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Recent Advances in Diagnostic Microbiology

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The past decade has seen a surge in the development of a variety of molecular diagnostics designed to rapidly identify or characterize medically important microorganisms. We briefly review important advances in molecular microbiology, and then discuss specific assays that have been implemented in clinical microbiology laboratories throughout the country. We also discuss emerging methods and technologies that will soon be more widely used for the prompt and accurate detection of the agents of infectious diseases.
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Clinical microbiology has entered a new era of diagnostic testing, in which molecular tests have transcended the confines of the basic science research laboratory and become important tools in the clinical laboratory. Traditional and cumbersome polymerase chain reaction (PCR) methods have given way to simpler, faster, and less costly techniques that can be used by smaller clinical laboratories that lack extensive training in molecular methods. The past decade has seen a surge in the development of various molecular diagnostics to rapidly identify or characterize infectious microorganisms, with each test designed to improve sensitivity, specificity, and/or turnaround times of conventional methods. A significant proportion of the recent developments described below are molecular tests. For this reason, a brief overview of molecular microbiology is provided, followed by a discussion of the most important advances in diagnostic testing that have been employed by key clinical microbiology laboratories throughout the country.

MOLECULAR MICROBIOLOGY

Molecular microbiology can be divided into three main categories: signal amplification, rapid cycle nucleic acid amplification, and a variety of methods of post-amplification analysis.

Signal amplification consists of the generation of a signal, usually in the form of light or color, which is formed when hybridization occurs between a probe and its complementary nucleic acid sequence ("the target"). Some of the first molecular assays to be commonly used in clinical microbiology laboratories consisted of oligonucleotide acid probes used for the direct detection of bacteria, mycobacteria, and fungi.[1]

In situ hybridization is another type of signal amplification reaction that uses the human eye as the complex detection device. When a fluorophore-labeled oligonucleotide probe is used to hybridize to its target sequence and the fluorescent signal is observed using a fluorescent microscope, the procedure is fluorescent in situ hybridization (FISH). Although in situ hybridization techniques have been used for many years in anatomic pathology and research laboratories, they are relatively new to the clinical microbiology laboratory.

A number of rapid cycle nucleic acid amplification assays are available for the rapid detection, differentiation, and, if needed, quantitation of microorganisms in clinical specimens. Most of them use PCR as the base chemistry, but other forms of nucleic acid amplification are also available in a rapid cycle or "real-time" format. Traditional PCR, which in the distant past took days to accomplish, has often been supplanted by faster and more user-friendly rapid cycle or real-time PCR methods. These assays are performed in a closed system, in which both amplification and detection occur within the same reaction vessel. Opening of the vessel and further manipulation of the amplicon are unnecessary, which greatly minimizes the risk of amplicon contamination of the laboratory and significantly diminishes the possibility of subsequent false positive amplification results. Although many of these are laboratory-designed and verified assays, several have been submitted and approved by the US Food and Drug Administration (FDA), and many more are under development or in clinical trials.

Monoplex PCR refers to the detection of a single target sequence, and this method is most commonly used in a qualitative manner, wherein the expected result is the presence or absence of a microorganism in

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a clinical specimen. For example, one may use this type of approach for the detection of *Mycobacterium tuberculosis, Legionella pneumophila,* or *Pneumocystis jiroveci* from respiratory specimens.[2-4] The technique is most advantageous for the rapid diagnosis of serious infections with microorganisms that are slow-growing, fastidious, or that cannot be cultivated in the routine laboratory. When multiple organisms cause similar or identical clinical manifestations, or an internal amplification control is desired, then a multiplex PCR reaction is needed (that is, two or more nucleic acid amplifications are performed in one assay). Multiplex assays that address the most likely causes of a particular disease (for example, community-acquired pneumonia or diarrhea) would be the most helpful and cost-effective way to diagnose the responsible pathogen in many instances. Multiplex assays have recently been developed for the viruses that cause meningitis, and two FDA-approved assays are available for the rapid detection of common respiratory viruses.[5,6]

