, the *in vitro* devices for HIV, HBsAg, and HCV manufactured/imported under a valid license are allowed to be used in Blood Banks as well as Pathology Laboratories as the criteria of sensitivity (%) and specificity (%) for evaluation of these tests for transfusion and diagnostic purposes are the same. The same order specifies that in order to ensure the quality of such kits manufactured in India, the state drug controllers may take random samples from the manufacturing site or the market, and send them to the NIB for testing. In effect,

stringent measures are being taken by the regulatory agency in India for licensing of the above-mentioned tests. But there remains a largely unaddressed area regarding the numerous immunodetection kits that are in existence in India, not only for other viruses, but also for various bacterial diseases (e.g., Widal test which is still commonly done in most laboratories, scrub typhus, brucellosis, leptospirosis, etc.) and parasitic infections, notably malaria. India accounts for approximately two-thirds of the confirmed cases of malaria reported in the South East Asia Region. In 2008, 1.5 million cases of malaria were confirmed [4]. Thus there is a huge market for malaria antigen detection kits, and numerous laboratories use the devices manufactured by Indian companies. But the quality of these kits remains controversial as they currently fall outside the purview of the government regulatory guidelines.

Any discussion on commercial methods in clinical microbiology will be incomplete without mentioning the nucleic acid detection tests. A few companies in India are manufacturing thermal cyclers, electrophoresis chambers, gel documentation systems, and transilluminators, with a gradual reduction in dependence on imported items. Many of these companies are also manufacturing the primers and the reaction mixtures. However, there is wide variation in the performance of these indigenous products (personal experience) and they need to be validated with a US FDA or Conformité Européenne (CE) approved imported product before routine use. The CDSCO has registered and approved a few imported products in this field for the detection and quantification of HIV-1, HCV, and HBV, but the cost factor has driven many of the laboratories to rely on locally manufactured consumables.

Tuberculosis remains a burning problem in India with a population of 1241 million. In the year 2011, it was estimated that globally there were 8.7 million incident TB cases [5] and India ranks first among the first five countries in terms of number of incident cases of approximately 2.2 million in 2011. To expedite the diagnostic turnaround time compared to culture on conventional solid medium, nucleic-acid-based tests for detection and drug sensitivity profiling are making rapid inroads in Indian laboratories. Almost all the imported kits for detection of *Mycobacterium tuberculosis* rely on the IS6110 primer sequence for the amplification procedure. But paradoxically, many of the *M. tuberculosis* strains from India, particularly from the four South Indian states of Andhra Pradesh, Tamilnadu, Karnataka, and Kerala may completely lack or may contain extremely low copy numbers of IS6110. This was first reported in 2001 from Kerala where it was found that almost 24% of the isolates did not contain a single copy of IS 6110 [3]; and their observation was later substantiated by similar studies from the other southern states of India. This is

Table 30.1 List of selected suppliers and selected domestic and imported products used in clinical microbiology laboratories in India

Test type	Product type	Indian	Imported
Bacteriology (aerobic/ anaerobic), mycology, and mycobacteriology	Culture Media (dehydrated, and ready to use types), identification disks, stains, reagents and chemicals for bacteriology, antibiotic disks and powder	Hi Media Laboratories Titan Media Micromaster Laboratories Tulip (Microexpress) Group Merck (India) Ltd Sisco Research Laboratories Pvt Ltd	BBL-Difco from Becton Dickinson (USA) Oxoid Limited UK (part of ThermoFisher Scientific) Remel (USA)** Bio-Rad (France) Hardy (USA)
	Rapid identification tests (manual/semi automated), and automated systems for blood culture, bacterial identification and antimicrobial susceptibility tests	Hi Media Laboratories.	API kits (bioMérieux), BBL Crystal (BD), Remel** BacT Alert System (bioMérieux SA, France) BACTEC System (BD, USA) MGIT system for Mycobacteria (BD, USA) Microscan System (Siemens, Germany)* Phoenix System (BD, USA) Vitek System (bioMérieux, France) TREK Diagnostic Systems (USA)** Biolog System (USA) MIDI (USA)
	Bacterial Serology	Widal Test: Span Diagnostic, Tulip Group Leptospira Antibody Test: J Mitra and Co. Ltd, Bio Standard Diagnostics (ELISA, immunochromatographic test) Brucella Antigen: Indian Veterinary Research Institute, Izzatnagar. (standard agglutination test) VDRL Antigen: Institute of Serology, Kolkata	Please refer to other references for immunodiagnostic products, tests, and antigen/ antibody testing. For immunoassay analyzers, please refer to other chapters in this book
Virology, mycoplasma, chlamydia, and rickettsia	Viral cultures: media, ingredients, and supplements	Hi Media Laboratories. Merck (India) Ltd	Sigma- Aldrich BBL Difco (BD) Oxoid** Remel**
	Viral immunodetection	Rapid immunochromatographic tests and ELISA. J Mitra and Company (India) Ltd (Western Blot test for HIV also available) Span Diagnostic Limited Transasia Biomedical Ltd. Qualipro Diagnostics (TULIP GROUP) Bhat Biotech India (P) Ltd Bio Standard Diagnostics (India) Ltd. Orchid Biomedical Systems (TULIP GROUP	Immunological tests for viruses, chlamydia, mycoplasma, and rickettsia are imported from USA, Germany, Italy, Australia, S.Korea, Taiwan, Singapore, and other countries
Nucleic acid amplification tests	Reagents, chemicals, and primers	Systronics (India) Limited Helini Biomolecules Genaxy Scientific Pvt. Ltd Hi Media (India) Chromous Biotech Pvt. Ltd. RAS Lifesciences Pvt. Ltd Merck Specialities Pvt. Ltd	Registered with CDSCO (ONLY FOR HIV-1, HBV, HCV) Versant 3.0 Assays (bDNA) (Siemens Medical Solutions, USA) Versant HIV 1 and HCV Genotype Assay Artus PCR Kits (Artus, Germany) Amplicor, CobasAmplicor, CobasAmpliscreen and CobasTaqMan Kits (Roche Molecular Systems, USA) Abbott Real Time calibrator, control, and amplification kits (Abbott Molecular Inc, USA)
	Thermal cyclers, electrophoresis chambers, gel documentation systems, and transilluminators	Helini Biomolecules Genaxy Scientific Pvt. Ltd Genetix Biotech Asia (P) Ltd Merck Specialities Pvt. Ltd	Real Time Quantitative PCR Qiagen (Germany) Roche Diagnostics, (Germany) Applied Biosystems, (USA) Focus diagnostics, (USA)
	Mycobacterial detection and drug sensitivity	Imported products and home brew analyte specific reagents used in this category	Line Probe Assay: Hain Life Sciences, Germany Xpert MTB/RIF: Cepheid HBDC (France)

Table 30.1 (Continued)

Test type	Product type	Indian	Imported
Parasitology	Quantitative Buffy coat test for malaria parasite	N/A	QBC Diagnostics, USA
	Immunodetection	Malaria parasite (rapid immunochromatographic (ICG), ELISA tests) J Mitra & Co. Ltd Bio Standard Diagnostics (India) Ltd Orchid Biomedical Systems, Zephyr (Tulip Group) Toxoplasma, Leishmania (Rapid ICG, ELISA) Bio Standard Diagnostics (India) Ltd	Filaria Card Test: Alere Medical <i>Giardia</i> , <i>Entameba</i> , <i>Cryptosporidium</i> , <i>Leishmania donovani</i> : various rapid kits imported from other countries. Refer to Chapter 15

*Please see Beckman Coulter.

**Please see ThermoFisher Scientific.

important when using *M. tuberculosis* PCR kits either from India or abroad since these may miss quite a few cases, particularly in the South India context.

In Table 30.1 we attempt to review many of the domestic and imported products used in clinical microbiology laboratories in India. This is a rapidly changing list of tests, devices, products, and instruments and we encourage the reader to consult all current sources of product information such as peer-reviewed journals, abstracts from scientific meetings, web sites, company product information and other contemporaneous sources of product information. We have attempted to include many of the commonly used products, companies, and vendors.

In conclusion, it can be said that a reasonably good regulatory network, and testing services are available in India which strives to maintain the quality of the commercial microbiological diagnostic kits, instruments, and products marketed in this country. However, post-license surveillance of the products and some immunodiagnostic tests constitute a grey area that needs to be strengthened.

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- 3 Indulakshmi Radhakrishnan, Manju YK, R Ajay Kumar, and Sathish Mundayoor. Implications of Low Frequency of IS6110 in Fingerprinting Field Isolates of *Mycobacterium tuberculosis* from Kerala, India. *J Clin Microbiol.* 2001; **39**: 1683.
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- <http://www.cdsco.nic.in> (Government regulatory guidelines.)
- <http://www.who.int> (Malaria and tuberculosis statistics.)

Clinical Microbiology *In Vitro* Diagnostics in Italy: Regulatory Requirements and Product Information

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31.1 Introduction

The European Directive for *in vitro* diagnostic (IVD) medical devices (IVDD 98/79/EC), which became operational in Member States by December 1998, was implemented in Italy in September 2000.

This Directive introduced common requirements in order to establish a uniform level of safety, quality, and performance of IVD devices. Thus since 1998, the Directive began circulation in the European Community, and therefore also in Italy, of IVDs compliant with the Directive and bearing the European Community marking (CE).

The Directive applies to IVD reagents, reagent products, calibrators, and instruments, including specimen containers, intended by the manufacturer to be used *in vitro* for the examination of samples from the human body, including tissues, blood, or other fluids, in order to provide information on the state of health of the patient.

The Italian Legislative Decree of 8 September 2000, n. 332 "Implementation of Directive 98/79/EC on *in vitro* diagnostic medical devices" includes the basic concepts underlying the Directive itself and the need to adopt measures to enable the free circulation within the European Community of goods, persons, services, and capital as follows:

- 1 the need to provide for certification procedures and control of IVD medical devices at the community level, to ensure the security, health protection, and operation of the devices that do not hamper trade within the European Community;
- 2 the need to harmonize national provisions on safety and health protection of patients, users and any third party interested in the use of IVDs and to ensure the free movement of such devices in the EU;
- 3 the need for IVD medical devices to ensure a high level of protection for patients, users, and third parties.

All these concepts entailed a major revision of the rules that previously prevailed in Italy: in fact, until the Implementation of Directive 98/79/EC, a system for the authorization and licensure regulation was provided only for IVDs for the diagnosis of hepatitis and of HIV, while the Legislative Decree n. 332 led to a system of certification and quality assurance based on specific procedures.

These procedures relate to the complexity and sensitivity of different products, involved appropriate governmental agencies, designated by the competent governmental authority (Ministry of Health), which grant the verification of compliance of *in vitro* diagnostic technical requirements as specified in the Directive.

31.2 National classification of medical devices

The National Commission of Medical Devices (CUD) was established by the Italian Finance Act 2003 (Art. 57, Law 27 December 2002, n. 289) with the task of defining and updating the repertoire of medical devices and to classify all products into classes and subclasses. The CUD defined the first national classification of medical devices (CND) (approved by the Italian Ministerial Decree of 22 September 2005).

The IVD medical devices, although falling within medical devices, were not included in the first draft of the CND. The CUD, nominated for the biennium 2005–2007, decided to proceed with a modification of the first version of the CND and complete it by including also the IVD medical devices not included in the first version. The updated version of the CND was approved by the CUD on November 29, 2006. The decree February 20, 2007 "Approving the National Classification of Medical Devices (CND)", defined by the Ministry of Health in

agreement with the State-Regions Conference, is published in the Official Journal (OJ) of the Italian Republic (similar to the Code of Federal Regulations, CFR, in the United States) n. 63 of March 16, 2007. The CND and subsequent updates are also published in the OJ, as well as in the portal of the Ministry of Health. The new CND in accordance with the guidelines for classification already defined in the first version and only partly expanded in the latter, has an alpha numeric structure that aggregates medical devices in “Categories”, “Groups”, and “Types”. The types reach different levels of detail.

All IVD medical devices were included within the CND in the specific category of “W”, which were classified by adopting the system in general classification proposed by the European Diagnostic Manufacturers Association (EDMA).

Diagnostic reagents were included in the group “W01” divided into the following types:

- W01 01: Clinical chemistry
- W01 02: Immunochemistry
- W01 03: Hematology/Histology/Cytology
- W01 04: Microbiology
- W01 05: Immunology of infectious diseases
- W01 06: Genetic tests.

The classification proposed by the EDMA has been further developed into the following groups:

- “W02” for Instrumentation IVD
- “W05” for IVD storage devices and supplies for general use.

In the Italian territory, entry is allowed into the market of IVD medical devices if it bears the CE mark to demonstrate compliance with law 332/2000 (Implementation of Directive 98/79/EC).

To receive the CE mark, any IVD medical device must meet the “essential requirements” set out in Appendix I to Legislative Decree 332/2000. Compliance with the essential requirements means that the devices are suitable for the established diagnostic purposes and have been designed and manufactured taking into account the generally recognized state of the art, so that their use does not compromise the health and safety of patients and users. Any risks relating to the use of the diagnostic device must be identified and brought to an acceptable level that must be demonstrated with the analysis of risks. These devices must achieve, where appropriate, acceptable performance particularly in analytical sensitivity, diagnostic sensitivity, analytical specificity, diagnostic specificity, accuracy, repeatability, and reproducibility, including control of known relevant interference and limits of detection.

Conformity assessment procedures are described in relation to the type of device: the different procedures are applied so as to be proportionate to the degree of potential risk based on the negative effect that could be produced when the medical device *in vitro* fails.

31.3 Assessment procedures for IVD medical devices

The *in vitro* diagnostic medical devices for the purpose of conformity assessment, are classified into four categories (Figure 31.1) based on the intended use specified by the manufacturer and on the risk to public health and/or for treating the patient.

1 Devices Appendix II – List A. Reagents and reagent products, including related calibrators and control materials, for:

- (a) the determination of the blood groups ABO system, rhesus (D,C,c,E,e), and anti-Kell;
- (b) the detection, confirmation, and quantification in human specimens of markers of HIV infection (HIV 1 and 2), HTLV I and II, and hepatitis B, C and D.

2 Devices Appendix II – List B. Reagents and reagent products, including related calibrators and control materials, for:

- (a) the determination of the blood groups anti-Duffy and anti-Kidd;
- (b) the determination of irregular RBC antibodies;
- (c) the identification and quantification in human specimens of congenital infections (e.g. rubella, toxoplasmosis and others);
- (d) the diagnosis of hereditary disease – phenylketonuria;
- (e) the determination of infection with cytomegalovirus and chlamydia;
- (f) the determination of the HLA tissue groups DR, A, B;
- (g) the determination of the tumor marker PSA;
- (h) the assessment of risk of trisomy 21.

Devices for self-testing, including related calibrators and control materials: device for measuring glucose in the blood.

3 Devices for self-testing: any device intended by the manufacturer to be used at home by nonexperts of diagnostic tests (except as described under category 2 for glucose self-testing).

4 Other types of devices: all other devices that do not belong to Appendix II and are not intended for self-testing. These products are the great majority of devices and have no direct risk to people tested since they are mainly used by properly trained professionals. Moreover the results of the analysis obtained with this type of reagents must be confirmed by other means.

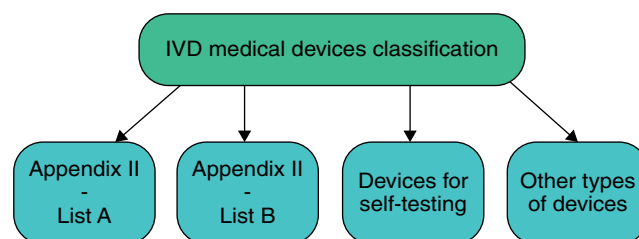


Figure 31.1 *In vitro* diagnostic medical devices classification.

Table 31.1 Tests, devices, and instruments manufactured and commonly used in Italy*

Bacteriology (aerobic/anaerobic)/ mycobacteriology	Virology	Mycology	Parasitology	Mycoplasma, chlamydia, rickettsia and miscellaneous
<p><u>Biolife</u> http://www.masciabrunelli.it/biolife/main.php Culture media (selective, enrichment, chromogenic, antibiotic) Swabs and transport systems Microbank (storage system) Rap ID Card (<i>Gardnerella vaginalis</i> identification) Identification tests for <i>Haemophilus</i> spp., <i>Salmonella</i> spp., <i>Listeria</i> spp.</p> <p><u>Copan</u> http://copanitalia.com/ WASP (Walk Away Specimen Processor) Swabs, broths and transport systems</p> <p><u>Dasit</u> http://www.dasit.it/web/index.asp?idfolder=66 <i>Helicobacter pylori</i> IgA, IgG (Pyloriset EIA immunoassay, MarBlot Western Blot) <i>Bordetella pertussis</i> IgG, IgA, IgM (EIA) <i>B. pertussis</i> MarBlot Western Blot Urine cultures media: dip slides and uricuit slides</p> <p><u>Diapro</u> https://www.diapro.it/ Immunoassay <i>H. pylori</i> antigen (stool)</p> <p><u>Diasorin</u> http://www.diasorin.com/it Liaison CLIA <i>Clostridium difficile</i> GDH <i>C. difficile</i> toxins A and B <i>H. pylori</i></p> <p><u>Diesse</u> http://www.diesse.it/ Robobact (automated system for Urine, Stool and Swabs coltures)</p> <p><u>Far Diagnostics</u> http://www.fardiag.com/ Strep A Card Strep B Card <i>H. pylori</i> antigen card (stool) <i>C. difficile</i> toxin A card</p>	<p><u>Dasit</u> http://www.dasit.it/web/index.asp?idfolder=66 EBV (EBV VCA IgG/M, EBV-NA, EBV- EA, EBV EBNA, EBV VCA) HSV (HSV1-2 IgG/M, MicroTrak HSV1-2) Rotavirus, Adenovirus (Diarlex)</p> <p><u>Diapro</u> https://www.diapro.it/ Giotto: HBsAg HIV Ab/Ag Combo HCV Ab Rubella IgG/IgM Measles IgG/IgM CMV IgG/IgM Dengue IgG/IgM Molecular biology: BKV Dna CMV Dna EBV Dna HBV Dna Enterovirus RNA HDV Rna HHV6 Dna HSV1 Dna HSV2 Dna JCV Dna VZV Dna</p> <p><u>Diasorin</u> http://www.diasorin.com/it Liaison/Murex CLIA/ELISA HIV HTLV VII HBV HCV HAV HEV HSV1/2 CMV EBV Rubella Parvovirus VZV Mumps Measles</p>	<p><u>Diasorin</u> http://www.diasorin.com/it Liaison ELISA Candida albicans IgA, IgG, IgM Liofilchem http://www.liofilchem.net/it/ Integral System Yeast Plus SensiQUATTRO <i>Candida</i></p> <p><u>Mascia Brunelli</u> http://www.masciabrunelli.it Immunoassay <i>Candida albicans</i> Muag Candi KIT (<i>C. albicans</i> identification)</p> <p><u>Radim</u> http://www.radim.com/Default.aspx Immunoassay <i>C. albicans</i> IgA, IgG, IgM <i>Aspergillus fumigatus</i> IgA, IgG, IgM</p>	<p><u>Dasit</u> http://www.dasit.it/web/index.asp?idfolder=66 <i>Cryptosporidium</i> (EIA, IFA) <i>Giardia</i> (EIA, IFA) <i>Entamoeba histolytica</i> (EIA)</p> <p><u>Diasorin</u> http://www.diasorin.com/it Liaison ELISA <i>Ascaris lumbricoides</i> IgG <i>Echinococcus</i> IgG <i>Entamoeba histolytica</i> IgG <i>Taenia solium</i> IgG <i>Trichinella spiralis</i> IgG <i>Toxocara canis</i> IgG <i>Leishmania</i> IgG Chagas (<i>Trypanosoma cruzi</i> IgG) <i>Schistosoma mansoni</i> IgG</p> <p><u>Diapro</u> https://www.diapro.it/ Immunoassay Malaria Ab <u>Mascia Brunelli</u> http://www.masciabrunelli.it Immunoassay Malaria Leishmania Filaria <i>Trichomonas</i> <i>Cryptosporidium</i> <i>Giardia</i></p> <p><u>Radim</u> http://www.radim.com/Default.aspx Immunoassay <i>Ascaris lumbricoides</i> IgG <i>Echinococcus</i> IgG <i>Entamoeba histolytica</i> IgG <i>Taenia solium</i> IgG <i>Trichinella spiralis</i> IgG <i>Toxocara canis</i> IgG <i>Leishmania</i> IgG Chagas (<i>T. cruzi</i>) IgG</p>	<p><u>Biolife</u> http://www.masciabrunelli.it/biolife/main.php Mycoview (identification system for Mycoplasma hominis and Ureaplasma spp.)</p> <p><u>Copan</u> http://copanitalia.com/ E-swab Fecal swab Uri swab UTM Viral transport Flocked swabs</p> <p><u>Dasit</u> http://www.dasit.it/web/index.asp?idfolder=66 <i>Chlamydia trachomatis</i> (IFA) <i>C. trachomatis</i> IgG, IgA (EIA) <i>Chlamydia pneumoniae</i> (IFA) <i>C. pneumoniae</i> IgA, IgG, IgA, IgM (EIA) <i>Mycoplasma pneumoniae</i> IgA, IgG, IgA, IgM (EIA) <i>Legionella</i> urinary antigen <i>Legionella</i> IgG (IFA) Syphilis (FTA ABS IFA, Marblot Western blot IgG/M)</p> <p><u>Diapro</u> https://www.diapro.it/ Giotto Toxo IgG/IgM Immunoassay ELISA IgG Meningococcus (serum and plasma) Syph IgM <i>C. pneumoniae</i> IgA, IgG, IgM <i>C. trachomatis</i> IgA, IgG, IgM Molecular biology <i>Chlamydia trachomatis</i> DNA <i>Toxoplasma gondii</i> DNA <i>L. pneumophila</i> DNA <i>T. cruzi</i> DNA</p>

<u>Liofilchem</u> http://www.liofilchem.net/it/ MIC test strip EnteroPluri-test Chromatic MH CoproSystem Plus UriSystem Chrom Sensi line (broth microdilution panels for AST) Pathogenic System BIOMIC <i>H. Pylori</i> antigen test Chromatic CRE (chromogenic media for carbapenem resistant Enterobacteriaceae) Mascia Brunelli http://www.masciabrunelli.it CLART (Clinical Array Technology) SeptiBAC+ (Gram-positive and fungi identification from positive blood culture) <i>Yersinia enterocolitica</i> , <i>C. difficile</i> and <i>C. jejuni</i> , <i>perfringens</i> , <i>Campylobacter coli</i> and <i>C. jejuni</i> , <i>Salmonella</i> , <i>Aeromonas</i> spp., <i>Shigella</i> spp., <i>Escherichia coli</i> Immunoassay <i>Leptospira</i> <i>Legionella pneumophila</i> <i>Gardnerella</i> <i>Neisseria gonorrhoeae</i> <i>Campylobacter</i> <i>Salmonella</i> <i>Shigella</i> <i>C. difficile</i> (toxin A + B) Strep A Strep B <i>Mycobacterium tuberculosis</i> (TBC) <i>Helicobacter</i> Ab, Ag Coli O157 Radim http://www.radim.com/Default.aspx Immunoassay <i>H. pylori</i> antigen (stool)	<u>Far Diagnostics</u> http://www.fardiag.com/ Adenovirus, rotavirus, duo adeno-rotavirus card Mascia Brunelli http://www.masciabrunelli.it CLART (clinical array technology) HPV Pneumovirus (adenovirus, metapneumovirus, parainfluenza, influenza (H1N1 included), RSVAB, bocavirus, coronavirus, rhinovirus and enterovirus) Enterhex (HSV1, HSV2, VZV, EBV, CMV, HHV6, HHV7, HHV8, poliovirus, enterovirus and coxsackievirus) Immunoassay Dengue IgG/IgM HAV IgM Adenovirus Rotavirus Norovirus Astrovirus Adenovirus Influenza A/B	<u>Diasorin</u> http://www.diasorin.com/it Liaison ELISA/CLIA <i>M. pneumoniae</i> IgG/IgM <i>C. pneumoniae</i> , <i>C. trachomatis</i> (IgA, IgG, IgM) <i>Treponema</i> screen <i>B. pertussis</i> toxin IgA, IgG, IgM <i>Brucella</i> IgG, IgM <i>C. tetani</i> IgG <i>C. burnetii</i> (Phase1 IgG, Phase2 IgG, IgM) <i>Corynebacterium diphtheriae</i> (IgG) <i>H. pylori</i> IgG, IgA <i>Borrelia</i> IgM Toxo IgG/IgM Diesse http://www.diesse.it/ CHORUS ENZY-Well Immunoenzymatic line (ELISA): Torch, Syphilis screen, CMV Screen, <i>Treponema</i> IgG and IgM, EBV Vca IgG and IgM, EBV EBNA IgG, EBV EA, <i>H. pylori</i> IgG and IgA Far Diagnostics http://www.fardiag.com/ Syphilis card Mononucleosis card ChlamyCheck Liofilchem http://www.liofilchem.net/it/ <i>Mycoplasma</i> System Plus A.F. Genital System Mascia Brunelli http://www.masciabrunelli.it Immunoassay Syphilis Ab Syphilis RPR Syphilis TPHA <i>C. trachomatis</i> <i>M. pneumoniae</i> Radim http://www.radim.com/Default.aspx ALISEI
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* Other tests, devices, and instruments available from the United States, the EU, and from other countries may be available for use in Italy. Please refer to other chapters in this text and to other companies and vendors in the Appendix of this book and from elsewhere in addition to current sources of information.

