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Molecular Microbiology

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ABSTRACT

Background

Nucleic acid (NA) amplification techniques are now commonly used to diagnose and manage patients with infectious diseases. The growth in the number of Food and Drug Administration—approved test kits and analyte-specific reagents has facilitated the use of this technology in clinical laboratories. Technological advances in NA amplification techniques, automation, NA sequencing, and multiplex analysis have reinvigorated the field and created new opportunities for growth. Simple, sample-in, answer-out molecular test systems are now widely available that can be deployed in a variety of laboratory and clinical settings. Molecular microbiology remains the leading area in molecular pathology in terms of both the numbers of tests performed and clinical relevance. NA-based tests have reduced the dependency of the clinical microbiology laboratory on more traditional antigen detection and culture methods and created new opportunities for the laboratory to impact patient care.

Content

This chapter reviews NA testing as it applies to specific pathogens or infectious disease syndromes, with a focus on those diseases for which NA testing is now considered the standard of care and highlights the unique challenges and opportunities that these tests present for clinical laboratories.

INTRODUCTION

Since the publication of the fifth edition of this textbook, significant changes have occurred in the practice of diagnostic molecular microbiology. Nucleic acid (NA) amplification techniques are now commonly used to diagnose and manage patients with infectious diseases. The growth in the number of Food and Drug Administration (FDA)—approved test kits and analyte-specific reagents (ASRs) has facilitated the use of this technology in clinical laboratories. Technological advances in NA amplification techniques, automation, NA sequencing, and multiplex analysis have reinvigorated the field and created new opportunities for growth. Simple, sample-in, answer-out molecular test systems are now widely available that can be deployed in a variety of laboratory and clinical settings.

Molecular microbiology remains the leading area in molecular pathology in terms of both the numbers of tests performed and clinical relevance. NA-based tests have reduced the dependency of the clinical microbiology laboratory on more traditional antigen detection and culture methods and created new opportunities for the laboratory to impact patient care. This chapter reviews NA testing as it applies to specific pathogens or infectious disease syndromes, with a focus on diseases for which NA testing is now considered the standard of care and highlights the unique challenges and opportunities that these tests present for clinical laboratories. A complete and current list of all FDA-cleared and FDA-approved microbial NA based tests can be found at http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm. Readers are directed to *Molecular Microbiology: Diagnostic Principles and Practice*, 3rd edition, for a more comprehensive and in depth examination of this dynamic and exciting discipline.[1]

VIRAL SYNDROMES

Human Immunodeficiency Virus 1

Human immunodeficiency virus 1 (HIV-1), the causative agent of the acquired immunodeficiency syndrome (AIDS), is an RNA virus belonging to the genus *Lentivirus* of the family *Retroviridae*. Replication of the virus is complex and involves reverse transcription of the RNA genome into a double-stranded DNA molecule or provirus, which is integrated into the host genome. HIV-1 enters the cell using CD4 as a receptor and CXCR4 or CCR5 as a coreceptor. In general, CCR5 coreceptors are found on macrophages, and CXCR4 coreceptors are found on T cells. Determining the cellular tropism of the virus has become important now that an antiretroviral drug targets the CCR5 coreceptor. The HIV-1 reverse transcriptase (RT) enzyme does not have proofreading capabilities, leading to the significant genetic diversity of HIV-1. Several distinct genetic subtypes or clades have been identified and are categorized into three groups: major (M), outlier (O), and N (nonmajor and nonoutlier). Recently, a new group P has been identified that is closely

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related to a gorilla simian immunodeficiency virus.[2] The major group is divided into nine clades (A—K) and circulating recombinant forms (CRFs), which are determined on the basis of sequence diversity within the HIV-1 *gag* and *env* genes.[3] Group M virus is found worldwide, with clade B predominating in Europe and North America, clade C in Africa and India, and clade E in much of Southeast Asia. The complex replication cycle and genetic diversity are two important factors that influence the design and interpretation of HIV-1 molecular assays.

The management of patients with HIV-1 infection was transformed by tests performed to measure the concentrations of HIV-1 RNA in blood (viral load testing) and tests for resistance to antiviral drugs. With these tools, it is possible to maximize the effectiveness of antiretroviral therapy (ART) for an individual.

The first HIV-1 viral load test was approved by the FDA in 1996 and rapidly became the standard of care for monitoring response to ART. Early studies found that patients who have higher viral loads progressed more rapidly to AIDS and death than those with low viral loads.[4-6]

Viral load testing is now widely accepted as a marker of response to ART. The guidelines for initiation of therapy based on viral load have changed as our understanding of disease progression at higher CD4 cell counts has improved. With the availability of newer more potent and less toxic drugs, ART is recommended for all HIV-infected individuals regardless of their CD4 cell count or viral load (DHHS Panel on Antiretroviral Guidelines, http://AIDSinfo.nih.gov). After treatment is initiated, viral load testing is crucial for monitoring response to therapy and should be measured in all HIV-infected individuals at the time of entry into care when therapy is initiated and at a regular interval (usually 3—4 months) while on therapy. The standard of care is to treat with a combination of antiretroviral drugs, which are classified based on their viral targets: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, integrase inhibitors (also known as integrase strand transfer inhibitors [INSTIs]), and CCR5 entry inhibitors. Current Department of Health and Human Services Panel on Antiretroviral Guidelines (http://aidsinfo.nih.gov/guidelines) recommend an initial regimen of two NRTIs and an NNRTI, a PI, or an INSTI. The pretreatment viral load values influence the treatment regimen because some regimens are less effective in patients with high viral loads. After initiation of appropriate therapy, there is typically a 2 $\log_{10}$ or greater decrease in viral load within 2 to 3 months. The goal for a patient is to achieve a viral load below the limit of detection of the most sensitive assays (20—50 copies/mL). Data have shown that the lower the absolute viral load, the better the clinical and virologic outcomes.[7,8] Guidelines recommend quantifying plasma HIV-1 RNA immediately before initiating therapy and 2 to 8 weeks later, with the goal to achieve an undetectable viral load level within 16 to 24 weeks of initiating therapy. It is important to determine early in the treatment course if there is suboptimal viral load suppression, so that factors affecting adherence can be assessed and, if needed, the regimen altered. After the initial response has been characterized, the viral load should be monitored every 3 to 4 months to ensure the response to therapy is sustained.

Of note, viral blips (defined as a detectable viral load usually <400 copies/mL) occur in successfully treated individuals and are not predictive of virologic failure.[9] For this reason, *virologic failure* is defined as a sustained viral load of more than 200 copies/mL.

Viral load testing also aids in the diagnosis of acute HIV-1 infection (the window period after infection that occurs before detectable antibody production), although the currently available viral load assays are not FDA approved for diagnostic purposes. During this period of early infection, patients typically have very high viral loads ranging from 10^5 to 10^7 copies/mL.[10] HIV RNA testing is recommended in the 2014 updated HIV diagnostic testing algorithm for patients who are reactive in a fourth-generation immunoassay but test negative in a supplemental HIV-1/HIV-2 antibody differentiation test, primarily to better detect acute HIV-1 infections.[11] However, only one HIV RNA test is FDA approved for diagnosis, the APTIMA HIV-1 qualitative assay (Hologic, San Diego, CA), and it is not widely available in clinical laboratories. Hospital laboratories would benefit from an FDA diagnostic claim for viral load testing that is done reflexively in the above diagnostic algorithm because verifying this claim at a local laboratory level is overly burdensome.

A proviral DNA test, AMPLICOR HIV-1 DNA polymerase chain reaction (PCR) (Roche Diagnostics), is available as a research use only (RUO) test that is helpful in neonates born to HIV-1—infected mothers receiving ART. Both antiretroviral agents and maternal antibody cross the placenta. Antiretroviral agents can suppress the replication of the virus in neonates, and RNA tests can be falsely negative early after birth. Maternal HIV-1 antibody can persist in neonates for up to 2 years after birth and is therefore not a reliable marker of neonatal infection.

Currently there are three commercially available, FDA-approved HIV-1 viral load assays. Two of these assays use real-time PCR, cobas AmpliPrep/cobas TaqMan HIV-1, version 2.0 (Roche Diagnostics), and m2000 RealTime System (Abbott Molecular); the third, Versant HIV-1 RNA 3.0 (Siemens), uses branched DNA signal amplification. The amplification method, gene targets, and dynamic ranges for these assays are given in Table 5.1. Newly emerging NA amplification tests (NAATs) and platforms have the potential for true point-of-care testing for HIV RNA,[12] and several (eg, Alere, BioHelix, Cepheid, and Iquum/Roche) are in commercial development. These tests may have application in detecting acute infections, confirming screening tests, and determining viral loads in resource-poor settings.

Most clinical laboratories are using one of the real-time PCR assays because of their lower limits of detection and quantification and increased dynamic range compared with older-generation PCR assays and the branched DNA assay. Genotype bias was a significant problem in some of the earlier viral load assays because the gene targets chosen were not highly conserved among the different HIV-1 subtypes. However, this problem has been addressed, and the current versions of the Roche and Abbott real-time PCR assays both accurately quantify group M, group O, and CRFs of HIV-1.[13] The intra-assay imprecision of HIV-1 viral load assays is approximately 0.12 to 0.2 $\log_{10}$ copies/mL. The biologic variation of viral load in patients not receiving therapy is

TABLE 5.1 Commercially Available Food and Drug Administration–Approved Viral Load Assays

Virus	Assay (Manufacturer)	Method	Gene Target	Dynamic Range
HIV-1	Versant 3.0 (Siemens)	Branched DNA	*pol*	75–500,000 copies/mL
	cobas Ampliprep/cobas TaqMan 2.0 (Roche)	RT-real-time PCR	*gag, LTR*	20–10,000,000 copies/mL
	RealTime (Abbott)	RT-real-time PCR	*int*	40–10,000,000 copies/mL
HCV	Versant 3.0 (Siemens)	Branched DNA	*5′ UTR*	615–7,700,000 IU/mL
	cobas Ampliprep/cobas TaqMan Test 2.0 (Roche)	RT-real-time PCR	*5′ UTR*	43–69,000,000 IU/mL
	RealTime (Abbott)	RT-real-time PCR	*5′ UTR*	12–100,000,00 IU/mL
HBV	cobas Ampliprep/cobas TaqMan Test 2.0 (Roche)	Real-time PCR	*Precore/core*	20–170,000,000 IU/mL
	RealTime (Abbott)	Real-time PCR	*Surface*	10–1,000,000,000 IU/mL
CMV	cobas Ampliprep/cobas TaqMan Test (Roche)	Real-time PCR	*UL54*	137–9,100,000 IU/mL
	artus RGQ MDx (Qiagen)	Real-time PCR	*MIE*	119–79,400,000 IU/mL

CMV, Cytomegalovirus; *HIV,* human immunodeficiency virus; *HBV,* hepatitis C virus; *HCV,* hepatitis B virus; *PCR,* polymerase chain reaction; *RT,* reverse transcriptase.

approximately 0.3 $\log_{10}$ copies/mL.[14] Consequently, changes in viral load must exceed 0.5 $\log_{10}$ copies/mL (a threefold change) to suggest a meaningful change in viral replication.

Viral load testing is routinely performed on plasma specimens, and ethylenediaminetetraacetic acid (EDTA) is the anticoagulant of choice. Acid–citrate–dextrose is also an acceptable anticoagulant, but blood anticoagulated in heparin is unacceptable for most tests. It is critical to handle clinical specimens properly to minimize the risk of RNA degradation during specimen collection and transport. Plasma should be separated within 6 hours of collection and ideally stored at −20°C, although plasma viral RNA is stable at 4°C for several days. For laboratories testing specimens collected at remote sites, sample handling can require careful attention. Special blood collection containers, or tubes, are available that contain a gel that provides a physical barrier between the plasma and cells after centrifugation. The tubes can be shipped without the need to transfer the plasma into a separate tube. Tubes should not be frozen before pouring off of the plasma because this may lead to falsely elevated viral load assays.[15,16]

Six general classes of antiretroviral drugs are used in clinical care: NRTIs, NNRTIs, PIs, fusion inhibitors, INSTIs, and CCR5 entry inhibitors. Viral resistance can occur with each of these drug classes, particularly when viral replication is not maximally suppressed during therapy. The current standard of care is to use regimens that contain a combination of drugs because resistance is less likely to occur on the complex regimens than on monotherapy.

The clinical utility of antiviral resistance testing in HIV-1–infected individuals is well established, and regularly updated guidelines for its use can be found at http://AIDSinfo.nih.gov. Current DHHS Panel on Antiretroviral Guidelines recommend that resistance testing be performed in the following situations: (1) before initiation of ART in treatment-naïve patients, (2) to guide the selection of active drugs when changing antiretroviral regimens, (3) for the management of suboptimal viral load reduction, and (4) in all pregnant women before the initiation of therapy.

HIV-1 resistance testing can be done by genotypic or phenotypic methods. Genotypic assays identify specific mutations or nucleotide changes that are associated with decreased susceptibility to an antiviral drug. Phenotypic assays are performed by the creation of a pseudoviral vector and measuring its replicative capacity in varying concentrations of drug and comparing it with replication of wild-type virus. Both genotypic and phenotypic methods are used clinically for assessing antiviral resistance in patients, with phenotypic testing usually reserved for drug-experienced patients with complicated resistance profiles.

This discussion is limited to automated sequencing methods for genotypic resistance because these are the methods used most often to inform treatment decisions. Currently available FDA-cleared assays will detect mutations in the RT and protease genes; modifications of existing assays are needed to detect resistance mutations associated with other classes of drugs such as integrase and fusion inhibitors.

The first step in genotypic assays is the isolation of HIV-1 RNA from plasma followed by reverse transcriptase polymerase chain reaction (RT-PCR) amplification and sequencing of RT and protease genes. Analysis of the results involves sequence alignment and editing, mutation identification by comparison with wild-type sequence, and interpretation of the clinical significance of the mutations identified. Most clinical laboratories performing genotypic resistance testing rely on commercial assays that provide reagents and software programs to assist with interpretation of the results. Two assays have been cleared by the FDA: the Trugene HIV-1 Genotyping Kit and OpenGene DNA Sequencing System (Siemens Healthcare Diagnostics) and the ViroSeq HIV-1 Genotyping System (Abbott Molecular).

Interpretation of genotypic resistance testing is complex. Interpretation of resistance mutations uses "rules-based" software that takes into account cross-resistance and interactions of mutations. The commercially available systems generate a summary report that lists the various mutations that have been identified in the RT and protease genes, and each drug is reported as resistant, possibly resistant, no evidence of

resistance, or insufficient evidence. A comprehensive discussion of the specific mutations associated with each antiretroviral drug and the interactions of mutations is beyond the scope of this chapter but is available from a variety of sources (eg, http://www.iasusa.org, http://hivdb.stanford.edu/).

A limitation of currently used genotypic and phenotypic assays is that they can detect only those mutants that make up at least 20% of the total viral population. Regimens chosen based on resistance testing may not always be effective because the minority populations will quickly predominate in the presence of drug. Drug selection pressure is also needed for some resistance mutations to persist at detectable concentrations in the viral population; when drug therapy is discontinued, the wild-type virus may quickly predominate. For this reason, it is recommended that specimens for resistance testing be obtained while the patient is on ART. The minimum viral load required for reliable resistance testing is approximately 1000 copies/mL. Because genotyping assays are especially sensitive to RNA degradation, care must be taken to properly handle the specimen after collection.

POINTS TO REMEMBER

Human Immunodeficiency Virus

- This RNA virus exhibits significant genetic diversity globally and within individual patients.
- HIV RNA is the earliest marker of infection.
- Viral load testing is a widely accepted marker of response to therapy with the goal of suppression to undetectable amounts.
- Special care should be taken in sample processing to avoid spurious increases in HIV viral load tests.
- HIV resistance genotyping should be performed on all therapy-naïve patients before initiation of ART and in selection of active drugs when changing therapy.

Hepatitis

Hepatitis C Virus

Approximately 3.2 million persons in the United States are living with active hepatitis C virus (HCV) infection, which is a major cause of chronic liver disease. After acute infection, 80% to 85% of individuals develop a chronic infection, and 2% to 4% of these individuals develop cirrhosis and end-stage liver disease, making end-stage liver disease secondary to HCV the most common indication for liver transplantation in the United States. Molecular tests for detection, quantification, and genotyping of HCV are standards of care for the diagnosis and management of patients with hepatitis C.

Hepatitis C virus is an RNA virus with a positive-sense, single-stranded genome of approximately 9500 nt encoding a single polyprotein of about 3000 amino acids. The long open reading frame is flanked at each end by short untranslated regions (UTRs). The genome structure is most similar to viruses of the family *Flaviviridae*, which includes many of the arthropod-borne viruses. As in other flaviviruses, the three N-terminal proteins of HCV (core, envelope 1 [E1]) and envelope 2 [E2]) are probably structural and the four C-terminal proteins (nonstructural 2, 3, 4, and 5) are thought to function in viral replication. HCV is classified within the family *Flaviviridae* in its own genus, *Hepacivirus*.

The 5′ UTR is a highly conserved region of 341 nt and has a complex secondary structure. It contains an internal ribosome entry site and is important in the translation of the long open reading frame. The 3′ UTR contains a short region that varies in sequence and length followed by a polypyrimidine stretch of variable length and finally a highly conserved sequence of 98 nt, which constitutes the terminus of the genome. The function of the 3′ UTR is not known but is thought to be essential for viral replication.

The E1 and E2 regions of HCV are the most variable regions within the genome at both the nucleotide and amino acid levels. Two regions in E2, called hypervariable regions 1 and 2 (HVR1 and HRV2, respectively), show extreme sequence variability, which is thought to result from selective pressure by antiviral antibodies. E2 also contains the binding site for CD81, one of the putative cell receptors or coreceptors for HCV.

The nonstructural regions 2 (NS2) and 3 (NS3) contain a zinc-dependent autoprotease that cleaves the polyprotein at the NS2—NS3 junction. The aminoterminal portion of the NS3 protein also is a serine protease that cleaves the polyprotein at several sites. The carboxyterminal portion of the NS3 protein has helicase activity, which is important for HCV replication. The NS4A protein is a cofactor for NS3 serine protease. The NS5B region encodes the RNA-dependent RNA polymerase, which replicates the viral genome. A region in NS5A has been linked to interferon-α (IFN-α) response and therefore is called the IFN-α—sensitivity determining region.

The first complete HCV genome sequence was reported by Choo et al in 1991.[17] As additional genome sequences from isolates from different parts of the world were determined and compared, it was evident that HCV exists as distinct genotypes with as much as 35% sequence diversity over the whole viral genome.[18] Much of the early literature on genotyping is confusing because investigators developed and used their own classification schemes. However, a consensus nomenclature system was developed in 1994. In this system, the genotypes are numbered using Arabic numerals in order of their discovery, and the more closely related strains within some types are designated as subtypes with lowercase letters. The complex of genetic variants found within an individual isolate is termed the “quasispecies.” The quasispecies results from the accumulation of mutations that occur during viral replication in the host.

The genotype and subtype assignments and nomenclature rules for HCV have recently been updated.[19] There are now 7 major genotypes and 67 subtypes of HCV recognized with another 20 provisional subtypes. HCV strains belonging to different genotypes differ at 30% to 35% of nucleotides and those that belong to the same subtype differ at fewer than 15% of nucleotides at the genome level.