Post-amplification analysis represents the third category in which there has been, and we predict will continue to be, advances in diagnostic molecular microbiology. Post-amplification analysis consists of a variety of methods that involve additional amplicon processing or analysis, in order to obtain more information than is available from the amplification reaction alone. Post-amplification analysis allows more accurate identification or characterization of a microorganism, often to the species level. Methods include melt curve analysis, reverse hybridization, DNA sequencing, and microarray analysis. Melt curve analysis consists of differentiating amplified DNA products after real-time PCR based on the probe dissociation characteristics. In brief, amplicons from a PCR reaction that are detected as fluorescently labeled oligonucleotide probes that are not consumed by the reaction (such as FRET or Eclipse probes) may yield additional information based on melt curve analysis. Oligonucleotide probes that hybridize to their target sequence with 100% complementarity will have a particular dissociation or melt curve that may be analyzed by heating the reaction mixture while monitoring fluorescence. If any nucleotide polymorphisms exist at the probe hybridization site, then the melt curve will be shifted. There have been amplification assays and probes designed that will afford microorganism differentiation through post-amplification melt curve analysis. This type of analysis has been used to detect and differentiate *M tuberculosis* from nontuberculous mycobacteria (Figure 1), and to distinguish the subtypes of herpes simplex virus (HSV). Whiley et al described an assay that detected both the BK and JC polyomaviruses but differentiated them from one another based on the unique melt curves generated.[7] Post-amplification melt curve analysis may also be accomplished using nonspecific DNA binders like SYBR green, or more recently with newer modifications of these chemistries that afford high-resolution melt curve analysis without the need for an oligonucleotide probe.

Reverse hybridization is a simple-to-perform method for detecting a variety of pathogens following either a multiplex or a broad-range amplification reaction. In brief, the amplicon is labeled and then applied to a nitrocellulose membrane strip that contains the probes for the microorganisms of interest; the amplicon will hybridize to the location on the nitrocellulose strip of the complementary probe. Thereafter, a chemical reaction demonstrates the location of the amplicon, and

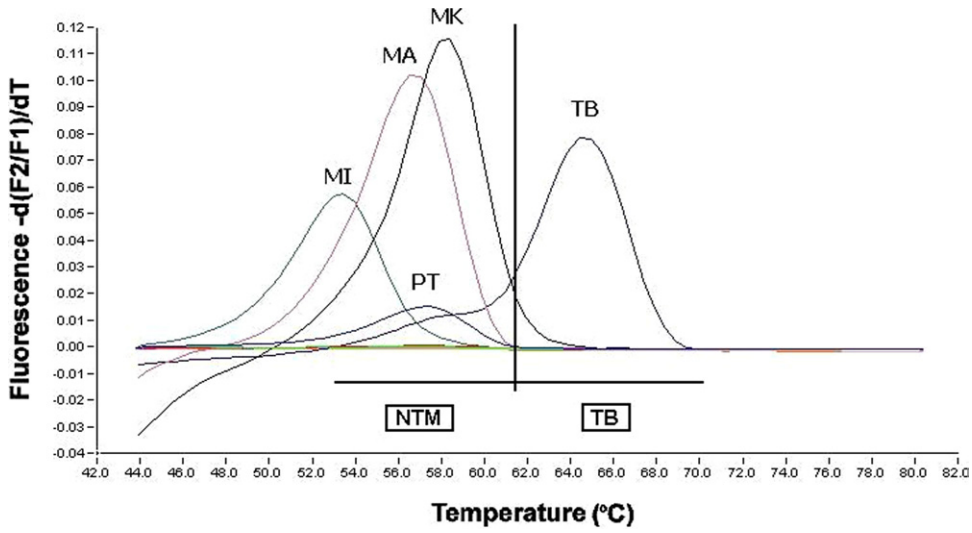

Figure 1. The post-amplification melt curve analysis of the broad-range mycobacterial PCR from formalin-fixed, paraffin-embedded tissue demonstrates that this patient (PT) has an infection caused by a nontuberculous mycobacteria (NTM). A post-amplification melt curve threshold is designated by the line between *M tuberculosis* (TB) and the NTM tested: *M kansasii* (MK), *M avium* (MA), and *M intracellulare* (MI). The cultures from this patient grew *M avium.*