The different assessment procedures are:

- For Appendix II – List A, the manufacturer must obtain from the Notified Body approval of its production facilities with strict controls and safeguards which also involve the design aspect. The term “Notified Body” refers to specific governmental entities that are authorized by the competent authorities of different EU members and appointed to carry out the certification procedures. These bodies are accredited for the certification of specific types of medical devices and for specific assessment procedures. The manufacturer may apply to any “Notified Body” accredited in Italy or another EU country, at its option. In Italy, the “Istituto Superiore di Sanità” (ISS) was authorized by the Ministry of Health as the Notified Body able to dispatch the procedures of evaluation of conformity and to release the conformity certificates for the IVDs included in Appendix II – Lists A and B and for self-testing devices.

The manufacturer can choose between two different procedures:

- (a) Declaration of Conformity EC: full quality assurance system, including the examination of design of the product and verification of the lot.
- (b) EC-Type Examination: EC declaration of conformity (production quality assurance) and verification of the lot.
- For Appendix II – List B, the manufacturer must obtain from the “Notified Body” approval of its production facilities with strict controls and safeguards, which also involve the design aspect. The manufacturer can choose between three different procedures:
 - (a) Declaration of Conformity (full quality assurance system)
 - (b) EC-Type Examination + EC Verification
 - (c) EC-Type Examination + EC Declaration of Conformity (production quality assurance).
- For devices for self-testing the manufacturer prepares a declaration of conformity and must obtain from the “Notified Body” the assessment of device design on issues that relate to its adaptability to non-professional users. The manufacturer may also choose the alternative procedures used for higher risk products.
 - (a) Declaration of Conformity
 - (b) Declaration of Conformity (full quality assurance system)
 - (c) EC-Type Examination + EC Verification
 - (d) EC-Type Examination + EC Declaration of Conformity (production quality assurance)
- For all other IVD medical devices the manufacturer will be able to CE mark the product and enter the market after making a declaration of conformity with the essential requirements (Appendix I) according to Appendix III of Directive 98/79/EC. In this declaration the manufacturer ensures and declares that its products meet the requirements of the Directive. The manufacturer shall comply with all technical

documents to demonstrate the safety of the manufactured device. This documentation must be available to the Competent Authority (“Notified Body”).

31.4 Registration for manufacturers of IVD medical devices

The manufacturers that place in Italy IVD medical devices in their own name are obliged to inform the Ministry of Health of all the necessary data to identify the devices (Article 10 of Legislative Decree 332/2000 concerning IVD medical devices) using the appropriate on-line forms (recommended) or sending data by fax or by mail (in this case, the sender must attach a cover letter).

All documents must be sent to:

Ministry of Health
General Directorate of Medical Devices
Pharmaceutical Services and the Safety of Care
Office IV

Via Ribotta 5 - 00144

Rome

Fax 0039 06 59943266

For IVD medical devices included in Appendix II and for devices for self-testing, also required is the submission of labels and instructions for use to the Ministry Department of Health. In order to facilitate the submission of such documentation, labels and instructions can be sent, as well as the other required information through online forms, by Certified Mail (dgfdm @ postacert.sanita.it) or by fax (0039 06 59943266).

If not located in a member state of the EU, the manufacturer who places devices on the market under its own name, must designate a representative in the European Union. The authorized representative must notify the Ministry in Italy all the data related to devices (article 10, c. 3 Leg. 332/2000).

As a transitional measure, until the establishment of the European database, manufacturers and/or representatives who are not established in Italy but already registered in their home country are required to submit data about the devices they intend to trade in the Italian territory (article 10, c. 6 D. Leg. 332/2000).

Table 31.1 contains products, devices, tests and instruments available for clinical microbiology laboratory diagnostic testing in Italy. Other products may also be available. The reader is encouraged to refer to other chapters in this book and to other companies and vendors (and their web sites) for additional current information.

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Clinical Microbiology *In Vitro* Diagnostics in Japan: Regulatory Requirements and Product Information

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32.1 The regulatory structure of pharmaceuticals and clinical trials in Japan

The current Japanese Pharmaceutical Affairs Law of 1960 (act no. 145) regulates licensed marketing business practice that routinely manufactures medicinal and pharmaceutical products and conducts national product distribution. Therefore, the regulated subjects of this Law are not research institutions such as universities, but rather commercial enterprises, including pharmaceutical companies. Under the regulation of the Pharmaceutical Affairs Law, clinical trials conducted for the purpose of marketing approval by the government (via the Minister of Health, Labour, and Welfare) are called “*chiken*,” (lit: treatment trials). Upon approval, an official price of the product is determined by the Government and then listed on the national health insurance price listing, which allows national medical institutions to use the product.

The Pharmaceuticals and Medical Devices Agency (PMDA) reviews the enforcement of *chiken*, as well as post-trial applications for marketing. Since the revision of the Japanese Pharmaceutical Affairs Law created several years ago, not only pharmaceutical companies, but also medical institutions, including academic hospitals, can also submit applications for investigator-initiated clinical trials to PMDA. However, there are exceptions for the regulatory requirements under the Japanese Pharmaceutical Affairs Law. With regards to novel molecular entities that are unapproved in any fields other than genetic and cellular therapies, the application and review process is not necessary if the new molecular entity is used as an investigational product for non-*chiken* clinical research (hereafter called “clinical research”, conducting development which is not for the purpose of marketing approval), conducted by institutions such as universities. The advantage

of this clinical research is that the expenses are covered by the national Special Healthcare Expenditure system if a subject participates in clinical research at a designated institution. For all clinical research, ethical guidelines are required. Nonetheless, it is still easier for investigators to conduct clinical research than *chiken* because the clinical research does not mandate good clinical practice (GCP). On the other hand, the clinical data obtained through clinical research is often considered insufficient evidence. Hence, the data cannot be used to evaluate new products for marketing approval in Japan and other countries.

Further information can be found on the following websites of the Ministry of Health, Labour, and Welfare and PMDA: Ministry of Health, Labour, and Welfare – <http://www.mhlw.go.jp/english/>; Pharmaceuticals and Medical Devices Agency in Japan – <http://www.pmda.go.jp/english/>

32.2 The regulatory structure of *in vitro* diagnostic testing in Japan

In vitro diagnostic (IVD) reagents are described in Article 2, paragraph 13 of the Pharmaceutical Affairs Law of 1960. The term “*in vitro* diagnostic reagent” used in this law refers to a test, device, or reagent not directly applied to the human or animal body and exclusively used in the diagnosis of disease.

Diagnostic tests for infectious diseases are classified based on their risk (see Table 32.1). In contrast to Class I items (low-risk tests and reagents) or Class II items (relatively low-risk tests and reagents), which require no regulatory review by PMDA and are instead cleared by third party certification, certain diagnostic tests for infectious diseases are classified into Class III (relatively high-risk tests and reagents), which requires approval from the Minister of Health, Labour, and Welfare. The

Table 32.1 Classification of *in vitro* diagnostic reagents in Japan

Class	Outline	Compliance with Standards	Application process required	Review authority
I	Reagents that have lower risks as diagnostic information tools for making diagnoses and lower potential to produce life-threatening experiences than Class III. Certified reference materials or standard measurement methods are given on the bulletin for the reagent, which provide convenient manufacture control and quality assurance management. Nonprescription reagents (over-the-counter) are excluded, e.g., GPT, GOT, ALP, glucose, LDH, HbA1c, IgG, cholesterol, estradiol	Compliant	Marketing notification	No review necessary
		Noncompliant	Marketing approval	Pharmaceuticals and Medical Devices Agency
II	Reagents that have relatively low risks as diagnostic information tools for making diagnoses and lower potential to produce life-threatening experiences than Class III. Nonprescription reagents (over-the-counter) are included, e.g., hematologic tests including hemoglobin and hematocrit, autoimmunology tests including anti-Sm antibody	Compliant	Marketing certification	Registered certifying agency
		Noncompliant	Marketing certification	Pharmaceuticals and Medical Devices Agency
III	Reagents that have relatively high risks as diagnostic information tools for making diagnoses and high potential to produce life-threatening experiences, e.g., cancer, diagnostic tests for infectious diseases including HIV and HCV tests, genetic diagnostic reagents including NAT test, microbiology tests	Compliant	Marketing approval	Pharmaceuticals and Medical Devices Agency
		Noncompliant	Marketing approval	Pharmaceuticals and Medical Devices Agency
		No standards	Marketing approval	Pharmaceuticals and Medical Devices Agency

approval criteria are divided into two categories: reagents for detection (e.g., qualitative and semiquantitative tests) and reagents for measurement (e.g., quantitative tests). There is a specific testing method, control reagent, and statistical method determined for each reagent. The differences in the criteria for reagents for detection and reagents for measurement are the number of specimens tested and their analytical method.

An applicant may determine in which class the product for consideration is classified. In cases where the appropriate class is difficult to determine, an applicant may consult the PMDA. The application categories are divided as follows (see below): (1) new products that are to detect or measure a new product; (2) products with undetermined approval criteria; (3) products which meet existing approval criteria; and (4) products which do not meet approval criteria. The category for each product should be determined based on its characteristics and results of clinical trials. The fees for determining approval are different depending on the application category.

Categories:

- 1 New product – application for marketing approval of IVD reagents that are to detect or measure a new product.
- 2 Nonstandard approval product – Class III IVD reagents with undetermined approval criteria (e.g., HIV, HCV, HDV, HTLV, genetic testing items for pathogens, human genetic testing items).
- 3 Conformity approval product – IVD reagents whose approval criteria have been determined and have also been met.

4 Nonconforming approval product – IVD reagents whose approval criteria have been determined, but on testing against these approval criteria, fail to meet them. All IVD testing for tests, devices and instruments, including domestic and imported products must follow these guidelines.

32.3 Marketing of IVD reagents in Japan

32.3.1 Marketing licenses

Marketing of IVD reagents is allowed only for licensed marketing approval holders who have submitted an application for marketing approval, marketing certification, or a notification of marketing on a product-by-product basis. These holders must receive approval or certification to start their practice. The regulations apply to all products used in Japan regardless of domestic or foreign origin. In addition to the marketing approval or certification, licensed marketing approval holders must also submit an application for quality management system (QMS) compliance inspection. Licensed marketing approval holders who wish to market IVD reagents are required to obtain a No. 2 type license for marketing.

The requirements for the No. 2 type license approval are as follows: complying with standards for quality control (GQP and QMS) and standards for postmarketing safety management (good vigilance practice; GVP). Marketing business licenses are granted by the prefectural governor at the location of the business's principal office following submission of their application. Marketing business licenses are valid for 5 years and must be renewed before the expiration date.

32.3.2 Foreign manufacturers

Foreign manufacturers whose facilities are located outside Japan and who wish to export IVD reagents to Japan are required to obtain relevant accreditation of foreign manufacturers. The accreditation of foreign manufacturers is granted by the Minister of Health, Labour, and Welfare on a facility-by-facility basis. The application process for accreditation may be conducted by proxy by a licensed marketing approval holder of the drug or medical device on behalf of a foreign manufacturer. Accreditation of foreign manufacturers is valid for 5 years. The accreditation needs to be renewed before the expiration date.

In vitro diagnostic reagents are classified by generic names that are given to each individual reagent. These generic names are determined based on the nomenclature for IVD reagents from the ISO/TC120 Global Medical Device Nomenclature (GMDN) project.

The approval criteria for Class III IVD reagents are described as follows:

1 Reagents for detection

- Testing methods
 - (a) Testing may be performed by the applicant or by other testing institutions at the applicant's request. The test results must be documented and signed by a test conductor or director.
 - (b) As a general rule, more than 50 samples must be collected for specimens with positive or negative results. Additionally, any specimens that are around the clinically standard concentration (cut-off value) must be included.
- Comparative control products: if approved or certified, IVD reagents are available as comparative control products; two or more IVD reagents must be selected as comparison. If multiple IVD reagents are available, reagents with several different measurement methods must be selected. In addition, for reagents for detection and reagents for semi-quantitative measurement, any reagents with different indicated value ranges due to the different number of steps required should not be compared to one another (e.g., a reagent determined as “–, 1+, 2+, 3+” should not be compared with another reagent determined as “–, 1+, 2+, 3+, 4+”).
- Statistical processing method: the concordance rate is calculated from data tested by comparison using an appropriate table (e.g., $m \times n$ contingency table). The concordance rate must be calculated as 90% or above when using this statistical method.

2 Reagents for measurement

- Testing methods
 - (a) Testing may be performed by the applicant or at other testing institutions at the applicant's request. The test results must be documented and signed by a test conductor or director.

- (b) As a general rule, more than 100 samples must be collected. The concentrations of specimens must be distributed throughout the measurable concentration range. Additionally, any specimens that are around the clinically standard concentration (standard value; cut-off value) must be included.

- Comparative control products: for approval or certification, two or more IVD reagents must be available as comparative control products. If multiple IVD reagents are available, and multiple measurement methods are available, two or more reagents with different methods must be selected.

The reagent is inappropriate to use as comparative control products if the y -intercept from the linear regression equation for the results of the measurement of the *in vitro* reagents that are to be compared is widely separated from zero.

- Statistical methods: a correlation coefficient and a regression coefficient are calculated by plotting the result of a comparative control reagent on the x -axis and the reagent under consideration on the y -axis for identical samples. The correlation coefficient must be 0.9 or greater, and the regression coefficient must trend between 0.9 and 1.1, when using this statistical method.

Table 32.1 shows a summary of each IVD reagent class, its application process, which is based upon whether the reagent meets its approval and certification criteria, and its reviewing authority.

32.4 Clinical efficiency study/correlation study

The contents of clinical studies vary depending upon whether the reagent is a new product or an existing product. If there is an existing reagent available as a comparator, a correlation study should be performed to determine whether or not it meets the approval criteria. If the product meets the approval criteria, the application for its marketing approval is submitted as a conformity approval product. If the product fails to meet the approval criteria, this application is submitted as a nonconforming approval product. The clinical usefulness of new products is evaluated by conducting a clinical efficiency study, since there are no existing IVD reagents available as a comparator.

Currently, the GCP, a standard for conducting clinical trials for medical products, is not required for IVD reagents, although compliance with GCP is preferred when clinical trials are conducted for these reagents. Clinical data obtained overseas may be used in applications for marketing approval. However, the raw data also must be retrievable in case of a request by the review institution, PMDA, for presentation, including a report on trial results and case reports.

Table 32.2 *In vitro* diagnostic reagents approved for infectious diseases in Japan (as of November 2011)*

Category	Generic name
Reagents for testing infection (non-viral)	<i>Chlamydia</i> Antigen Kit
	<i>Chlamydia</i> Nucleic Acid Kit
	<i>Chlamydia</i> Antibody Kit
	<i>Chlamydia</i> Immunoglobulin G Kit
	<i>Chlamydia</i> Immunoglobulin A Kit
	<i>Chlamydia</i> Immunoglobulin M Kit
	Anti-streptolysin O Kit
	Anti-streptokinase Antibody Kit
	Group A <i>Streptococcus</i> of Polysaccharide Antibody Titer Kit
	Anti- <i>Streptococcus pyogenes</i> DNase B Kit
	Anti-streptococcal-hyaluronidase Kit
	Group A beta-hemolytic <i>Streptococcus</i> Antigen Kit
	<i>Neisseria gonorrhoeae</i> Antigen Kit
	<i>Neisseria gonorrhoeae</i> Nucleic Acid Kit
	Endotoxin Kit
	<i>Salmonella typhi</i> Antibody Kit
	<i>Bordetella pertussis</i> Antigen Kit
	<i>Bordetella pertussis</i> Antibody Kit
	<i>Helicobacter pylori</i> Antigen Kit
	<i>Helicobacter pylori</i> Urease Kit
	<i>Helicobacter pylori</i> Antibody Kit
	<i>Leptospirosis</i> Kit
	<i>Treponema</i> Antibody Kit
	<i>Treponema</i> Immunoglobulin M Antibody Kit
	Non- <i>Treponema</i> Lipid Antibody Kit
	<i>Borrelia</i> Antibody Kit
	<i>Mycoplasma</i> Antibody Kit
	<i>Clostridium difficile</i> Kit
	Rickettsia Kit
	<i>Tsutsugamushi</i> disease Kit
	<i>Tsutsugamushi</i> disease Antibody Kit
	<i>Cryptococcus neoformans</i> Kit
	<i>Candida</i> Kit
	<i>Aspergillus</i> Kit
	Toxoplasma Antibody Kit
	Toxoplasma Immunoglobulin M Kit
	Toxoplasma Immunoglobulin G Kit
	<i>Entamoeba histolytica</i> Antibody Kit
	<i>Mycobacterium</i> Nucleic Acid Kit
	<i>Mycobacterium</i> Antibody Kit
	<i>Mycobacterium</i> Antigen Kit
	<i>Escherichia coli</i> O157 Antigen Kit
	<i>Escherichia coli</i> H7 Antigen Kit
	<i>Escherichia coli</i> Verotoxin Kit
	<i>Escherichia coli</i> O157 LPS Antibody Kit
	(1→3)-β-D-glucan Kit
	<i>Neisseria meningitidis</i> Capsular Polysaccharides Antigen Kit
	<i>Streptococcus</i> Antigen Kit
	Thermostable Direct Hemolysin of <i>Vibrio parahaemolyticus</i> Kit
	<i>Brucella</i> Kit
	Anti- <i>Anisakis</i> Immunoglobulin G, Immunoglobulin A Antibody Kit
	<i>Mycoplasma</i> Antigen Kit

Table 32.2 (Continued)

Category	Generic name
Reagents for testing infection (nonviral)	<i>Mycobacterium leprae</i> specific Antibody Kit
	Anti- <i>Mycobacterium</i> Antibody Kit
Reagents for testing viral infection	Malignant Malaria HRP-2n Kit
	<i>Legionella</i> Kit
	Interferon-γ Release Assay Kit
	<i>Streptococcus pneumoniae</i> Kit
	<i>Streptococcus pneumoniae</i> Capsule Antigen Kit
	Procalcitonin Kit
	<i>Mycoplasma</i> Nucleic Acid Kit
	<i>Legionella</i> Nucleic Acid Kit
	Hepatitis A Virus Antibody Kit
	Hepatitis A Virus Immunoglobulin M Kit
	Hepatitis B Virus Core Antibody Kit
	Hepatitis B Virus Core Immunoglobulin M Kit
	Hepatitis B Virus e Antigen Kit
	Hepatitis B Virus e Antibody Kit
	Hepatitis B Virus/e Antigen/Antibody Kit
	Hepatitis B Virus Surface Antigen Kit
	Hepatitis B Virus Surface Antibody Kit
	Hepatitis B Virus pre-S2 Antigen Kit
	HBs Antigen • Albumin Receptor Conjugates Kit
	Hepatitis B Virus Core Nucleic Acid Kit
	Hepatitis C Virus Nucleic Acid Kit
	Hepatitis C Virus Antigen Kit
	Hepatitis C Virus Core Protein Kit
	Hepatitis C Virus Antibody Kit
	Hepatitis C Virus Immunoglobulin G Kit
	Hepatitis C Virus Core Antibody Kit
	Hepatitis C Virus Grouping Kit
	GOR Antibody Kit
	Hepatitis C Virus Antibody Confirmation Kit
	Hepatitis D Virus Antibody Kit
	Papilloma Virus Nucleic Acid Kit
	Adenovirus Kit
	Herpes Simplex Virus Immunoglobulin G Kit
	Herpes Simplex Virus Immunoglobulin M Kit
	Herpes Simplex Virus Antibody Kit
	Herpes Simplex Virus Type 1 Kit
	Herpes Simplex Virus Type 2 Kit
	Varicella • Herpes Zoster Virus Antibody Kit
	Varicella • Herpes Zoster Virus Antigen Kit
	Varicella • Herpes Zoster Virus Immunoglobulin G Kit
	Varicella • Herpes Zoster Virus Immunoglobulin M Kit
	Cytomegalovirus Antigen Kit
	Cytomegalovirus Antibody Kit
	Cytomegalovirus Immunoglobulin G Kit
	Cytomegalovirus Immunoglobulin M Kit
	Epstein-Barr Virus Antibody Kit
	Epstein-Barr Virus Immunoglobulin G Kit
	Epstein-Barr Virus Immunoglobulin A Kit
	Epstein-Barr Virus Immunoglobulin M Kit
	Polio Virus Kit

Table 32.2 (Continued)

Category	Generic name
Reagents for testing viral infection	Coxsackie Virus Kit
	Echovirus Kit
	Japanese Encephalitis Virus Antibody Kit
	Rubella Virus Antibody Kit
	Rubella Virus Immunoglobulin G Kit
	Rubella Virus Immunoglobulin M Kit
	Influenza Virus Kit
	Neuraminidases derived from Influenza
	Type A Virus and Influenza Type B Virus Kit
	Parainfluenza Virus Kit
	RS Virus Kit
	Measles Virus Kit
	Measles Virus Antibody Kit
	Measles Virus Immunoglobulin G Antibody Kit
	Measles Virus Immunoglobulin M Antibody Kit
	Mumps Virus Kit
	Human T-cell Lymphotropic Virus Type I Antibody Kit
	Human T-cell Lymphotropic Virus II Antibody Kit
	Human T-cell Lymphotropic Virus I Antibody Confirmation Kit
	Human T-cell Lymphotropic Virus II Antibody Confirmation Kit
	Human T-cell Lymphotropic Virus I/II Antibody Confirmation Kit
	Human Immunodeficiency Virus 1 Nucleic Acid Kit
	Human Immunodeficiency Virus 1 p24 Antigen Kit
	Human Immunodeficiency Virus 1 p24 Antigen • HIV Antibody Kit
	Human Immunodeficiency Virus 1 Antibody Kit
	Human Immunodeficiency Virus 2 Antibody Kit
	Human Immunodeficiency Virus 1 Antibody Confirmation Kit
	Human Immunodeficiency Virus 2 Antibody Confirmation Kit
	Human Immunodeficiency Virus Antibody Kit
	AIDS related Virus Antibody Kit
	HIV Structural Protein Specific Antibody Kit
	Reovirus Kit
	Reovirus Antibody Kit
	Rotavirus Kit
	Rotavirus Antibody Kit
	SV5 Virus Kit
	SV5 Virus Antibody Kit
	SV41 Virus Kit
	Enterovirus Kit
	Parvovirus Kit
	Human Parvovirus B19 Immunoglobulin G Antibody Kit
	Human Parvovirus B19 Immunoglobulin M Antibody Kit
	Hepatitis B Virus DNA Polymerase Kit
	SARS Coronavirus Nucleic Acid Kit
	Hepatitis E Virus Antibody Kit
	Influenza Virus Nucleic Acid Kit
	Swine Influenza (H5 Subtype) Virus Nucleic Acid Kit
	Herpes simplex Virus Kit

Table 32.2 (Continued)

Category	Generic name
Culture, nucleic acid, serologic testing reagents for identification	Norovirus Antigen Kit
	Hepatitis B Virus DNA Polymerase Gene Mutations Detection Kit
	Hepatitis B Virus Core related Antigen Kit
	Hepatitis B Virus Surface Nucleic Acid Kit
	Hepatitis B Virus Antigen Epitope Kit
	Human Papilloma Virus Nucleic Acid Typing Kit
	Culture Identification • General
	Bacteria Kit
	Non-fermenting Gram-negative Rod Kit
	<i>Staphylococcus</i> Kit
	<i>Mycobacterium tuberculosis</i> complex
	Rifampin Resistance Gene Identification Kit
	Nucleic Acid Identification, <i>Staphylococcus aureus</i> Kit
	Nucleic Acid Identification, <i>Mycobacterium</i> spp. Kit
	Nucleic Acid Identification, <i>Legionella</i> spp. Kit
	Nucleic Acid Identification, General
	Bacteria Kit
	MRSA Coagulase Type Identification • <i>Staphylococcus</i> Coagulase Type Identification Kit
	Culture Identification, Fungus Kit
	Culture Identification, <i>Mycobacterium</i> Kit
	Culture Identification, Niacin Test Kit
	Anaerobe Biochemical Identification Kit
	<i>Streptococcus</i> Kit
	Influenza Type B Kit
	<i>Salmonella</i> Kit
	<i>Shigella</i> Kit
	<i>Escherichia coli</i> Kit
	<i>Yersinia</i> Kit
	<i>Klebsiella</i> Kit
	<i>Serratia marcescens</i> Kit
	Pseudotuberculosis Bacterial Typing Kit
	<i>Pseudomonas</i> Kit
	<i>Flavobacterium</i> spp. Typing Kit
	<i>Vibrio parahaemolyticus</i> Serologic Typing, <i>Vibrionaceae</i> Serologic Typing Kit
	<i>Vibrio cholerae</i> Kit
	Influenza Capsule Typing • <i>Haemophilus</i> Capsule Typing Kit
	Heat-resistant <i>Clostridium perfringens</i> Type A Typing Kit
	<i>Campylobacter</i> Kit
	Serologic Typing, <i>Alcalescens dispar</i> Kit
	Serologic Typing, Fungus Kit
	Bacterial Toxins Detection Kit
Drug sensitivity testing reagents	Drug Sensitivity Testing (General Bacteria, Disk Diffusion Method) Kit
	Drug Sensitivity Testing (General Bacteria, Broth Dilution Method) Kit
	Drug Sensitivity Testing (<i>Mycobacterium</i>) Kit
	Drug Sensitivity Testing (Fungus) Kit

* Please note that other tests, devices and instruments manufactured in the United States, the EU, and other countries (such as rapid tests and instruments for *Enterobacteriaceae*, non-*Enterobacteriaceae*, yeasts, parasites, viruses, blood cultures, and antimicrobial susceptibility testing) may be used in healthcare settings in Japan. Please refer to other chapters in this book and vendors and their web sites for additional information.