Hepatitis C virus genotypes 1, 2, and 3 are found throughout the world, but there are clear differences in their distribution.[20] HCV subtypes 1a, 1b, 2a, 2b, 2c, and 3a are responsible for more than 90% of infections in North and South America, Europe, and Japan. In the United States, type 1 accounts for approximately 70% of the infections with equal distribution between subtypes 1a and 1b. Viral genotype does not correlate with disease progression.[21,22]

Detection of HCV RNA in serum or plasma by NA amplification methods is important for confirming the diagnosis of HCV, distinguishing active from resolved infection, assessing the virologic response to therapy, and screening the blood supply. These tests are incorporated into diagnostic algorithms for hepatitis C proposed by the Centers for Disease Control and Prevention (CDC),[23] American Association for the Study of Liver Diseases,[24] and National Academy of Clinical Biochemistry.[25]

The detection of HCV RNA in the plasma or serum is the earliest marker of infection, appearing 1 to 2 weeks after infection and weeks before elevation of liver enzymes or the appearance of anti-HCV antibodies. Approximately 80% of individuals infected with HCV will be chronically infected with the virus. In antibody-positive individuals, HCV RNA tests can distinguish active from resolved infections. In patients with a high pretest probability of infection, a positive serologic screening test result is usually confirmed with a test for HCV RNA rather than the recombinant immunoblot assay (RIBA). This strategy is cost-effective and more informative than using the RIBA to confirm positive antibody screening tests in a diagnostic setting.[26] However, with the discontinuation of the HCV RIBA by the manufacturer in 2012, all reactive HCV antibody screening tests should be followed by FDA-approved HCV RNA testing.[27]

Hepatitis C virus RNA testing also is helpful for the diagnosis of infection in infants born to HCV-infected mothers because of the persistence of maternal antibody and in immunocompromised or debilitated patients who may have blunted serologic responses. An HCV RNA test also should be used for patients suspected of having an acute infection and in patients with hepatitis of no identifiable cause.

Hepatitis C virus RNA tests are the most reliable means of identifying patients with active HCV infection. A negative HCV RNA test result in a serologically positive individual may indicate that the infection has resolved or that the viremia is intermittent. Up to 15% of chronically infected individuals have intermittent viremia and, as a result, a single negative HCV RNA determination may not be sufficient to exclude active infection when the index of clinical suspicion is high.[28] In these individuals a second specimen should be collected and tested.

The use of anti-HCV antibody tests to screen the blood supply has dramatically reduced the risk of transfusion-associated HCV infection in developed countries. The risk in the United States from blood that is negative for anti-HCV antibodies is less than 1 in 103,000 transfused units.[29] To drive the risk of infection from transfusion even lower, blood donor pools currently are tested for the presence of HCV RNA.[30] The serologic screening tests for HCV have a 70-day window period of seronegativity, and antigen detection tests are not yet available for blood product screening. HCV RNA testing is estimated to reduce the detection window by 25 days and reduce the number of transfused infectious units from 116 to 32 per year.[31]

Assays for the detection and quantification of HCV core antigen in serum have recently been commercially developed but are not yet FDA cleared for diagnostic use.[32-36] These tests significantly shorten the serologically silent window period using seroconversion panels, and their performance correlates closely with RNA detection tests in blood donors. However, the analytical sensitivity is less than most RNA tests, at approximately 10,000 IU/mL. The analytical sensitivity of the core antigen test is too high to be used in the monitoring of late events during and after treatment. Antigen detection may represent a cost-effective alternative to HCV RNA testing to distinguish active from resolved infections in resource-poor settings.

Hepatitis C viral load testing is useful in pretreatment evaluations of patients being considered for therapy because a viral load of less 600,000 IU/mL is one of several predictors of achieving a sustained virologic response.[37,38] Other factors associated with achieving a sustained response to therapy include the absence of cirrhosis, age younger than 40 years, female gender, white race, viral genotype 2 or 3, and presence of two copies of the C allele at position rs12979860 near the gene for IFN lambda 3 (*IFNL3*, *IL28B*).[39,40]

Hepatitis C viral load does not predict disease progression and is not associated with severity of liver disease.[41] This is in sharp contrast to HIV-1, in which the viral load is the principal factor determining the rate of disease progression. Monitoring HCV viral load in untreated patients is not warranted and should be discouraged. Until recently, the standard therapy for patients with chronic HCV infection was pegylated IFN-α in combination with ribavirin administered for either 48 weeks for HCV genotype 1, 4, 5, and 6 infections or for 24 weeks for HCV genotype 2 and 3 infections. Sustained virologic response (SVR) rates were attained in 40% to 50% of patients with genotype 1 and in 80% or more of those with genotype 2 and 3 infections. SVR was defined as the absence of detectable HCV RNA in plasma or serum as determined with a test that has a limit of detection of 50 IU/mL or less and is considered a virologic cure.

The first direct-acting antivirals (DAAs) for treatment of hepatitis C were approved by the FDA in 2011. Both are NS3/4A serine protease inhibitors, boceprevir (BOC) (Merck) and telaprevir (TVR) (Vertex). These DAA agents are used in combination with pegylated IFN-α and ribavirin. Triple therapy for genotype 1 infections has led to approximately a 30% increase in SVR over the previous standard of care therapy in all patient subgroups. TVR also has activity against genotype 2 infections but not against genotype 3 infections. BOC appears to have activity against both genotypes 2 and 3 infections. However, neither drug should be used to treat patients with genotype 2 and 3 infections because SVR rates with pegylated IFN-α and ribavirin alone are much higher.[42]

The important time points for response-guided therapy are at 8, 12, and 24 weeks for BOC and 4, 12, and 24 weeks for TVR. Treatment with all three drugs should be stopped if HCV RNA is greater than 100 IU/mL at week 12 or detectable at week 24 for BOC triple therapy and if HCV RNA is greater than 1000 IU/mL at weeks 4 or 12 or detectable at week 24 with TVR.

The goal of treatment is a SVR, defined as no detectable HCV RNA in serum or plasma by a highly sensitive assay (limit of detection $\leq$10–15 IU/mL) 6 months after the end of treatment. Patients who achieve a SVR have little or no chance of virologic relapse of their disease.

In 2013, two more potent DAAs were approved by the FDA: sofosbuvir (Gilead), a NS5B polymerase inhibitor,[43]

and simeprevir (Johnson & Johnson) a second-generation protease inhibitor.[44] Sofosbuvir was approved in combination with pegylated IFN-α and ribavirin for treatment of genotypes 1 and 4 and in combination with ribavirin alone for genotypes 2 and 3. Simeprevir was approved by the FDA for treatment of genotype 1 infections in combination with pegylated IFN-α and ribavirin but only for patients with genotype 1 who have not failed therapy with first-generation protease inhibitors.

Monitoring of viral load during treatment does not affect management decisions with a sofosbuvir-based regimen because treatment failure is almost exclusively caused by relapse.[43] However, given the expense of the drugs and the potential risk of viral resistance with inappropriate use, viral load testing at week 4 and at the end of treatment (either week 12 or 24 depending on the regimen) seems prudent.

The viral load should be determined at weeks 4, 12, and 24 to assess treatment response and possible cessation of therapy in patients treated with simeprevir, pegylated IFN-α, and ribavirin. Discontinuation is warranted for patients who are unlikely to achieve a SVR based on the virologic response during treatment. If HCV RNA is greater than 25 IU/mL at week 4, the entire regimen should be discontinued. If the HCV RNA is greater than 25 IU/mL at week 12 or 24 after the simeprevir has been completed, the pegylated IFN-α and ribavirin should be discontinued.[44]

Numerous other DAAs have been developed and are currently in clinical trials. These include NS3/4A protease inhibitors, NS5B polymerase inhibitors, and inhibitors of host cell proteins required for HCV replication. The most current recommendations for all aspects of HCV treatment can be found at http://www.hcvguidelines.org.

Currently, three FDA-approved HCV viral load assays are available commercially. Two of these assays use real-time PCR, cobas AmpliPrep/cobas TaqMan, version 2.0 (Roche Diagnostics), and m2000 RealTime System (Abbott Molecular); the third uses branched DNA signal amplification, Versant 3.0 (Siemens). The amplification method, gene targets, and dynamic ranges for these assays are given in Table 5.1. The commercially available assays are calibrated against a World Health Organization (WHO) international calibration standard and report results in IU/mL. The first version of the cobas TaqMan HCV test had a genotype bias particularly against genotype 4 samples. The second version of the assay has been modified to enhance its ability to accurately quantify all of the major HCV genotypes.[45,46]

The development of the WHO first international HCV RNA standard and its acceptance by the manufacturers of HCV RNA assays as a calibrator was a significant advance in HCV RNA quantification.[47] However, despite the implementation of an international standard, HCV RNA measurements are not equivalent between the different assays.[48,49] Therefore, patients should ideally be tested with the same assay during the course of their treatment to minimize the potential for patient management errors.[50]

Although a number of baseline factors are predictive of response to treatment of chronic hepatitis C infection, HCV genotype is a strong and consistent predictor for achieving a SVR to pegylated IFN-α and ribavirin. In the large clinical trials of combination therapy with pegylated IFN-α and ribavirin, only 30% of patients infected with genotype 1 had a SVR compared with 65% of patients infected with genotypes 2 or 3.[37,38]

The determination of HCV subtypes has no clinical relevance in patients treated with IFN-α and ribavirin, but different treatment durations based on viral load kinetics are recommended for patients with different HCV genotypes. However, the emergence of resistant variants and virologic breakthrough were more common in patients infected with HCV subtype 1a than 1b when treated with TVR triple therapy.[51] HCV subtyping may play a role in helping to select treatment regimens and predict the development of resistance to DAA drugs. In addition, triple therapy with a protease inhibitor is not recommended for patients infected with genotypes 2 and 3.

Antiviral-resistance mutations that cluster around the catalytic site of the NS3/4A serine protease emerge during protease inhibitor therapy and are associated with failure and relapse.[42] Similar resistant variants are detected in both BOC- and TVR-treated patients, suggesting that cross-resistance occurs with these protease inhibitors. Also antiviral-resistant variants are found in about 5% of patients before treatment but do not appear to impact response to either protease inhibitor. Currently, there is no role for antiviral resistance genotyping at baseline or during treatment with the protease inhibitors.[52]

Several mutations in the NS3/4A protease are associated with reduced susceptibility to simeprevir. One of the most common and clinically relevant mutations is the substitution Q80K. This mutation may be present at baseline in approximately 30% of patients with genotype 1a and is associated with lower SVR rates. For patients with genotype 1a, Q80K mutation testing is recommended and patients with this variant should be offered other treatment options.[53]

A variety of laboratory-developed and commercial assays are used for HCV genotyping. The methods include NA sequencing, reverse hybridization, subtype-specific PCR, DNA fragment length polymorphism, heteroduplex mobility analysis, melting curve analysis, and serologic genotyping. Currently, there is only one FDA-approved HCV genotyping assay, the Abbott RealTime HCV Genotype II assay.[54] It uses real-time PCR and multiple hydrolysis probes to amplify and differentiate genotypes 1 to 6 and subtype 1a and 1b. The 5′ UTR is the target to identify the genotype and the NS5B is the target for subtyping genotype 1 samples. The results from this test have shown good overall agreement with direct sequencing methods. However, relatively high rates of indeterminate results and inability to distinguish all genotype 1 subtypes are limitations.

A commercially available reverse hybridization line probe assay is the most commonly used method for genotyping HCV among clinical laboratories participating in the HCV proficiency-testing surveys of the College of American Pathologists. This reverse hybridization assay was developed by Innogenetics (Fujirebio Europe) and is now marketed as the Versant HCV Genotype 2.0 Assay by Siemens. In this line probe assay (LiPA), biotinylated PCR products from the 5′ UTR and core regions of the HCV genome are hybridized under stringent conditions with oligonucleotide probes attached to a nitrocellulose strip: 19 type- and subtype-specific probes interrogate the 5′ UTR and an additional three probes interrogate the core region. The core region probes

were added to provide better discrimination of subtypes 1a and 1b and genotype 6.[55] Hybridized PCR products are detected with a streptavidin—alkaline phosphatase conjugate. The pattern of reactive lines defines the genotype and in some cases the subtype. The assay discriminates among genotypes 1a, 1b, 2a/c, 2b, 3a, 3b, 3c, 3k, 4a/c/d, 4b, 4e, 4h, 5a, and 6a/b. The Versant HCV Genotype 2.0 Assay correctly identifies genotypes and distinguishes subtypes 1a and 1b when compared with sequencing but may not be able to adequately identify the other subtypes.[56] Mixed genotype infections can be recognized as unusual patterns of hybridization signals. However, the LiPA requires a considerable amount of amplicon for typing, and the assay may regularly fail when the viral load is less than 10^4 copies/mL.

Sequence analysis of amplified subgenomic sequences is the most definitive way to genotype HCV strains. Genotyping schemes based on sequencing variable genes such as E1, Core, and NS5B provide enough resolution to determine types and subtypes.[57,58] The 5′ UTR is too highly conserved to discriminate all subtypes reliably. Genotyping methods targeting highly variable regions have higher failure rates because of primer mismatches and failed amplification reactions. Sequencing reactions can be performed directly on PCR products or on cloned amplicons. Mixed infections with multiple genotypes may be missed by sequence analysis. Definitive detection of mixed infections requires analysis of a large number of clones. Cloning may, however, emphasize artifactual nucleotide substitutions introduced by the DNA polymerase during amplification or by selection during the cloning procedure and is generally not practical for clinical laboratories.

A standardized direct sequencing system for HCV genotyping is commercially available (Siemens). The Trugene HCV 5′NC genotyping kit targets the 5′ UTR (nt 96 to 282) and employs proprietary single-tube chemistry.[59] This method can be used with the 244-base-pair amplicon generated by either the Roche Amplicor HCV or Amplicor HCV Monitor tests as the sequencing template after a column purification step.[60] The sequencing chemistry produces bidirectional sequences. The software acquires the sequence data, and each pair of forward and reverse sequences is combined. A reference sequence library module contains approximately 200 sequences from the six major genotypes and 24 subtypes of HCV. The software automatically aligns the patient HCV sequence with the reference sequences in the library and reports type, subtype, and closest isolate determinations. The Trugene HCV 5′NC genotyping system is a reliable method for determining HCV genotypes, but similar to all approaches targeting the conserved 5′ UTR, cannot reliably distinguish all HCV subtypes.[60,61]

The practice of using sequence analysis of a single subgenomic region for HCV genotyping has been challenged by the description of naturally occurring intergenotypic recombinants of two HCV genotypes.[62-65] The recombinant forms have been detected in patients in Russia (genotypes 2k and 1b), Vietnam (genotypes 2 and 6), and France (genotypes 2 and 5), as well as in experimentally infected chimpanzees (genotypes 1a and 1b).

A novel HCV genotyping method using a solid phase electrochemical array was developed by GenMark Diagnostics. The method uses sequence specific capture of a PCR amplicon from the HCV 5′ UTR by surface-bound oligonucleotide capture probes formed within a preassembled monolayer with electrochemical detection using ferrocene-labeled oligonucleotide signal probes. High genotype concordance between the GenMark and LiPA HCV tests was observed; however, there were minor discrepancies in genotype 1 subtype identifications by the two tests due to differences in the regions of the HCV genome interrogated.[66]

The widespread use of tests not cleared by the FDA for HCV genotyping has placed an increased burden on clinical laboratories to verify the performance characteristics of these tests before clinical use. When validating HCV genotyping tests, laboratories should take advantage of the published evaluations and commercially available genotype panels to streamline the verification process.

The College of American Pathologists has a well-established proficiency testing program for laboratories performing tests for detection, quantification, and characterization of HCV RNA. These surveys have shown a steady improvement in the performance of laboratories over time that probably reflects progress in both the available technologies and laboratory practices.

POINTS TO REMEMBER

Hepatitis C Virus

- There are approximately 3.2 million people living with active HCV infections in the United States. HCV is a common cause of chronic liver disease.
- HCV viral load does not predict progression or severity of liver disease but is important in distinguishing active from resolved infections and as a widely accepted marker of response to therapy.
- Although there are seven major HCV genotypes and numerous subtypes that differ in geographical distribution, genotype 1 infections predominate in much of the developed world and have, historically, been the most difficult to successfully treat using IFN-α and ribavirin.
- Treatment response rates for genotype 1 infections have dramatically improved with the FDA clearance of DAA agents that target HCV proteases and polymerase.
- Currently, there is no role for HCV resistance genotyping in guiding therapy, but that may change as the number of DAA agents increases.

Hepatitis B Virus

Hepatitis B virus (HBV) is a small enveloped DNA virus belonging to the family *Hepadnaviridae* and causes transient or persistent (chronic) infection of the liver. This family is divided into two genera, orthohepadnavirus and avihepadnavirus, which infect mammals or birds as natural hosts, respectively.[67] They possess a narrow host range determined by the initial steps of viral attachment and entry. Approximately 2 billion people have serologic evidence of hepatitis B, and of these, approximately 350 million people have chronic infections.[68] Depending on viral and host factors, the outcomes of infection with HBV range from clearance to mild or severe chronic hepatitis (CHB) to the development of cirrhosis or hepatocellular carcinoma (HCC).[69,70] The evolution of increasingly sensitive methods for HBV DNA detection and its accurate quantification has created an important role for

molecular testing in routine patient care. Improved methods for sequencing and single mutation detection have led to tests that document mutational change in the viral genome. Currently, NA testing plays a critical role in the overall care of HBV-infected patients.

The HBV genome is a 3.2-kilobase relaxed circular, partially double-stranded DNA molecule. It has four partially overlapping open reading frames encoding the viral envelope (pre-S and S), nucleocapsid (precore and core), polymerase, and X proteins. After binding to hepatocytes, the virion is taken up into the cell by endocytosis and uncoated. The partially double-stranded DNA genome is converted to a covalently closed circular DNA (cccDNA) in the cell nucleus. The cccDNA is used as a template for transcription of the pregenomic RNA (pgRNA) and messenger RNA in the cell nucleus. The pgRNA moves into the host cell cytoplasm and serves as the template for translation of the HBV RT as well as the core protein by the cellular translational proteins. Concurrently, the HBV RT reverse transcribes the pgRNA to a new circular DNA molecule. Early in the replication cycle, some of the newly synthesized genomes will circulate back to the nucleus to maintain and increase the pool of cccDNA.[71]

Although HBV is a DNA virus, it replicates by an RT that lacks proofreading activity and, as a result, is prone to errors. The overlapping open reading frames of the genome limit the types of mutations that can be tolerated. However, variations in HBV sequences have been detected in almost all regions of the genome. Consequently, HBV exists as quasispecies, and different patients may be infected with different strains and genotypes.

Seven phylogenetic genotypes (A through H) of HBV have been identified, most of which have distinct geographic distribution. Genotypes are defined by intergroup divergence of greater than 8% in the complete genome nucleotide sequence. All known genotypes have been found in the United States with the prevalence of A, B, C, D, and E to G being 35%, 22%, 31%, 10%, and 2%, respectively.[72] Recent data suggest that HBV genotype plays an important role in the progression of HBV-related liver disease as well as response to IFN-α and pegylated IFN-α; however, HBV genotyping is not necessary in routine clinical practice.[73]

Serologic assays with high sensitivity, specificity, and reproducibility have been developed to detect HBV antigens and their respective antibodies. This complicated system of serologic markers is used for diagnosis of HBV infection and to define the phase of infection, degree of infectivity, prognosis, and patient's immune status. The presence of HBV DNA in the serum is a marker of viral replication in the liver and has replaced hepatitis B e antigen (HBeAg) as the most sensitive marker of viral replication. HBeAg is the extracellular form of the hepatitis B core protein. Molecular assays to quantify blood HBV DNA are useful for the initial evaluation of HBV infections, monitoring of patients with chronic infections, and assessing the efficacy of antiviral treatment.[71,73] In addition, U.S. blood donors are routinely screened for HBV DNA by qualitative tests to detect donors in the early stage of infection.[74] Antiviral resistance mutations are detected by molecular methods that identify known mutations associated with drug resistance.