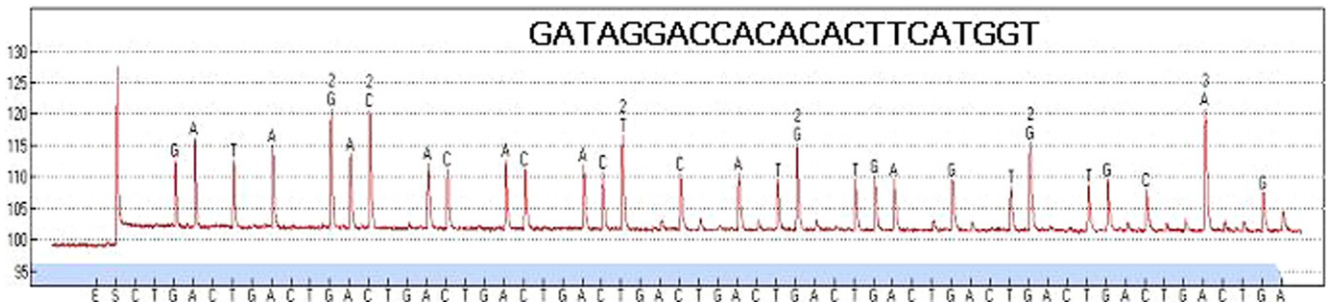

Figure 2. Pyrosequencing may be used after broad-range mycobacterial PCR to identify the most common types of mycobacteria. This sequence was used to characterize the mycobacteria present as a member of the *M chelonae/M abscessus* complex.

the identification of the microorganism of interest is thereby obtained. This technology has been used to detect common and unusual mycobacteria, human immunodeficiency virus (HIV) and hepatitis C virus (HCV) genotypes, and more frequent causes of fungal infections.[8-11]

DNA sequencing likewise has important uses in diagnostic microbiology, including identification and characterization of organisms to the species, as well as strain level differentiation (eg, multilocus sequence typing [MLST]); it may also be used to detect resistance-associated mutations from bacteria, fungi, and viruses. This method of post-amplification analysis consists of determining the nucleic acid sequence of the amplicon, usually through either Sanger or pyrosequencing (Figure 2). Once a sequence of an unidentified microorganism is determined, it is queried against a genetic database to find a match. DNA sequencing is becoming, if it is not already recognized as such, the method of choice for identifying *Mycobacterium* sp., *Nocardia,* and other aerobic actinomycetes.[12,13]

Microarray technology, which has been used for discovery by researchers for many years, has come to the clinical laboratory. Research tools, which have the capability to detect thousands of reactions simultaneously, have been pared down to address complex diagnostic problems that usually have one of numerous possible answers. These have been used to differentiate numerous microbial pathogens within a group, as well as to identify a large variety of genetic determinants of drug resistance. FDA-approved microarray-based assays are currently available for the genetic characterization of patients with cystic fibrosis, and for the detection and differentiation of respiratory viruses, discussed in more detail below.

There have been many advances in traditional and molecular microbiology within the past few years, and there are many more on the horizon. What follows is a brief review of some of the assays that likely will have an impact for patients with hematologic disorders who are at risk for opportunistic infections.

BACTERIA AND YEAST

Rapid Cycle PCR

Staphylococcus aureus and Methicillin Resistance

Staphylococcus aureus is one of the most common virulent bacterial pathogens, and it is responsible for a significant proportion of nosocomial infections. Two molecular tests have received FDA-approval for *S aureus* detection: the BD GeneOhm IDI-MRSA/StaphSR (BD, Sparks, MD) assay and the GeneXpert MRSA (Cepheid, Sunnyvale, CA). Both IDI-MRSA and GeneXpert have been approved for methicillin-resistant *S aureus* (MRSA) screening from nasal swabs, whereas StaphSR, which detects and differentiates MRSA and methicillin-susceptible *S aureus* (MSSA), has been cleared for use on positive blood cultures.

IDI-MRSA is a qualitative multiplex real-time PCR assay that is MRSA-specific (that is, the target encompasses sequences within *SCCmec*—the mobile genetic element that contains the *mecA* gene—and *orfX*).[14] The ability to detect the *mecA* gene that is associated with *S aureus* but not with coagulase-negative staphylococci is advantageous when testing clinical specimens, particularly nasal swabs containing microbiota that may include coagulase-negative staphylococci. An internal amplification control is included in order to detect inhibition.