32.5 Marketing approval

In vitro diagnostic reagents for infectious diseases are classified as Class III. Therefore, their application for marketing approval must be submitted to the PMDA, and the Minister of Health, Labour, and Welfare is required to approve the application.

In general, the following data is required for application. Different data may be required for submission depending upon the type of product.

- Data concerning the origin of the IVD product, the background of its discovery and the condition of use overseas
- Data concerning setting of specifications
- Data concerning stability
- Data concerning performance
- Data concerning risk analysis
- Data concerning the manufacturing method
- Data concerning clinical trial results to demonstrate clinical effectiveness and safety

Any trials that are prepared for application for marketing approval must be performed in a reputable facility with appropriate equipment and staff members. The facility also needs to demonstrate appropriate management.

In vitro diagnostic reagents play an important role in public health, thus application for pre-approval clinical trials is required after the application is submitted for marketing approval. The National Institute of Infectious Diseases conducts pre-approval clinical trials in cooperation with the PMDA.

The following IVD reagents require pre-approval clinical trials: (i) products listed in the Japanese Standards of Antibodies for the determination of blood type; (b) products related to blood transfusion (syphilis, HBV gene, HBs antigen, HCV, HDV, HIV, and HTLV); (c) products which are important in public health (HAV, rubella virus, *Chlamydia trachomatis* gene and antigen; and (iv) products designated by administrative authorities.

Table 32.2 shows the IVD reagents for infectious diseases available in Japan as of November 2011. Other kits, reagents, tests, devices, and instruments may also be available in Japan. Please refer to other chapters in this book and vendors and their web sites for additional information.

32.6 National Health Insurance coverage of IVD reagents

With regards to the insurance repayments for IVDs, the medical cost payment is regulated and scored based on the medical care cost equation table (Special Care Fees), all values of which are determined by the government. The cost includes the personal cost for physicians, nurses, and technicians who perform the tests, the cost of the reagent and disposables, depreciation cost for medical equipment, service charges, and the cost of linen. The medical reimbursement system is revised biannually.

The cost is identical throughout the country, regardless of the type of insurance. Licensed marketing approval holders need to submit an application for national health insurance coverage to the Ministry of Health, Labour, and Welfare following marketing approval.

There are three different classes of applications for national health insurance coverage: E1, E2, and E3.

- E1 (existing): both the measuring item and measuring method are the same as an existing reagent.
- E2 (new method): the measuring item is the same as an existing reagent, but its measuring method is different from any existing reagents.
- E3 (new item): the measuring item is new.

This classification is different from the classification of IVD reagents for marketing approval. For example, an IVD reagent classified into Class III is classified into E1 if it already has the same measuring item and measuring method as an existing reagent. The measuring items refer to items listed on the medical care cost equation table.

An application for national health insurance coverage may be submitted to the Health Insurance Bureau, Economic Affairs Division of the Ministry of Health, Labour, and Welfare. The Division also accepts consultation in advance of application submission. The required documentation varies depending upon its classification. E1 is a class where both the measuring item and measuring method are the same as an existing reagent. The application for E1 must include submission of an application for national health insurance coverage, along with a copy of certification for marketing approval under the Pharmaceutical Affairs Law, as well as a copy of its application. The approval is granted if the applicant does not receive any notifications from the division within 20 business days.

For the application for E2, the number of tests, price of the reagent, desired payment fee and its justification, an outline of sales forecasting, and data comparing its usefulness with existing reagents must be submitted along with the application.

The Health Insurance Bureau, Economic Affairs Division confirms the contents of the submitted application. For E2 and E3, the Division interviews the applicant to review usefulness for the national health insurance system as well as any medical economic impact. If the health insurance bureau medical economics division determines that the reagent is suitable for national health insurance coverage, the applicant is notified of the medical cost for payment under the name of the head of Health Insurance Bureau, Medical Economics Division of the Ministry of Health, Labour and Welfare.

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Clinical Microbiology *In Vitro* Diagnostic Medical Devices in the Republic of Korea: Regulatory Requirements and Product Information

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The Medical Devices Act (August 13, 2013) regulates the production, import, and selling of all medical devices and instruments in the Republic of Korea. Enforcement Decree (March 23, 2013) and Enforcement Regulation (April 1, 2014) of the Medical Device Act deal with specific regulations and requirements. Most devices and instruments used in the clinical microbiology laboratory are regulated by the Medical Device Act, the Enforcement Decree of the Medical Device Act, and Enforcement Regulations of the Medical Device Act. Diagnostic reagents are also regulated by related laws and regulations.

In the past, diagnostic reagents were not regulated by the Medical Devices Act. Instead, reagents used with manual procedures including enzyme-linked immunosorbent assay (ELISA) were regulated by the Act for medicine and medical supplies (December 2, 2011). For reagents used in automatic analyzers, it was enough to register with the Korea Pharmaceutical Traders Association (KPTA) when the reagents were imported from abroad; and when made by domestic manufacturers they could be sold freely without any restriction.

In 2009, the whole world was challenged due to the appearance of the H1N1 influenza virus, which was raging fiercely in Korea as well as other Asian countries. At that time, many new reagents for polymerase chain reaction (PCR), real-time PCR (RT-PCR), and rapid antigen tests were used to diagnose H1N1 influenza. The flood of unverified instruments and reagents became a serious problem, and the importance of safety management for instruments and products for *in vitro* diagnosis (IVD) was recognized. Consequently, in 2011, the members of the Korean Food and Drug Administration (KFDA) recruited a Taskforce team to control products for IVD. The team revised the Medical Devices Act, and products for IVD were reclassified as medical devices (July 28, 2011).

All analytical products for IVD are categorized into four grades according to their purpose and the risk of disease. There are some differences by grade in the documents required for permit applications and declarations.

The analytical products used for general purpose diagnosis are included in Grade 1. This grade covers IVD reagents for staining or culturing in clinical microbiology.

Grade 2 includes products for IVD except those in Grades 1, 3, and 4. Grade 2 includes IVD reagents and media for antibiotic susceptibility. This includes IVD reagents for infectious diseases to detect relatively low infectivity pathogen such as *Salmonella* (Widal test), HEV, *Helicobacter pylori*, *Saccharomyces*, *Clostridium difficile*, and *Giardia lamblia*.

Grade 3 contains the widest range of test items, including many materials used in immunologic and molecular tests for infectious diseases. This grade includes analytical products for IVD of life-threatening or highly transmissible diseases. Thus, the products are those used in tests that can have a decisive effect on treatment, determination of disease stage, or patient monitoring. The first category in Grade 3 includes measures of viral load, genotyping, subtyping, and serotyping of HIV, HBV, HCV, and HTLV. The next category includes items used to diagnose sexually transmitted diseases, diseases that can cause fetal death or severe fetal disability, infectious diseases affecting the cerebrospinal fluid (CSF) and blood, and to detect legally designated infectious pathogens other than “high risk pathogens”, and infectious agents with moderate infectivity. The covered pathogens are as follows: *Toxoplasma*, rubella, cytomegalovirus, herpes simplex virus, EBV, enterovirus, measles virus, parvovirus B19, herpes zoster, *Cryptococcus neoformans*, *Neisseria meningitidis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Treponema pallidum*, HPV, HPV genotype,

Trichomonas vaginalis, Hendra virus, malaria, prion disease, *Mycobacterium tuberculosis* complex, adenovirus, and rotavirus.

Grade 4 includes products used for screening of blood for transfusion, or organs for transplantation such as the antigens, antibody assays, and nucleic acid amplification methods. This grade includes IVD reagents for the diagnosis of HIV, HBV, HCV, and HTLV.

The KFDA implemented the guideline (November 25, 2011, revised in March 23, 2013) for evaluation of analytical products for IVD. It is required that an application form be submitted for production and importation, and the data needed for the technical document are listed. The application form should include the proprietary name, design and dimensions, source material, manufacturing method, purpose and method of use, directions for use, quantity per pack, storage requirements, use-by date, test specification, and manufacturer. The data needed for evaluation of the technical document are as follows: details of development, principles and method of measurement, status of use in Korea and abroad, source material and manufacturing method, storage requirements and the expiration date, test specifications, and rules for safe handling. The requirements are the same for domestic and foreign products.

Most instruments and reagents used in the clinical laboratory in Korea are no different from those used in the United States because many of them are imported from abroad. So, it is sufficient to describe representative instruments for various companies. For example, four major automated systems for bacterial identification and susceptibility tests commonly used in Korea are the VITEK 2 system, the MicroScan system, the Sensititre system, and the Phoenix system, although traditional biochemical kits such as the API system are still used (please refer to other chapters in this book for supplementary information on these and other products and vendor information). For mycobacterial culture, the BACTEC MGIT 960 system and the BacT/ALERT MB system are commonly used in the clinical laboratory, although other products and systems may be available. Viral markers can be divided into immunologic tests using antigen/antibody reactions and molecular tests. There are many instruments from many companies for the detection of viruses using immunologic methods. These include the Architect series of Abbott Diagnostics, modular analytics of Roche Diagnostics with the Elecsys assay, and the ADVIA Centaur System and IMMULITE of Siemens. The VIDAS system of bioMérieux is also commonly used. The COBAS Ampliprep/CobasTaqMan and Abbott Molecular m2000 real-time systems are also commonly used in Korea for viral markers, including those for HBV, HCV, and HIV-1. The reader is referred to other chapters in this book for additional products, devices and instruments that may also be used in healthcare facilities in the Republic of Korea.

Although many instruments and reagents are imported from abroad, we have several domestic corporations that produce many kinds of reagents related to infectious disease identification using molecular biology techniques (Table 33.1). Several products and instruments are listed in the table and discussed below; however, this is a snapshot of some of the products that this author is familiar with and does not include an exhaustive review of all products used in Korea. Please refer to other chapters in this book for additional information and consult current literature and vendor information for supplementary information.

LG Life Science (Seoul, Korea) distributes a number of clinical laboratory kits and instruments. For some time LG has sold ELISA kits for HBsAg, anti-HBs, HCV, HIV, malaria, and syphilis. In recent years, the company has developed several real-time PCR kits using a SLAN or SLAN 96-P real-time PCR detection system for diagnosis of infectious diseases. These include TB/NTM, CT/NG, HPV screening, *Clostridium difficile* toxins A and B, HBV Quantitative, HCV Quantitative, and respiratory viruses. Moreover, the company has developed the GenoBlot Assay using Advasure GenoLine Scan and AdvanSure GenoLine Station (AdvanSure GenoBlot Assay, Seoul, Korea). The kits for the GenoBlot Assay are the Mycobacteria GenoBlot Assay for identification of 25 mycobacteria and the MDR-TB GenoBlot Assay for the detection of *M. tuberculosis* complex and its resistance to rifampin and isoniazid. The HPV GenoBlot Assay can identify 35 types of HPV. The AdvanSure GenoLine Station is a fully automated instrument from test processing to result interpretation, and the AdvanSure GenoLine Scan is a strip scanner for test result scanning and interpretation.

Panagene (Daejeon, Korea) provides a custom peptide nucleic acid (PNA) oligomer. This is an artificially created nucleic acid with a polyamide backbone. Based on PNA oligomers, the company has focused on the development of molecular diagnostic kits. The main technologies are PNAqPCR, PNA FMCA, PANArray, and PNAclamp. The PNAqPCR is real-time PCR combined with a sequence-specific PNA probe. This is useful for multiplexing and quantitative analysis with high sensitivity and specificity. The PNA FMCA provides PNA probe-based fluorescence melting curve analysis and is useful for mutation detection by a single probe. The PANArray is a microarray using PNA probes. This technique is useful for screening of many samples quickly. The PNAclamp technique is used to amplify selectively the mutated target DNA sequence only. This company has a wide-ranging product inventory, and it provides the PNAqPCR TB/NTM Detection Kit and PANArray HPV Genotyping Chip.

Bioneer Corp. (Daejeon, Korea) provides molecular diagnostics by developing nucleic acid extraction and real-time PCR instruments, as well as molecular

Table 33.1 Manufacturers and a Sampling of their Commercial Products in the Republic of Korea*

Manufacturer	Technology	Products
LG Life Sciences. Ltd http://www.lgls.com Tel:+82-2-6924.3652 Fax:+82-2-6924-3062	Real-time PCR and multiplex real time PCR: AdvanSure Real-time PCR kits GenoBlot Assay AdvanSurGenoBlot Assay	TB/NTM (<i>M. tuberculosis</i> and NTM), CT/NG (<i>N. gonorrhoeae</i> and <i>C. trachomatis</i>), HPV screening (detection of 35 HPV types), CD (<i>C. difficile</i> toxin A and B), HBV quantitative, HCV quantitative, RV (14 respiratory viruses) Mycobacteria GenoBlot Assay, MDR-TB GenoBlot Assay, HPV GenoBlot Assay
PANAGENE, Inc http://www.panagene.com Tel:+82-42-861-9295 Fax:+82-42-861-9297	PAN (peptide nucleic acid) qPCR PNA FMCA (PNA probe-based fluorescence melting curve analysis): PANArray, PNAClamp	PNAqPCR TB/NTM detection kit: detection and differentiation of <i>M. tuberculosis</i> and NTM PANArray HPV genotyping chip: detection of 32 HPV types
BIONEER Corp http://eng.bioneer.com Tel:+82-42-930-8777 Fax:+82-42-930-8688	Real-time PCR AccuPower RT-PCR Kits AccuPower technology Dual-HotStart technology Exicycler and ExiPrep 16Dx	HBV/HCV/HIV-1 blood screening, HBV quantitative, HCV quantitative, HIV-1 quantitative, New Inf A (H1N1) and Inf A, MTB and NTM, MP (<i>M. pneumoniae</i>), CP (<i>C. pneumoniae</i>) CT and NG (<i>C. trachomatis</i> and <i>N. gonorrhoeae</i>), STI 8A-Plex and STI 8B-Plex (eight sexually transmitted infections), norovirus, CMV, EBV, and BKV
Biosewoom Inc. http://www.biosewoom.com Tel:+82-2-498-2340 Fax:+82-2-498-1189	PCR, real-time PCR	MTB, NTM and MTB, HSV 1 and 2, CMV, hepatitis B virus quantification, hepatitis C virus quantification, <i>M. tuberculosis</i> , NTM-ID, CMV quantification, EBV, EBV quantification, BK virus, BK virus quantification, influenza A, B, RV detection (13 respiratory virus), seasonal flu H1N1, H3N2 detection, H1N1 Novel One-Step, CT and NG, MH and TV, MG andUU, enterovirus one-step, HSV I and II detection
STANDARD DIAGNOSTICS, Inc http://www.standardia.com Tel:+82-31-8014-6741 Fax:+82-31-8014-6799	POCT: Rapid Antigen test	HBsAg, anti-HBs, HBeAg, HCV, HAV IgM, HIV 1/2 3.0, influenza Ag, RSV, Strep A, TB, TBAg MPT64, malaria Ag P.f/Pan, malaria P.f/P.v, <i>Leptospira</i> , Hantaan virus, <i>Tsutsugamushi</i> , syphilis 3.0, <i>Chlamydia</i> , <i>H. pylori</i> , rotavirus, Rota/Adeno, tetanus, rubella IgG
Seegene http://www.seegene.com Tel:+82-2-2240-4080 Fax:+82-2-2240-4040	ACP technology, PCR: DPO technology(Seeplex ACE detection kit) Real-time PCR: READ Technology (Anyplex) Real-time PCR: TOCE technology (AnyplexII)	RV(7, 12, 15), PneumoBacter, STD, diarrhea, HSV2, meningitis, VanR, MTB/NTM, CT/NG, CMV quantitation, FluA/B typing, HPV28, RB5, STI-7, RV16, MTNB/MDR/XDR

* Other tests, devices, products and instruments manufactured in the United States, the EU, and elsewhere may also be available. Please refer to other chapters in this book and other vendors and their web sites for additional products and information.

diagnostic reagents and kits. The company uses a unique technique of AccuPower technology for qPCR and RT-qPCR. Dual-HotStart technology is based on an enzyme-mediated HotStart method, so it can reduce nonspecific cDNA synthesis and amplification. ExiPrep16DX is an instrument for automated nucleic acid extraction and purification. Exicycler 96 is a real-time PCR system using Light Tunnel technology patented by Bioneer. The ExiStation™ is a set of two instruments composed of three ExiPrep 16DX and one Exicycler 96. There are many kits for detection of bacteria and viruses, including HBV, HCV, HIV-1, MTB and NTM, CMV, EBV, and BKV.

Biosewoom Inc. (Seoul, Korea) provides molecular diagnostics based on the *TaqMan* probe assay and melting curve analysis. The major products are associated with infections by bacteria and viruses, and include HBV

quantification, HCV quantification, MTB and NTM, CMV, EBV, BK virus, CT and NG, and enterovirus.

Standard Diagnostics, Inc. (Yongin-si, Kyonggi-do, Korea) is developing rapid point of care testing (POCT) kits using antigens and antibodies. Alere Healthcare acquired this company a few years ago. The main products are POCT kits for hepatitis markers, tuberculosis, malaria, sexually transmitted disease, and diarrheal diseases.

Seegene (Seoul, Korea) is a relatively new corporation providing unique molecular techniques, such as dual priming oligonucleotide (DPO), real amplicon detection (READ), and TOCE technology. It is useful for multiplex PCR with high sensitivity and specificity. Many kinds of kits have been introduced covering respiratory pathogens, human papilloma virus, diarrheal pathogens, drug resistance, tuberculosis, STD pathogens, and meningitis pathogens.

Please refer to other chapters in this book, and other vendors and their web sites for other products, devices and instruments manufactured and distributed by companies based in the United States, the EU, and other countries.

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Clinical Microbiology *In Vitro* Diagnostic Medical Devices in South Africa: Regulatory Requirements and Product Information

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34.1 Regulatory requirements

There are currently few regulations controlling the sale and use of medical devices and diagnostic tests in South Africa. The exception is electromedical devices or those emitting radiation, which would be regulated by the Directorate of Radiation Control and which falls under the Department of Health (DoH). A license needs to be obtained from the DoH (Directorate: Radiation Control) and needs to have the Conformité Européenne (CE) mark of approval.

At the time of this writing the South African DoH is in the process of drafting proposals to regulate both medical devices and diagnostic tests. This process commenced in 2008 when the Government proposed amendments and made recommendations for a new regulatory authority for health products. These recommendations, known as the Green–Thompson report, were incorporated as amendments to the Medicines and Related Substances Act (Act 101 of 1965) and passed by Parliament in 2008. These amendments, however, have yet to be implemented.

A bill recently came before the Portfolio Committee on Health in September, 2014 (Bill 6B) and after formal discussions and public hearings, the Committee adopted the bill in August, 2015. The bill will create the South African Health Products Regulatory Authority (SAHPRA), which will replace the existing Medicines Control Council (MCC). The current MCC Committee will be replaced by a separate board which will oversee the work of SAHPRA. The new national medicines regulatory authority will have a mandate that will include management of the registration, regulation and control of medicines, complementary medicines, clinical trials, active pharmaceutical ingredients, medical devices and *in vitro* diagnostic (IVD) devices. The above bill was adopted by the National Assembly of Parliament and will be transmitted to the National Council of Provinces for

concurrence as of this writing (January, 2016; the reader is referred to *in session*, Volume 15, issue 7, 2015, the monthly publication of the Parliament of the Republic of South Africa). After review of the bill, it will return to Parliament for final ratification and then signed into law by the President of South Africa. As a result of the potential passing of this bill and its implementation, the regulation and control of medical products in the Republic of South Africa may change significantly in the upcoming months.

SAHPRA and its CEO will report to the Minister of Health and the decision-making process in SAHPRA will be like that of the US Food and Drug Administration (FDA). In addition to the regulation of medicines, SAHPRA's scope of authority will include regulation of complementary medicines, foodstuff, cosmetics, disinfectants, and medical devices (including *in vitro* diagnostic tests). It is also envisaged that there will be "mutual recognition" agreements between SAHPRA and other regulatory authorities like the US FDA to expedite registration times for medicines and devices.

The new regulation, when fully active, will require the licensing of all medical devices, with the exception of custom-made devices. Devices will include all invasive and noninvasive equipment, those that use electricity as a source of power, combination devices, and those manufactured using animal or human cells, tissues or a derivative thereof, sterilization or disinfection apparatus, equipment used for contraception or the prevention of sexually transmitted diseases, as well as those used for *in vitro* diagnostics. Devices that are already on the market in South Africa will also need to be registered within a specified time. It is expected that once a device has been certified, companies will be allowed to sell the device locally. Licenses will have to be renewed every 5 years.

In the absence of fully implemented regulations, the South African Medical Device Industry Association (SAMED), a not for profit organization that represents the medical device association in South Africa, was established in 1985. Its members have developed a code of ethical marketing and business practices to regulate the relationship between medical device and diagnostics companies and healthcare professionals.

Affiliated to SAMED is SALDA (the Southern African Laboratory Diagnostics Association), formally constituted in 2008 with 43 members and represents the interests of the Southern Africa *In Vitro* Diagnostics (IVD) industry, www.samed.org.za. A full list of their members is available on their respective web sites (www.samed.org.za and salda.org.za). Additionally, a potential source of information on all laboratory related products is a publication entitled *LMS-Laboratory Marketing Spectrum* [1].

Most recently, the South Africa Department of Health (DoH) has published, “Draft Regulations Relating to Medical Devices and *In vitro* Diagnostic Medical Devices

(IVDs)” in the South African Government Gazette Notice 37579 (April, 2014) requesting further comments from interested parties. SAMED has submitted detailed comments to the Registrar of the Medicines Control Council and further developments are forthcoming.

34.2 Product information

Many products, tests, devices and instruments manufactured in the United States, the EU, and in other countries may be used in healthcare facilities in South Africa. Some domestic companies and vendors, in addition to those from other countries can be found in the Appendix. The user should consult current company web sites and local publications, periodicals and scientific journals for products used in clinical/diagnostic microbiology.

Reference

- 1 *LMS-Laboratory Marketing Spectrum* (published quarterly). www.lmsmagazine.co.za

Clinical Microbiology *In Vitro* Diagnostic Medical Devices in the UK: Regulatory Requirements and Product Information

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35.1 Background

The regulatory framework for the introduction and supply of commercial microbiological tests in the UK (Table 35.1) is laid out in European Commission Directive 98/79/EC, which pertains to *in vitro* diagnostic medical devices (IVDMDs). As a result the requirements are the same for all European Union countries; variation occurs as a result of national implementation and the nature of the appropriate authority, referred to as the 'notified body'. In the UK there is a single authority, the Medicines and Healthcare Products Regulatory Agency (MHRA: www.mhra.gov.uk). The MHRA is a government agency with responsibility for standards of safety, quality, and performance.

The purpose of the Directive and its essential requirements is to ensure that the microbiological test does not compromise the health and safety of patients and users and is manufactured to a sufficient standard to achieve the performance specified for the stated purpose.

35.2 Definitions

Commercial microbiological tests fulfill the requirements of the definition of an IVDMD:

"any medical device which is a reagent, reagent product, calibrator, control material, kit, instrument, apparatus, equipment, or system, whether used alone or in combination, intended by the manufacturer to be used *in vitro* for the examination of specimens, including blood and tissue donations, derived from the human body, solely or principally for the purpose of providing information:

- Concerning a physiological or pathological state, or
- Concerning a congenital abnormality, or

- To determine the safety and compatibility with potential recipients, or
- To measure therapeutic measures."