The initial evaluation of patients found to have hepatitis B surface antigen (HBsAg) in serum should include routine liver tests and a variety of virologic tests, including HBV DNA testing.[73] Chronic HBV infection is a disease of variable course, and establishing baseline laboratory values at the time of diagnosis is important clinically for the tracking of disease progression over time and to evaluate candidates for liver biopsy. Monitoring disease activity in chronically HBV-infected patients is best done by measuring aminotransferase (ALT) at regular intervals in HBeAg-positive patients. However, serial HBV DNA testing is recommended in HBeAg-negative patients. The determination of serum HBV DNA (viral load) is important in the pretreatment evaluation and monitoring of therapeutic response in patients with chronic infection.[73] Currently, therapy for chronic HBV infection does not eradicate the virus and has limited long-term efficacy. The decision to treat should be based on ALT elevations; the presence of HBeAg or HBV DNA (or both); viral load of greater than 2000 IU/mL; the presence of moderate disease activity and fibrosis on liver biopsy; and virologic testing to exclude concurrent infections with hepatitis D virus (HDV), HCV, and HIV. The treatment goals for chronic hepatitis B are to achieve sustained suppression of HBV replication and to prevent further progression of liver disease. Parameters used to indicate treatment response include normalization of serum ALT, decrease in serum HBV DNA, and loss of HBeAg with or without detection of anti-HBeAg. Currently, there are eight FDA-approved therapies for chronic HBV infection: IFN-α, pegylated IFN-α2a, four nucleoside analogs (lamivudine, telbivudine, entecavir, and emtricitabine), and two nucleotide analogs (adefovir and tenofovir). Several factors predict a favorable response to IFN treatment with the most important being high ALT and low serum HBV DNA viral load, which are indirect markers of immune clearance.

Therapy usually does not eradicate the virus because the covalently closed circular form of the HBV genome is difficult to eliminate from the liver and the existence of extrahepatic reservoirs of HBV. Endpoints of treatment have traditionally been clearance of HBeAg, development of anti-HBe antibodies, and undetectable serum HBV DNA using insensitive hybridization assays with detection limits of approximately 10^6 genome copies/mL. Achieving these endpoints usually is accompanied by resolution of liver disease as evidenced by normalization of ALT and decreased inflammation on liver biopsy. The response usually is sustained at long-term follow-up. Nevertheless, most responders continue to have detectable HBV DNA when sensitive NA amplification tests are used. Responses to antiviral therapy are categorized as biochemical, virologic, or histologic and as on therapy or sustained off therapy.[73]

Several variations in the nucleotide sequence of HBV have important clinical consequences. An important mutation in the gene encoding HBsAg is a glycine-to-arginine substitution at codon 145 (G145R) in the conserved "a" determinant, which causes decreased affinity of the HBsAg for anti-HBs antibodies.[75] HBV with this mutation has been found in children of HBsAg-positive mothers who develop HBV infection despite vaccination and an adequate anti-HBs antibody response after vaccination, as well as in liver transplant recipients who have recurrent infection despite administration of HBV immune globulin.[76,77] These immune escape mutants have raised concern about vaccine

efficacy and serologically silent infections. The G145R mutation has been reported in many countries and is responsible for 2% to 40% of vaccine failures. Although there is diminished binding to anti-HBs antibodies, the vast majority of S mutants can be readily detected with the current generation of HBsAg tests. Thus, an initial concern that widespread use of HBV immune globulin and vaccination would result in HBV mutants that would escape detection in the HBsAg test was unfounded.

Mutations in the basal core promoter and the precore genes affect the synthesis of HBeAg and commonly arise under immune pressure.[78] The most common basal core promoter mutation has a dual change of A to T at nt 1762 (T1762) and G to A at nt 1764 (A1764) that diminishes the amount of mRNA and hence HBeAg secretion.[79] The predominant precore mutation is a G to A change at nt 1896 (A1896), which leads to premature termination of the precore protein at codon 28, thus preventing the production of HBeAg.[80] The A1896 mutation is infrequent in North America and Western Europe but is geographically widespread. This geographic variability in frequency is related to the predominant genotypes in a geographic region because the mutation is found only in genotypes B, C, D, and E.

The A1896 mutation was first reported in patients with chronic active hepatitis or fulminant hepatitis. However, the A1896 mutation also can be present in asymptomatic carriers, and viruses with this mutation replicate no more efficiently than wild-type HBV. Thus, the pathophysiologic significance of this mutation is unclear.[81] However, the clinical picture of persistent HBV replication and active liver disease in HBeAg-negative patients appears to be increasingly prevalent, and in some regions, the A1896-mutant virus may be more prevalent than the wild-type virus.

Therapy for chronic hepatitis B requires long courses of treatment with nucleoside or nucleotide analogs. A major concern with long-term therapy is the development of antiviral resistance by mutation in one or more domains of the gene encoding the HBV polymerase. The rate at which resistant mutants are selected is related to pretreatment serum HBV DNA viral load, rapidity of viral suppression, duration of treatment, and prior antiviral exposure. The incidence of genotypic resistance also varies with the sensitivity of the methods used to detect resistance mutations and the patient population tested.

Typically, when a patient experiences a virologic breakthrough, defined as an increase in serum HBV DNA greater than 1 $\log_{10}$ above nadir after achieving a virologic response during continued treatment, HBV resistance genotyping should be performed. The standardized nomenclature of HBV antiviral resistance mutations in the polymerase is shown in Table 5.2.[71,82,83] No HBV mutations are associated with resistance to IFN-α or pegylated IFN-α2a.

There are a number of commercially available tests for quantification of HBV DNA in serum and plasma, but only two tests have United States-In Vitro Diagnostics (US-IVD) regulatory status. These tests—the cobas AmpliPrep/cobas TaqMan (Roche) and the Real-time (Abbott) HBV tests—both use real-time PCR (see Table 5.1). The others produced by Cepheid, Qiagen, and Siemens are CE (*Conformité Européenne*, meaning European Conformity) marked and RUO kits or ASRs in the United States.

TABLE 5.2 Antiviral Agents and the Hepatitis B Virus Polymerase Mutations Associated With Resistance

Antiviral Agent	Drug Class	Resistance Mutations
Lamivudine	Nucleoside analog (cytidine)	(L180M + M204V/I/S), A181V/T, S202G/I
Telbivudine	Nucleoside analog (dTTP)	M204I, A181T/V
Entecavir	Nucleoside analog (2-deoxyguanosine)	T184S/C/G/A/I/L/F/M, S202G/C/I, M250V/I/L
Emtricitabine	Nucleoside analog (cytidine)	M204V/I
Adefovir	Nucleotide analog (dATP)	A181V/T, N236T
Tenofovir	Nucleotide analog (dATP)	A194T, N2263T, A181V/T

dATP, Deoxyadenosine triphosphate; *dTTP,* deoxythymidine triphosphate.

A WHO international HBV standard was first created in 2001 in response to the recognized need to standardize HBV DNA quantification assays.[84] However, despite the availability of HBV DNA standards, the various quantitative assays usually have different conversion factors for copies to IU/mL, which may reflect their different amplification and detection chemistries. Laboratories should report HBV viral load test results in IU/mL as both $\log_{10}$ transformed and arithmetic values. HBV is included in the hepatitis viral load proficiency testing surveys available from the College of American Pathologists.

Two HBV genotyping systems are commercially available as RUO kits. Innogenetics (Fujirebio Europe) offers three different line probe assays for (1) HBV phylogenetic genotyping; (2) detection of precore mutations; and (3) detection of all relevant lamivudine, emtricitabine, telbivudine, adefovir, and entecavir resistance mutations as well as known compensatory mutations.[85,86] All assays use PCR to amplify portions of the relevant genes to produce a biotinylated product. The PCR products are denatured and hybridized to a series of informative probes immobilized on a nitrocellulose strip. The hybrids are visualized on the strip after addition of streptavidin—alkaline phosphatase and a colorimetric substrate. The mutations are identified by the colored patterns of PCR product hybridization to the probes. The line probe assays typically have better sensitivity for detection of sequence variants than direct Sanger sequencing.

The TRUGENE HBV genotyping test (Siemens) uses two fluorescently labeled DNA primers and PCR to amplify a portion of the HBsAg gene and overlapping polymerase gene, with bidirectional sequencing of these amplicons, and a software module that includes a sequence database that identifies the phylogenetic genotype (A to H) as well as mutations associated with resistance to the nucleoside and nucleotide analog drugs.[87] The sequence ladder is resolved on a polyacrylamide slab gel. The total analysis time is approximately 8 hours, including the time required for DNA extraction and purification.

The standardized nomenclature for reporting of HBV antiviral resistance mutations shown Table 5.2 should be used when resistance genotyping is performed. The inability of genotyping assays to detect minor populations of circulating HBV is a significant technical issue. In general, direct sequencing is limited to resolution of populations that are more than 20% of the viral population.

Transplant Recipients

Cytomegalovirus

Cytomegalovirus (CMV), a member of the *Herpesviridae* family, is an enveloped, double-stranded DNA virus. It has a large (240-kb) genome with approximately 95% DNA homology among different strains. CMV usually causes asymptomatic or minor infections in immunocompetent individuals but remains an important pathogen in immunocompromised individuals, including persons with AIDS, transplant recipients, and those on immune-modulating drugs. Primary infection is usually asymptomatic in immunocompetent persons, although a small percentage of individuals with CMV infection may develop a syndrome similar to mononucleosis. After primary infection, a lifelong latent infection is established that does not cause clinical symptoms. However, if an infected individual becomes immunocompromised, the virus can reactivate, leading to a wide variety of clinical syndromes.

The most severe CMV infections are seen in patients who acquire their primary infection while immunocompromised. In persons with AIDS, CMV disease rarely occurs when the CD4+ cell count is above 100 cells/mm^3; the most common clinical presentations are retinitis, esophagitis, and colitis. In transplant recipients, the occurrence and severity of CMV disease are related to the CMV serostatus of the organ donor and recipient, the type of organ transplanted, and the overall degree of immunosuppression. For example, CMV disease tends to be more severe in lung transplant recipients than in renal transplant recipients. For all types of solid organ recipients, the most severe disease occurs when CMV-seronegative recipients receive an organ from a CMV-seropositive donor and the primary CMV infection occurs while the person is immunosuppressed. In contrast, CMV-seropositive recipients of hematopoietic stem cells from CMV-seronegative donors are at highest risk of CMV diseases after hematopoietic stem cell transplant (HSCT). CMV disease can also occur in seropositive individuals, whether they receive an organ from a seropositive or seronegative donor. Clinical findings associated with CMV disease in transplant recipients are diverse and include interstitial pneumonitis; esophagitis and colitis; fever; leukopenia; and, less commonly, retinitis and encephalitis.

The diagnosis of CMV disease represents a challenge because latent infections are common. Immunocompromised individuals can have an asymptomatic, clinically insignificant, low-level, persistent infection that must be distinguished from clinically important active CMV disease. The distinction can be challenging when sensitive molecular assays are used that can detect small amounts of CMV DNA in clinical specimens.

Traditionally, the diagnosis of CMV disease relied on the detection of CMV from clinical specimens by the use of cell culture techniques in human diploid fibroblasts. Although considered the gold standard, these conventional culture methods are labor intensive and have a turnaround time (TAT) of 1 to 3 weeks. In addition, the assays lack adequate sensitivity for detecting CMV in blood specimens. The rapid shell-vial culture method can provide results in 1 to 2 days and is useful for detection of CMV in tissue, respiratory, and urine specimens. However, this method may also fail to detect CMV in blood. For many years, laboratories relied on a CMV antigenemia assay, which detects the matrix protein pp65 in circulating polymorphonuclear white blood cells. This semiquantitative assay is more rapid than culture, and the number of CMV antigen-positive cells correlates with the likelihood of CMV disease, but the assay is labor intensive, subjective, and nonstandardized and, consequently, is no longer done in most clinical laboratories.

Considering the limitations of culture, there is great interest in using NA testing for the detection and quantification of CMV DNA in blood specimens. The clinical uses of CMV molecular assays are diverse and include (1) initiation of preemptive therapy, (2) diagnosis of active CMV disease, and (3) monitoring of response to therapy. Preemptive therapy identifies a group of individuals at higher risk for developing CMV disease. For example, all members of the group would be tested for the presence of CMV DNA in their blood or plasma, and only those testing positive would be treated. Therapy is administered before development of symptoms in an attempt to prevent the onset of active disease. By contrast, with prophylactic therapy, all patients in the group are treated, without further stratification of risk, thus involving treatment of a greater number of patients. Preemptive therapy has become the standard of care for management of HSCT recipients.

Molecular assays are useful to diagnose active CMV disease because CMV DNA concentrations are higher in patients with active CMV disease than in those with asymptomatic infection.[88-91] Quantitative PCR from plasma or whole blood is now commonly used to diagnose CMV disease and monitor response to therapy. Until recently, there have been no FDA-approved CMV viral load tests, and laboratories used a variety of different laboratory-developed tests for detection and quantification of CMV DNA. Therefore, the threshold or viral load cutoff at which a diagnosis is made or preemptive therapy is begun has varied among institutions and transplant populations. Because a universal viral load cutoff for defining CMV disease has not been established, health care providers should rely on the trend of viral load values over time rather a value obtained at a single point in time for diagnosis and patient management.

After active CMV disease has been diagnosed, molecular assays are useful in monitoring response to therapy. Viral load values decrease rapidly after appropriate antiviral therapy is begun, and CMV DNA is cleared from the plasma within several weeks of initiation of therapy.[92-94] Failure of viral loads to decrease promptly should raise concerns of possible treatment failure because persistently elevated concentrations of CMV DNA during therapy indicates therapeutic resistance. Molecular assays can also identify patients at risk for relapsing CMV infection. In solid organ transplant recipients, patients with a detectable viral load after completing 14 days of ganciclovir therapy for CMV infection are at increased risk of relapse. The rate of decline in CMV

DNA after initiation of therapy can be also used to predict risk of relapse of CMV infection.[94]

Cytomegalovirus DNA concentrations are also useful in assessing the risk of developing CMV disease in persons with AIDS. Detection of CMV DNA in plasma is associated with increased risk of developing CMV disease and increased risk of death. In addition, each $\log_{10}$ increase in viral load (ie, each 10-fold increase in concentration) has been associated with a threefold increase in the risk of developing CMV disease.[95]

Currently, there are only two FDA-approved CMV viral load assays (see Table 5.1): CAP/CTM CMV test (Roche) and artus CMV RGQ MDx (Qiagen). Both are based on real-time PCR, are calibrated against the WHO CMV standard, and consequently report results in international units per milliliter. The Roche test amplifies a portion of *UL54* gene and the Qiagen test amplifies a portion of the *MIE* gene. Both assays have similar lower limits of quantification, but the Qiagen assay has a 10-fold greater dynamic range. Despite the availability of an international standard and of FDA-approved assays, there is still considerable interlaboratory variability of results in CMV proficiency testing surveys.[96]

Epstein-Barr Virus

Epstein-Barr virus (EBV) is a double-stranded DNA virus belonging to the *Herpesviridae* family. The seroprevalence of EBV is greater than 95% among adults older than the age of 40 years, and primary infection is followed by lifelong latency with reactivation of infection in immunocompromised hosts. In transplant recipients, EBV infection may cause malaise, fever, headache, and sore throat, but it is also associated with posttransplantation lymphoproliferative disease (PTLD), a significant cause of morbidity and mortality. PTLD is a spectrum of lymphocytic proliferation that ranges from benign lymphocytic hyperplasia to potentially fatal malignant lymphoma. The process is often multicentric and may involve the central nervous system (CNS), eyes, gastrointestinal (GI) tract (with bleeding and perforation), liver, spleen, lymph nodes, lungs, allograft, oropharynx, and other organs. Clinical presentations vary and include, but are not limited to, adenopathy, fever (including "fever of unknown origin"), abdominal pain, anorexia, jaundice, bowel perforation, GI bleeding, renal dysfunction, liver dysfunction, pneumothorax, pulmonary infiltrates or nodules, and weight loss.

The pathogenesis of PTLD involves the exponential proliferation of B cells as a result of uncontrolled EBV infection. Risk factors include a donor and recipient serologic mismatch (eg, donor positive/recipient negative), a high degree of immunosuppression (particularly the use of antilymphocyte therapy for rejection), and a high EBV viral load.[97] Most cases of PTLD occur during the first year after transplant, and the cumulative incidence ranges from 1% to 2% in HSCT and liver transplant recipients and up to 11% to 33% in intestinal or multiorgan transplant recipients.[98]

Treatment of EBV-related lymphoproliferative disease is challenging. After lymphoproliferative disease is established, antiviral treatment is not effective, and immunosuppression must be reduced. Murine humanized chimeric anti-CD20 monoclonal antibody (rituximab) has been helpful in some cases; some patients require chemotherapy, radiation therapy, or both. Adoptive immunotherapy using donor-derived cloned EBV-specific cytotoxic T cells may be useful for prophylaxis and treatment of lymphoproliferative disease in allogeneic HSCT and solid organ transplant recipients.

Increases in EBV viral load may be detected in patients before the development of EBV-associated PTLD[99-102]); viral loads typically decrease with effective therapy. Whereas a high EBV DNA viral load is a strong predictor for the development of PTLD, low-level EBV viral load occurs relatively frequently and may resolve without intervention.[103,104] To complicate the matter, some pediatric liver and heart transplant recipients may exhibit chronic high EBV viral loads.[105,106] Available assays lack standardization, and the optimal assay technique, sample type (ie, whole blood, lymphocytes, plasma), and sampling schedule are not defined. Nevertheless, EBV viral load assays are generally sensitive, specific, precise, linear across a wide dynamic range, rapid, reasonably inexpensive, and, overall, useful in patient care.[107] Although there are no defined "trigger points" predictive of PTLD, persistently detectable concentrations of EBV DNA by PCR (cutoffs vary between programs) typically result in a thorough evaluation for PTLD (eg, computed tomography of the chest, abdomen, and pelvis).

Epstein-Barr virus viral load testing is also indicated in transplant recipients who present with lymphadenopathy, fever, or other signs and symptoms suggestive of lymphoproliferative disease. A high EBV load should trigger the search for mass lesions or organ dysfunction pinpointing potential sites of disease, which should be biopsied.

Currently, there are no FDA-approved EBV viral load tests available. A wide variety of commercially available primers and probes for different gene targets are used in the laboratory developed tests (LDTs) deployed in clinical laboratories.[108] There is no consensus on the best target gene or specimen type (whole blood, white blood cells, or plasma); however, the first WHO international standard for EBV DNA was developed to address variation between assay results attributable to calibration (http://www.nibsc.org/documents/ifu/09-260.pdf).