The BD GeneOhm StaphSR test also is a multiplex real-time PCR assay, consisting of the same targets for MRSA as has IDI-MRSA but with the addition of primers that amplify a target sequence specific for *S aureus.*[15] This modification affords the differentiation of MSSA and MRSA within 2 hours after a blood culture bottle signals positive. In a study of 300 blood cultures positive for gram-positive cocci, Stamper et al found the *S aureus* PCR component of the assay 98.9% sensitive and 96.7% specific compared to bacterial culture, whereas the MRSA component was 100% sensitive and 98.4% specific compared to culture and susceptibility testing.[15] Overall negative predictive value (NPV) and

positive predictive value (PPV) were 99.5% and 93.6%, respectively.

The GeneXpert MRSA test is another FDA-approved molecular test for MRSA screening from nasal swabs. The GeneXpert real-time PCR platform is a fully automated, walk-away system that requires minimal expertise and hands-on time. The total assay time is approximately 75 minutes. Although it is clearly the leading technology with respect to ease-of-use, it is more expensive at $35-$55 per test. A comparison between the GeneXpert MRSA and IDI-MRSA tests showed comparable sensitivity and specificity for MRSA detection from nasal swabs[16] The limits of detection of this test from nasal specimens was found to be only 58 colony-forming units (CFU) per swab compared to direct culture at 171 CFU per swab.[17]

There are several validated assays for *S aureus* and the *mecA* gene. We have used the *sa442* gene target for many years to detect *S aureus*[18-20]; this may be multiplexed with a *mecA* gene PCR, or the *mecA* gene PCR may be performed separately. Although these assays have proven to be highly sensitive and specific for detecting *S aureus* and the *mecA* gene, limitations include the inability to distinguish a mixture of MSSA and methicillin-resistant coagulase negative staphylococci (MRSE) (the *sa442* PCR would be positive and the *mecA* PCR would detect the *mecA* gene in the MRSE, and this could be interpreted as MRSA).

Clostridium difficile

The cell cytotoxicity assay is presently considered the standard for *Clostridium difficile* testing.[21] However, rapid enzyme immunoassay (EIA) testing has been used more commonly in clinical laboratories, due to its faster turnaround time and ease-of-use. The EIA tests vary widely in sensitivity (50%-99%) and specificity (70%-100%), depending on the study and the reference standard.[22] Ticehurst et al showed the sensitivity of one EIA to be as low as 36% when they compared this test to the two-step diagnostic algorithm for *Clostridium difficile*–associated disease (CDAD) consisting of a screening glutamate dehydrogenase (GDH) by solid-phase EIA (C diff Check-60; Techlab, Blacksburg, VA), followed by a confirmatory cell culture cytotoxicity neutralization assay (CCNA) for those specimens that test positive with the GDH assay. Sensitivity for screening GDH EIA was 96% with a negative predictive value of >99%. This approach rapidly screened out the negative specimens (75%-80% of samples); confirmatory testing was then performed on the remaining 20% by CCNA. Negative results could be reported on the same day, whereas the GDH-positive specimens had to be incubated for another 2 days before CCNA results could be finalized.[23] Gilligan also found a low sensitivity of 59.5% for the Premier (Meridian Biosciences, Cincinnati, OH) toxin A and B EIA compared to the two-step diagnostic algorithm. The use of the screening GDH followed by CCNA enhanced the detection of *C difficile*–positive specimens by 40% in this study.[24]

Although the GDH EIA test is easy to perform and gives rapid results, the CCNA portion of the assay is labor-intensive, requiring both filtration and centrifugation steps, and takes 18 to 48 hours of incubation before results can be finalized. Several laboratory-validated real-time PCR assays have subsequently been developed that demonstrate superior sensitivity compared to EIA; these also have a fast turnaround time and are easy to use.[25-27] In a prospective multicenter study, EIA, an enzyme-linked fluorescent assay, a laboratory-verified real time-PCR assay targeting the *tcdB* gene, and CCNA were compared; PCR had the highest concordance with CCNA (the reference standard) at 71.4% and was recommended as the preferred method for diagnosing CDAD.[25] In another study, Peterson and colleagues, using clinical criteria as part of their reference standard, demonstrated that a real-time PCR was significantly more sensitive than EIA (sensitivity 73.3%) with sensitivity, specificity, PPV, and NPV of 93.3%, 97.4% 75.7%, and 99.4%, respectively.[22]