The Directive places IVDMDs into four categories (see Chapter 31 (Italy) for additional information) in order of perceived risk; most microbiological tests will be in the lowest risk categories and will consequently be acceptable for manufacturer declaration of fulfilment of the provisions of the Directive with no involvement by MHRA as the notified body.

The role of the MHRA as the notified body is that of a third party independent certification organization designated by the UK Government, as the competent authority, to supervise performance of conformity testing procedures.

The manufacturer is the person with responsibility for design, manufacture, packaging and labelling of a device, or who assembles, packages, processes, refurbishes and/or labels one or more ready made product, with the intention of marketing them under their own name. As such, they are required to inform MHRA of changes to their products and quality systems.

Healthcare institutions that develop and manufacture assays for their own use (in-house tests) are exempt from the requirements of the Directive as long as the use of the IVDMD is intrinsic to the operation of the health function of the institution and is retained within the bounds of the legal entity. However, it should be noted that at the time of writing, the draft Revision Regulation is under negotiation by the European Commission and there is an expectation that the exemption for healthcare institutions will be modified with conditions to ensure patient safety.

Table 35.1 Tests, devices, and instruments available in the United Kingdom*

Manufacturer/supplier	Bacteriology (aerobic/anaerobic)/mycobacteriology	Virology	Mycology	Parasitology	Mycoplasma, Chlamydia, rickettsia and miscellaneous
Abbott Abbott House Vanwall Business Park Vanwall Road Tel. 01628 773355	Abbott produces a range of reagents and equipment	Abbott Molecular's m2000sp Viral load Hepatitis B (HBsAg, HBsAb, HBeAg and others) Hepatitis C <i>Treponema pallidum</i> TPPA HIV Ag/AC HTLV CMV G+M AXSYM/Architect platforms			<i>C. difficile</i> Toxins A&B Vancomycin Resistant <i>Enterococcus</i> Van-R <i>M. tuberculosis</i> PCR RealTime CT/NG assay is an <i>in vitro</i> polymerase chain reaction (PCR) assay for the direct, qualitative detection of the plasmid DNA for <i>C. trachomatis</i> and the genomic DNA of <i>N. gonorrhoeae</i>
BioConnections Victoria Business Park, Knypersley ST8 7PP Tel. 01782 516010 Fax 01782 510733 welcome@bioconnections.co.uk	Extensive range of bacterial and fungal culture media both pre-poured and powders Identification kits, antimicrobial sensitivity testing discs and automation, e.g. carbapenem resistance in both <i>Enterobacteriaceae</i> and <i>Pseudomonas aeruginosa</i>	Rotavirus, norovirus and adenovirus		<i>Cryptosporidium</i> and <i>Giardia</i>	
bioMérieux UK Grafton Way, Basingstoke RG22 6HY Hampshire Tel. (44) 1256.461881 Fax (44) 1256.816863	UF500 UF 1000 Cytology of urine sample Bact/ALERT 3D Culture media PREVI Colour Gram Gram staining PREVI Isola Conventional culture media Columbia agar + 5% sheep blood Columbia CNA agar + 5% sheep blood Chocolate agar + PolyViteX (PVX) Specific media Campylosel agar BCSA agar (selective isolation of <i>Burkholderia cepacia</i>) Yersinia CIN agar BCP agar Mac Conkey agar Hektoen agar Mannitol Salt 2 agar (selective isolation of staphylococci) Mueller Hinton agar Mueller Hinton agar blood VITEK 2 Identification and antibiotic susceptibility testing E test Antibiotic susceptibility testing	VIDAS Serology Rubella Ig G/M CMV IgG CMV IgM CMV IgG Hepatitis A IgM Hepatitis B : HBsAg, HBcAb, HBcAb-IgM and others Anti HIV-1 (M &O) Anti HIV-2 total Ig HIV-1 P24 Ag Measles IgG/M	Fungi identification Vitek card YST Culture media <i>Albicans ID</i> Sabouraud Antifungal susceptibility Vitek card ATB FUNGUS3 A OR AST-YS01 E test NucISens easyMAG extractor	VIDAS Serology <i>Toxoplasma gondii</i> Ig G/M/	Specific media Culture A7 Mycoplasma agar

Alere Limited Pepper Road Hazel Grove Stockport Cheshire SK7 5BW, UK Tel. 0161 483 5884 Fax 0161 483 5778 Out of hours Technical Support 07917 091 581	Alere Determine HIV-1/2 Dengue: blood Detection of dengue NS1 antigen +IgG/.M BinaxNOW RSV BinaxNOW Influenza A and B	<i>C. difficile</i> testing BinaxNOW <i>S. aureus</i> BinaxNOW <i>Strep A</i> BinaxNOW <i>Legionella</i> Urinary Antigen BinaxNOW <i>S. pneumoniae</i> Urinary Antigen BinaxNOW Malaria
Meridian Products distributed by Launch Diagnostics Limited Ash House, Ash Road, New Ash Green, Longfield, Kent DA3 8JD enquiries@launchdiagnostics.com	Immunofluorescence Serology <i>Legionella pneumophila</i> Rapid test stool immunoassay <i>Helicobacter pylori</i> antigen	<i>Cryptococcus neoformans</i> Antigen Latex Agglutination System in Serum, Cerebrospinal Fluid
Diasorin S.p.A. Products distributed by Launch Diagnostics Limited Ash House, New Ash Green, Longfield, Kent. DA3 8JD enquiries@launchdiagnostics.com	LIASON ELISA <i>Borrelia burgdorferi</i> IgG <i>Borrelia burgdorferi</i> IgM Syphilis IgG/M	<i>C. difficile</i> EIA for Toxins A and B
BIO-RAD Products distributed by Launch Diagnostics Limited Ash House, Ash Road, New Ash Green, Longfield, Kent. DA3 8JD enquiries@launchdiagnostics.com	LIASON ELISA EBV IgM VCA IgG EBNA IgG Parvovirus B19 IgG Parvovirus B19 IgM VZV IgG VZV IgM GENSCREEN HIV1 +2 enzyme immunoassay Molecular biology CFX Real-Time PCR Detection System HSV VZV HHV6 Parvovirus B19 JC-BK polyomavirus Enterovirus Chikungunya virus Dengue Measles Mumps Toscana West Nile Lymphocytic choriomeningitis Influenza	LIASON ELISA <i>Mycoplasma pneumoniae</i> IgM
	Serology <i>Brucella melitensis</i> Rose Bengal and Wright stains <i>Salmonella</i> Molecular biology CFX Real-Time PCR Detection System <i>Coxiella burnetii</i> <i>Bartonella henselae</i> , <i>B. quintana</i> <i>Rickettsia conorii</i> , <i>R. typhi</i> , <i>R. africae</i> , <i>R. slovaca</i> , <i>R. mongolitimonae</i> , <i>R. felis</i> , <i>R. prowazekii</i> <i>Tropheryma whippelii</i> <i>Chlamydia trachomatis</i> <i>Streptococcus pneumoniae</i> <i>Neisseria meningitidis</i> <i>Mycoplasma pneumoniae</i> <i>Bordetella pertussis</i>	Culture media Sabouraud Chloramphenicol Gentamicin Agar Antifungal susceptibility FUNGITEST Kit Molecular biology CFX Real-Time PCR Detection System <i>Pneumocystis</i> <i>Plasmodium</i>

(Continued)

Table 35.1 (Continued)

Manufacturer/supplier	Bacteriology (aerobic/anaerobic)/ mycobacteriology	Virology	Mycology	Parasitology	Mycoplasma, Chlamydia, rickettsia and miscellaneous
Bruker UK Ltd Banner Lane Coventry CV4 9GH Tel. 024 7685 5200 Fax 024 7646 5317 Contact Us by Email Customer Support	Mass spectrometry identification microflex LT	Parainfluenza Parechovirus Human respiratory syncytial virus Hantavirus Norovirus Rotavirus Japanese encephalitis virus Yellow fever	Mass spectrometry identification microflex LT		
E&O Labs Burnhouse, Bonnybridge Tel. +44 (0)1324 840 404 info@eolabs.com www.eolabs.com	Pre-poured culture and bottled media Blood and serum supplements Plastics and containers				
Lab Mode Ltd 409 Centennial Avenue Elstree Borehamwood WD6 3TN Tel. 01908 768000 Fax 01908 614967	Suppliers of laboratory equipment, class 1, 2, and 3 laboratory microbiological safety cabinets Centrifuges and others				
Sigma-Aldrich Company Ltd. The Old Brickyard New Road Gillingham Dorset SP8 4XT Tel. 0800 717181 Fax 0800 378785 ukorders@sial.com	Suppliers of chemicals Antibodies Cell Culture Bioactive small molecules Probes and primers General molecular biology reagents Chromatography HPLC				

Siemens Diagnostics Sir William Siemens Square Frimley Camberley Surrey GU16 8QD Tel. +44 (0)1276 696000	Automated sample preparations using WASP Microscan ID and antibiotic susceptibility testing	Evolyser (Tecan) Mumps IgG Mumps IgM Measles IgG Measles IgM Parvovirus B19 IgG/M HSV-1/2 IgG HSV-1 IgG HSV-2 IgG HSV-1/2 IgM VZV IgG VZV IgM		
Oxoid Limited Wade Road Basingstoke Hampshire RG24 8PW Tel. 44 (0) 1256 841144 Fax 44 (0) 1256 814626 For information on obtaining products: oxoid.orders@thermofisher.com	Extensive range of bacterial and fungal culture media both pre-poured and powders Identification kits, antibiotic susceptibility testing discs and automation, e.g. Signal Blood Culture System <i>C.difficile</i> toxin latex Identification of enteropathogenic <i>Campylobacter</i> spp. from solid culture media Dryspot <i>Legionella</i> Latex Test Staph Latex Supply Remel products <i>Salmonella</i> , <i>Shigella</i> agglutination sera and others	EIA for adenovirus, herpes virus, norovirus and rotavirus		
Becton Dickinson The Danby Building Edmund Halley Road Oxford Science Park Oxford Oxfordshire OX4 4DQ Tel. +44 1865 748844 Fax +44 1865 781528 Web: www.bd.com/uk enquiriesukirl_bdds@europe.bd.com	BACTEC Blood culture aerobic and anaerobic bottles BACTEC MGIT 960 System <i>Mycobacteria</i> BD Phoenix Identification and antibiotic susceptibility testing BD EpiCenter IT solution The BD Innova Advanced Specimen Processor Poured media and ID systems BBL CHROMagar Orientation Medium/Columbia CNA Agar (Biplate) 9 BBL CHROMagar 0157 10 BBL CHROMagar <i>S. aureus</i> 11 BBL CHROMagar MRSA II 12 BBL CHROMagar <i>Candida</i>	Rapid test Chromatographic immunoassay in nasal swab Influenza and Respiratory VIPER HPV HSV1 & HSV2	BD MAX Molecular	BD MAX Molecular systems MRSA <i>C difficile</i> Enterics, <i>Salmonella</i> , <i>Shigella</i> , <i>Campylobacter</i> , <i>E. coli</i> 0157 <i>Phuomocystis</i> Open channels VIPER <i>Chlamydia</i> /GC

(Continued)

Table 35.1 (Continued)

Manufacturer/supplier	Bacteriology (aerobic/anaerobic)/ mycobacteriology	Virology	Mycology	Parasitology	Mycoplasma, Chlamydia, rickettsia and miscellaneous
	13 BD Sabouraud GC Agar/ BBLCHROMagar <i>Candida</i> Medium/ (Biplate) 14 BBL CHROMagar <i>Salmonella</i> 15 BBL CHROMagar <i>Salmonella</i> / XLD Agar (Biplate) 16 BD Easy Plates – innovative packaging for BD's Prepared Plated Media 18 BBL CHROMagar Proof Sources				
Cepheid UK Jupiter House Mercury Park Wooburn Green Buckinghamshire HP10 0HH UK Tel. +44 (0) 1628 564000 Fax +44 (0) 1628 564001 sales@cepheideurope.co.uk	Molecular biology GeneXpert Range of NAATs	Influenza A and Influenza B Enterovirus			Molecular biology GeneXpert Enteric panel; <i>Salmonella</i> , <i>Shigella</i> , <i>Campylobacter</i> , <i>E. coli</i> 0157 <i>S. aureus</i> MRSA <i>Streptococcus agalactiae</i> detection in vaginal swab <i>Clostridium difficile</i> in stool <i>Mycobacterium tuberculosis</i> rifampicin resistance Vancomycin resistance
Qiagen UK Qiagen House, Fleming Way, Crawley West Sussex RH10 9NQ UK	Molecular diagnosis Easy one DNA extraction kit Artus range of diagnostic kits, e.g., <i>Borrelia</i> , <i>Mycobacteria</i> , <i>Chlamydia</i> / GC/malaria	Molecular diagnosis Easy one RNA-DNA extraction kit Artus range of diagnostic kits, e.g., HCV/EBV/HAV/ Parvo/SARS/Enterovirus			Molecular diagnosis Easy one DNA extraction kit
Launch Diagnostics Limited Ash House, Ash Road, New Ash Green, Longfield, Kent. DA3 8JD, England. enquiries@launchdiagnostics. com	Wide range of diagnostics and instrumentation DS2 Alegria – for automated laboratory diagnostics Mart Microbiology B.V. microbiology equipment such as anaerobic jar systems Distributors of a comprehensive range of assays, e.g., products from: EuroClone Fujirebio FastTrack Detect-Ready Meridian MKL Diagnostics Omega Diagnostics Savoy Diagnostics Sekisui Diagnostics point of care	Distributors of a comprehensive range of assays, e.g., products from: Biorad CerTest Biotech Diamedix DiaSorin EuroClone Fujirebio Meridian Omega Diagnostics Savoy Diagnostics Virion\Seirion	Distributors of a comprehensive range of assays, e.g. products from: CerTest Biotec IVD Research Inc		Distributors of a comprehensive range of assays

ThermoFisher http://www.lifetechnologies.com/uk/en/home/brands/applied-biosystems.html	Applied Biosystems 3130 Genetic Analyzer Various sequencing equipment and sequencing solutions Rt PCR qPCR				
Serosep Ltd Annacotty Business Park, Annacotty, Co. Limerick, Ireland. Tel +353 61 358190 info@serosep.com	EntericBio Product Range Enteric Real Time PCR <i>Salmonella/Shigella/Campylobacter/E coli</i> 0157 toxins VT 1 and 2 <i>Clostridium difficile</i> toxin B <i>Cryptosporidium</i> <i>Giardia</i>				
A Menarini Diagnostics Tel. 0118 944 4100 Fax 0118 944 4111 Marketing Dept Fax: 0118 944 4123 enquiries@menarini diag.co.uk	SediMAX, Diagnostic automated urine analyser				
Luminex The Netherlands Krombraak 13-15 4906 CR Oosterhout Tel. +31-162-408-333 Fax +31-162-408-337	Gastrointestinal diseases panel using multiplex bead system: wide range of bacteria, virus and parasites Respiratory Diseases panel using multiplex bead system Common viral pathogens	Influenza A, influenza A subtype H1, influenza A subtype H3, influenza B, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), rhinovirus, and adenovirus, parainfluenza virus 1, 2, and 3			
Pro-Lab Diagnostics 3 Bassendale Road Bromborough, Wirral, Merseyside CH62 3QL Tel. +44 (0)151 353 1613 Fax +44 (0)151 353 1614 uksupport@pro-lab.com	Pro-Lab Diagnostics offer a comprehensive line of microbiology, EIA/Latex reagents/stains and chemicals Bacterial culture loops, storage vials, and autoclave deodorant				
Roche Diagnostics Ltd Charles Avenue RH15 9RY Burgess Hill UNITED KINGDOM +44-1444-256 000 http://www.roche.co.uk/portal/uk/diagnostics	Wide range of diagnostics Cobas 4800 and LightCycler Molecular <i>Chlamydia</i> /GC <i>Mycobacteria</i> /MRSA/VRE	LC 480 Viral load EBV Adenovirus CMV HHV6	LC 480 <i>Candida albicans</i> , <i>C. glabrata</i> , <i>C. krusei</i> , <i>C. parapsilosis</i> , <i>C. tropicalis</i> <i>Aspergillus</i> sp. Dermatophytosis Other fungi	LC 480 <i>Cryptococcus neoformans</i> <i>Taenia solium</i> <i>Giardia</i> <i>Histoplasmosis</i> <i>Plasmodium falciparum</i> , <i>P. ovale</i> , <i>P. malariae</i> <i>Toxoplasma gondii</i> <i>Pneumocystis jirovecii</i> <i>Dirofilaria</i>	

Table 35.1 (Continued)

Manufacturer/supplier	Bacteriology (aerobic/anaerobic)/ mycobacteriology	Virology	Mycology	Parasitology	Mycoplasma, Chlamydia, rickettsia and miscellaneous
<p>The United Kingdom National Culture Collection (UKNCC)</p> <p>Culture Collections</p> <p>Public Health England</p> <p>Microbiology Services</p> <p>Porton Down</p> <p>Salisbury</p> <p>SP4 0JG</p> <p>www.hpacultures.org.uk</p> <p>culturecollections@phe.gov.uk</p> <p>Tel. +44 (0)1980 612512</p> <p>Fax +44 (0)1980 611315</p>	<p>The UKNCC member's collections offer a culture/cell supply service. The organisms that are supplied include actinomycetes, algae, animal cells, bacteria, cyanobacteria, filamentous fungi, nematodes, protozoa, mycoplasma, viruses and yeasts.</p> <p>Supply of pure named organisms</p> <p>Reference strains and cell lines</p> <p>Starter cultures</p> <p>Production strains</p> <p>Plant and human pathogens (subject to permits)</p> <p>Microbial resistance test strains</p> <p>DNA and extracts derived from authenticated strains</p>				
<p>Advisory Committee on Dangerous Pathogens (ACDP)</p> <p>HSE ACDP Secretariat</p> <p>1.2 Redgrave Court, Merton Road, Bootle, Merseyside, L20 7HS</p> <p>acdpp@phe.gov.uk</p>	<p>ACDP advises the Health and Safety Executive (HSE), and Ministers for the Department of Health and the Department for the Environment, Food and Rural Affairs for England and their counterparts in Scotland, Wales & Northern Ireland, as required, on all aspects of hazards and risks to workers and others from exposure to pathogens. The ACDP produces guidance on a range of dangerous pathogens-related subjects. Documents are available as a paper copy from The Stationery Office or HSE Books or online; a full, current list can be found on the Department of Health (DH) website. The electronic documents can also be accessed from the HSE's website</p>				

* Many other products manufactured in the United States, the EU, and across the world may also be used in healthcare settings in the UK.

35.2.1 Inclusions and exclusions

Application of the Directive to individual components of the diagnostic process relates to the specific intent of the manufacturer in production of the item. For example:

- 1 Specimen receptacles – will be in the scope of the Directive where they have been produced with the primary intent for the containment of specimens derived from the human body for diagnostic evaluation, e.g., blood tubes, microbiological swabs.
- 2 General laboratory reagents – are likely to be out of the scope of the Directive unless they are prepared specifically for use in IVD examination.
- 3 Accessories to IVDMDs – whilst these may not be specifically manufactured for this purpose, or provide diagnostic information, they are required to enable the IVDMD to fulfill its purpose and so are in the scope for the Directive, e.g., bar code readers.
- 4 Devices for performance monitoring – may be used alongside a test to monitor performance in respect of sensitivity and specificity and are appropriate in terms of clinical need. These are not subject to conformity testing/CE marking procedures but the manufacturer must follow procedures as described in the Directive and notify the MHRA.
- 5 Reference material – calibrators and control materials needed to establish and verify performance are within the scope of the Directive. Internationally certified and EQA (external quality assurance) materials are not.

35.3 Conformity assessment

A manufacturer wishing to place their products on the market under the Directive must undergo the conformity testing process and, depending on the category of the product, must engage with the MHRA to validate the certification process. Certification leads to Conformité Européenne (CE) marking of the product and the route depends on classification of devices detailed in Regulation 40 (article 9 of EU Directive). The majority of microbiological tests will require self-documentation and no involvement of the MHRA.

A key step in this process is evidence of conformity to recognized standards; and there are European standards harmonized under the Directive. However, the manufacturer may show evidence of compliance to other standards, for example international standards, in support of conformity.

Following release of an IVDMD to market, the manufacturer has an obligation to the MHRA regarding post-production and implementation vigilance including reporting of any corrective actions. In turn the MHRA, on behalf of the UK Government, must inform the European Commission and other member states of withdrawal, prohibition, or restricted use of an IVDMD as a result of compromise to health and safety of patients or users.

35.4 Other considerations

- 1 Documentation and information must be in English unless the end user is not in the UK or if MHRA has specifically authorized use of another Community language(s).
- 2 Manufacturers with a registered place of business in the UK that make a device available for performance evaluation must register with MHRA. Alternatively, if the manufacturer does not have a registered place of business they must designate an authorised representative.
- 3 Manufacturers must be aware of and abide by other regulations, for example (but not exclusively):
 - The Health & safety at Work Act 1974
 - The HIV Testing Kits and Services Regulations 1992
 - The Radioactive Material (Road Transport)(Great Britain) Regulations 2002
 - Packaging Requirements for the Royal Mail System.

Bibliography

In vitro Diagnostic Medical Devices Directive 98/79/EC. http://ec.europa.eu/health/medical_devices/regulatory-framework/legislation/index_en.htm

MHRA: *Guidance on the in vitro diagnostic medical devices Directive 98/79/EC*. <http://www.mhra.gov.uk/home/groups/es-era/documents/publication/con007521.pdf>

APPENDIX

Manufacturers, Distributors and Vendors

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In this Appendix are listed many of the manufacturers, distributors, and vendors described and listed within the chapters of this book. We have listed them in alphabetical order, and have not grouped them with respect to the products they offer or by country of origin. Some chapters, particularly the international chapters may have the companies and vendors listed at the end of their specific chapter. These will not be duplicated in this Appendix.

When reviewing and evaluating vendors for selected products, tests, instruments or devices, the reader is referred to the specific chapter for a discussion of which vendors offer the product in which the reader is interested. The authors have made every effort to include the relevant companies, however, there may be additional manufacturers, vendors, distributors, and companies that may offer similar products. The reader is encouraged to seek alternative sources for additional companies and vendors, particularly those outside the United States. It is always important to seek current

information, especially from new companies or those that have merged or bought by other companies.

We attempt here to list the vendor address, telephone number, toll-free telephone number (if available), fax number, and web site and e-mail address (when available). Only those companies that offer clinical microbiology products are listed here. For general laboratory products and information, and laboratory testing services, please refer to other sources.

We encourage the reader to contact the editor or publisher with any errors, changes, or additional vendors that should be added or corrected in future versions of this manual. In addition, as emphasized in other parts of this manual, the users of any kit, reagent, instrument, device, or product must evaluate the performance of each product in their laboratory or setting, investigate the US Food and Drug Administration (or other pertinent regulatory authority) review and approval status, and use the product within its stated shelf life and for the recommended clinical uses.

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Web: <http://www.diasorin.com>

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See ThermoFisher Scientific listing

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see Meridian Diagnostics

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See ThermoFisher Scientific Corporation

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See Diasys Corp listing

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Inverness Medical

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Iris International -Beckman Coulter

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Labsystems-

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Medical Packaging Corp.

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See Remel listing

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See ThermoFisher Scientific

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 CDC *see* Centers for Disease Control and Prevention
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 CE *see* Conformité Européenne
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- PMA *see* premarket amendment
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- RFP *see* Requests for Proposal
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- RTI *see* respiratory tract infections
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- SALDA *see* Southern African Laboratory Diagnostics Association
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- VPI *see* Virginia Polytechnic Institute
- VRE *see* vancomycin-resistant *Enterococcus*
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- VZV *see* varicella zoster virus

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- WGS *see* whole genome sequencing
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Figure 3.7 Biolog System. (Courtesy of Biolog.)

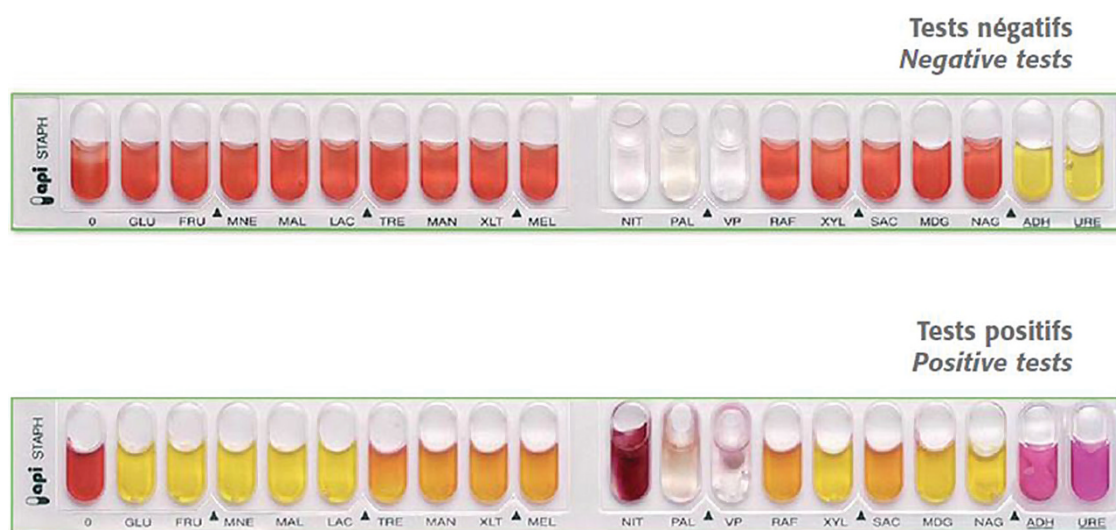


Figure 3.8 API Staph. (Courtesy of bioMérieux.)