The definitive diagnosis of PTLD requires biopsy. Tissues from patients with EBV-associated lymphoproliferative disease may show monoclonal, oligoclonal, or polyclonal lesions. The diagnosis of EBV-associated lymphoproliferative disease requires demonstration of EBV DNA, RNA, or protein in biopsy tissue. In situ hybridization targeting EBER1, EBER2, or both is the gold standard assay for determining whether a lymphoproliferative process is EBV related. Commercial systems for EBER in situ hybridization are available from Ventana, Leica, Dako, Invitrogen, and Biogenex.[107]

BK Virus

BK virus (BKV) is a member of family *Polyomaviridae*, which also includes JC virus (JCV) and simian virus 40 (SV40). It is an enveloped, double-stranded DNA virus that shares approximately 70% sequence homology with JCV and SV40. Seroprevalence reaches nearly 100% in early childhood, generally after an asymptomatic primary infection (although fever and nonspecific upper respiratory tract symptoms may occur).[109] Seroprevalence declines to 60% to 80% in adulthood. After primary infection, the virus can remain latent in many sites, most notably the epithelium of

the urinary tract and lymphoid cells, until an immunosuppressed state allows reactivation and replication of the virus. Replication of BKV in immunocompromised hosts may be asymptomatic or cause organ dysfunction, affecting the kidney, bladder, or ureter. BKV disease in the urinary system manifests as hemorrhagic or nonhemorrhagic cystitis and with ureteric stenosis in bone marrow and solid organ transplant recipients.[110] It also causes polyomavirus-associated nephropathy (PVAN) in renal transplant recipients.[111]

Hemorrhagic cystitis (HC) is a cause of morbidity and occasional mortality in patients undergoing bone marrow transplantation.[112] The manifestations range from microscopic hematuria to severe bladder hemorrhage leading to clot retention and renal failure. Its incidence varies from 7% to 68% of bone marrow transplant recipients. Although mild HC usually resolves with supportive care, severe HC may require bladder irrigation, cystoscopy, and cauterization.[113] BKV was observed in early studies to be associated with the development of HC during bone marrow transplantation; however, later studies using sensitive PCR assays showed that BKV DNA could be detected in the blood and urine of patients with or without HC.[114-116] Recently, quantitative assays for BKV DNA in urine have demonstrated that patients with HC have higher peak BK viruria and larger total amounts of BKV excreted during bone marrow transplantation compared with asymptomatic patients.[117,118]

Although BKV was first isolated from the urine of a renal transplant recipient in 1971,[119] the association between nephropathy and the presence of BKV in renal transplant recipients was not reported until 1995.[120] BKV replication in renal allografts can lead to progressive graft dysfunction and, potentially, graft failure. Although the recognition of PVAN in renal transplant recipients coincided with the use of newer immunosuppressive drugs such as tacrolimus, sirolimus, and mycophenolate mofetil, risk factors for development of PVAN have not been elucidated.[121] The prevalence of PVAN ranges from 1% to 10% in kidney transplant recipients with loss of allograft function in about one third to half of these cases.[121] The disease appears to result from reactivation of BKV infection in the donor allograft.

The signs and symptoms of PVAN are mild and nonspecific, often with only a gradual increase in serum creatinine over weeks as the allograft loses function.[122] A definitive diagnosis of PVAN is obtained through histopathology of the biopsied kidney; the characteristic PVAN pattern includes viral cytopathic changes in epithelial cells and interstitial inflammation and fibrosis. However, these changes are not pathognomonic for PVAN, and most centers use immunohistochemical staining with antibodies specific for polyomavirus proteins or in situ hybridization to confirm the diagnosis.[121] Because of the focal nature of the nephropathy and the possibility of sampling error, a negative biopsy does not rule out PVAN. Biopsy of the kidney is an invasive procedure that is impractical for serial monitoring, early diagnosis, and clinical management of patients with PVAN. Other less invasive diagnostic methods for PVAN have also been assessed. Urine cytology may reveal renal epithelial cells with intranuclear viral inclusion bodies, termed *decoy cells.*[123] The sensitivity and specificity of decoy cells for diagnosing PVAN is 99% and 95%, respectively, but the positive predictive value varies between 27% and 90%. Quantification of BKV DNA or mRNA in urine by NA amplification methods has been proposed as a method to monitor changes in BKV replication.[124-127] However, physiological changes of urine constituents and use of different urine fractions may give rise to considerable variation in viral load values that may complicate the identification of diagnostic thresholds and quantitative cross-sectional and longitudinal studies.[111] PCR methods for detection and quantitation of BK viremia have emerged as clinically useful tools in the diagnosis and management of PVAN because viremia precedes development of nephropathy in almost all cases.[128-131]

In 2005, an expert interdisciplinary panel recommended the use of either urine cytology or NA amplification tests to screen renal transplant recipients for BK viruria every 3 months up to 2 years posttransplant or when allograft dysfunction occurs or biopsy is performed.[121] Patients with positive screening test results should have an adjunct quantitative NA amplification test performed using urine or plasma. Patients with urine DNA loads of more than 10^7 copies/mL or plasma DNA loads of more than 10^4 copies/mL that persist for more than 3 weeks have presumptive PVAN and a renal biopsy should be performed to confirm the diagnosis.

Reducing the intensity of maintenance immunosuppression is the primary intervention in patients with PVAN. No effective antiviral agents for BKV are available, but low-dose cidofovir has been used for treatment of cases not amenable or refractory to decreased maintenance immunosuppression.[121] Viral load in urine or plasma should be monitored every 2 to 4 weeks to gauge the effectiveness of the intervention.

Currently, real-time PCR is the method of choice for BKV DNA quantification because of its simplicity and wide dynamic range of 6 to 7 $\log_{10}$ virus copies/mL. Such high concentrations in plasma are uncommon but can exceed 10^{12} copies/mL. Although BK viral load tests have become a standard of care for diagnosis and monitoring of patients with PVAN, there is neither consensus in the design of PCR assays nor recognized standard reference material. As a consequence, the assays developed by different laboratories may give markedly different results, requiring individual laboratories to establish and verify their own clinical threshold values.

Polymerase chain reaction assay design is complicated by the high degree of homology between the genomes of the different human polyomaviruses. Gene targets for BKV-specific assays include coding sequences for VP1, large T antigen, and agnoprotein because these sequences are sufficiently variable among human polyomaviruses.[130]

BKV is classified into seven subtypes based on phylogenetic analysis of full-genome sequences, subtypes Ia, Ic, II, III, IV, V, and VI.[132] Hoffman et al[133] compared seven *Taq*-Man real-time PCR primer—probe sets in conjunction with two different reference standards to quantify BKV DNA in urine samples. They observed substantial disagreement among assays attributable both to features of the primer and probe design and to choice of reference material. The most significant source of error were primer and probe mismatches caused by BKV subtype polymorphisms, primarily among subtype III and IV isolates. However, they found less subtype bias among the seven assays for the more common subtypes Ia, V, and VI. The assay that provided the

most reliable measure of all subtypes included a mixture of primers and probes that targeted both the VP1 and large T antigen sequences.

SEXUALLY TRANSMITTED INFECTIONS

Chlamydia trachomatis and *Neisseria gonorrhoeae*

Testing for *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) is discussed together because several of the available NA amplification tests (NAATs) for these pathogens are multiplex assays. Although both CT and NG can cause a variety of clinical infections, the focus here is on genital infections.

Detection of CT is a challenging and important public health issue. CT is a major cause of sexually transmitted infections (STIs), with an estimated 1 million cases occurring annually among sexually active adolescents and young adults in the United States.[134] More than half of the infections are asymptomatic.[135] Even when symptomatic, the diagnosis can be missed as the manifestations are protean. In men, CT infection may present as urethritis, epididymitis, prostatitis, or proctitis.[136,137] and as cervicitis, endometritis, and urethritis in women, with 10% to 40% of infections in women progressing to pelvic inflammatory disease (PID) if untreated.[138,139] Related complications include chronic pelvic pain, ectopic pregnancy, and infertility. In the United States, CT infection is the likely cause of most secondary infertility in women. In pregnant women, there is the additional risk of transmitting the infection to the newborn during labor and delivery, leading to pneumonia or conjunctivitis in the newborn.

N. gonorrhoeae infection, too, may present in various ways, and the clinical presentations overlap with those of CT. Men may have acute urethritis with discharge, epididymitis, prostatitis, and urethral strictures. In women, NG infection can produce cervicitis, which, if left untreated, can lead to PID, abscesses, or salpingitis.

Traditional methods for the diagnosis of CT infection include cell culture, antigen detection by immunofluorescence, enzyme immunoassay (EIA), and nonamplified NA probes. These traditional methods have been replaced in most laboratories by amplified NA tests, which provide greater sensitivity in detecting CT from genital specimens. For NG, which was traditionally diagnosed based on culture methods that relied on selective culture media, NA testing does not offer significant improvement in sensitivity compared with culture when culture is performed under appropriate conditions. NG is highly susceptible to extreme temperatures and desiccation, which can lead to decreased sensitivity of detection by culture, particularly when specimen transport is required before culture.[140] NA testing for NG offers a sensitive and reliable alternative to culture since it is easier to maintain the integrity of the target NA than it is to maintain the organism's viability.

In addition to high diagnostic and analytical sensitivity and specificity, NA testing offers several advantages over conventional culture and antigen detection methods for the diagnosis of CT and NG. Testing for both pathogens can be done on a single specimen, and for some multiplex assays, testing is performed in a single reaction. Unlike the infectious organism itself, the DNA and RNA of NG and CT are quite stable in commercial transport devices, thus accounting for some of the increased diagnostic sensitivity of these assays compared with culture. The stability of NA avoids the necessity of immediate transport to the laboratory, and specimens may be stored refrigerated or at room temperature before transport. Transport and storage requirements vary among tests, so it is important to refer to the package insert for specific details. An additional advantage of NA testing is the use of urine specimens, which for women allows testing to be done without the need for a pelvic examination. In men, urine offers a convenient and diagnostically sensitive alternative to collection with a urethral swab and increases the likelihood that asymptomatic men will agree to be tested.

NAATs for the detection of CT and NG from clinical specimens use a variety of specimens, including cervical and vaginal swabs, urethral swabs, and urine from both asymptomatic and symptomatic individuals. Not all assays are cleared by the FDA for use in the United States for all conditions and age ranges, and the current assays are not FDA-cleared for oropharyngeal, rectal, or conjunctival specimens. However, many of these tests have been assessed for diagnosing infections in multiple extragenital anatomic sites in men, women, and children. Current CDC guidelines for laboratory-based detection of CT and NG recommend NAATs for oropharyngeal and rectal specimens and for use in evaluating cases of adult and pediatric sexual abuse.[141] However, use of these tests outside of the FDA-approved indications requires that laboratories establish the specifications for performance characteristics according to Clinical Laboratory Improvement Amendments (CLIA) regulations. Performance characteristics vary among assays (details are available in the package inserts), but some general comments can be made. The diagnostic sensitivity of the tests varies according to the specimen type and whether the patient is asymptomatic or symptomatic. Interpretation of the results of NA testing for CT can be challenging because many studies have shown these assays to be more diagnostically sensitive than culture, which was previously used as the gold standard for clinical trials. For men, the diagnostic sensitivity of testing urine specimens is nearly equivalent to that of testing urethral swabs. A limited volume (20—50 mL) of first-passed urine is preferred because larger volumes will lead to a decreased concentration of the organism in the sample and thus reduced diagnostic sensitivity. With proper specimen collection, male urethral swabs and urine specimens have a sensitivity of nearly 100% for the detection of NG or CT infection. For women, vaginal and cervical swab specimens provide the highest sensitivity for the detection of NG and CT infection, with many studies showing a sensitivity of 90% to 95%. Vaginal swabs are preferred because they are easier to collect. Urine specimens can be used, but they generally result in a lower diagnostic sensitivity than cervical swabs (75% —85%). An alternative to urine testing in women is the use of self-collected vaginal swabs, which have been shown in some studies to have a diagnostic sensitivity that is equal to that obtained with cervical swabs; several commercial tests have been cleared for use with vaginal swabs.

Decisions regarding the selection of a specific amplification test for the detection of CT and NG should not be based solely on the cost of reagents. Other key factors to consider include test performance characteristics, such as diagnostic sensitivity and specificity, and applicability for urine and

swab specimens in both symptomatic and asymptomatic individuals. Ideally, the test should include an internal control, particularly if a crude lysate is used in the assay. Other factors to consider are degree of automation, ease of use, work flow issues, and space and equipment needs.

Historically, for several of the NG assays, reduced specificity was due to presence of the gene target in nongonococcal *Neisseria* spp.[142,143] Currently, only the ProbeTec tests (Becton-Dickinson) produce false-positive results with commensal species including *Neisseria lactamica*, *Neisseria subflava*, and *Neisseria cinerea* (Table 5.3). None of the NAATs for CT have known biological false-positive results because of presence of the gene target in other organisms. Other sources of false-positive results include carryover contamination of amplified product and cross-contamination during specimen collection, transport, or processing. Concerns over these issues have led to consideration of supplemental testing for all CT- or NG-positive specimens using alternative target tests because false-positive results can have psychosocial and medicolegal ramifications.[144] However current recommendations do not advise confirming all positive NAATs unless otherwise indicated in the package insert or for tests with known cross-reactivity with commensal *Neisseria* spp.[141] False-positive results in a low-prevalence population can significantly reduce the predictive value of a positive result.

Because DNA can persist in urine samples for up to 3 weeks after completion of therapy, test of cure using NA testing is discouraged. If this must be done, then testing should be delayed for at least 3 weeks after therapy is completed to allow time for clearance of the DNA of the pathogen.

False-negative results from inhibition of amplification are a consideration for both NG and CT testing and can occur with both cervical swabs and urine specimens. Inhibition rates may vary considerably depending on the amplification and NA extraction methods used. For tests using a crude lysate (eg, ProbeTec), inhibition rates tend to be higher than those seen with the APTIMA Combo test, which uses a target capture method to purify NA. For assays that test a crude lysate, it is useful to amplify another NA sequence as an internal control (or "amplification control") to assess for inhibition of amplification. Results are reported as negative for NG or CT only when amplification of the internal control is documented.

A conserved, cryptic plasmid is found in more than 99% of strains of CT and contains the gene target for several NAATs. However, a new variant (nv) strain of CT emerged in Sweden in 2006 with a 377-base-pair deletion in the cryptic plasmid, which contained the target for several of the CT tests. This deletion led to false-negative results with some but not all of the tests that targeted the cryptic plasmid.[145] The current versions of all the NAATs for CT have been modified to detect the nv strain of CT. Obviously, tests that target the sequences contained on the cryptic plasmid will not detect the rare strain of CT that lacks the plasmid.

Performing CT and NG testing on liquid cytology media is a matter of interest because a single specimen can be used for cervical cancer screening (Papanicolaou [Pap] and human papillomavirus [HPV] tests) and for CT or NG testing.[146] The latter two tests are performed on the liquid specimen that remains after completion of the PAP and HPV testing. However, several drawbacks to this approach must be considered. The instruments used to prepare PAP tests were not designed to control for cross-contamination during processing, and this may lead to false-positive results. CT and NG testing are performed after the PAP smear and HPV testing are complete, delaying diagnosis and treatment of CT or NG infection. Moreover, the remaining specimen may be inadequate to complete CT and NG testing, thus requiring the patient to make a return visit for collection of an additional sample. Removing an aliquot for CT and NG testing before Pap testing is performed ("pre-aliquoting") may be helpful in overcoming some of these issues, provided adequate volume of sample remains for PAP and HPV testing. This approach does not completely remove the risk of cross-contamination, so specimens must be handled in a manner consistent with procedures used in molecular laboratories. In addition, not all NAATs for CT and NG have been FDA approved for use with liquid cytology media, and those that

TABLE 5.3 Amplification Methods and Target Regions for Food and Drug Administration–Cleared Nucleic Acid Amplification Tests for Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

Assay (Manufacturer)	Method	*C. trachomatis* Target	*N. gonorrhoeae* Target
Abbott RealTime CT/NG (Abbott)	Real-time PCR	Two distinct regions in cryptic plasmid	*Opa* gene region
Aptima COMBO 2 assay (Hologic/Gen-Probe)	Transcription-mediated amplification	23S rRNA region	16S rRNA region
Aptima CT assay		16S rRNA region	
Aptima GC assay			Distinct 16s rRNA region
BD ProbeTec Q^x CT Amplified DNA assay	Strand displacement amplification	One region in the cryptic plasmid	
BD ProbeTec Q^x GC Amplified DNA assay			Chromosomal pilin gene-inverting protein homologue*
Xpert CT/NG test (Cepheid)	Real-time PCR	One distinct chromosomal region	Two distinct chromosomal regions
cobas CT/NG test (Roche)	Real-time PCR	One cryptic plasmid and one chromosomal region	DR-9A and DR-9B regions

*False-positive test results with some commensal *Neisseria* spp. may occur.
CT, Chlamydia trachomatis; *NG, Neisseria gonorrhoeae*; *PCR*, polymerase chain reaction.

have may not have been cleared for both types of media (Hologic PreservCyt and BD SurePath).

POINTS TO REMEMBER

Chlamydia trachomatis* and *Neisseria gonorrhoeae

- NAATs are the recommended test method for detection of CT and NG genital tract, oropharyngeal, and rectal infections, but they have not been cleared by the FDA for the latter two infections.
- Routine repeat testing of NAAT-positive specimens is not recommended because this practice does not improve the positive predictive value of the test.
- Positive reactions with nongonococcal *Neisseria* spp. have been reported with some NAATs and the use of an alternative target NAAT might be needed to avoid false-positive results for NG.
- CT and NG DNA can persist in samples from successfully treated patients for up to 3 weeks; therefore, tests of cure using NAATs are discouraged.

Trichomonas vaginalis

Trichomoniasis is an STI caused by the protozoan *Trichomonas vaginalis*. Although *T. vaginalis* infection is not a reportable disease in the United States, it is the most prevalent nonviral STI in the United States.[147] *T. vaginalis* infection may present as vaginitis in women and urethritis in men; however, it is frequently asymptomatic. Infection with *T. vaginalis* may also cause additional adverse health outcomes, including PID in women, as well as infertility and increased incidence of HIV transmission in women and men. Current recommendations for *T. vaginalis* diagnosis and treatment can be found at http://www.cdc.gov/std/tg2015/default.htm and available diagnostic tests range from simple microscopy to NAATs.

Microscopic examination of vaginal fluid or urethral discharge for *T. vaginalis* (wet mount) in the clinic is the most commonly used test. It has low sensitivity (51%−65%) but with experienced observers can be highly specific.[148] Culture has long been considered the gold standard test, but it requires special medium and 5 days to complete. However, recent studies indicate that the sensitivity of culture may be as low as 75% to 96%.[148] Pap tests are not suitable for routine screening or diagnosis because of low sensitivity.[149] There is a single rapid antigen test for *T. vaginalis* (OSOM, Sekisui Diagnostics) that is FDA cleared for use as a point-of-care test for female patients. Test specifications include sensitivity of 82% to 95% and specificity of 97% to 100%.[150]

The Affirm VPIII Microbial Identification test is an FDA-cleared test that uses nonamplified NA probes to detect three organisms associated with vaginitis: *T. vaginalis*, *Gardnerella vaginalis*, and *Candida albicans*. Its sensitivity and specificity for detection of *T. vaginalis* are 63% and 99.9%, respectively.[151] NAATs are the most sensitive tests available for detection of *T. vaginalis*. A variety of LDT NAATs are more sensitive than the previous gold-standard test of culture but with a more rapid analysis time. Currently, there are two FDA-cleared NAATs for detection of *T. vaginalis* from female patients only. The APTIMA *Trichomonas vaginalis* assay (Hologic/Gen-Probe) detects *T. vaginalis* RNA by transcription-mediated amplification, and its sensitivity and specificity are both 95% to 100%.[148,151,152] It also offers the opportunity to test for *T. vaginalis* from the same sample submitted for CT and NG testing with their APTIMA Combo 2 assay because the test runs on the same platform. The BD Probe Tec TV Q^x Amplified DNA assay detects *T. vaginalis* using strand displacement amplification on the Viper system with performance characteristics similar to the APTIMA assay.[153] Tests for CT and NG can also be performed on the Viper system.

The laboratory diagnosis of trichomoniasis remains challenging particularly in men. Considerations for selection of diagnostic methods should include testing location, analysis time, performance characteristics, and the cost to perform the test.

Herpes Simplex Virus

Herpes simplex virus (HSV) is a double-stranded DNA virus surrounded by a lipid glycoprotein envelope. HSV persists as a latent infection in specific target cells despite the host immune response, often resulting in recurrent disease. Genital herpes is a chronic viral infection. Two serotypes of HSV have been identified, HSV-1 and HSV-2. Most cases of recurrent genital herpes in the United States are caused by HSV-2. However, an increasing proportion of anogenital herpetic infections in some populations is now attributed to HSV-1. HSV-1 is usually associated with oral lesions. The CDC estimates that 776,000 new HSV-2 infections occur each year in the United States. Most genital herpes infections are transmitted by persons unaware of their infections. Up to 90% of persons seropositive for HSV-2 antibody have not been diagnosed with genital herpes. However, many have mild or unrecognized disease, and probably most, if not all, shed virus from the genital area intermittently.

Clinical diagnosis of HSV is insensitive and nonspecific; therefore, the clinical diagnosis of genital herpes should be confirmed by laboratory testing. Many infected persons do not experience the multiple vesicular or ulcerative lesions typical of genital herpes. Both virologic and type-specific serologic tests are used to confirm the diagnosis.[154]

Cell culture and NAATs are the preferred virologic tests for persons who seek diagnosis and treatment of genital ulcers or other mucocutaneous lesions. The sensitivity of viral culture is low, especially for recurrent lesions, and declines rapidly as lesions begin to heal. NAATs for HSV DNA are increasingly used in many laboratories, and several tests are now cleared by the FDA for anogenital specimens.[155,156] NAATs are the preferred tests for detecting HSV in spinal fluid for diagnosis of HSV infection of the CNS and are discussed later in this chapter. Both culture and NAATs should determine whether the infection is due to HSV-1 or HSV-2 because recurrences and asymptomatic shedding are much less frequent for HSV-1 than for HSV-2 genital infections.[157] Failure to detect HSV by culture or NAAT does not rule out HSV infection because viral shedding is intermittent. The use of Tzanck preparations or Pap tests to detect cytologic changes produced by HSV are insensitive and nonspecific and should not be used for genital HSV infections.

Serologic tests detect type-specific and nonspecific antibodies to HSV that develop during the first several weeks to

few months after infection and persist indefinitely. Type-specific serologic tests based on antigens specific for HSV-1 (gG1) and HSV-2 (gG2) are commercially available. Because almost all HSV-2 infections are sexually acquired, type-specific HSV-2 antibody indicates anogenital infection. However, the presence of HSV-1 antibody does not distinguish anogenital from orolabial infection. Type-specific HSV serologic assays might be useful in the following scenarios: (1) recurrent genital symptoms or atypical symptoms with negative HSV PCR or culture, (2) clinical diagnosis of genital herpes without laboratory confirmation, and (3) a patient whose partner has genital herpes. HSV serologic testing should be considered for persons presenting for an STI evaluation (especially for those persons with multiple sex partners), persons with HIV infection, and men who have sex with men at increased risk for HIV acquisition.[158]

Human Papillomavirus

Human papillomaviruses are small, double-stranded DNA viruses that infect squamous epithelium, subverting normal cell growth and potentially leading to squamous cell carcinoma (SCC). HPV is not a single virus but a family of more than 150 related viral genotypes that are distinguished based on sequence analysis of the L1 region of the viral genome. Anogenital HPV infections are common in both men and women. It is estimated that more than 24 million men and women in the United States are currently infected with HPV. HPV is an STI; it is most common among sexually active young women ages 15 through 25 years. In one study, cervicovaginal HPV was found in up to 43% of sexually active college women during a 3-year period.[159] Infections, however, are usually transient, and progression to cancer requires persistence of viral infection over several years. The types of HPV that are spread through sexual contact are classified as low or high risk for progression to malignancy, and there are multiple types. Infections with low-risk HPV such as types 6 and 11 can lead to benign genital warts or condyloma acuminata and have a low likelihood of progressing to malignancy. In contrast, high-risk types such as types 16, 18, and 45 are associated with development of SCC of the anogenital region and oropharynx. Currently, there are 14 high-risk (HR) HPV types recognized. The cervix is particularly affected, and worldwide, cervical SCC continues to cause significant morbidity and mortality (5% of cancer deaths).

Productive infections usually result in cytologic and histologic changes, including cellular and nuclear enlargement, nuclear hyperchromasia, and perinuclear halos (koilocytosis). These changes can be identified on a stained smear of cells collected from the cervix (the “Pap smear,” developed by Dr. George Papanicolaou in the 1940s) or in a biopsy taken during colposcopy or a loop electrosurgical excision procedure. The Pap smear has been used very successfully to identify women with cervical cancer and, more important, for the detection of precursor lesions, so that biopsy or excision can be performed to remove the lesion earlier in the disease process before metastasis can occur. With the introduction of liquid cytology media and automated cytology processors, the procedure is more appropriately called the Pap test because “smears” are no longer used.

The histologic types of squamous precursor lesions are divided into three categories: mild dysplasia, or cervical intraepithelial neoplasia (CIN1); moderate dysplasia, or CIN2; and severe dysplasia, or carcinoma in situ, or CIN3. In the Bethesda System for Cytologic Classification, squamous precursor lesions are divided into low- and high-grade squamous intraepithelial lesions (LSIL and HSIL). LSIL corresponds with CIN1, and HSIL corresponds to CIN2 and CIN3. Frequently, the cytologic evaluation demonstrates mildly atypical cells that do not meet these criteria and are referred to as atypical squamous cells of undetermined significance (ASCUS); these cells may correspond to an early HPV infection. The prevalence of ASCUS on Pap tests is approximately 5% to 10%, with rates as high as 20% reported in sexually active women.

Screening for cervical cancer with cytology testing has been very effective in reducing cervical cancer in the United States. For many years, the approach was an annual Pap test. In 2000 the Hybrid Capture 2 (HC2) test (Qiagen/Digene) for detection of HR HPV types was approved by the FDA for screening women who had ASCUS detected by the Pap test to determine the need for colposcopy. At the time, the Hybrid Capture 2 test was the only FDA-approved test available. In 2003, the FDA approved expanding the use of this test to include screening preformed in conjunction with a Pap test for women over the age of 30 years, referred to as “co-testing.” Co-testing allows women to extend the testing interval to 5 years if both test results are negative.[160] In 2014, the FDA approved the use of an HR HPV test (cobas HPV test, Roche) for primary cervical cancer screening for women older than the age of 25 years, without the need for a concomitant Pap test. When using the HR HPV test as the primary screening test, a Pap test is performed only when specific HR HPV types are detected (HPV-16 and -18 are excluded). Colposcopy is performed without an intervening Pap test in women who test positive for HPV-16 and -18. This algorithm was based primarily on the results of a single large FDA registration study for the cobas HPV test.[161] Interim clinical guidance is available for the use of primary HR HPV testing in cervical cancer screening.[162] However, there is still considerable debate about whether co-testing or HR HPV as a primary screening test is the optimal approach for cervical cancer screening.[163]

Four tests for the detection of HR HPV types have been cleared by the FDA for use in the United States: HC2 test, Cervista HPV HR (Hologic/Gen-Probe), cobas HPV test (Roche), and Aptima HPV test (Hologic/Gen-Probe). In addition, two different FDA-cleared tests to specifically identify HPV types 16 and 18 (Cervista) and types 16 and 18/45 (Aptima) are available. The features of these tests are compared in Table 5.4. All of these tests have been cleared by the FDA for use with ThinPrep PreservCyt liquid-based cytology medium (Hologic) but not with the other commonly used SurePath medium (Becton-Dickinson).

The HC2 test relies on hybridization of a RNA probe to the HPV DNA followed by use of an antibody for capture of the duplex (RNA-DNA) hybrids and then detection with chemiluminescent signal amplification. The test uses a pool of RNA probes spanning the entire genome that are specific for 13 HR HPV types. The specific type is not identified. The test uses a 96-well microtiter plate format and can be performed manually or with the semiautomated Rapid Capture system (Qiagen) for reagent and plate handling. It is also cleared for use on Digene specimen transport media (STM). The HC2 test has been used in several large studies and reproducibly demonstrates high sensitivity of 93% to 96%, but false-positive results occur as a result of cross-reaction with low-risk HPV types.[164]

TABLE 5.4 Features of Food and Drug Administration–Cleared High-Risk Human Papillomavirus Tests

Feature	HC2	Cervista	cobas	Aptima
Technology	Hybrid capture	Cleavase/Invader	Real-time PCR	Transcription-mediated amplification
Target(s)	Multigene	L1, E6, E7	L1	E6, E7 mRNA
LOD and clinical cutoff	5000 copies/reaction	1250–7500 copies/reaction	300–2400 copies/mL	20–240 copies/reaction
Cross reaction with low-risk types	6, 11, 40, 42, 53, 66, 67, 70, 82/82v	67, 70	None reported	26, 67, 70, 82
Internal control	None	Human histone 2 gene	Human β-globin gene	Process
HPV-16/-18 genotyping	No	Yes (separate test)	Yes (integrated)	Yes (separate test also includes type 45)
Automation	Semiautomated and automated	Semiautomated and automated	Automated (cobas 4800)	Automated (Tigris and Panther)
Sample type (volume)	ThinPrep (4 mL), sample transport medium	ThinPrep (2 mL)	ThinPrep (1 mL)	ThinPrep (1 mL)
Prealiquot required	No	No	Yes	Yes
Expanded STI menu	CT/NG	None	CT/NG, HSV 1/2	CT/NG, TV
Primary screening indication	No	No	Yes	No

CT, *Chlamydia trachomatis*; *HSV*, herpes simplex virus; *LOD*, limit of detection; NG, *Neisseria gonorrhoeae*; *PCR*, polymerase chain reaction; *STI*, sexually transmitted infection; *TV*, *Trichomonas vaginalis*

The Cervista HPV HR assay also uses a signal amplification method that is based on cleavase/invader technology and detects the same 13 high-risk types as HC2 test plus type 66. A combination of DNA probes and invader oligonucleotides targeting the L1, E6, and E7 sequences and secondary fluorescently labeled probes are divided into three phylogenetically related reactions that are performed on 96-well microtiter plates. Unlike the HC2 assay, this assay includes an internal control with each reaction. Both assays have detection limits of around 3000 to 5000 genome copies per milliliter. The Cervista HPV HR assay has less cross-reactivity with low-risk types. Studies comparing the two assays demonstrate concordance of 82% to 88%.[165] However, the Cervista test may have poor specificity compared with other tests for HR HPV.[166,167] The Cervista HPV-16 and -18 genotyping test uses the same cleavase/invader technology.

The cobas HPV Test is the first real-time PCR method approved by the FDA for cervical cancer screening.[161] It uses a multiplexed primer and hydrolysis probe assay to individually detect both HPV types 16 and 18 simultaneously with the 12 other HR HPV types using different fluorescently labeled probes. The assay includes detection of the human β-globin gene as an internal control for extraction and amplification adequacy. The cobas 4800 system uses automated bead-based NA extraction and PCR assembly. The sensitivity and specificity is similar to the HC2 and Cervista HR HPV assays. Currently, this is the only FDA-cleared test that has an indication for primary screening.

The Aptima HPV assay targets the viral mRNA for the E6/E7 HPV genes for the 14 HR HPV types. The E6 and E7 genes of HR HPV types are known oncogenes. Proteins expressed from E6-E7 polycistronic mRNA alter cellular p53 and retinoblastoma protein functions, leading to disruption of cell-cycle check points and genome instability. Targeting the mRNA of these oncogenic elements may be a more effective approach to detect cervical disease than detection of HPV genomic DNA.[168] The APTIMA HPV Assay involves three main steps, which take place in a single tube: target capture, target amplification by transcription-mediated amplification, and detection of the amplicons by the hybridization protection assay. The assay also incorporates an internal control to monitor NA capture, amplification, and detection, as well as operator or instrument error. Unlike the internal controls used in the Cervista and Roche assays, it does not assess specimen adequacy (cellularity). An adjunctive test to detect and differentiate HPV type 16 and 18/45 based on the same principle described is also available from Hologic/Gen-Probe.

POINTS TO REMEMBER

Human Papillomavirus

- Most HPV infections in women are transient and progression to cervical cancer requires persistent infection with one of the 14 HR oncogenic types of which 16, 18, and 45 are most common.
- HR HPV testing is recommended in conjunction with the Pap test for cervical cancer screening in women older than 30 years of age (co-testing).
- Co-testing allows women to extend the testing interval to 5 years if both test results are negative because HPV DNA is more sensitive than the Pap test for detection of women with significant cervical lesions.
- Currently, there are four FDA-cleared tests for HR HPV types, each based on a different amplification method: hybrid capture, cleavase/invader, real-time PCR, and transcription mediated amplification.

RESPIRATORY TRACT INFECTIONS

Viruses

The viruses that infect the respiratory tract consist of large and diverse groups that cause disease in humans, and new ones continue to be discovered. The more common viruses that infect humans include influenza A and B, parainfluenza virus (PIV) types 1 to 4, respiratory syncytial virus (RSV), metapneumovirus, adenoviruses (>50 different types), rhinoviruses (>100 different types), and coronaviruses (4 types). The disease spectrum ranges from the common cold to severe life-threatening pneumonia. It can be difficult to differentiate the viral origin based on signs and symptoms alone, and treatment options vary depending on the viral etiology. Infection with these viruses has demonstrated the potential for global public health threats of epidemic and pandemic proportions. The 1918 influenza A pandemic, human deaths caused by infection with avian influenza A H5N1 in 1997,[169] the severe acute respiratory syndrome (SARS) coronavirus outbreak in 2003, the 2009 pandemic caused by the novel multiply-reassorted (swinelike) influenza A H1N1, and the emergence of Middle East respiratory syndrome (MERS) coronavirus in 2012 on the Arabian peninsula are all reminders of the potential threats to human health posed by novel respiratory viruses.[170] Detection of emerging respiratory viruses will require multiple modalities, but molecular methods have been crucial to their discovery and characterization and in the development of diagnostic tools.

Acute respiratory viral infection (1) is a leading cause of hospitalization and death in infants and young children; (2) contributes to problems of asthma exacerbation, otitis media, and lower respiratory tract infection; and (3) contributes to acute disease in immunocompromised and elderly patients. Rapid diagnosis aids in effective treatment (eg, with antiviral medications such as oseltamivir for influenza A virus infection) and management (eg, reduction in inappropriately prescribed antibiotics for viral infection and infection control).

Rapid antigen-based EIAs provide short TATs (minutes) but are hampered by poor diagnostic sensitivity compared with culture methods or molecular assays and low positive predictive values, especially when the prevalence is low. Direct fluorescent antibody (DFA) detection assays for viral antigens on centrifuged cellular material from nasopharyngeal swabs, aspirates, or wash specimens demonstrate greater rates of detection than the rapid antigen assays and provide results in a relatively short time frame of 2 to 4 hours. Detection rates, however, are lower for antigen detection methods than for NAATs.

Cell culture methods, although slower than antigen detection methods, have been considered the gold standard for detection of a wide range of viral pathogens. In recent years, culture methods have been optimized for detection by combining multiple cell lines and improving the TAT from weeks to days through the use of shell-vial spin amplification cultures. Here, the patient's specimen is concentrated onto cells grown on a coverslip, and fluorescent antibody detection is performed after 16 to 24 hours of incubation instead of waiting for the development of a cytopathic effect. Although this has hastened the time to detection, 1 to 2 days is still required along with significant technologist labor, and it is not quite as sensitive as molecular methods of detection.

Molecular detection of respiratory viruses offers several advantages over traditional virologic culture or antigen detection. Most important, analytical sensitivity of molecular assays, primarily using PCR or real-time PCR, is consistently better than that of traditional methods.[171-174] Results from molecular testing are more accurate, and thus the patient benefits from the most appropriate treatment decision; also, infection control practitioners can more effectively implement strategies to prevent or reduce nosocomial transmission. Molecular assays can be designed to detect a wide range of viral pathogens, including viruses that are difficult to culture.

Despite the advantages of NAATs for respiratory virus detection, their adoption by clinical laboratories was initially slow because of the limited capacity of real-time PCR assays for multiplexing. There are numerous LDTs and FDA-cleared real-time PCR assays capable of detecting one to three different viruses in single reactions, but to provide comprehensive coverage for respiratory viruses, a panel of such assays needed to be deployed, an approach not practical for most laboratories.

Currently, there are several FDA-cleared multiplexed respiratory virus panels capable of detecting up to 20 different viral targets, thus providing simplified approaches to comprehensive diagnostics for respiratory viruses.[175] See Table 5.5 for an overview of the important parameters of these respiratory virus panels. These tests are truly transformative for the laboratory in that they can replace the combination of limited multiplex NAATs, antigen detection tests, and culture-based methods that were traditionally used in clinical laboratories to detect respiratory viruses and thus dramatically increase diagnostic yield.

The xTAG Respiratory Viral Panel (RVP) v1 assay is a multiplexed RT-PCR—based assay with fluorescently color-coded microsphere (bead) hybridization for simultaneous detection and identification of 12 respiratory viruses and subtypes.[175] The multiplexed RT-PCR primers amplify conserved regions of the viruses, and the products are labeled with biotin-containing deoxynucleotides in a second target-specific primer extension reaction. The extension product has a proprietary tag sequence incorporated for hybridization to the virus-specific probe on the color-coded bead. After hybridization, phycoerythrin-conjugated streptavidin is bound by the biotin-labeled primer extension products, and the fluorescent signal is quantified on the Luminex xMAP instrument. The instrument contains two lasers: one for identification of the microbe by a color-coded bead and the other for detection of the phycoerythrin signal attached to the primer extension product. The data are recorded as mean fluorescent intensities, and the software analyzes the data and reports the positive results. The assay includes a separate lambda phage amplification control and an MS-2 bacteriophage internal control for extraction and amplification. The original version of the assay was modified to reduce the number steps and analysis time (RVP-Fast), but it does not include the parainfluenza viruses, and it is not as sensitive overall as its predecessor. Because there are a number of post-PCR processing steps in both versions of the test, care must be taken to avoid amplicon cross-contamination and false-positive results.

BioFire Diagnostics developed a PCR instrument called the FilmArray and an associated reagent pouch that together are capable of simultaneously detecting multiple organisms in

TABLE 5.5 Parameters of Different Food and Drug Administration–Cleared Respiratory Virus Panels

Parameter	Luminex xTAG RVPv1	Luminex xTAG RVP-Fast	FilmArray	eSensor
Amplicon detection method	Fluorescence-labeled bead array	Fluorescence-labeled bead array	Melting curve analysis	Voltammetry
On-board sample processing	No	No	Yes	No
Post-PCR manipulation	Yes	Yes	No	Yes
Hands-on-time (min)	70	45	3	55
Throughput	High	High	Low	Moderate
Analysis time (hr)	7	4	1	6
Total time to results (hr)*	9	6	1.1	8
Complexity	High	High	Low	Moderate
Pathogens detected	ADV INF A (H1, H3) INF B MPV RSV (A, B) RV/EV PIV (1, 2, 3)	ADV INF A (H1, H3) INF B MPV RSV RV/EV	ADV INF A (H1, H3, 09H1) INF B MPV RSV RV/EV PIV (1, 2, 3, 4) COV (HKU1, NL63, 229E, OC43) *Bordetella pertussis* *Chlamydophila pneumoniae* *Mycoplasma pneumoniae*	ADV (B/E, C) INF A (H1, H3, 09H1) INF B MPV RSV (A, B) RV PIV (1, 2, 3,)

*Includes the time required for nucleic acid extraction.