Figure 3.10 Binax NOW *Streptococcus pneumoniae* antigen test kit. (Courtesy of Alere, Inc.)



Figure 3.14 Thermo Scientific™ ProSpect™ STEC (Shiga Toxin *E. coli*) Microplate Assay. (Photo courtesy of ThermoFisher Scientific, copying is prohibited.)



Figure 3.12 Meridian Bioscience Illumigene System. (Courtesy of Meridian Bioscience, Inc.)



Figure 3.15 Immunocard STAT! EHEC Kit. (Courtesy of Meridian Bioscience, Inc.)



Figure 3.13 Premier EHEC ELISA. (Courtesy of Meridian Bioscience, Inc.)

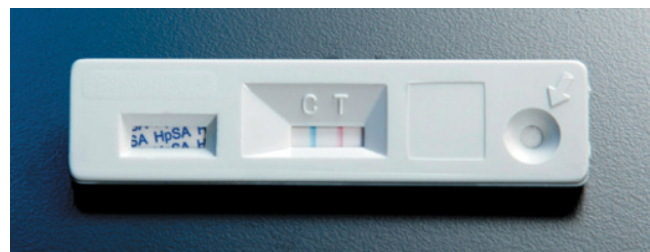


Figure 3.17 *H. pylori* Immunocard STAT! Antigen Kit. (Courtesy of Meridian Bioscience, Inc.)



Figure 3.18 Verigene System. (Courtesy of Nanosphere, Inc.)



Figure 3.19 Biofire System. (Courtesy of Biofire Diagnostics Inc.)



Figure 4.1 WEE-TABS stand-alone chromogenic tests. Each tube contains one tablet and a small volume of bacterial suspension. (Courtesy of Key Scientific Products.)

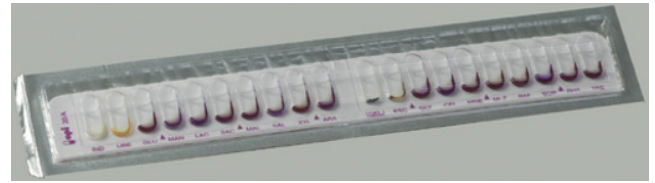


Figure 4.2 API 20A test strip. Miniaturized cupules are inoculated with a bacterial suspension and after 24–48 h of incubation, biochemical reactions are visualized. (Courtesy of bioMérieux.)

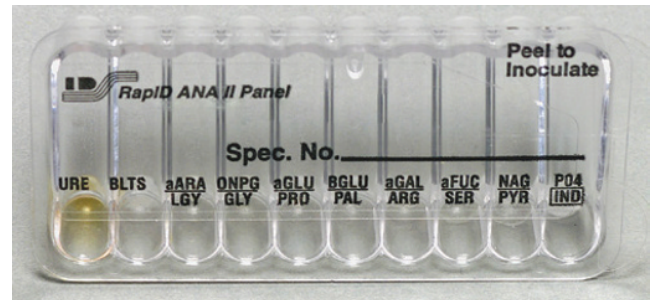


Figure 4.3 Thermo Scientific™ RapID™ ANA II System. This 4-h rapid test system for the identification of anaerobes contains 10 wells for testing the performance of 18 preformed enzymatic tests. (Photo courtesy of ThermoFisher Scientific, copying is prohibited.)

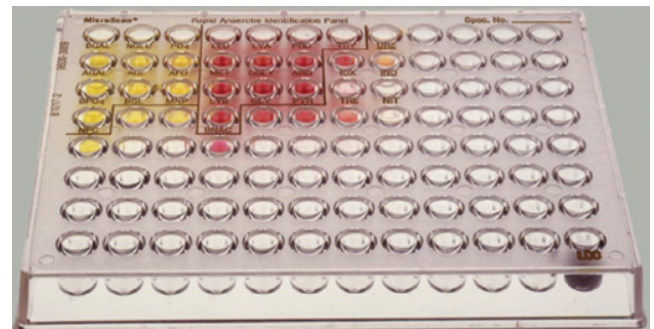


Figure 4.5 MicroScan Rapid Anaerobe Identification Panel. This 96-well microdilution tray contains dehydrated substrates and is incubated for 4 h aerobically. (© 2015 Beckman Coulter, Inc., all rights reserved, used with permission.)

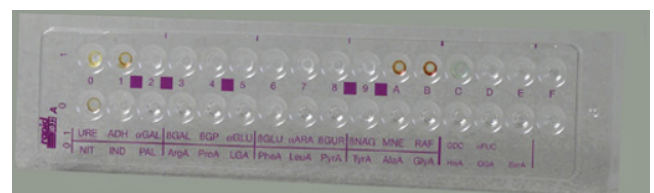


Figure 4.6 Rapid ID 32A. A plastic strip with 32 cupules, 29 of which contain dehydrated substrates for rapid biochemical characterization of anaerobes, is incubated for 4 h aerobically. (Courtesy of bioMérieux.)



Figure 4.8 Microflex LT. (Courtesy of Bruker Daltonics.)



Figure 4.13 ImmunoCard *C. difficile* GDH. (Photograph courtesy of Meridian Bioscience, Inc.)



Figure 4.12 *C. Difficile* Tox-B cytotoxin assay. (Photograph courtesy of Alere, Inc.)



Figure 4.14 ImmunoCard Toxins A&B. (Photograph courtesy of Meridian Bioscience, Inc.)



Figure 4.16 Premier Toxins A&B test. (Photograph courtesy of Meridian Bioscience, Inc.)



Figure 4.17 Thermo Scientific™ Xpect™ Toxin A/B test. (Photo courtesy of ThermoFisher Scientific, copying is prohibited.)



Figure 4.18 *C. Difficile* Tox A/B II. (Photograph courtesy of Alere, Inc.)

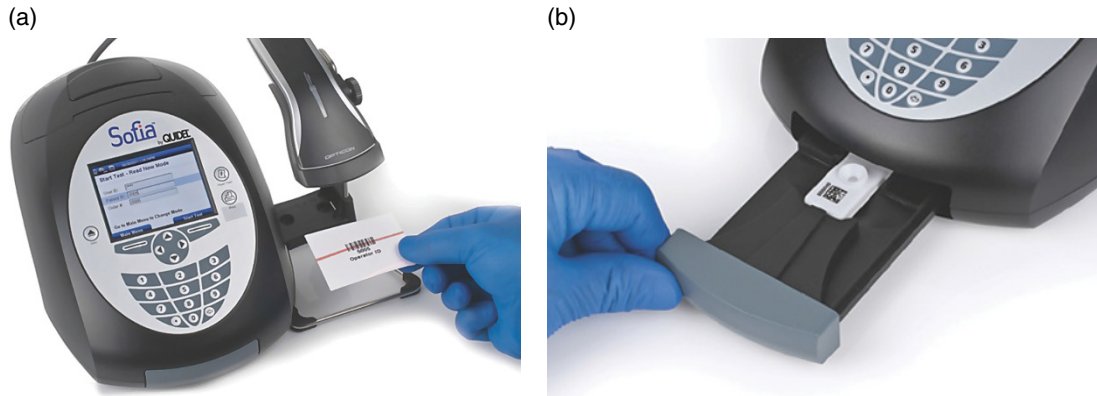


Figure 5.6 (a) Sofia FIA instrument. (b) Inserting cartridge into Sofia Reader. (Courtesy of Quidel Corporation.)



Figure 5.7 DHI D³ Ultra staining kit. (Courtesy of Quidel Corporation.)

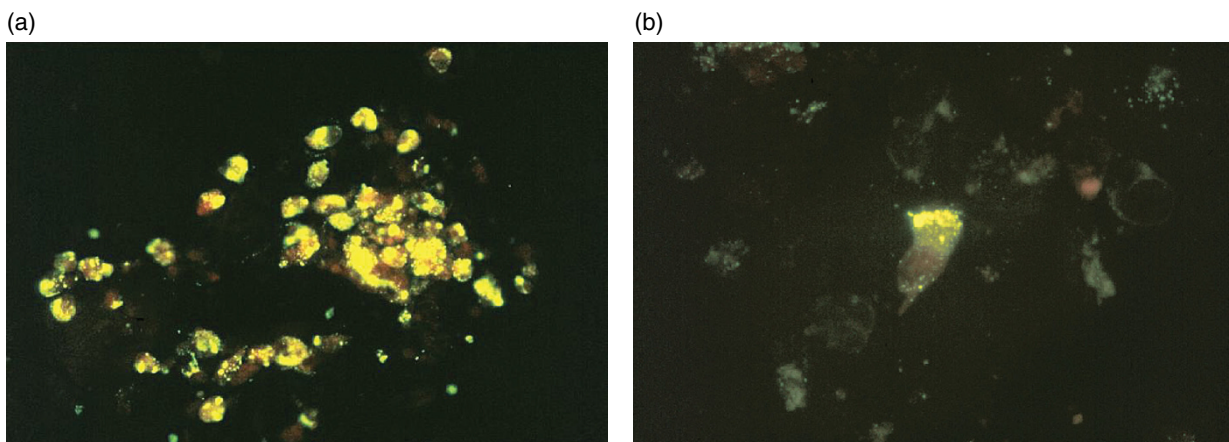


Figure 5.8 (a) Microscopic detection of influenza A in a NP specimen by DFA. (b) Microscopic detection of RSV in a NP specimen by DFA.

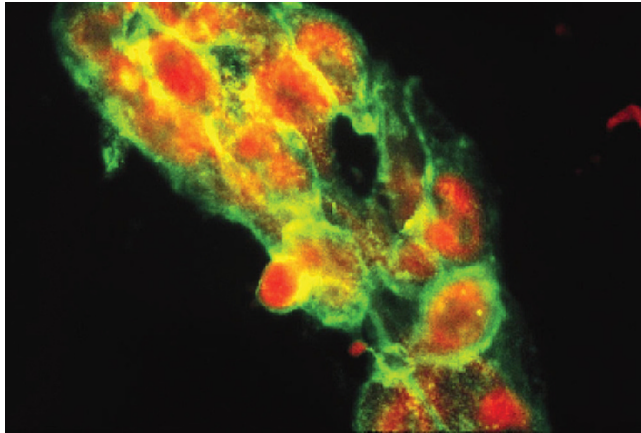


Figure 5.9 Microscopic detection of VZV in a skin scraping by DFA.

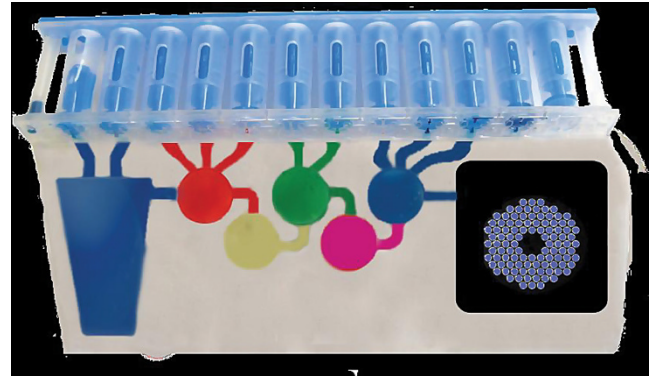


Figure 6.3 Biofire FilmArray pouch. (Courtesy of Biofire Diagnostics, Inc.)



Figure 6.1 The Luminex 100/200 detection platforms. (Image provided by Luminex Corporation.)



Figure 6.4 Biofire FilmArray instrument. (Courtesy of Biofire Diagnostics, Inc.)



Figure 6.2 GenMark e-sensor platform. (Image provided courtesy of GenMark Diagnostics.)



Figure 6.5 Xpert Flu. (Courtesy of Cepheid.)

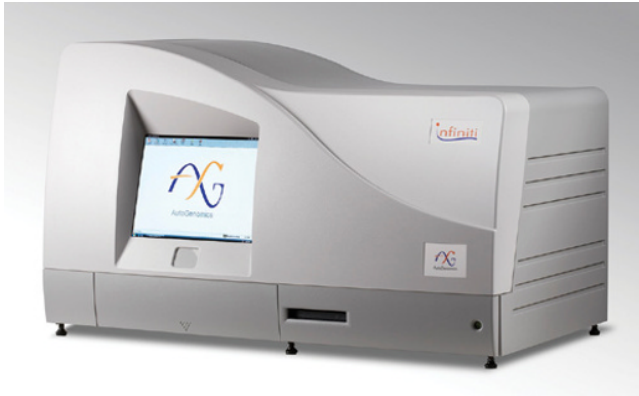


Figure 6.6 Infiniti RVP System. (Reproduced by permission of Autogenomics, Inc.)



Figure 6.7 Luminex Magpix. (Image provided by Luminex Corporation.)

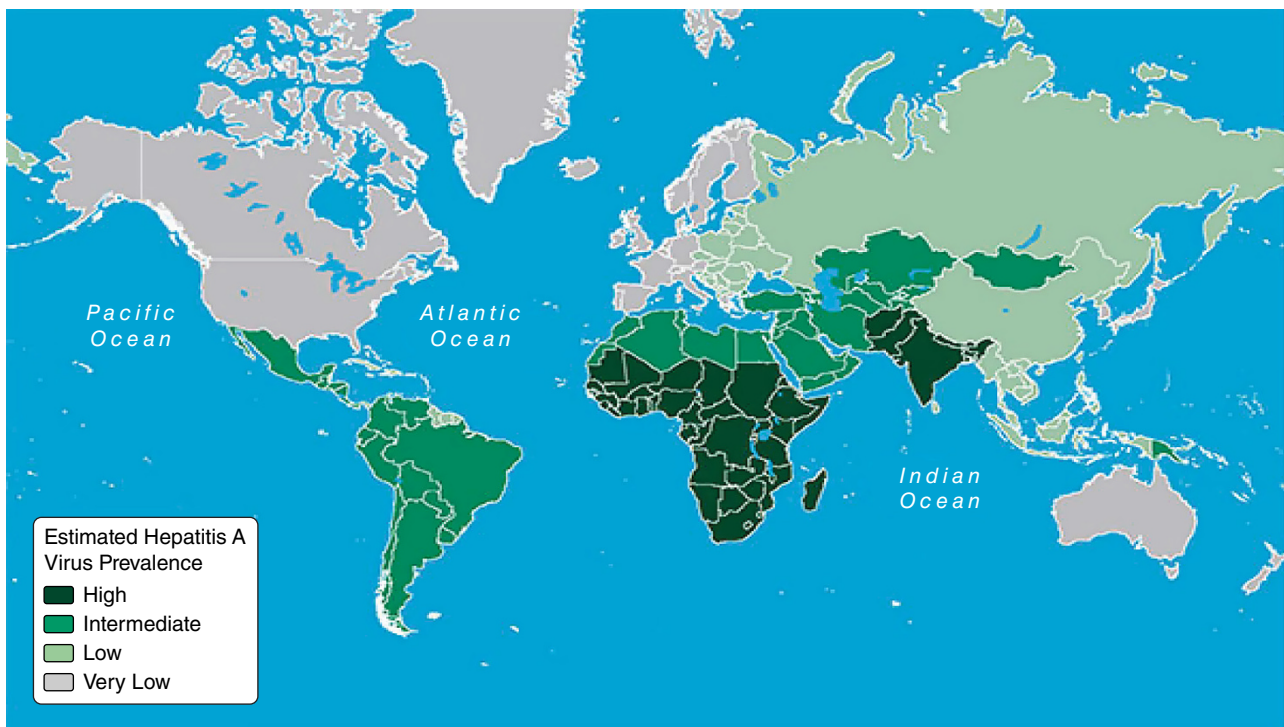


Figure 7.1 Hepatitis A seroprevalence worldwide. (Source: <http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/hepatitis-a.htm>)

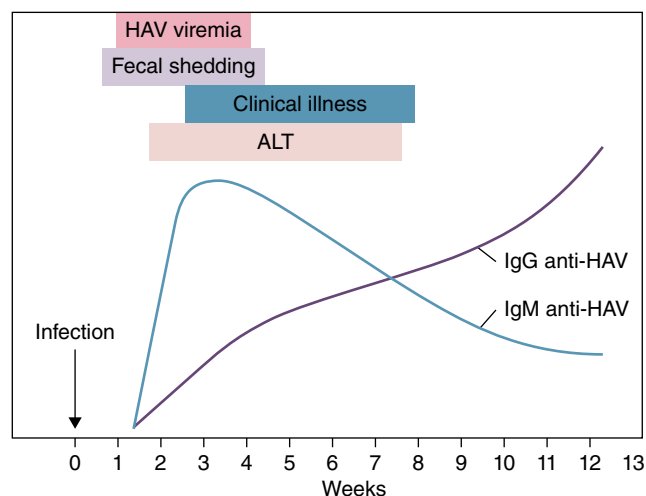


Figure 7.2 The clinical, virologic, and serologic course of acute hepatitis A virus (HAV) infection. ALT, alanine aminotransferase. (Reproduced with permission from Mandell, Douglas, and Bennetts *Principles and Practice of Infectious Diseases*, 7th Edition, 2010, M.P. Curry and S. Chopra, Acute Viral Hepatitis, 1577–1592, Churchill Livingstone/Elsevier, Philadelphia, PA.)

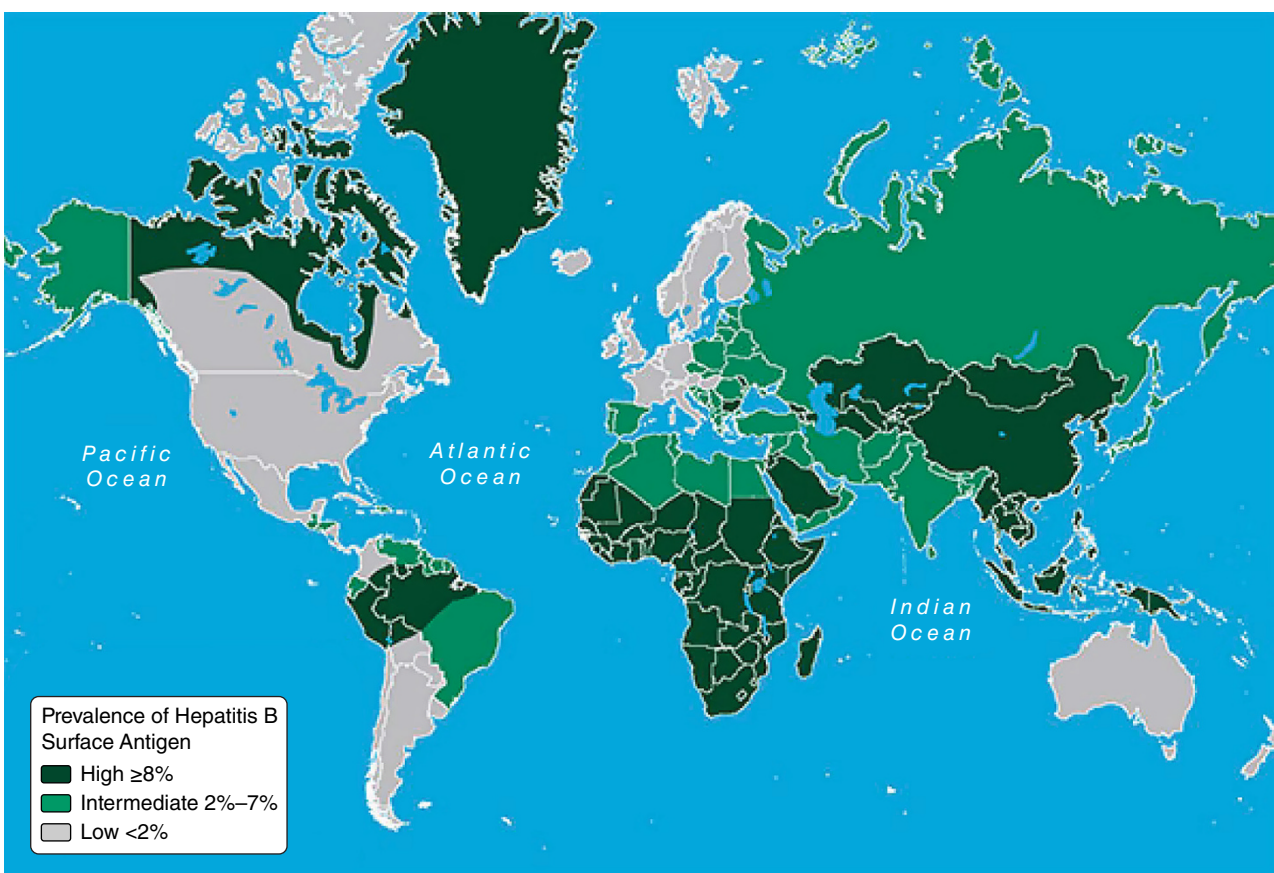


Figure 7.3 Hepatitis B seroprevalence worldwide. (Source: <http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/hepatitis-b.htm>)

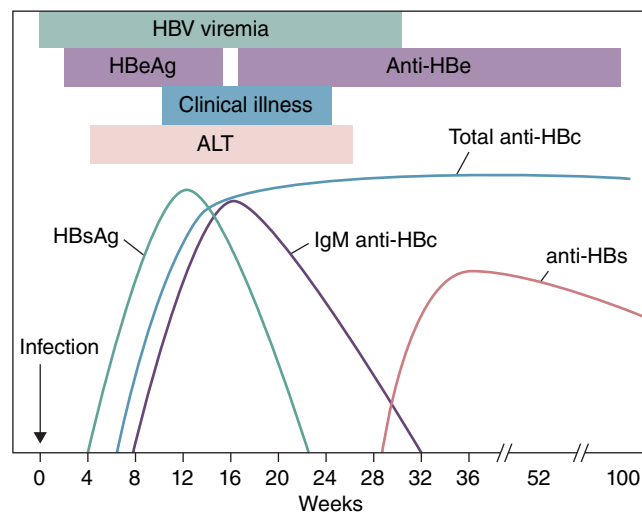


Figure 7.4 The clinical, virologic, and serologic course of acute hepatitis B virus (HBV) infection. ALT, alanine aminotransferase; HBc, hepatitis B core; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; IgG, immunoglobulin G; IgM, immunoglobulin M. (Reproduced with permission from Mandell, Douglas, and Bennetts *Principles and Practice of Infectious Diseases*, 7th Edition, 2010, M.P. Curry and S. Chopra, Acute Viral Hepatitis, 1577–1592, Churchill Livingstone/Elsevier, Philadelphia, PA.)