ADV, Adenovirus; *COV*, coronavirus *EV*, enterovirus; *INFA*, influenza A virus; *INFB*, influenza B virus; *MPV*, metapneumovirus; *PCR*, polymerase chain reaction; PIV, parainfluenza virus; *RSV*, respiratory syncytial virus; *RV*, rhinovirus.

the same sample. The FilmArray pouch contains freeze-dried reagents to perform NA purification; reverse transcription; and nested, multiplex PCR followed by high-resolution melting analysis. The FilmArray Respiratory Panel (RP) was designed for simultaneous detection and identification of 17 viral and 3 bacterial respiratory pathogens (see Table 5.5). The test is initiated by loading water and an unprocessed patient nasopharyngeal swab specimen mixed with lysis buffer into the FilmArray RP pouch. The pouch is then placed into the FilmArray instrument. The software has a simple interface that requires only identification of the specimen and pouch barcode to initiate a run. Multiplexed two-stage RT-PCR followed by high-resolution melting analysis of the target amplicons is used to detect each of the panel analytes.[176] Results are reported in an hour; currently, the instrument is designed to test a single sample per run, though multiple instruments can be linked. Because it is a completely closed system, false-positive results caused by amplicon cross-contamination are not an issue.

The eSensor system (GenMark Dx) uses electrochemical-detection-based DNA microarrays.[177] These microarrays are composed of a printed circuit board consisting of an array of 76 gold-plated electrodes. Each electrode is modified with a multicomponent, self-assembled monolayer that includes presynthesized oligonucleotide capture probes. NA detection is based on a sandwich assay principle. Signal and capture probes are designed with sequences complementary to immediately adjacent regions on the corresponding target DNA sequence. A three-member complex is formed between the capture probe, target sequence, and signal probe based on sequence-specific hybridization. This process brings the 5′ end of the signal probe containing electrochemically active ferrocene labels into close proximity to the electrode surface. The ferrous ion in each ferrocene group undergoes cyclic oxidation and reduction, leading to loss or gain of an electron, which is measured as current at the electrode surface using alternating-current voltammetry. Higher-order harmonic signal analysis also facilitates discrimination of ferrocene-dependent faradic current from background capacitive current.

The eSensor cartridge consists of a printed circuit board, a cover, and a microfluidic component. The microfluidic component includes a diaphragm pump and check valves in line with a serpentine channel that forms the hybridization channel above the array of electrodes. The eSensor instrument consists of a base module and up to three cartridge-processing towers, each with eight slots for cartridges. The cartridge slots operate independently of each other. The throughput of a three-tower system can reach 300 tests in 8 hours. A respiratory pathogen panel for the eSensor system that detects 14 different types and subtypes of respiratory viruses (see Table 5.5) is FDA cleared.[178] Because this test requires post-PCR manipulations of the sample, care must be taken to avoid false-positive tests caused by amplicon cross-contamination.

The Verigene system (Nanosphere) uses PCR amplification and gold nanoparticle-labeled probes to detect target NA hybridized to capture oligonucleotides arrayed on a glass

slide. Silver signal amplification is then performed on the gold nanoparticle probes that are hybridized to the captured DNA targets of interest. The Verigene reader optically scans the slide for silver signal, processes the data, and produces a qualitative result. A test for detection of influenza A virus, influenza A virus subtype H3, influenza A virus subtype 2009 H1N1, influenza B, and RSV subtypes A and B is cleared by the FDA for the Verigene system.[179] The system is capable of much higher-order multiplexing and a respiratory panel that detects 13 viral and 3 bacterial targets has been developed and is available in the United States as an RUO product.

Molecular testing for respiratory viruses will likely continue to include tests designed to detect a limited number of viruses of particular importance (eg, influenza A and B viruses and RSV), as well as tests that detect a broad array of viruses because there are clinical needs for both types of tests. The use of comprehensive respiratory virus panels greatly increases diagnostic yield and the ability to detect mixed viral infections. However, the clinical significance of mixed infections is not well documented or understood. In addition, there are test options that range from simple, "sample in answer out" systems to complex tests that require multiple manual steps, meeting different niches for various clinical laboratory settings. In fact, point-of-care molecular testing for respiratory viruses is now possible with the recent development of a CLIA-waived test for influenza A and B viruses (Alere). It delivers results in 15 minutes and can be performed by nonlaboratory personnel, and its performance characteristics are similar to those of NAATs performed in laboratories.[180]

Mycobacterium tuberculosis

Mycobacterium tuberculosis causes a wide range of clinical infections, including pulmonary disease; miliary tuberculosis; meningitis; pleurisy, pericarditis, and peritonitis; GI disease; genitourinary disease, and lymphadenitis. *M. tuberculosis* infection was in steady decline in the United States with an all-time low in the late 1990s, when the number of reported cases began to increase.[181] This resurgence was related to the AIDS epidemic, homelessness, and a decreased focus on tuberculosis control programs. The infection rate continues to rise in foreign-born persons as the result of immigration from countries with a high prevalence of *M. tuberculosis* infection. This increase in *M. tuberculosis* infection has focused considerable attention on the development of assays for its rapid diagnosis; molecular methods are at the center of this effort.

Conventional tests for laboratory confirmation of tuberculosis include acid-fast bacilli (AFB) smear microscopy, which can produce results in 24 hours, and culture, which requires 2 to 6 weeks to produce results.[182,183] Although rapid and inexpensive, AFB smear microscopy is limited by its poor sensitivity (45%–80% with culture-confirmed pulmonary tuberculosis cases) and its poor positive predictive value (50%–80%) for tuberculosis in settings in which nontuberculous mycobacteria are commonly isolated.[183-185]

Compared with AFB smear microscopy, the added value of NAATs include (1) their greater positive predictive value (>95%) than AFB smear-positive specimens when nontuberculous mycobacteria are common and(2) their ability to rapidly confirm the presence of *M. tuberculosis* in 60% to 70% smear-negative, culture-positive specimens.[183-187] Compared with culture, NAATs can detect the presence of *M. tuberculosis* in specimens weeks earlier for 80% to 90% of patients suspected to have pulmonary tuberculosis ultimately confirmed by culture.[184,186,187] These advantages can impact patient care and tuberculosis control efforts, such as by avoiding unnecessary contact investigations or respiratory isolation for patients whose AFB smear-positive specimens do not contain *M. tuberculosis*.

The CDC recommends that NAATs be performed on at least one (preferably the first) respiratory specimen from each patient suspected of pulmonary tuberculosis for whom a diagnosis of tuberculosis is being considered but has not yet been established and for whom the test result would alter case management or tuberculosis control activities.[188] NAATs can also be used to inform the decision to discontinue airborne infection isolation precautions in health care settings.[189,190] NAATs do not replace the need for culture; all patients suspected of tuberculosis should have specimens collected for mycobacterial culture.[188]

Currently, two FDA-approved NAATs are available for direct detection of *M. tuberculosis* in clinical specimens: the Amplified *Mycobacterium tuberculosis* Direct test (MTD test, Hologic/Gen-Probe) and the Xpert MTB/RIF assay (Cepheid). The MTD test is based on transcription-mediated amplification of ribosomal RNA and can be used to test both AFB smear-positive and smear-negative respiratory specimens. The Xpert MTB/RIF assay uses real-time PCR to detect the DNA of *M. tuberculosis* and the mutations in the *rpoB* gene associated with rifampin resistance in sputum specimens. Rifampin resistance most often coexists with isoniazid resistance so detection of rifampin resistance serves as a marker for potentially multidrug-resistant *M. tuberculosis* strains. Similar to the other assays developed by Cepheid, the Xpert MTB/RIF assay uses a disposable cartridge that automates the NA extraction, target amplification, and amplicon detection in conjunction with the GeneXpert Instrument System. Sensitivity and specificity of the Xpert MTB/RIF assay for detection of *M. tuberculosis* appear to be comparable to other FDA-approved NAATs for this use. Sensitivity of detection of rifampin resistance was 95% and specificity 99% in a multicenter study using archived and prospective specimens from subjects suspected of having tuberculosis.[191]

Because the prevalence of rifampin resistance is low in the United States, a positive result indicating a mutation in the *rpoB* gene should be confirmed by rapid DNA sequencing for prompt reassessment of the treatment regimen and followed by growth-based drug susceptibility testing.[190] The CDC offers these services free of charge.

Bordetella pertussis

The genus *Bordetella* is composed of eight species, four of which can cause respiratory disease in humans: *B. bronchiseptica*, *B. holmesii*, *B. parapertussis*, and *B. pertussis*. Whooping cough, or pertussis, is a highly contagious respiratory disease caused by *B. pertussis*. Despite widespread childhood vaccination, more than 28,660 cases were reported in the United States in 2014. (http://www.cdc.gov/pertussis/downloads/pertuss-surv-report-2014.pdf). The reported cases represent only the "tip of the iceberg" with an estimated 800,000 to 3.3 million cases occurring in the United States annually. Although pertussis occurs

most often in children younger than 1 year of age, the incidence in older children has increased substantially in recent years. Adolescents and adults, in whom immunity wanes several years after prior infection or vaccination, transmit the organism to susceptible infants. Pertussis in older children and adults is usually characterized by prolonged cough without the inspiratory whoop or posttussive vomiting that typically is observed in infants.

B. parapertussis may be responsible for up to 20% of pertussis-like disease, more often in young children.[192] Illness is generally milder than that caused by *B. pertussis*. *B. bronchiseptica* is an infrequent cause of disease in humans, usually occurring in immunocompromised individuals. Cases usually have exposure to farm animals or pets, which serve as the natural hosts for *B. bronchiseptica.*[193] *B. holmesii* is the most recently recognized species to be associated with pertussis-like illness in humans.[194] All four species play a significant role in human respiratory disease, and they should be considered in the design of NAATs for patients with pertussis-like disease.[195]

The laboratory diagnosis of pertussis has been fundamentally transformed in the past 2 decades. Culture and DFA staining of nasopharyngeal secretions are now largely replaced by NAATs in clinical laboratories. Although culture is specific for diagnosis, it is relatively insensitive. The fastidious nature and slow growth of the *B. pertussis* make it difficult to isolate. Although DFA staining can provide rapid results, it is neither sensitive nor specific. Serologic testing can be useful late in the disease when the organism may not be detectable by culture or NAAT and in the investigation of outbreaks, but the tests are not standardized, so the results may be difficult to interpret.

NAATs are important tools for the diagnosis of pertussis with enhanced sensitivity and rapid turnaround compared with culture and are now considered standard of care, but they can give both false-positive and false-negative results as discussed later. A variety of LDTs primarily based on real-time PCR with different performance characteristics are deployed in clinical laboratories. Currently, there are only two FDA-approved NAATs for *B. pertussis*, one a standalone test based on loop-mediated amplification (Meridian Biosciences) and the other as part of a respiratory panel (BioFire Diagnostics).

A number of different gene targets have been used in NAATs for *Bordetella* spp., some of which are shared among the different species.[195] Most NAATs are based on detection of multicopy insertion sequences (IS), which can increase the sensitivity of the tests. IS*481* is the most validated target for *B. pertussis*, but it can also be found in *B. holmesii* and *B. bronchiseptica*; therefore, tests based on this target alone are of limited value, particularly when used in an outbreak setting. IS*1001* is found in *B. parapertussis* and *B. bronchiseptica* but not in *B. holmesii*. IS*1002* is found in *B. pertussis* and *parapertussis* but not in *B. holmesii* or *bronchiseptica*. Multiplex PCR targeting all three ISs may allow detection and differentiation of the major pathogens that infect humans, *B. pertussis*, *B. parapertussis*, and *B. holmesii*.

A number of assays based on single copy gene targets have also been described.[195] The promoter region of the pertussis toxin operon is often used in diagnostic assays. However, it is also present in *B. parapertussis* and *B. bronchiseptica*, but because of mutations in the promoter region, it is not expressed. It is not found in *B. holmesii*. The mutations in the promoter region found in *B. parapertussis* and *B. bronchiseptica* can be exploited in real-time PCR assays that use post-amplification analysis by melting temperature to distinguish the amplicons from the different species. Pertactin, filamentous hemagglutinin, adenylate cyclase, REC A, flagellin, and BP3385 gene sequences are also shared among the different species. BP283 and BP485 gene targets are reported to be specific for *B. pertussis.*[196] With the exception of the pertussis toxin gene, none of the other single gene targets has been extensively validated in diagnostic assays.

The positive predictive value of NAATs remains their biggest challenge. IS481-based tests will detect *B. holmesii* and *B. bronchiseptica,* which for clinical and epidemiologic purposes are considered biological false-positive results. Environmental contamination with *B. pertussis* DNA in patient clinics has been identified as a source of pseudo-outbreaks of disease.[197] The positive predictive value of NAATs for *B. pertussis* can be increased by amplifying gene targets not shared by other species, using multiplex assays or a two-tiered approach to confirm positives, creating an indeterminate range for assays that target multicopy ISs, segregating "clean" and "dirty" areas in the clinic and the laboratory, and testing only symptomatic patients.[195] Further guidance for health care professionals for the use and interpretation of NAATs for *B. pertussis* can be found on the CDC's website (http://www.cdc.gov/pertussis/clinical/diagnostic-testing/diagnosis-pcr-bestpractices.html).

BLOODSTREAM INFECTIONS

Positive Blood Culture Identification

One of the most important functions of clinical microbiology laboratories is the detection of bloodstream infections. Using conventional grow-based systems, when the blood culture system signals positive, typically within 12 to 72 hours of incubation for most pathogens, the blood culture broth is Gram stained and then subcultured to solid medium. When colonies grow on this medium, identification and antimicrobial susceptibility tests are performed. This typically takes an additional 24 to 48 hours to complete after the blood culture signals positive. Direct inoculation of conventional identification and susceptibility tests using positive blood culture broth can reduce the time required to obtain results by eliminating the subculture to solid medium, but this practice is not FDA approved for automated identification and susceptibility test systems.

A variety of NA-based tests have been developed to expedite identification of organisms in positive blood cultures. FDA-approved tests include peptide NA fluorescent in situ hybridization (PNA FISH) probes, real-time PCR assays for detection of single or limited numbers of pathogens, and high-order multiplex blood culture identification panels based on nested PCR and gold nanoparticle microarrays.[198] Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) uses proteomics rather than genomics to identify pathogens based on the mass spectrum of proteins found in the microorganisms. It has been applied to the direct identification of microorganisms from

TABLE 5.6 Key Features of Rapid Blood Culture Identification Methods

Feature	Nested Multiplex PCR FilmArray	Gold Nanoparticle Microarray	PNA FISH	MALDI-TOF MS
Inclusivity*	+++	+++	+	++++
Hands on time	2 min	5 min	5 min	30 min
Time to result	1 hr	2.5 hr	30 min	35 min
Technical complexity	+	++	++	+++
Antibiotic resistance genes (*n*)	Yes (3)	Yes (9)	No	No
Reagent cost	$$$$	$$$	$$	$

*Relative ability to identify common bloodstream pathogens.

MALDI-TOF MS, Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; *PCR*, polymerase chain reaction; *PNA FISH*, peptide nucleic acid fluorescent in situ hybridization.

positive blood culture bottles.[199] The key features of these methods are listed in Table 5.6.

PNA FISH probes are DNA probes in which the negatively charged sugar phosphate backbone is replaced by a noncharged peptide backbone. This results in rapid binding to DNA targets because there is no electrostatic repulsion with the target.[200] PNA FISH probes are available for rapid identification of *Staphylococcus aureus* and coagulase-negative staphylococci; *Enterococcus faecalis* and other enterococci; *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*; and *Candida albicans*, and/or *C. parapsilosis*, *C. tropicalis* and *C. glabrata* and/or *C. krusei* (AdvanDx). The most recent protocol involves approximately 5 minutes of hands-on time and 30 minutes for results. Access to a fluorescence microscope with a special filter is required to read the stained slides.

A number of laboratory-developed NAATs for direct identification of single or a limited number of organisms directly from blood cultures have been described, but in general, they have not gained widespread acceptance in clinical laboratories. The number of commercially available assays for this application is also limited. *S. aureus* bacteremia requires prompt microbiologic diagnosis and appropriate antibiotic administration. Vancomycin is the standard treatment for suspected *S. aureus* bacteremia because in most centers, 50% or more of isolates are methicillin-resistant *S. aureus* (MRSA); however, it is less effective than methicillin for treating methicillin-susceptible *S. aureus* (MSSA) strains. Therefore, it is not surprising that many of the methods for rapid identification of bloodstream pathogens focused on the differentiation of MRSA from MSSA. Two FDA-approved real-time PCR assays for detection and differentiation of MRSA and MSSA directly from positive blood cultures are the BD GeneOhm StaphSR (BD Diagnostics) and the Xpert MRSA/SA BC (Cepheid) assays.[201,202] Each assay has limitations in accurately differentiating MRSA from MSSA largely because of assay design and selection of gene targets. See this chapter's section on antibacterial drug resistance for more details.

Two high-order multiplex assays have been approved by the FDA for identification of microorganisms from blood culture bottles, the Verigene Gram-Positive and Gram-Negative Blood Culture Tests (Nanosphere) and the FilmArray Blood Culture Identification (BCID) Panel (BioFire Diagnostics).[203-205] The organisms and antibiotic resistance genes included in each of the panels are listed in Box 5.1.

The FilmArray BCID panel uses the same technology as the respiratory panel described previously to detect 24 genus- or species-specific targets including gram-positive and gram-negative bacteria, *Candida* spp., and thee antibiotic resistance genes in approximately 1 hour.[205] This panel identifies from 80% to 90% of all positive blood cultures and provides important information about resistance to methicillin in staphylococci, vancomycin resistance in enterococci, and carbapenemase production in enteric gram-negative rods.

The Verigene BCID panels use Nanogold microarray technology to identify organisms from positive blood culture bottles without NA amplification. The gram-positive or gram-negative panel (or both) is chosen based on the results of the Gram stain that is performed when the bottle signals positive. The gram-positive panel detects 12 genus- or species-specific targets and 3 antibiotic resistance genes, and the gram-negative panel detects 8 genus- or species-specific targets and 9 antibiotic resistance genes in about 2.5 hours with minimal hands-on time. The gram-positive panel detects *mecA*, *vanA*, and *vanB* genes, and the gram-negative panel detects six different β-lactamase genes. A Verigene yeast blood culture panel is in development that will include *C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, C. gattii,* and *Cryptococcus neoformans.*

The FilmArray and Verigene panels provide comprehensive approaches to rapid identification of the vast majority of blood pathogens and important information about susceptibility of these pathogens to antibiotics. When coupled with active antimicrobial stewardship program interventions, the results of these tests will likely have a positive impact on the clinical outcomes of patients with sepsis.[206]

Direct Pathogen Detection

The methods discussed in the preceding section provide opportunities to expedite the identification of microorganisms when a blood culture signals positive and as such represent significant advances. However, blood cultures require 1 to 5 days of incubation before they are positive, and this timeline is inconsistent with the need to obtain rapid answers to inform treatment decisions in patients with sepsis. Direct detection of pathogens in blood without the need for culture would be ideal but presents a number of challenges. Specimen preparation, enrichment for pathogen DNA, and integration of the front-end specimen preparation with a back-end molecular analysis that identifies virtually all pathogens are major obstacles to success. Also, highly sensitive molecular methods for direct detection of microbial DNA in blood

BOX 5.1 Comparison of Organisms and Antibiotic Resistance Genes Included in the FilmArray and Verigene Blood Culture Identification Panels

FilmArray

Gram Positive
Enterococcus spp.
Listeria monocytogenes
Staphylococcus spp.
  S. aureus
Streptococcus
  S. agalactiae
  S. pneumoniae
  S. pyogenes
Gram Negative
Acinetobacter baumannii
Haemophilus influenzae
Neisseria meningitidis
Pseudomonas aeruginosa
Enterobacteriaceae
  Enterobacter cloacae complex
  Escherichia coli
  Klebsiella pneumoniae
  K. oxytoca
  Proteus spp.
  Serratia marcescens
Yeast
Candida albicans
C. glabrata
C. krusei
C. parapsilosis
C. tropicalis
Antibiotic Resistance Genes
mecA
vanA/B
bla_{KPC}

Verigene

Gram Positive
Staphylococcus spp.
  S. aureus
  S. epidermidis
  S. lugdunensis
Streptococcus spp.
  S. anginosus group
  S. agalactiae
  S. pneumoniae
  S. pyogenes
Enterococcus faecalis
E. faecium
Listeria spp.
Antibiotic Resistance Genes
mecA
vanA
vanB

Gram Negative
E. coli/Shigella
Klebsiella pneumoniae
K. oxytoca
Pseudomonas aeruginosa
Acinetobacter spp.
Proteus spp.
Citrobacter spp.
Enterobacter spp.
Antibiotic Resistance Genes
bla_{KPC}
bla_{NDM}
$bla_{CTx\text{-}M}$
bla_{VIM}
bla_{IMP}
bla_{OxA}

present significant challenges for validation given the known limitations of the sensitivity of culture, the current gold standard.

The Roche SeptiFast system has been available longer than any other method for direct detection of microorganisms in the blood.[207] It uses real-time PCR performed on a LightCycler instrument that targets the ribosomal internal transcribed spacer region. The target DNA is amplified in three parallel, multiplex, real-time PCR assays for detection of 10 gram-positive, 10 gram-negative, and 5 fungal pathogens. Melting curve analysis is used to reliably differentiate the pathogens. The assay is technically complex, requires large amounts of hands-on time, and has an analysis time of about 6 hours. Several evaluations have reported lower sensitivities and specificities in clinical settings when compared with blood cultures.[208-211]

Another molecular approach to direct detection of pathogens in blood and other body fluids has been developed by Ibis Biosciences, a subsidiary of Abbott Molecular. This method combines broad-range PCR with electrospray ionization time-of-flight mass spectrometry (PCR/ESI-MS). The technology has been described in great detail elsewhere,[212] but briefly, it works by coupling conserved-site PCR reactions that are able to amplify shared genes from diverse microorganisms to ESI-MS. Measurement of amplicon mass provides species-specific signatures that can be matched to known signatures in a database. After PCR amplification, ESI-MS analysis is performed on the amplicon mixtures, and the A, G, C, and T base compositions are compared with a database of known base compositions derived from existing sequence data. This technology accurately identifies diverse microbes from blood, other body fluids, and tissues in research settings.[213] The PCR/ESI-MS system was evaluated for the direct detection of bacteria and *Candida* spp. in the blood of 331 patients with suspected bloodstream infections and was found 83% sensitive and 94% specific compared with culture.[214] Replicate testing of the discrepant samples by PCR/ESI-MS resulted in increased sensitivity (91%) and specificity (99%) when confirmed infections were considered true positives. Ibis/Abbott has developed an automated and integrated platform for PCR/ES-MS analysis of clinical samples called IRIDICA. A bloodstream infection assay for this new platform that identifies up to 500 different organisms and four antibiotic resistance genes is currently in clinical trials.

Another novel technology that shows promise for direct detection of pathogens in blood is T2 magnetic resonance (T2MR)–based biosensing.[215] T2 Biosystems has developed an assay to directly identify *Candida* spp. in the blood of patients with suspected candidemia. In this assay, the *Candida* cells are lysed, their DNA is amplified by PCR, and the amplified product is detected directly in the whole-blood matrix by amplicon-induced agglomeration of superparamagnetic nanoparticles. Nanoparticle clustering yields changes in the T2 (spin-spin) relaxation time, making it detectable by magnetic resonance. A small portable T2MR instrument for rapid and precise T2 relaxation measurements has been designed for standard PCR tubes. The T2Dx instrument automates all of the steps in the assay with approximately 5 minutes of hands on time, and results are available within 3 to 5 hours. The T2 *Candida* panel is FDA approved, and the clinical trial data showed an overall sensitivity and specificity of 91.1% and 99.4%, respectively.[216] T2 Biosystems has a panel in development for direct detection of bacteria in blood.

CENTRAL NERVOUS SYSTEM

Herpes Simplex Virus

Herpes simplex virus types 1 and 2 produce various clinical syndromes involving the skin, eye, CNS, and genital tract. Although NA testing has been used to detect HSV DNA in all of these clinical manifestations, this discussion focuses on the use of HSV PCR for the diagnosis of CNS infections because NA amplification testing is widely viewed as the standard of care for diagnosis.

Herpes simplex virus causes both encephalitis and meningitis. In adults, whereas HSV encephalitis is usually attributable to infection with HSV type 1, HSV meningitis is most commonly caused by HSV type 2. HSV encephalitis is a severe infection with high morbidity and mortality; treatment with acyclovir reduces the mortality rate from approximately 70% in those with untreated infection to 19% to 28%. Neurologic impairment is common (≈50%) in those who survive.[217] HSV encephalitis may reflect primary infection or reactivation of latent infection. HSV meningitis is usually a self-limited disease that resolves over the course of several days without therapy. In some patients, the disease may recur as a lymphocytic meningitis over a period of years.[218]

Neonatal HSV infection occurs 1 in 3500 to 1 in 5000 deliveries in the United States. It is most commonly acquired by intrapartum contact with infected maternal genital secretions and is usually HSV type 2. In newborns, three general presentations of the disease are known: skin, eye, and mouth disease, which account for approximately 45% of infections; encephalitis, which accounts for 35%; and disseminated disease, which accounts for 20%. Because disseminated disease is often associated with neurologic disease, CNS disease occurs in about 50% of newborns with neonatal HSV infection.

Herpes simplex virus encephalitis cannot be distinguished clinically from encephalitis caused by other viruses such as West Nile Virus, St. Louis encephalitis virus, and Eastern equine encephalitis virus. Historically, the gold standard for the diagnosis of HSV encephalitis required brain biopsy with identification of HSV by cell culture or immunohistochemical staining. This approach provided high sensitivity (99%) and specificity (100%), but it required an invasive procedure, and several days elapsed before results were available. Viral culture of cerebrospinal fluid (CSF) has a sensitivity of less than 10% for the diagnosis of HSV encephalitis in adults. Tests that measure HSV antigen or antibody in CSF have diagnostic sensitivities of 75% to 85% and diagnostic specificities of 60% to 90%.[217] Because of the limitations of conventional methods, there was interest in assessing the clinical utility of PCR for the detection of HSV DNA from CSF of patients with encephalitis. The two largest studies compared HSV PCR on CSF specimens versus brain biopsy in patients with suspected HSV encephalitis.[219,220] The sensitivity and specificity of PCR were greater than 95%, and the sensitivity of HSV PCR did not decrease significantly until 5 to 7 days after the start of therapy. PCR is positive early in the course of illness, usually within the first 24 hours of symptoms, and in some individuals, HSV DNA can persist in the CSF for weeks after therapy is initiated.

The clinical utility of HSV PCR has also been established for the diagnosis of neonatal HSV infection. In one study, HSV DNA was detected in the CSF of 76% (26 of 34) of infants with CNS disease; 94% (13 of 14) of those with disseminated infection; and 24% (7 of 29) of infants with skin, eye, or mouth disease.[221] The persistence of HSV DNA in the CSF of newborns for longer than 1 week after therapy initiation is associated with a poor outcome.[222] Based on these findings, detection of HSV DNA in CSF by PCR has become the standard of care for the diagnosis of HSV encephalitis and neonatal HSV infection. In newborns with disseminated disease, HSV DNA may be detected in serum or plasma specimens and is useful diagnostically in newborns if it is not possible to do a lumbar puncture. Although the sensitivity of HSV PCR is high, it is not 100%, so a negative PCR test result may not rule out neurologic disease caused by HSV, particularly if the pretest probability is high. In this situation, it is important to consider repeat testing.

As with HSV encephalitis, HSV meningitis cannot be distinguished clinically from other viral meningitides, although recurrence of viral meningitis is a strong clue that HSV may be the etiologic agent. Unlike HSV encephalitis, HSV meningitis has not been the subject of large studies evaluating the clinical utility of PCR for diagnosis. Nonetheless, because the sensitivity of viral culture of CSF specimens is only 50%, HSV PCR of CSF is commonly used in the evaluation of meningitis and has been described as accurate in anecdotal reports.[223]

Several molecular tests for the detection of HSV DNA from genital specimens have been cleared by the FDA, but only one, the Simplexa HSV 1 and 2 Direct Kit (Focus Diagnostics), has been cleared for use with CSF specimens. Several companies provide primers and probes as ASRs, which can be used as components in LDTs.

Molecular tests are often designed to detect HSV types 1 and 2 with equal sensitivity. Distinguishing between HSV types 1 and 2 may not be necessary because the clinical management of CNS disease is the same for both infections. Primers used for the detection of HSV DNA commonly target the polymerase, glycoprotein B, glycoprotein D, or thymidine kinase genes. It is important that the primers not amplify DNA from other herpesviruses that are associated with neurologic disease; these include cytomegalovirus, varicella zoster virus, human herpes virus type 6, and Epstein-Barr virus.

Herpes simplex virus PCR assays need low detection limits (several hundred copies per milliliter of specimen) to be useful in evaluating neurologic disease. This is particularly true for

the diagnosis of meningitis, in which CSF concentrations of DNA tend to be lower than those seen with encephalitis. HSV neurologic disease rarely occurs in individuals without an increased CSF white blood cell count or protein concentration. Caution should be exercised in applying this generalization to immunocompromised individuals because they may not mount a typical inflammatory response to HSV infection. Although HSV PCR of CSF specimens is clearly the gold standard for the diagnosis of neurologic disease, results should be interpreted with caution because neither sensitivity nor specificity is 100%. Test results should always be interpreted within the context of the clinical presentation of the patient. If results do not correlate with the clinical impression, repeat testing should be performed 3 to 7 days later because initial negative PCR results can occur in a small but notable number of patients with confirmed HSV encephalitis.

Enterovirus

The enteroviruses (EVs) are a diverse group of single-stranded RNA viruses belonging to the *Picornaviridae* family. Currently, human EVs are divided into seven species: EV A to D and rhinoviruses A to C. The EV species A to D contain viruses formerly referred as coxsackieviruses, EVs, polioviruses, and echoviruses. The genus Parechovirus (PeV) comprises 16 different serotypes that were originally thought to be echoviruses. Although the genomic organization is similar to EVs, the origin of PeVs is uncertain. Numerous clinical presentations are seen with EV and PeV infections, including acute aseptic meningitis, encephalitis, exanthems, conjunctivitis, acute respiratory disease, GI disease, myopericarditis, and sepsis-like syndrome in neonates. Diagnoses typically are based on clinical presentation and NAATs.

Virus culture methods have several drawbacks, including the requirement to inoculate multiple cell lines because no single cell line is optimal for all EV types, the inability to grow some EV types in cell culture, the limited diagnostic sensitivity of cell culture (65%—75%), and the long TAT of 3 to 8 days for those EVs that do grow in cell culture.[224] The long TAT for culture means that results are rarely available in a time frame to influence clinical management. NAATs offer several important advantages over cell culture, including improved sensitivity and TAT. As a result, NA testing is considered the new gold standard for the diagnosis of aseptic meningitis and neonatal sepsis syndrome caused by EV and PeV infections.

Two methods are used for the detection of enteroviral RNA from clinical specimens: RT-PCR and NA sequence—based amplification. The primers used in clinical testing most commonly target the highly conserved 5′ URT of the genome and detect polioviruses and EVs.[225] These primers do not detect parechoviruses, although these viruses can cause aseptic meningitis. In general, molecular assays have good detection limits ranging from 0.1 to 50 tissue culture infectious doses ($TCID_{50}$) per test. The assays are quite specific, but sequence similarities may allow amplification of some types of rhinoviruses.[226,227] Currently, two tests for the detection of EVs from CSF specimens have been cleared by the FDA: the NucliSENS EasyQ Enterovirus (bioMérieux) and Xpert EV (Cepheid). However, the NucliSENS Easy Q EV assay is no longer commercially available. The Xpert EV test has a sensitivity of 97% and a specificity of 100% for the diagnosis of enteroviral meningitis.[228] The Xpert test has the advantage of being very simple to perform: The specimen and reagents are added to a cartridge, which is inserted into the instrument. NA extraction, amplification, and detection are fully automated, and results are available within about 2.5 hours. The system permits random access, which allows for on-demand testing.

Nucleic acid testing for the diagnosis of enteroviral infection has been evaluated in a variety of clinical studies, with testing showing sensitivities equal to or greater than that of cell culture, a high specificity, and faster TATs than cell culture. Several studies have suggested that the use of molecular methods for the diagnosis of enteroviral infection in infants and pediatric patients can lead to an overall cost savings by reducing the use of antibiotics and imaging studies.[229-231] To maximize the benefits for patient care and cost savings, testing should be available on a daily basis.

As mentioned earlier, many EV molecular assays detect rhinoviruses, and most detect polioviruses. These two factors can lead to unexpected and misleading positive results when respiratory or stool specimens are tested. The diagnosis of EV meningitis should be based on testing of CSF specimens, and sepsis syndrome is best diagnosed in neonates by testing serum, plasma, or CSF samples.

GASTROENTERITIS

Clostridium difficile

Clostridium difficile is a gram-positive spore-forming anaerobic bacillus that is frequently found in the stool flora of healthy infants but is rarely found in the stool flora of healthy adults and children older than 12 months. The organism is acquired by ingesting spores, which survive the gastric acid barrier and germinate in the colon. Alteration of the intestinal flora with the use of antibiotics facilitates colonization of the intestinal tract. After being colonized, patients may develop symptoms of diarrhea or colitis. Most strains of *C. difficile* make two toxins: toxin A and toxin B; the regulatory proteins TcdR and TcdC control expression of the toxin A (*tcdA*) and B (*tcdB*) genes. These toxins are responsible for symptomatic disease; strains that lack these toxin genes do not cause diarrhea or colitis. Toxin B may be more important for production of disease than toxin A.[232] Detection of these toxins or of their activity is essential in diagnostic tests for *C. difficile*—associated disease. An additional toxin, the binary toxin, has been described in some strains of *C. difficile*, and recent reports have suggested that strains encoding the binary toxin (CDT) have a deletion in the *tcdC* gene, leading to overexpression of toxins A and B (ribotype 027), and are causing outbreaks of more severe disease.[233]

C. difficile is a frequent cause of antibiotic-associated diarrhea and colitis both in the hospital and community. In hospitals, the risk of infection increases with the length of hospital stay, and use of antimicrobial therapy greatly increases the likelihood of acquiring *C. difficile* colitis. *C. difficile* causes a spectrum of disease ranging from asymptomatic carrier state to fulminant, relapsing, and fatal colitis. Diarrhea may be mild to severe. Pseudomembranous colitis is a classic presentation of *C. difficile* disease, and toxic megacolon may also be seen. Although clindamycin, penicillins, and cephalosporins

have commonly been associated with disease, almost all antibiotics can cause similar disease.

Various non-NA tests are available for the diagnosis of *C. difficile* infection. Culture of the organism alone is not helpful in the diagnosis because there needs to be confirmation that the organism produces toxins. The cell culture cytotoxicity test neutralization assay (CCNA), which detects the cytopathic effect of toxin B, is considered the gold standard for the diagnosis of clinically important *C. difficile* infection. The test is highly sensitive and specific but is labor intensive and technically demanding. The TAT of 1 to 3 days limits its clinical utility.[234] The most commonly used tests are EIAs and lateral flow devices that detect toxin A, toxin B, or both. Overall these tests have lower sensitivities (45%–95%) and specificities (75%–100%) than the cytotoxicity test. In general, EIAs that detect both toxins A and B are preferred because some strains may not produce toxin A. An alternative testing approach is detection of the common antigen glutamate dehydrogenase (GDH). The test does not distinguish between toxigenic and nontoxigenic strains and cannot be used alone for the diagnosis of *C. difficile* disease. A positive result needs to be confirmed with the cytotoxicity test, a toxin EIA, or a NAAT for the detection of toxin B gene. The GDH test is a useful screening test because it has a high negative predictive value. One study evaluated a two-step approach using the GDH test as the initial screen followed by a CCNA for antigen-positive specimens to confirm the presence of toxin. A negative antigen test result was more than 99% predictive of a negative CCNA.[235] A limitation of this approach is the delay in obtaining a result because of the long TAT of the CCNA test. More recently, multistep algorithms using GDH, toxin EIA, and NAATs have been deployed in clinical laboratories.[236]

In view of the limitations of traditional methods, molecular tests are a good alternative for the diagnosis of *C. difficile* infection. The first NAAT for detection of *C. difficile* in stool was approved in 2009. At the time of this writing, 15 different platforms are FDA approved and available for testing using a variety of methods, including real-time PCR, loop-mediated amplification, helicase-dependent amplification, and microarray technology. Some platforms are designed for low-volume laboratories and on-demand testing, and others are more amenable to high-volume, batch mode testing. These assays detect a variety of gene targets, including *tcdA, tcdB*, *cdt*, and Δ117 deletion in *tcdC,* the latter two as surrogates for identification of the ribotype 027 strain.

Although NAATs have replaced other methods in clinical laboratories for diagnosis of *C. difficile* infection and have very high negative predictive values and analytical and clinical sensitivities, there are concerns about their specificity and positive predictive value because they will detect colonization as well as infection.[236] As mentioned earlier, some laboratories have implemented multistep diagnostic test algorithms using GDH, toxin EIA, and NAAT; however, these algorithms con complicate and delay final results, may not be reimbursed, and may ultimately be less cost effective than NAAT alone. Regardless of how a laboratory chooses to deploy NAAT testing, it should be limited to only patients with diarrhea to increase the pretest probability of disease and thus help mitigate concerns about detecting patients who are asymptomatically colonized with toxigenic strains.