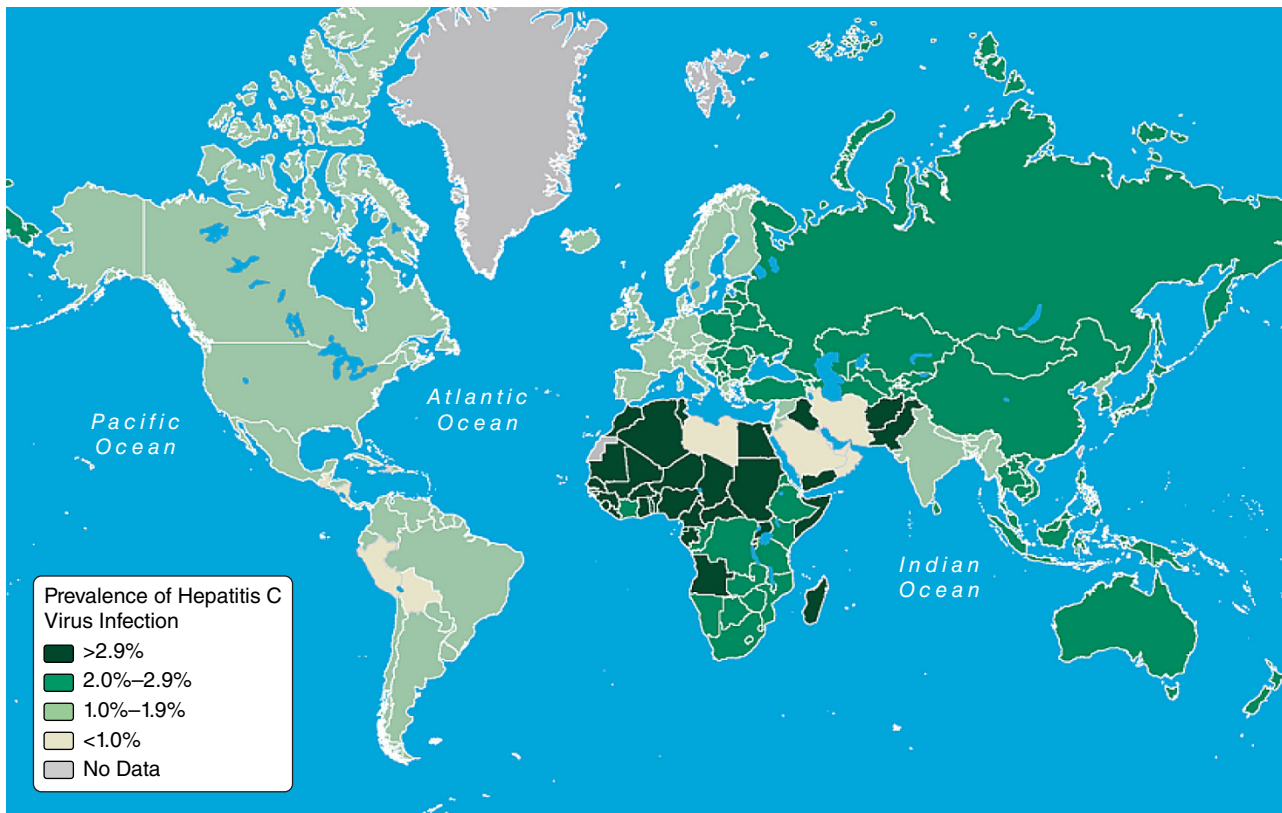


Figure 7.5 Hepatitis C seroprevalence worldwide. (Source: <http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/hepatitis-c.htm>)

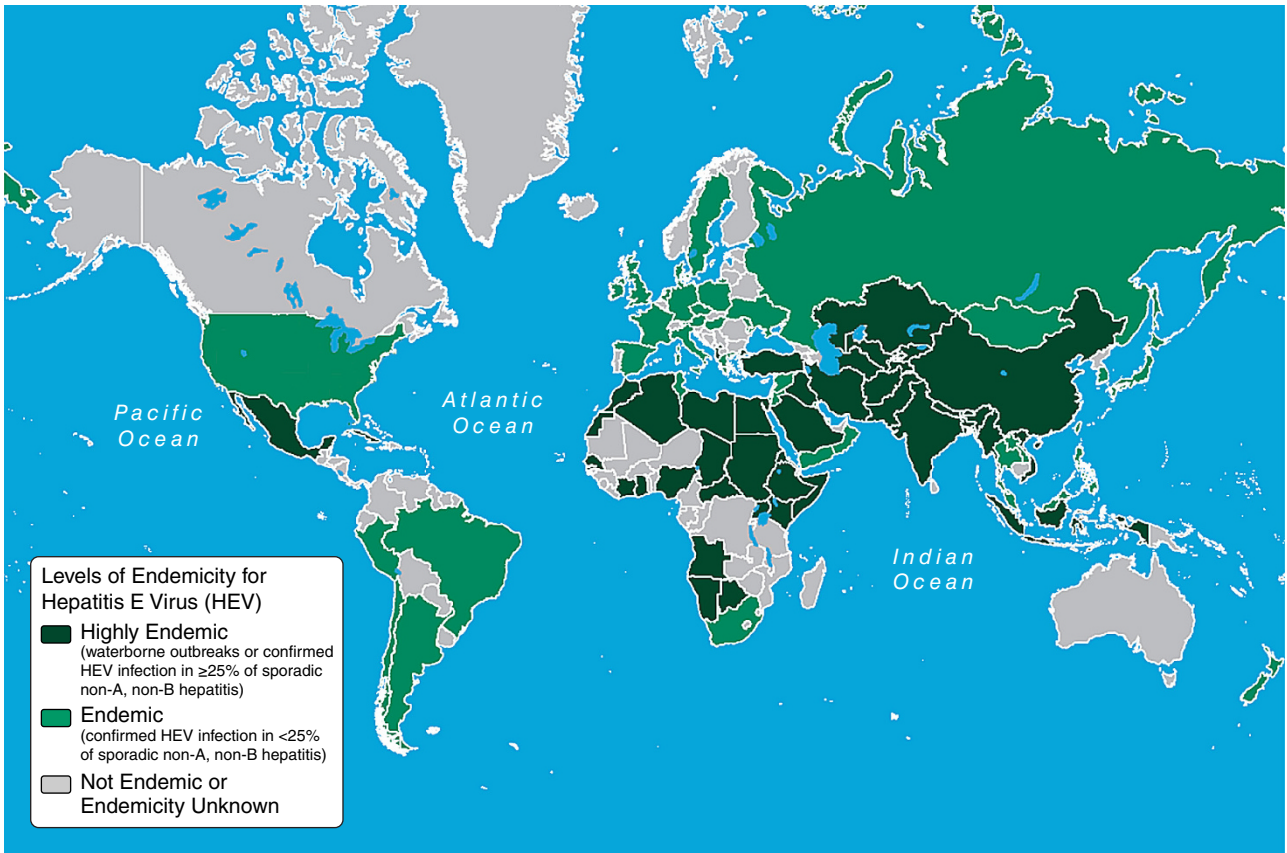


Figure 7.7 Hepatitis E seroprevalence worldwide. (Source: <http://wwwnc.cdc.gov/travel/yellowbook/2012/chapter-3-infectious-diseases-related-to-travel/hepatitis-e.htm>)

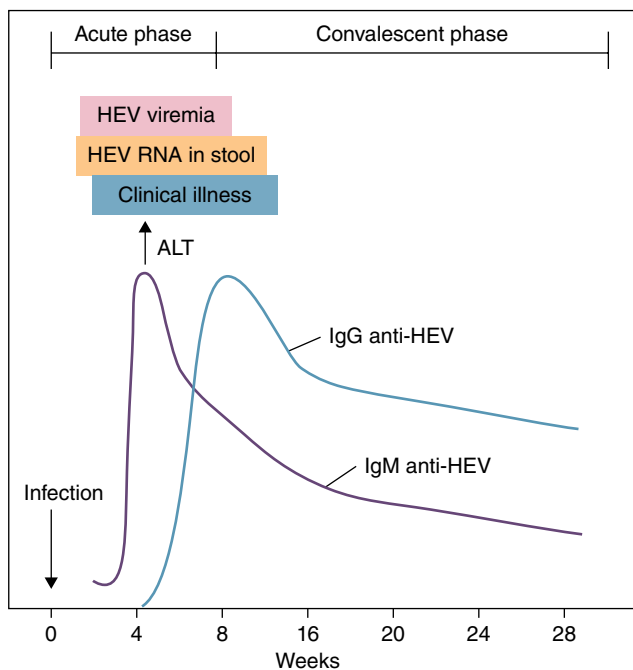


Figure 7.8 Time course of clinical and serologic events during acute hepatitis E virus (HEV) infection. ALT, alanine aminotransferase; IgG, immunoglobulin G; IgM, immunoglobulin M. (Reproduced with permission from Mandell, Douglas, and Bennett's *Principles and Practice of Infectious Diseases*, 7th Edition, 2010, M.P. Curry and S. Chopra, Acute Viral Hepatitis, 1577–1592, Churchill Livingstone/Elsevier, Philadelphia, PA.)



Figure 10.1 Cobas System. (Courtesy of Roche Diagnostics Corporation.)



Figure 10.2 The m2000 System. (Courtesy of Abbott.)



Figure 10.5 The BD Viper System. (Courtesy and © Becton, Dickinson and Company.)



Figure 12.1 MYCOSCREEN PLUS (ELITech).

MYCOSCREEN PLUS is a commercial kit for specimen transport, enumeration, and identification of *Ureaplasma* spp. and *M. hominis* in the urogenital tract. The kit consists of UMMt liquid transport medium and UMMlyo lyophilized growth medium in microplate wells. Detection of growth of the organisms is based on color change in liquid medium containing urea and arginine. Species identification is based on differential inhibition by erythromycin and lincomycin. This product is available in the United States. (Photograph courtesy of ELITechGroup, Inc.)



Figure 10.3 The GeneXpert platform. (Courtesy of Cepheid.)



Figure 12.2 MYCOFAST US (ELITech). MYCOFAST US is a commercial kit for the detection, identification, and enumeration of *Ureaplasma* spp. and *M. hominis* in clinical specimens. The kit consists of microplates containing lyophilized growth substrates and antimicrobials. Detection of growth of the organisms is based on color change in liquid medium containing urea and arginine. Species identification is based on differential inhibition by erythromycin, trimethoprim/sulfamethoxazole, and lincomycin. This product is available in the United States. (Photograph courtesy of ELITechGroup, Inc.)



Figure 10.4 The Tigris DTS System. (Courtesy of Hologic, Inc.)



Figure 12.3 ImmunoCard (Meridian Bioscience, Inc.). The Meridian ImmunoCard is a qualitative, membrane-based EIA that can be performed in 10 min or less to detect the presence of IgM antibody directed against *M. pneumoniae*. A positive test is indicated by development of a blue color in both of the upper reaction ports. (Photograph courtesy of Meridian Bioscience, Inc.)



Figure 12.4 illumigene (Meridian Bioscience, Inc.). The illumigene is a loop-mediated isothermal amplification assay (LAMP) that enables detection of *M. pneumoniae* in up to ten respiratory specimens within 1 h after extracted DNA is set up in the incubator/reader. (Photograph courtesy of Meridian Bioscience, Inc.)

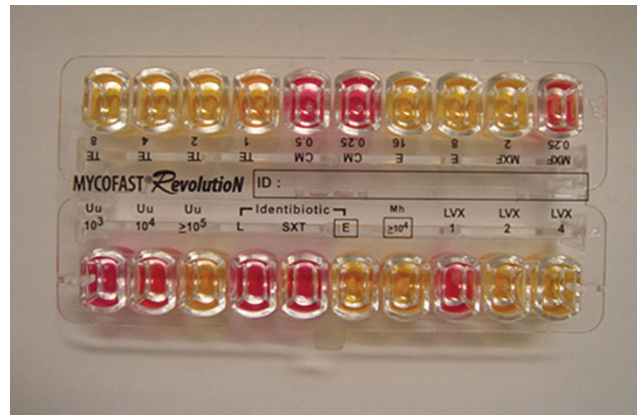


Figure 12.5 MYCOFAST RevolutionN (ELITech). Mycofast *RevolutionN* is a commercial kit for identification, enumeration, and antimicrobial susceptibility testing of *M. hominis* and *Ureaplasma* spp. that can be used directly with clinical specimens. The kit consists of microplates containing lyophilized growth substrates and antimicrobials. Detection of growth of the organisms is based on color change in liquid medium containing urea and arginine. Species identification is based on differential inhibition by erythromycin, trimethoprim/sulfamethoxazole, and lincomycin. Susceptibility testing is provided for levofloxacin, moxifloxacin, erythromycin, clindamycin, and tetracycline. This product is currently available in Europe, but not in the United States. (Photograph courtesy of ELITechGroup, Inc.)

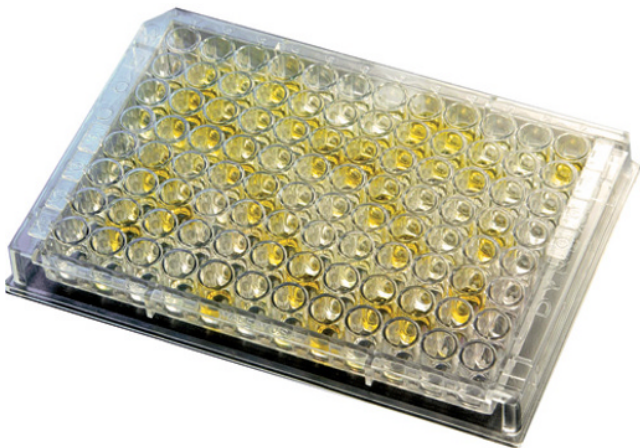


Figure 13.2 Enzyme linked immunoassay for cryptococcal antigen. (Courtesy of Meridian Bioscience, Inc.)



Figure 13.3 Cryptococcal antigen Lateral Flow Assay. (Courtesy of IMMY.)



Figure 13.4 Platelia *Aspergillus* Antigen assay kit. (Courtesy of Bio-Rad Laboratories, Inc.)

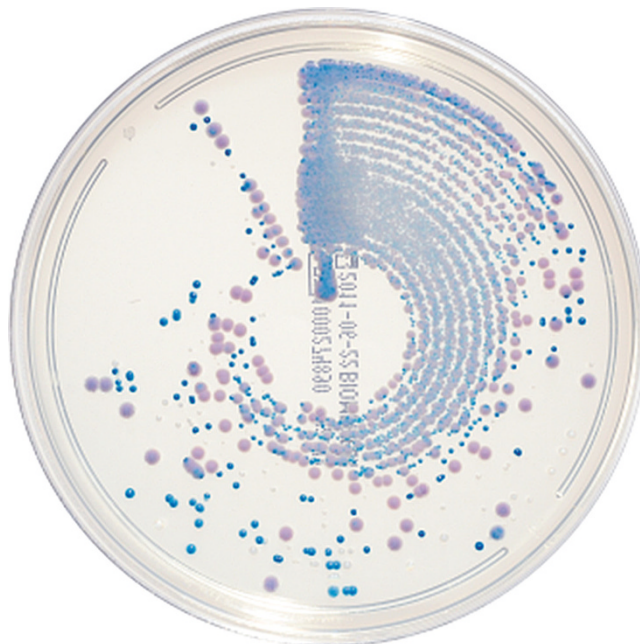


Figure 13.6 ChromID *Candida* agar. (Courtesy of bioMérieux.)

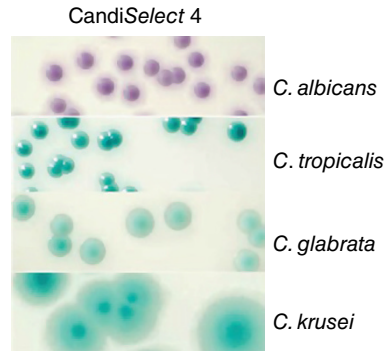


Figure 13.7 Different appearance of *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. krusei*. (Courtesy of Bio-Rad Laboratories, Inc.)



Figure 13.10 GlabrataQuick. (Courtesy of Hardy Diagnostics.)

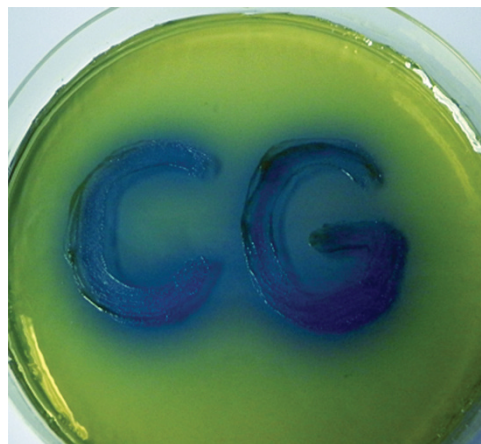
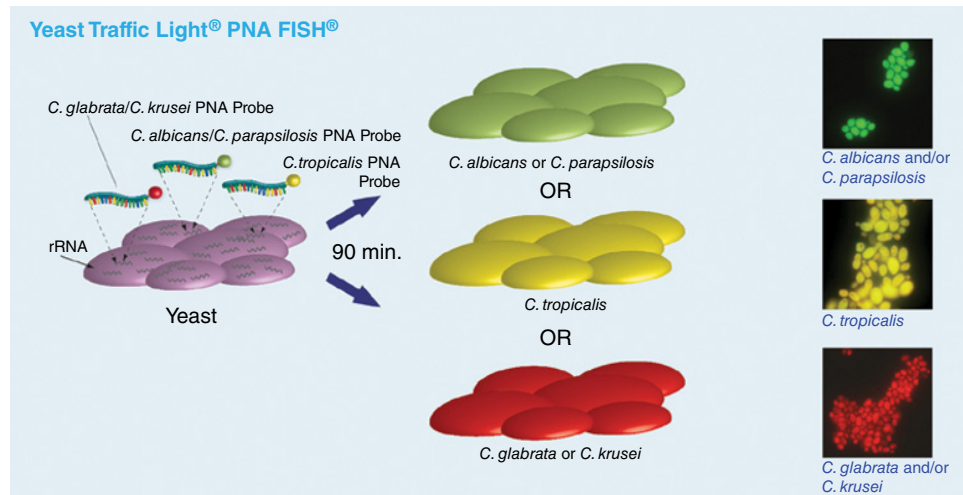


Figure 13.11 CGB agar for the differentiation of *C. gattii* (positive) from *C. neoformans*. (Courtesy of S. Moser.)

(a)



(b)

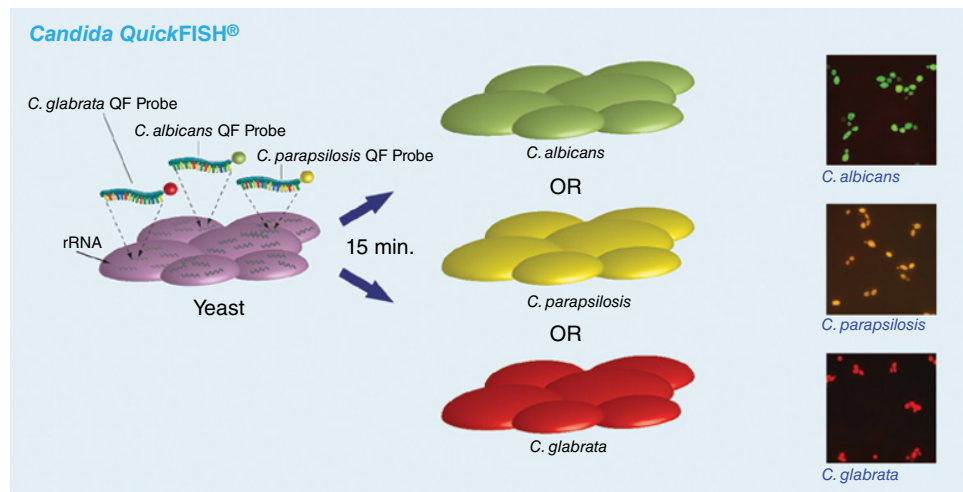


Figure 13.16 (a) Yeast Traffic Light PNA FISH. (b) *Candida* QuickFISH. (Courtesy of AdvanDx.)



Figure 15.12 *E. HISTOLYTICA* II Immunoassay for the true pathogen, *Entamoeba histolytica*. (Courtesy of TECHLAB, Inc.)



Figure 15.23 FE-5 semiautomated sampling and examination system for fecal concentration sediments. (Courtesy of Apacor Ltd USA, Brooklyn and New York.)



Figure 15.24 Detailed view (from Figure 15.23) of the glass viewing chamber that holds a portion of the concentration sediment and rests on the microscope stage. The fecal material is viewed through the microscope focused on the contents in the chamber. (Courtesy of Apacor Ltd USA, Brooklyn and New York.)

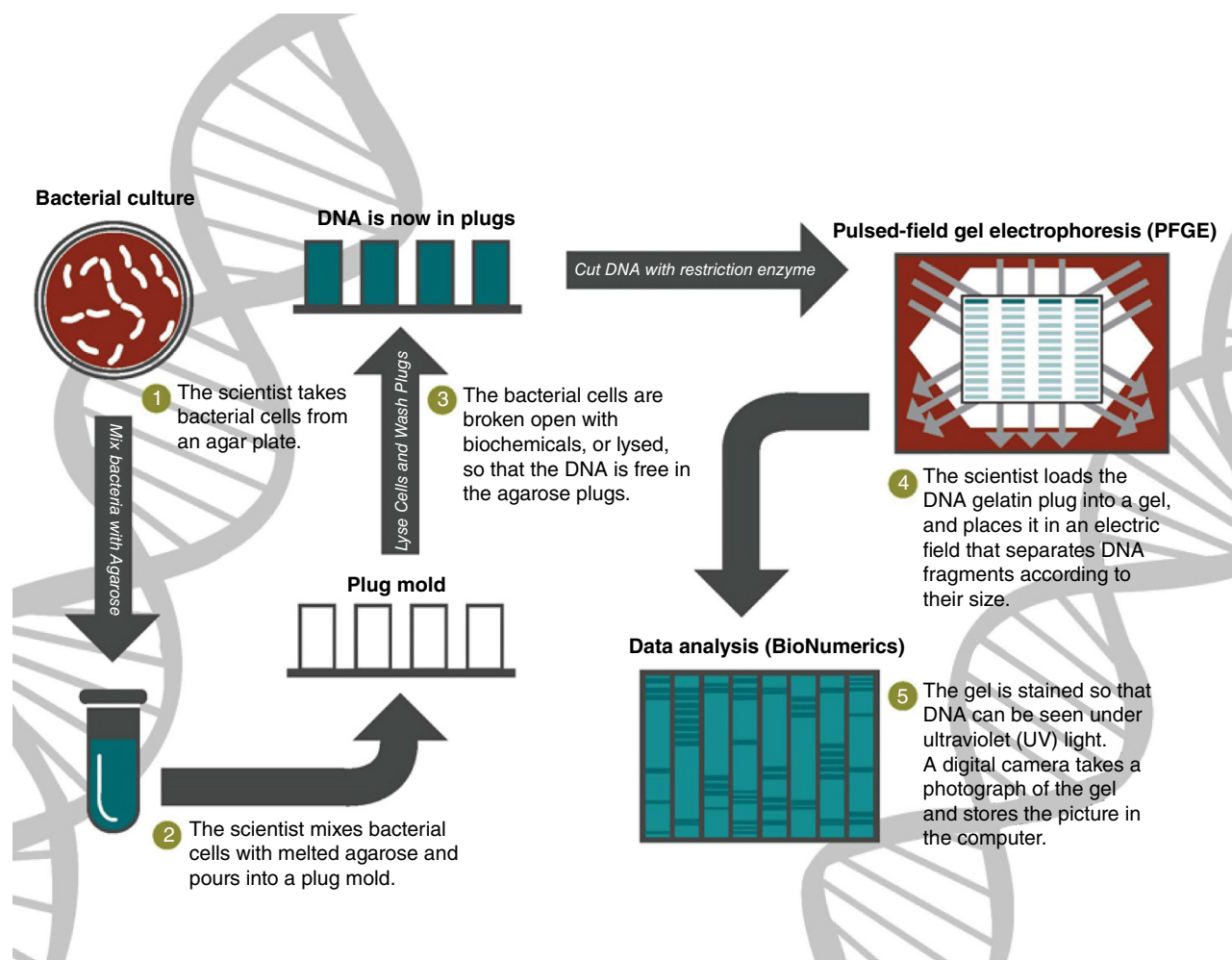


Figure 18.1 Simplified Process of Pulsed-Field Gel Electrophoresis. This schematic developed by the Centers for Disease Control explains the PFGE process from bacterial culture to data analysis. (Source: <http://www.cdc.gov/pulsenet/pathogens/protocol-images.html>).