POINTS TO REMEMBER

Clostridium difficile

- *C. difficile*–associated disease spectrum ranges from mild antibiotic-associated diarrhea to life-threatening toxic megacolon and occurs both in hospitals and the community.
- NAATs for detection of the toxin B gene of *C. difficile* have several advantages over traditional methods for diagnosis, including increased analytical and clinical sensitivity, high negative predictive value, and decreased analysis time.
- Concerns about the specificity and positive predictive value of NAATs have led some laboratories to adopt multistep diagnostic algorithms to help mitigate the problem of detecting patients asymptomatically colonized with toxigenic strains of *C. difficile.*

Gastrointestinal Pathogen Panels

Infectious gastroenteritis (IGE) is a leading cause of global morbidity and mortality. Diarrheal disease disproportionally affects developing nations, but IGE remains a significant problem in industrialized countries as well. Each year, approximately 178.8 million cases of IGE occur in the United States, resulting in 474,000 hospitalizations and 5000 deaths.[237] IGE is associated with a diverse array of etiologic agents, including bacteria, viruses, and parasites. Clinical presentation does little to aid with a specific etiologic diagnosis because diarrhea is the predominant symptom regardless of the etiology. Accurate identification of the etiology of IGE provides important information that impacts individual patient management, infection control, and public health interventions.

Common diagnostic practice in the United States requires that providers choose among a variety of tests, including antigen detection tests, culture, ova and parasite microscopic examination, and single-target NAATs, for detection of the responsible organism or toxin. In addition, the selection of tests may be informed by patient's age, severity of disease, immunocompromised state, duration and type of diarrhea, travel history, and time of year.[238] Often the clinician is unsure of what pathogens are included in each test and consequently may miss testing for specific pathogens of interest. In the laboratory, the battery of tests required to detect all possible pathogens is laborious and expensive to maintain, can require special expertise, and may have an unacceptably long TAT. In addition, the conventional microbiologic tests have limited sensitivity for many of the major pathogens.

The application of NA amplification methods could have significant impact on the diagnosis, treatment, and understanding of the epidemiology of IGE.[239] At the time of this writing, there were five FDA-approved enteric pathogen panels. A comparison of their key features is shown in Table 5.7. The systems use a variety of different technologies and differ in the number and types of targets included in the assay and the overall platform design and throughput. The Prodesse ProGrastro SSCS assay (Hologic/Gen-Probe) uses real-time PCR to detect and differentiate among *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp., as well as Shiga toxin 1 *(stx1)* and Shiga toxin 2 *(stx2)* genes as indicators of Shiga-toxin producing *E. coli* in two separate master mixes.[240]

TABLE 5.7 Comparison of Different Food and Drug Administration–Approved Enteric Pathogen Panel Platforms

Feature	ProGastro SSCS	BD MAX EBP	Verigene EP	xTag GPP	FilmArray GI
Technology	Real-time PCR	Real-time PCR	PCR and gold nanoparticle microarray	PCR and bead array	Nested PCR and melting curve analysis
Automation	Separate extraction, manual PCR setup	Sample to result	Sample to result	Separate extraction, manual PCR setup, post-PCR amplicon transfer	Sample to result
Throughput	Batch (16/thermal cycler)	Batch (24)	1/run	Batch (limited by extractor)	1/run
Analysis time (hr)	3	1.5	2	4	1
Targets	5 (3 bacteria, 2 toxins)	4 (3 bacteria, 1 toxin)	9 (5 bacteria, 2 toxins, 2 viruses)	14 (8 bacteria, 3 viruses, 3 protozoa)	21 (12 bacteria, 5 viruses, 4 protozoa)
Relative cost/test	$$	$	$$$	$$$	$$$

PCR, Polymerase chain reaction.

Separate NA extraction and manual PCR setup are required. The BD MAX EBP (BD Diagnostics) uses a single real-time PCR master mix to detect essentially the same pathogens and toxins: *Salmonella* spp., *Shigella* spp. or enteroinvasive *E. coli*, *Campylobacter* spp., and *stx1*/*stx2*. However, the BD MAX automates all of the steps from sample preparation to target amplification and detection.[241]

Other systems have been developed to expand the panel of bacteria detected and include viral and protozoal pathogens. The Luminex xTAG GPP uses multiplex endpoint PCR and liquid bead array to detect and differentiate eight bacteria, three viruses, and three protozoa, including *Campylobacter* spp., *C. difficile* (toxins A and B), *E. coli* 0157, enterotoxigenic *E. coli*, Shiga-like toxin producing *E. coli*, *Salmonella* spp., *Shigella* spp., *Vibrio cholera*, adenovirus 40/41, norovirus GI/GII, rotavirus A, *Cryptosporidium* spp. *Entamoeba histolytica*, and *Giardia* spp.[242] This system provides for high throughput but requires a separate NA extraction step and post-PCR amplicon manipulation, which can lead to false-positive results caused by amplicon carry-over cross-contamination. It also has the longest analysis time of the available systems.

The Verigene uses multiplex PCR and a gold nanoparticle microarray to detect five bacteria, two toxins, and two viruses, including *Campylobacter* spp., *Salmonella* spp., *Shigella* spp. *Vibrio* spp., *Yersinia enterocolitica*, stx1, stx2, norovirus, and rotavirus.[243] It is a simple to use "sample in, answer out" system, but it has limited throughput because only one sample per instrument can be run at a time.

The FilmArray uses nested multiplex PCR and melting curve analysis to detect 12 bacteria, 5 viruses, and 4 protozoa, including *Campylobacter* spp., *C. difficile* toxin A/B, *Plesiomonas shigelloides*, *Salmonella* spp., *Vibrio* spp., *V. cholerae*, *Y. enterocolitica*, enteroaggregative *E. coli*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, Shiga-like toxin-producing *E. coli*, *E. coli* 0157, adenovirus F 40/41, astrovirus, norovirus GI/GII, rotavirus A, sapovirus, *Cryptosporidium* spp., *Cyclospora cayetanensis*, *E. histolytica*, and *G. lamblia.*[244] This is the most comprehensive enteric pathogen panel currently available. Similar to all of the FilmArray products, it is simple to use and provides results in about 1 hour. Its chief limitations are low throughput and high cost.

Laboratories can choose from a variety of test platforms based on whether a more focused or broader approach to IGE pathogen detection is desired. Also, the technical complexity and required throughput are important variables that may influence the approach chosen for this application. Current stool test algorithms using conventional methods typically require clinicians to consider which pathogens might be associated with the disease and choose among a variety of tests to ensure that all pathogens are covered. It is not surprising that this piecemeal approach often fails to yield positive results. The use of comprehensive pathogen panels dramatically increases diagnostic yield, but with this comes the unique challenge of interpreting the results from patients with multiple pathogens detected. In the FDA clinical trial of the FilmArray GI Panel, at least one potential pathogen was detected in 53.5% of specimens, and among these, multiple potential pathogens were detected in 32.9%.[244] Asymptomatic infections with *C. difficile*, *Cryptosporidium* spp., and *G. lamblia* are not uncommon, and some of the other IGE pathogens such as *Salmonella* spp. and norovirus can be shed for weeks after resolution of symptoms. Comprehensive panels consolidate testing platforms for agents of IGE and substantially reduce, but not completely eliminate, the need for culture because isolates are needed for epidemiologic surveillance and occasionally for antibiotic susceptibility testing.

ANTIBACTERIAL DRUG RESISTANCE

The detection of antibiotic resistance is one of the most important functions of the clinical microbiology laboratory. This has traditionally been done by phenotypic methods.

However, the delays inherent in phenotypic tests can lead to delays in appropriate therapy and adverse clinical outcomes. Molecular methods offer faster alternatives for detection of antibiotic resistance, but the genotypic approach has its own set of challenges because of the complexity of antibiotic resistance. In addition, the detection of a resistance gene may not necessarily imply phenotypic resistance if the gene is expressed at low levels or is not functional. Advances in technology and our understanding of the genetics of antibiotic resistance will likely make the use of molecular detection for antibiotic resistance more widespread in the future. This section focuses on the commonly used resistance targets, currently MRSA, vancomycin-resistant enterococci (VRE), and β-lactamases in gram-negative bacteria.

Because *S. aureus* is among the most common cause of bacterial infections in the industrialized world, particular attention has been focused on assays to rapidly differentiate MRSA from MSSA for diagnosis of infection and surveillance for infection control purposes. Molecular assays that recognize MRSA based on detection of a single target detect the junction between the staphylococcal cassette chromosome *mec* element (SCC*mec*), which carries the *mecA* resistance and other genes, and the flanking *orfX* gene.[245] This assay design has several limitations, including false-negative results caused by SCC*mec* variants and false-positive results caused by MSSA strains that carry SCCmec remnants lacking the *mecA* gene, sometimes referred to as "empty cassettes," or that carry SCCmec with a nonfunctional *mecA* gene.[245-247] An alternative approach to molecular detection of MRSA combines a *mecA* target and a second gene target specific for *S. aureus* such as *sa442, nuc, femA-femB, spa,* or *Idh1*.[248] At the time of this writing, there were five companies with FDA-approved assays for molecular detection of MRSA or MRSA and MSSA (BD Diagnostics, Cepheid, Elitech, Roche, and bioMérieux). In addition to these stand-alone assays, molecular detection of MRSA is incorporated into the blood culture identification panels discussed previously. These assays are intended for use in surveillance testing or to assist in the diagnosis of infections. Depending on the platform, the tests can be run on demand or in batches. Studies indicate that use of molecular methods for rapid identification of patients who are colonized with MRSA may be a cost-effective infection control strategy.[249,250]

Enterococci are commensal residents of the GI tract and female genital tract that account for about 10% of hospital-acquired infections. The vast majority of enterococcal infections are caused by *Enterococcus faecalis* and *E. faecium* and occur primarily in patients requiring long-term care. The emergence of VRE in hospitals is concerning because vancomycin is often used empirically to treat a wide variety of infections. Infection with VRE is associated with increased morbidity and mortality because of the propensity of VRE to infect patients already at high risk for comorbidity.[251]

In the United States, about 30% of enterococci are resistant to vancomycin. High-level vancomycin resistance in enterococci occurs via acquisition of mobile transposable elements carrying the *vanA* or *vanB* genes. *E. faecium* is more frequently resistant to vancomycin than *E. faecalis,* and *vanA* is more commonly found than *vanB* in resistant strains. As with MRSA, the rapid detection of VRE colonization to prevent health care–associated infections is widely recommended.[252] Molecular assays work well for this application.[253,254] Three FDA-approved molecular assays are marketed for rapid detection of VRE from perianal and rectal swabs. The BD GeneOhm and IMDx assays detect both *vanA* and *vanB,* and the Cepheid assay detects *vanA* alone. All are based on real-time PCR, but only the Cepheid assay is designed for on-demand testing. Aside from rare reports of *vanA* in *S. aureus* and *Streptococcus* spp., detection of *vanA* is highly specific for VRE. However, *vanB* can be found in a wide variety of commensal nonenterococcal bacteria, so detection of *vanB* requires confirmation of VRE by culture. As with *mecA*, assays for detection of *vanA* and *vanB* have also been incorporated in the commercially available blood culture identification panels.

One of the greatest threats to our antibiotic formulary is the emergence of β-lactamases in gram-negative bacteria with the capabilities of hydrolyzing broad-spectrum penicillins, cephalosporins, and carbapenems. These enzymes include extended-spectrum β-lactamases (ESBLs), AmpCs, and carbapenemases. The accurate detection of these zymes is important for both treatment decisions and infection control purposes. Detection of these organisms harboring these broad-spectrum β-lactamases by phenotypic methods is imperfect.[255,256] A rapid, inexpensive, multiplex molecular assay to detect the genes encoding these enzymes would be clinically useful but presently is an unmet need. One of the biggest challenges to molecular detection is the great diversity of β-lactamases, with more than 200 described ESBLs and numerous classes of carbapenemases, including *K. pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase (NDM), Verona integrin-encoded metallo-β-lactamase (VIM), imipenem metallo-β-lactamase (IMP), and oxacillinase (OXA). An additional challenge is that the detection of the gene(s) does not provide information about copy number and expression, which are important to phenotypic expression of resistance to β-lactam and carbapenem antibiotics.

A number of LDTs and RUO kits have been developed for molecular detection of a variety of broad-spectrum β-lactamase genes that range from single target assays to detect KPC to highly multiplexed assays detecting multiple ESBLs, multiple AMPCs, KPC, NDM, VIM, IMP, and OXA-48.[257-262] Both the Biofire FilmArray and Verigene BCID panels include assays to detect KPC, and the Verigene panel detects genes encoding for five additional broad-spectrum β-lactamases, CTX-M, NDM, VIM, IMP, and OXA.

HUMAN MICROBIOME AND METAGENOMICS

Microbial inhabitants outnumber our own body's cells by about 10 to 1 and at a genomic level have 100-fold greater gene content than the human genome. Interest in elucidating the role of resident organisms in human health and disease has flourished over the past decade with the advent of new technologies for interrogating complex microbial communities. The microbiome is the totality of microbes, their genetic information, and the milieu in which they interact.[263] It includes bacteria, fungi, viruses and phages, and parasites, but most of the emphasis to date has been on the bacterial component of the microbiome. However, progress is being made toward defining the human virome and the role that it plays in complex microbial communities.[264]

Metagenomics refers to the concept that a collection of genes sequenced from the environment could be analyzed in a way analogous to the study of a single genome. It has been facilitated by advances in NA sequencing technology, and this technology has permitted the study of microbial communities directly in their natural environment, thus bypassing the need for isolation and cultivation of individual species. We now know that the majority of microorganisms from the human body cannot be cultured in vitro. Most taxonomic metagenomic studies have used targeted sequencing of the phylogenetically informative regions of the 16s rRNA gene from bacteria because it has long been the gold standard method for bacterial identification, and there are large sequence databases and sophisticated analysis tools available.[265] However, 16s rRNA gene sequencing does not provide enough information for comprehensive microbiome studies. To overcome the limitations of single gene-based amplicon sequencing, researchers have used whole-genome shotgun sequencing on massively parallel sequencing platforms. Whole-genome approaches permit identification and annotation of diverse sets of microbial genes that encode many different biochemical or metabolic functions, thus providing functional metagenomic information.

In 2007, the Human Microbiome Project was launched by the National Institutes of Health with the overarching goal of developing tools and resources for characterization of the human microbiome and to relate it to human health and disease.[266-268] Initial microbiome comparisons across 18 different body sites confirmed high interindividual variation with four phyla of bacteria, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* predominating across all body sites.[269] Additionally, the composition of the gut microbiome is most often characterized by smooth abundance gradients of key organisms and does not cluster individuals into discrete microbiome types.[268] However, the microbiome at other body sites such as the vagina can show such clustering.[266] An important point that emerged from these early studies is that although the microbial communities varied among individuals, the metabolic pathways encoded by these organisms were consistently present, forming a functional "core" to the microbiome at all body sites.[268,270,271] Although the pathways and processes of this core were consistent, the specific genes associated with these functions varied.

Alterations of the microbiome in many different disease states have been described.[263] A complete review of this topic is beyond the scope of this chapter, but some specific examples are given in Table 5.8. Establishing a causal link between microbiome changes and a specific disease often is challenging because most studies have been observational, the disease entities themselves may not be well defined, and the pathogenesis may be multifactorial.

However, it seems clear that future approaches in medical microbiology will be shaped in part by developments in metagenomics and human microbiome research. The identification of single agents of infection will be supplemented by techniques that will determine the relative composition of microbiomes in the context of different infections and other disease states. Recent evidence that recurrent *C. difficile* infections can be treated by reconstituting the normal colon microbiota in the patient by transferring feces from a normal donor is a good example of how a better understanding of changes in the microbiome composition can lead to effective treatments options.[272] Differences in the composition of the microbiome that may cause or contribute to noninfectious diseases may offer new opportunities for clinical microbiology laboratories to impact other areas of medicine. Finally, metagenomic techniques will facilitate the discovery of previously unrecognized pathogens and increase our understanding of how changes in the microbiome may contribute to infectious diseases.

TABLE 5.8 Association of Human Disease With Changes in the Microbiome

Disease	Relevant Change
Psoriasis	Increased ratio of *Firmicutes* to *Actinobacteria*[280]
Reflux esophagitis	Esophageal microbiota dominated by gram-negative anaerobes; gastric microbiota with low or absent *Helicobacter pylori*[281,282]
Obesity	*Reduced ratio of Bacteroidetes to Firmicutes*[283,284]
Childhood-onset asthma	Absent gastric *H. pylori* (especially cytotoxin-associated gene genotype[285,286]
Inflammatory bowel disease (colitis)	Increased *Enterobacteriaceae*[287]
Functional bowel diseases	Increased *Veillonella* spp. and *Lactobacillus* spp.[288]
Colorectal carcinoma	Increased *Fusobacterium* spp.[289,290]
Cardiovascular disease	Gut-dependent metabolism of phosphatidylcholine[291]

Modified from Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. *Nat Rev Genet* 2012;13:260–270.

FUTURE DIRECTIONS

Molecular microbiology will continue to be one of the leading growth areas in laboratory medicine. The number of applications of this technology in clinical microbiology will continue to increase, and the technology will increasingly be deployed in clinical laboratories as it becomes less technically complex and thus more accessible. However, now more than ever, clinical and financial outcomes data will be needed to justify the use of this often expensive technology in an era of declining reimbursement and increased cost consciousness.

The clinical utility of molecular testing for infectious diseases is now well established, and the gap between the availability of FDA-cleared and -approved tests and clinical need is improving. However, the pending enhanced oversight of LDTs and restriction of the use of RUO and IUO reagents and systems by the FDA could limit the ability of laboratories to develop tests to meet clinical needs not met by IVD products (http://www.fda.gov/downloads/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm416684.pdf).

Although considerable progress has been made in recent years, other important needs remain unmet, including the availability of international standards and traceable and commutable calibrators that can be used for assay verification and validation. These materials, when widely available, should improve agreement of the results between or among different tests and aid in the establishment of their clinical utility. Another need is for the continued development of

effective proficiency testing programs that will help ensure that the results of molecular tests are reliable and reproducible among laboratories.

Digital PCR is the next advance in the evolution of quantitative PCR methods. Digital PCR has many applications, including detection and quantification of low numbers of pathogen sequences. It can provide a lower limit of detection than real-time PCR methods with better precision at very low concentrations. As opposed to relative quantification, digital PCR provides absolute quantification with no need for reference standards. Currently, digital PCR is used as a research tool, but it may find applications in clinical laboratories to resolve ambiguous results obtained with quantitative real-time PCR assays or for creating accurate viral reference standards as the technology becomes less costly.[273,274]

To a great extent, the future of molecular microbiology depends on automation. Many of the available tests are labor intensive, with much of the labor devoted to tedious sample processing methods. Several fully automated systems for molecular diagnostics have been developed for high- and midvolume laboratories, but most suffer from a limited test menu. To increase access to molecular tests, simple, affordable, fully automated, random access platforms with broad test menus are needed, particularly for laboratories that have a low- and midvolume of testing. NA testing for infectious diseases at the point of care is beginning to enter clinical practice in developed and developing countries, particularly for applications that require short TATs and in settings where a centralized laboratory approach is not feasible.[275]

The use of multiplex NA-based assays to screen at-risk patients for panels of probable pathogens remains a goal for molecular microbiology. Several such tests are currently available, but success to date has been limited by technical complexity of some systems. The development of simple, multiparametric technologies is key to providing molecular tests with the same broad diagnostic range provided by culture and other conventional methods for syndromic diagnosis of infectious diseases.

Metagenomic studies have provided new insights into the human microbiome and alterations in these communities of microorganisms have been linked to a number of disease states. With the continued decrease in the cost of massively parallel NA sequencing and the increasing availability of the necessary bioinformatics tools, it is likely that our understanding of the human microbiome will result in novel microbiome-focused diagnostics and clinical interventions. In addition, the massively parallel sequencing platforms that have enabled metagenomics will be increasingly used in epidemiological investigations[276,277] and new pathogen discovery.[278,279]

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