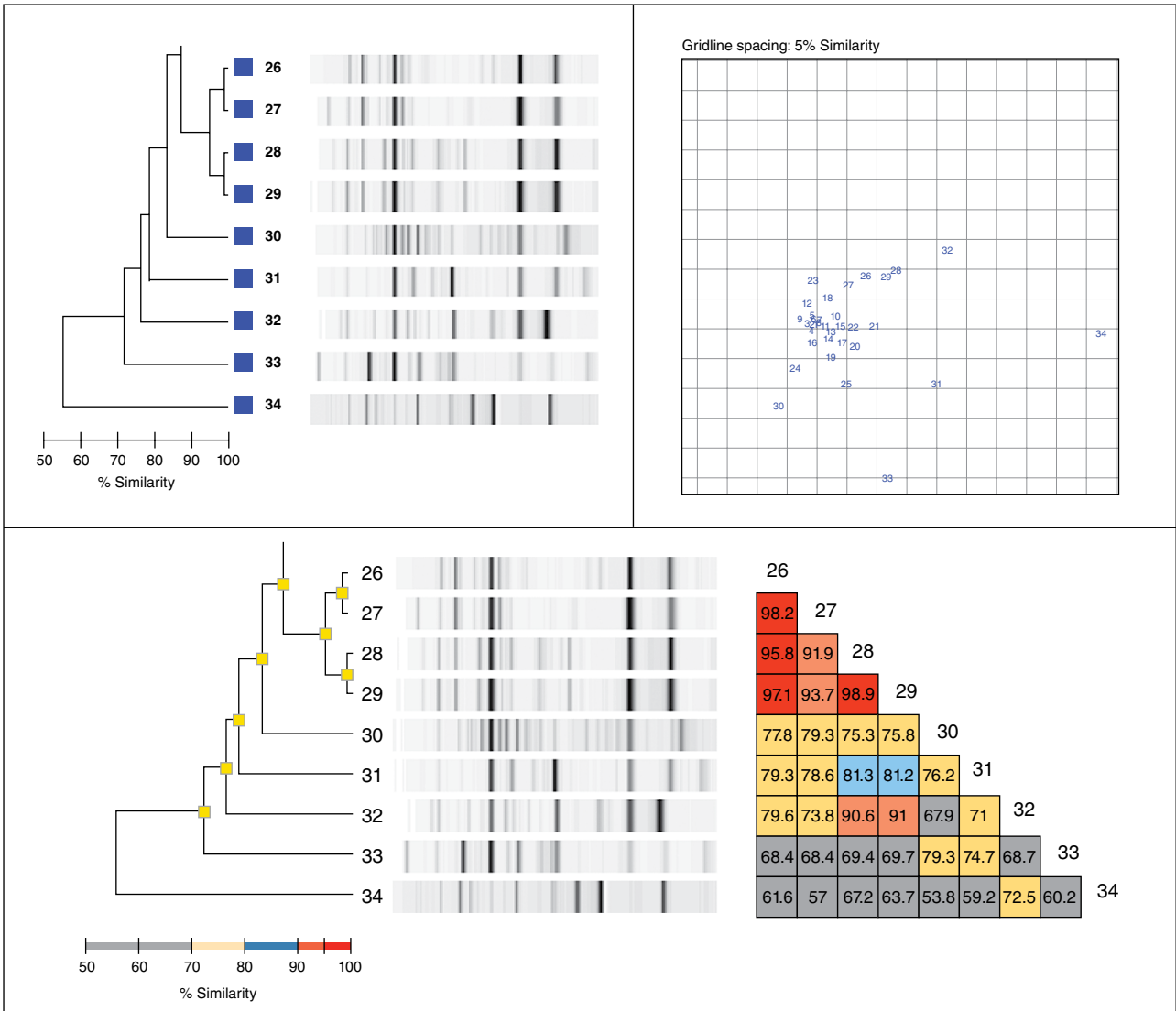


Figure 18.2 DiversiLab Report Components. This DiversiLab report is an example of an automated analysis and reporting system for molecular strain typing that provides several options for visualizing relatedness of clones including: (a) upper left, a dendrogram, (b) upper right, a scatterplot, and (c) lower, a similarity matrix. (Courtesy of bioMérieux.)

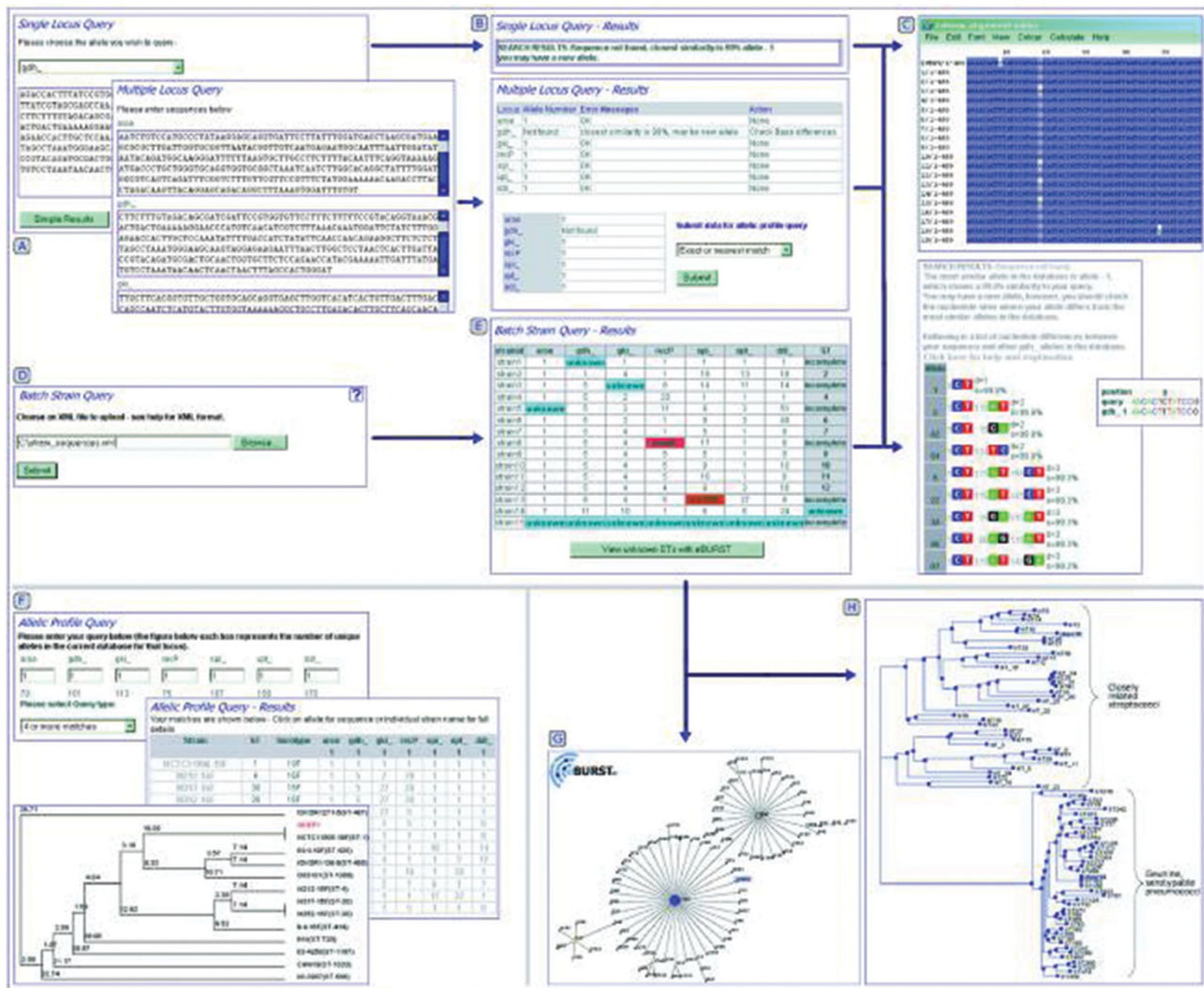


Figure 18.3 Overview of Multi Locus Sequencing Typing Workflow. Various details of the MLST process are presented in this workflow originally described in Aanensen et al. 2005 [1]. (Source: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1160176/>). (See manuscript and website for expanded view and details.)

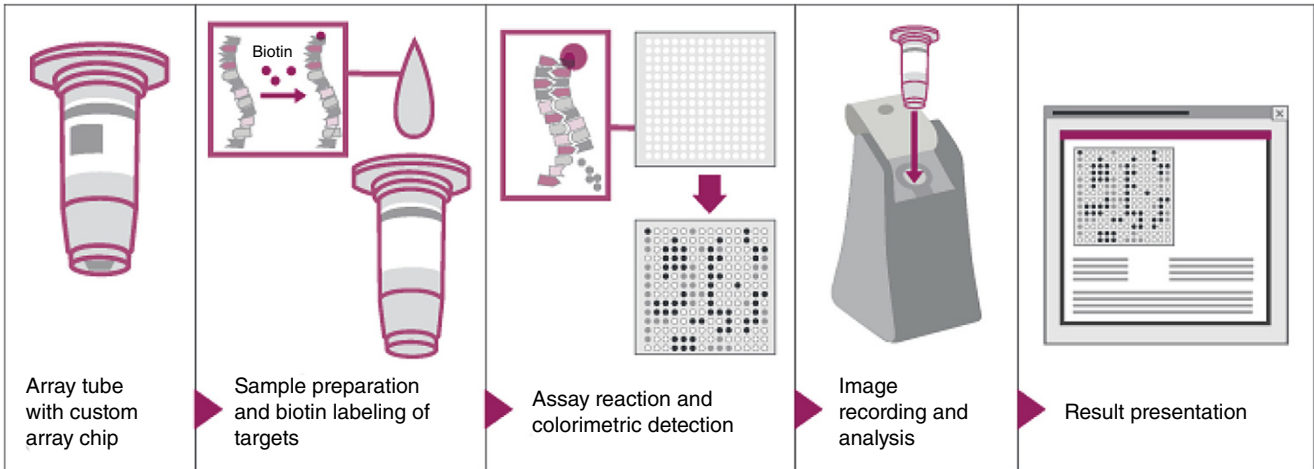


Figure 18.4 ArrayTube Technology. Custom arrays can also serve as effective methods for molecular strain typing as shown in this explanation of the ArrayTube system. (<http://alere-technologies.com/en/products/lab-solutions/platform-components/arraytube-at.html>). (Courtesy of Alere, Inc.)

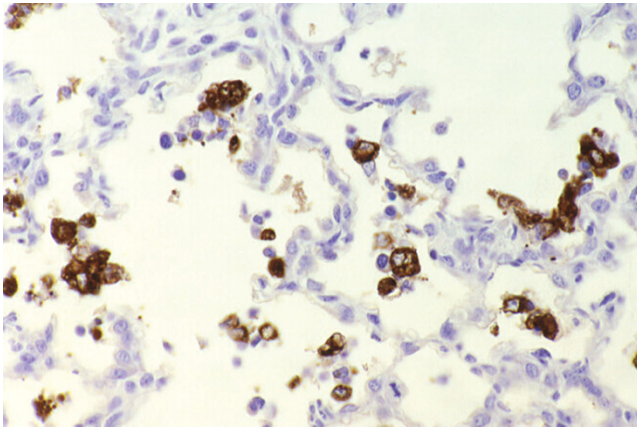


Figure 19.1 Immunohistochemical detection (IHC) of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the alveolar macrophages of a lung from a pig infected with PRRSV. Alveolar macrophages show dark brown staining of PRRSV antigen. Avidin–biotin peroxidase complex staining, hematoxylin counterstain. (Courtesy of Dr. Patrick G. Halbur, Iowa State University.)

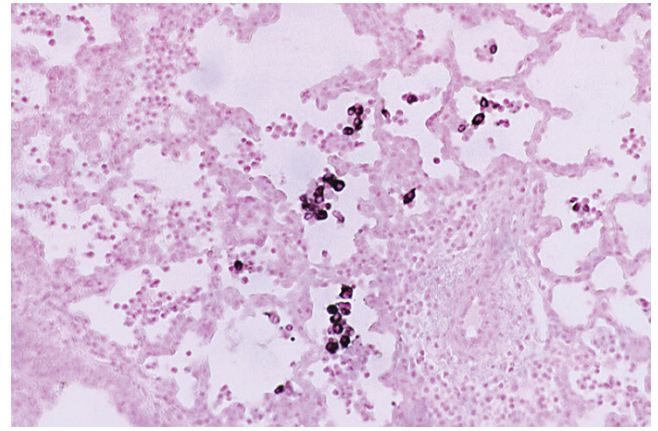


Figure 19.2 *In situ* hybridization detection (ISH) of porcine reproductive and respiratory syndrome virus (PRRSV) nucleic acid in the alveolar macrophages of a lung from a pig infected with PRRSV. PRRSV nucleic acid is detected in the cytoplasm of macrophages. ISH with a digoxigenin-labeled PRRSV riboprobe detected by anti-digoxigenin–alkaline phosphatase complex, nuclear fast red counterstain (Courtesy of Dr. Patrick G. Halbur, Iowa State University.)

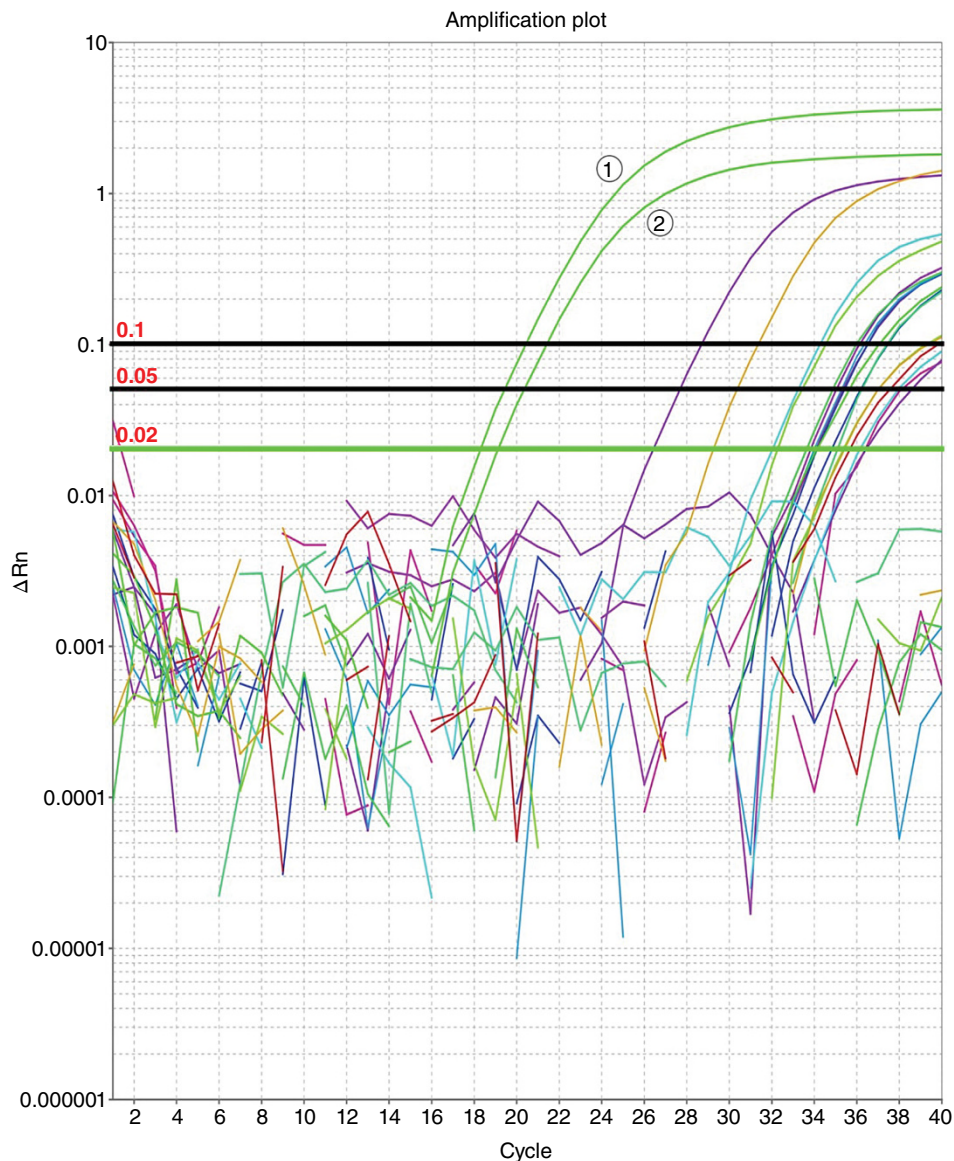


Figure 19.3 Detection and differentiation of type 1 and type 2 porcine reproductive and respiratory syndrome virus (PRRSV) nucleic acids by real-time PCR using a commercially available PRRSV RT-PCR kit. The lines indicate different thresholds: black 0.1 line = type 2 PRRSV; black 0.05 line = type 1 PRRSV; green 0.02 line = internal control. 1 = type 2 PRRSV positive control curve; 2 = type 1 PRRSV positive control curve.

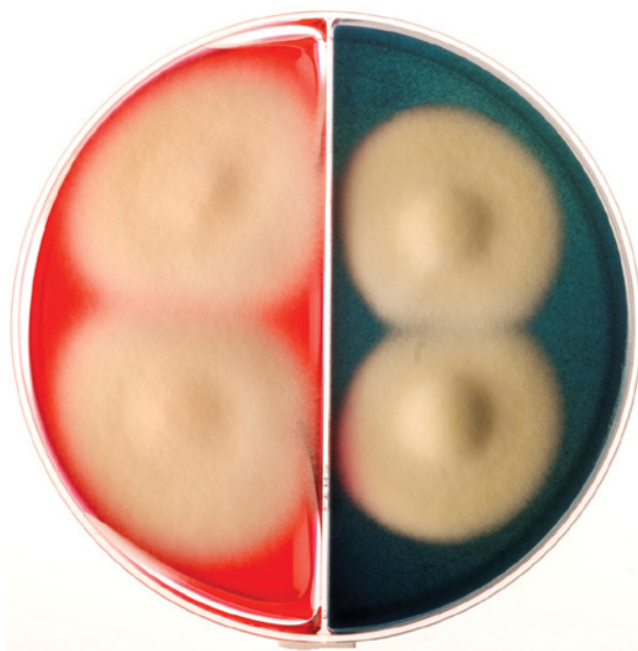


Figure 19.6 *Trichophyton* culture on Derm-Duet II, DTM/ESA (Dermatophyte Test Media/Enhanced Sporulation Media).
(Courtesy of Hardy Diagnostics, www.HardyDiagnostics.com, used with permission.)

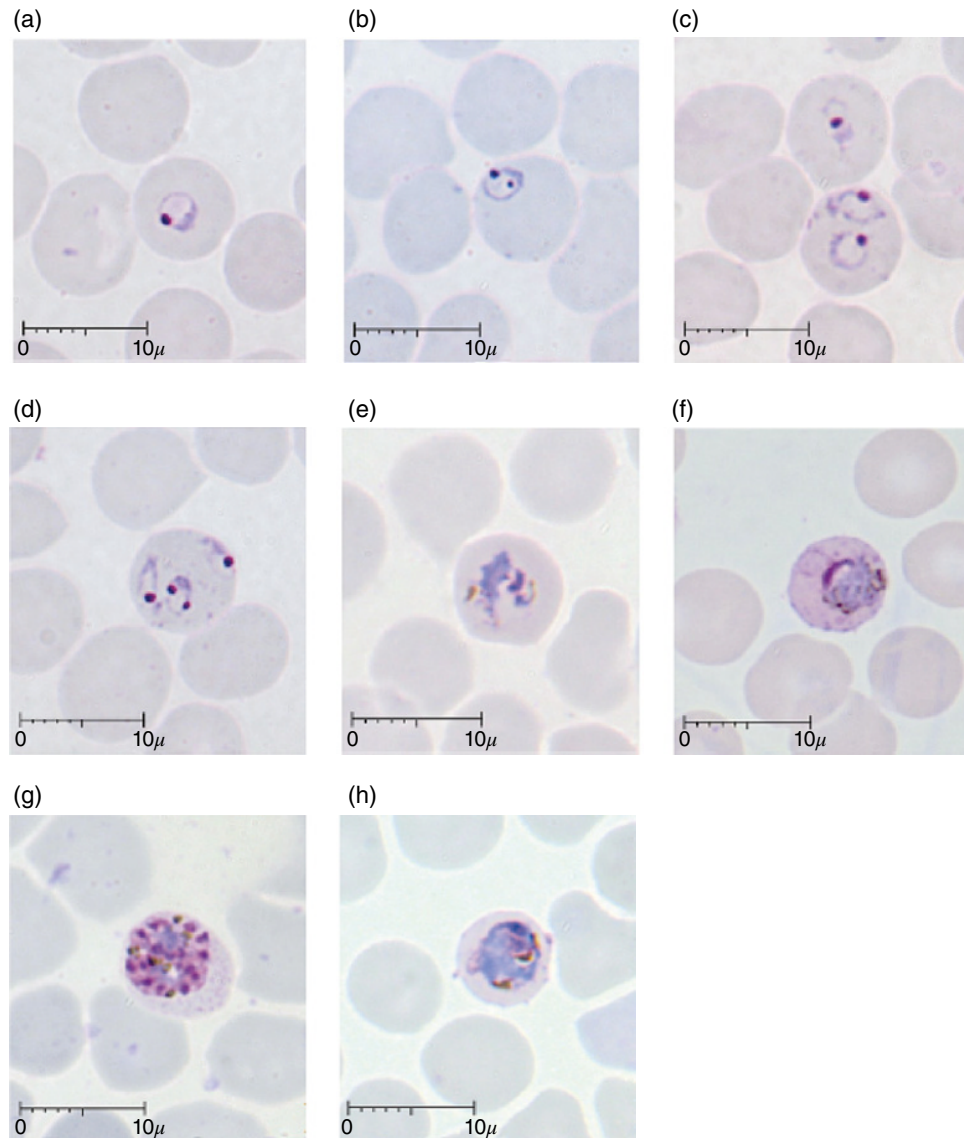


Figure 21.1 Giemsa-stained thin blood films of different stages of *P. knowlesi* infection in human red blood cells (RBC). Early trophozoite (a), early trophozoite with double chromatin dots (b), single RBC with multiple early trophozoites (c), appliqué forms of early trophozoites (d), late trophozoites (e), mature trophozoites (f), schizonts (g), and gametocytes (h). (Adapted from Lee et al. [154], <http://www.ncbi.nlm.nih.gov/pmc/articles/pmc2676309/>. used under CC-BY 2.0; <http://creativecommons.org/licenses/by/2.0/>.)



Figure 22.1 Vitek 2 system. (Courtesy of bioMérieux.)



Figure 22.3 MicroScan WalkAway *plus* system. (© 2015 Beckman Coulter, Inc., all rights reserved, used with permission.)

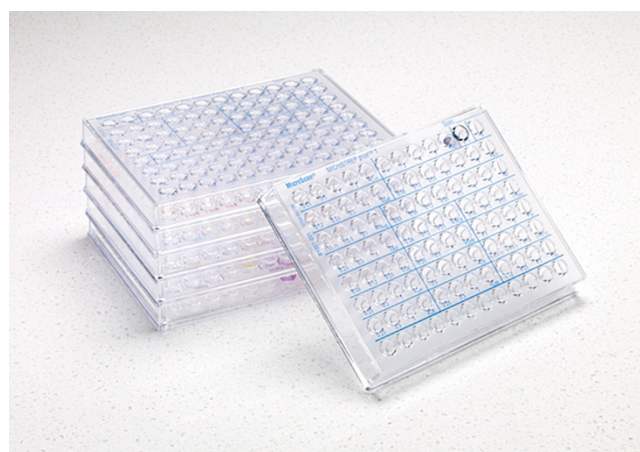


Figure 22.4 MicroScan Combo ID and AST panels. (© 2015 Beckman Coulter, Inc., all rights reserved, used with permission.)



Figure 22.5 Phoenix system. (Courtesy and © 2015 Becton, Dickinson and Company.)

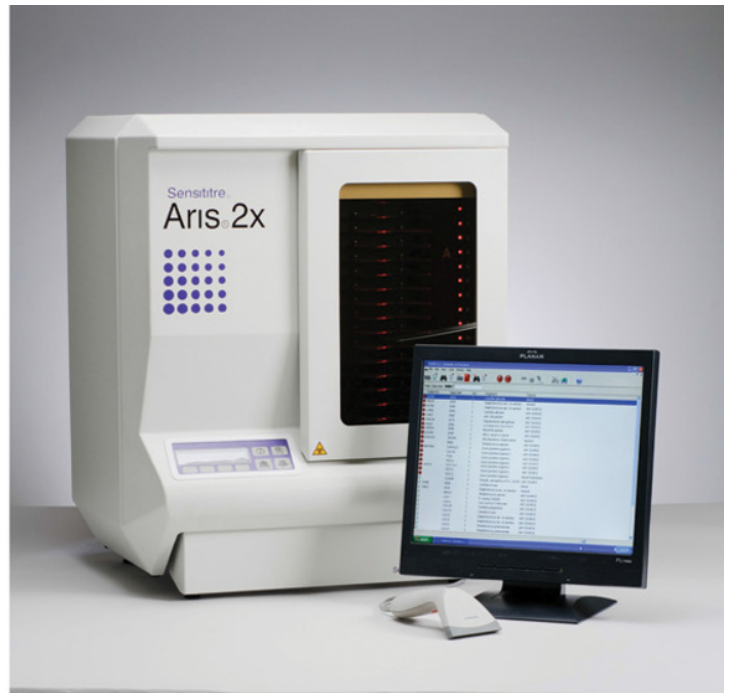


Figure 22.7 Thermo Scientific™ Sensititre™ ARIS™ 2X ID/AST System. (Photos courtesy of ThermoFisher Scientific, copying is prohibited.)

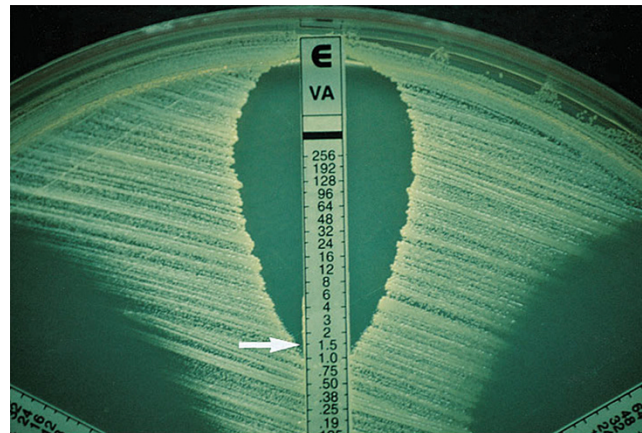


Figure 22.9 Etest. (Courtesy of bioMérieux.)



Figure 24.5 Bio-Rad's QX100 Droplet Digital PCR system (ddPCR) is a novel way to accomplish quantitative PCR. (Courtesy of Bio-Rad Laboratories, Inc.)

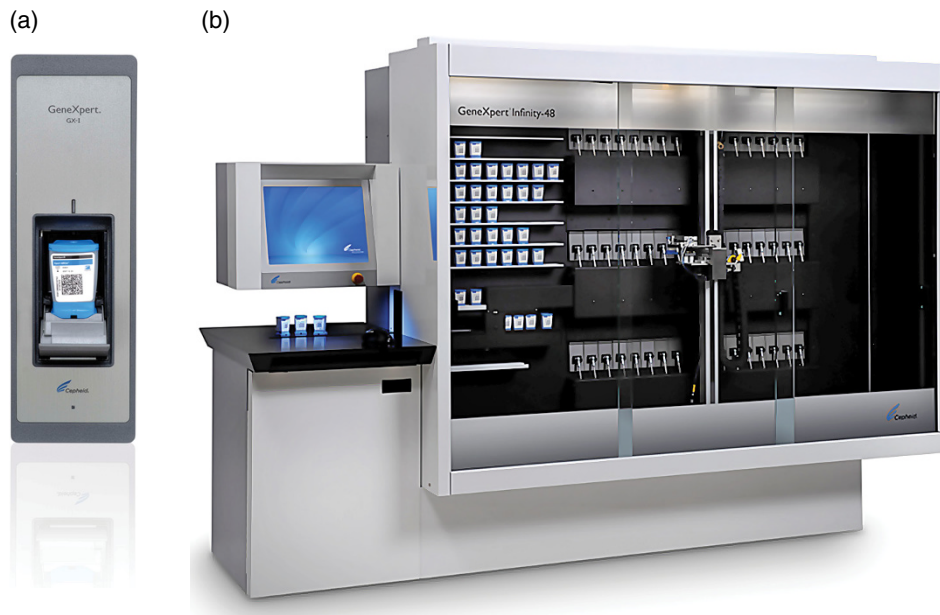


Figure 24.6 Cepheid GeneXpert systems range in throughput, from (a) one-cartridge to (b) a 96-cartridge instrument. (Courtesy of Cepheid.)

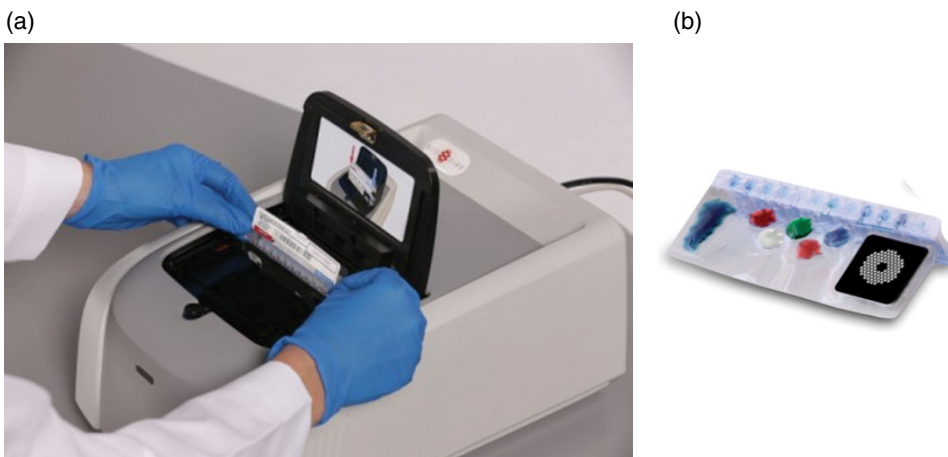


Figure 24.7 (a) BioFire Diagnostics' FilmArray instrument and (b) freeze-dried reagent pouches. (Courtesy of BioFire Diagnostics, Inc.)

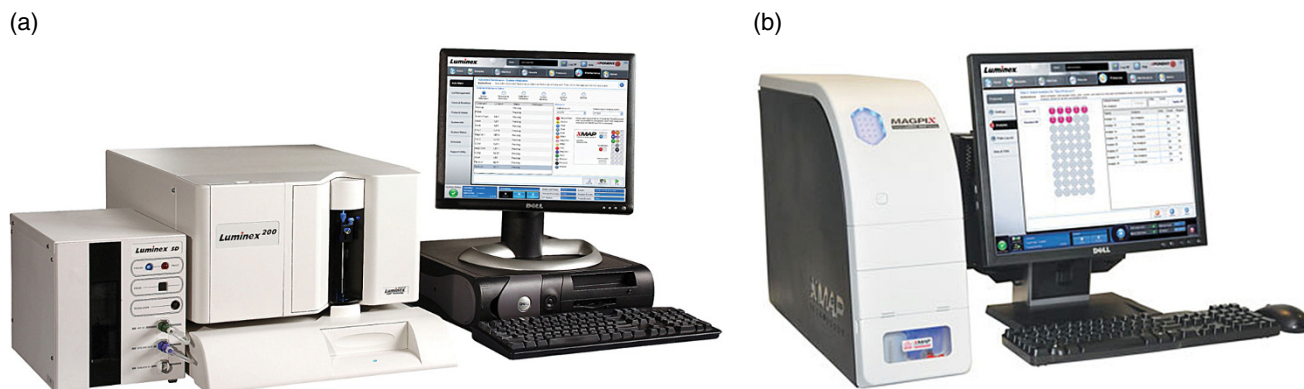


Figure 24.8 (a) The Luminex 100/200 system and (b) the Luminex MAGPIX instrument are used for detection of targets downstream to multiplex PCR. (Images provided by Luminex Corporation.)

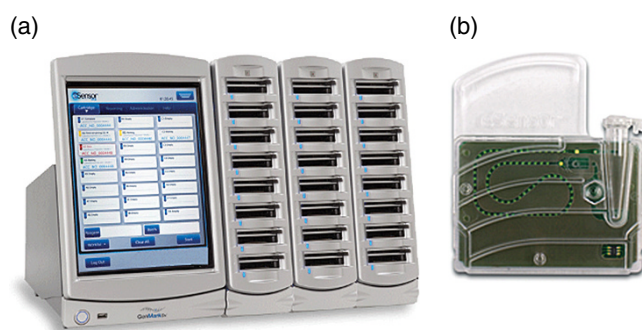


Figure 24.9 (a) The GenMark XT-8 system for detection of multiplex PCR products and (b) the test cartridge. (Images provided courtesy of GenMark Diagnostics.)



Figure 24.12 Loop-mediated isothermal amplification of DNA (LAMP assay) can be performed on Meridian's Illumigene instrument. (Courtesy of Meridian Bioscience, Inc.)

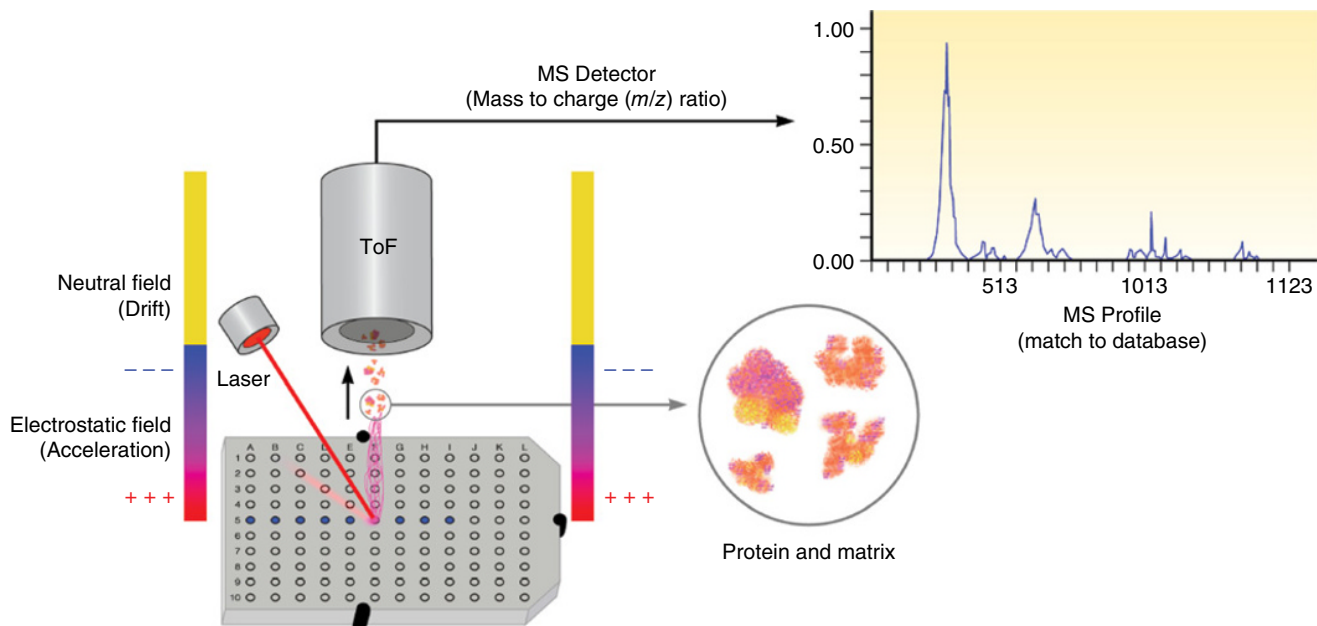


Figure 24.14 Schematic for MS analysis of ionized microbiological isolates and clinical material. Once appropriately processed, samples are added to the MALDI plate, overlaid with matrix and dried, then the sample is bombarded by the laser. This bombardment results in the sublimation and ionization of both the sample and matrix. These generated ions are separated based on their mass-to-charge ratio via a ToF tube, and a spectral representation of these ions is generated and analyzed by the MS software, generating an MS profile. (Copyright © American Society for Microbiology [Clinical Microbiology Reviews, Volume 26, Number 3, 2013, pp. 547–603] doi: 10.1128/CMR.00072-12.)

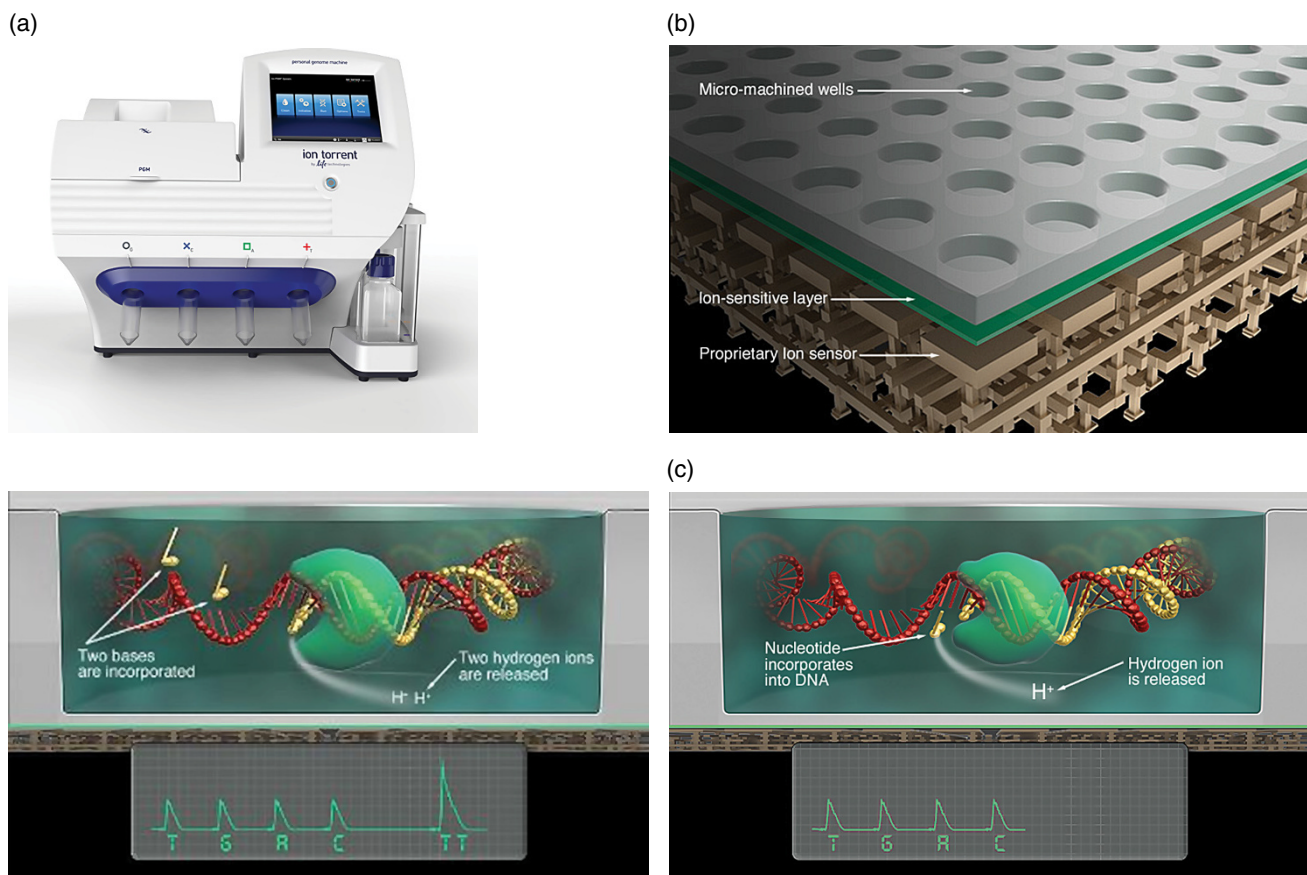


Figure 24.19 (a) The Ion Torrent Personal Genome Machine (Ion PGM™ System) Sequencer from Life Technologies utilizes (b) an ion sensor to identify the type and number of nucleotides in a sequence, as depicted in (c). (Photo courtesy of ThermoFisher Scientific, copying is prohibited.)

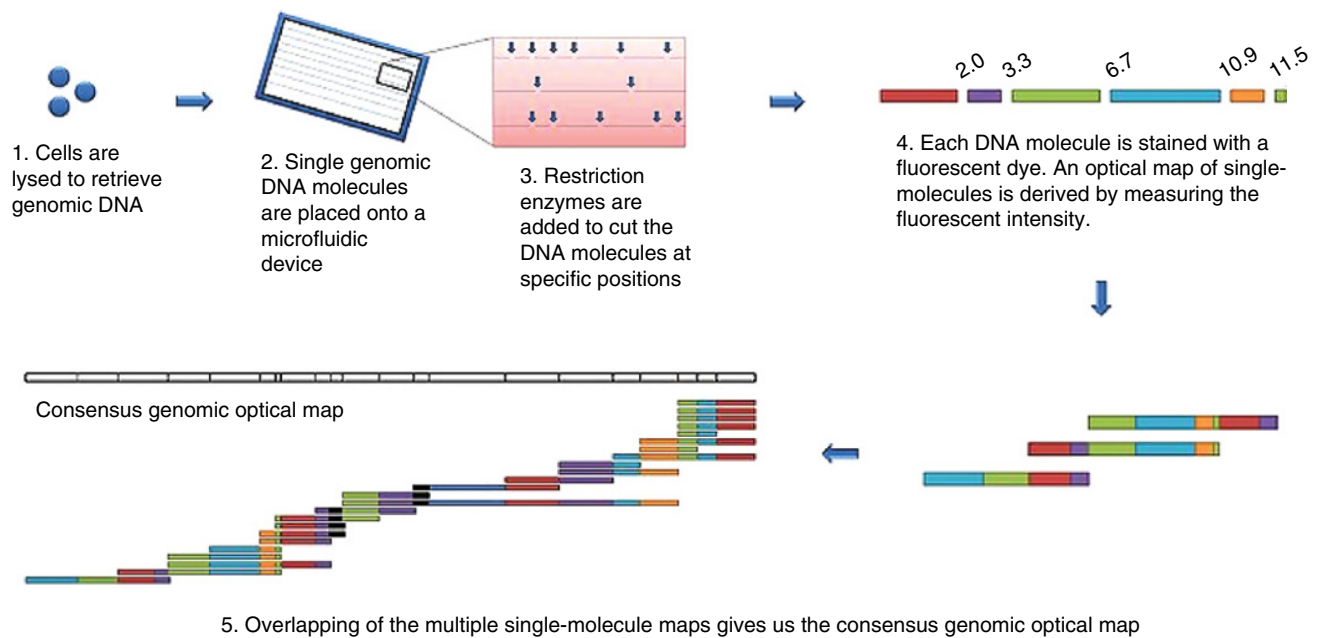


Figure 24.21 Overview of procedural steps for optical mapping. (Source: Chan and Wang, Used with permission, <http://upload.wikimedia.org/wikipedia/commons/e/e1/opticalmapping.jpg>; used under CC-BY 3.0, <http://creativecommons.org/licenses/by/3.0/deed.en>.)

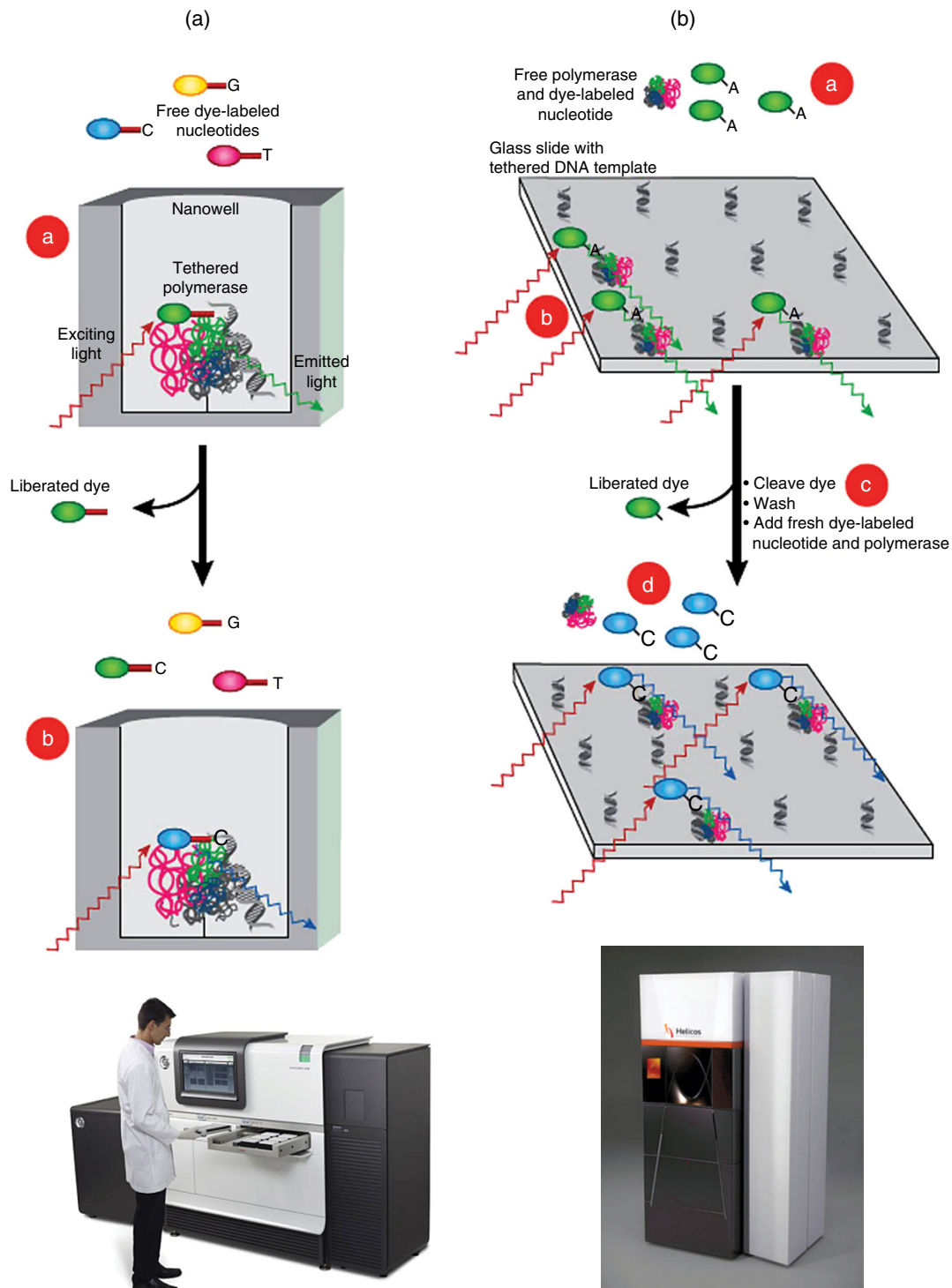


Figure 24.22 Single-molecule sequencing schemes: (a) Pacific Biosciences SMRT sequencer and (b) Heliscope Bioscience's HeliScope Genetic Analysis System represent novel research technologies. No applications are currently available for clinical use. (Osheroich 2010, reproduced with permission of Nature Publishing Group.)

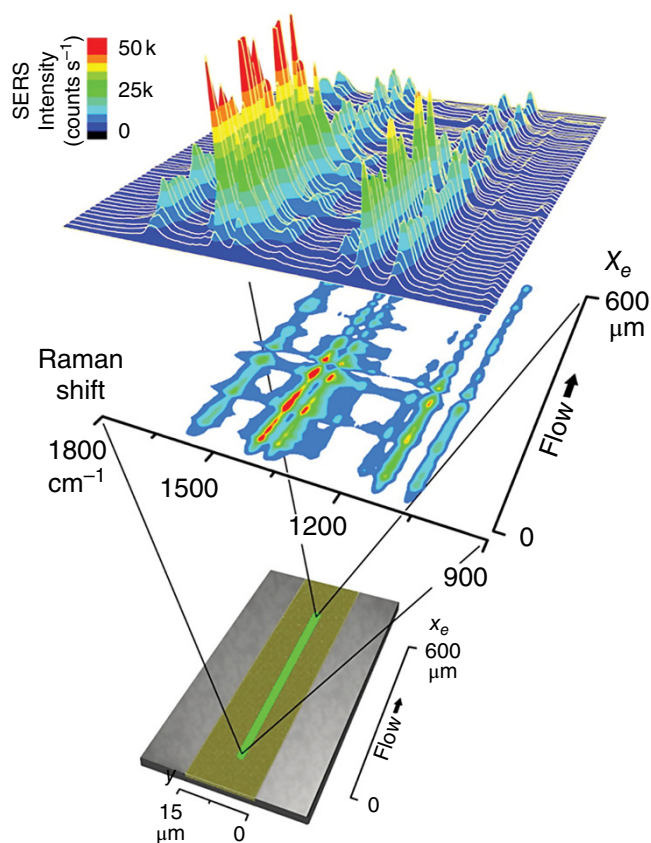


Figure 24.25 For surface enhanced Raman spectroscopy (SERS) a typical series of SERS spectra, obtained by the stepwise measurement of Raman scattering along the microchannel in 10- μm increments, is shown as a function of position down the streamwise direction of the microchannel. (Free-surface microfluidic control of surface-enhanced Raman spectroscopy for the optimized detection of airborne molecules, PNAS, Vol. 140, No.48, pp. 18898–18901, Nov. 7, 2007. Copyright (2007) National Academy of Sciences, USA.)

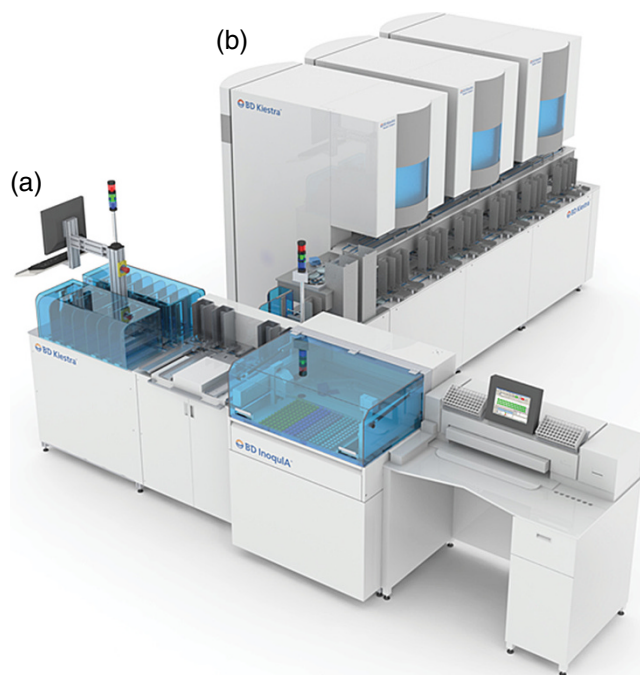


Figure 24.26 (a) Becton Dickinson's InoqulaA with (b) smart incubators attached. (Courtesy and © Becton, Dickinson and Company.)



Figure 24.27 COPAN's Walk-Away Specimen Processor (WASP) is part of the WASPLab system for automated microbiology. (Courtesy of Copan Diagnostics.)

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