Most recently, the FDA has approved a real-time PCR assay for CDAD detection, the BD GeneOhm Cdiff Assay (BD GeneOhm, San Diego, CA), which amplifies a highly conserved region of the *tcdB* gene. This assay was compared to CCNA (Wampole C difficile Toxin B Test [TOX-B]; Techlab) and to toxigenic culture.[28] The overall agreement was high (94.8%) between the PCR assay and CCNA. Sensitivity and specificity of the BD assay were 83.6% and 98.2%, respectively, compared to toxigenic culture. Real time-PCR proved to be more sensitive than CCNA, which had 67.2% sensitivity and 99.1% specificity in detecting *C difficile* from fecal specimens. We believe these new testing algorithms and new approaches to *C difficile* testing represent significant advances in clinical microbiology. In the future, clinicians will likely be offered tests that will more reliably help them to confirm or exclude enteritis caused by *C difficile.*

Chromogenic Media

Chromogenic media are rapid culture-based tests that can provide an alternative to molecular testing in terms of cost, need for technical expertise, and equipment. They have been used for the detection of MRSA, *Enterococcus*, group B streptococcus, and *Candida* species.[29,30] These selective media contain a proprietary colorless chromogenic substrate(s) that is cleaved by enzymes produced by the microorganism of interest. This chemical reaction results in a product that is colored, which thereby imparts a color to the entire colony as it grows on the medium. Additional selectivity may be obtained through the incorporation of antibiotics. Direct identification of the organism from pri-

mary culture is thus possible within 24 hours in some instances, but some products require 48 hours of incubation if the color is not present or is not fully developed.

ChromID (bioMerieux, Marcy l'Etoile, France) is a selective, chromogenic medium for *S aureus* that targets the alpha-glucosidase enzyme and incorporates cefoxitin (4 mg/L). The growth of MSSA is inhibited by the cefoxitin, whereas the MRSA grows as green-colored colonies. CHROMagar MRSA (CHROMagar Microbiology, Paris, France; BD Diagnostics, Erembodegem, Belgium) also contains cefoxitin but has a different chromogenic reaction that yields rose to mauve MRSA colonies. The sensitivities of these assays vary somewhat between media and incubation, but the specificities are uniformly high for 24 hours of incubation.[16,31-33] A comparison of two chromogenic media, MRSA ID (bioMérieux, La Balme et Craponne, France) and CHROMagar MRSA, with oxacillin screening agar performed on swabs submitted for nasal screening, demonstrated sensitivities of 77% for MRSA ID and 73% for CHROMagar MRSA at 24 hours. Specificity was 98% for both media at 24 hours, but declined to 94% for MRSA ID and 90% for CHROMagar MRSA at 48 hours.[34] The bacteria that caused false positives on the MRSA ID included *Enterobacter* sp. and coagulase-negative staphylococci (CoNS), whereas on CHROMagar MRSA they were streptococci, CoNS, and *Corynebacterium* spp. In general, an extended incubation for these chromogenic media results in increased sensitivity but decreased specificity at >24 hours for MRSA detection. Hence, some authors recommend additional confirmatory testing in the form of Gram stain and latex agglutination tests to improve specificities, especially if incubation times >24 hours are used.[16,34] It is also important to note that if this chromogenic media alone is used for MRSA screening, as many as 18% of the MRSA-colonized patients may be missed compared to real-time PCR.[35]

Chromogenic media are relatively new methods that afford the rapid detection of select isolates in a culture-based format. It suffers from many of the limitations of culture and may not be as sensitive as nucleic acid amplification, but the assay also has many of the benefits of culture (for example, the organisms are available for subsequent testing, such as susceptibility testing or typing). These products provide options to microbiology laboratories that are not yet ready to offer molecular diagnostic testing.

Peptide Nucleic Acid FISH

As briefly described above, traditional FISH uses fluorophore-labeled oligonucleotide probes that bind to genetic targets within the cell. Peptide nucleic acid probes (PNA) are used in the same manner, but they are chemically unique and believed by some to have advantages over DNA oligonucleotide probes with respect to penetration across the intact cell walls and cell membranes of microorganisms. There are several commercially available PNA FISH assays for use on blood culture bottles that signal positive and have a particular organism morphotype present. Test results are obtained within approximately 2.5 hours.[36-38] These assays have been adapted by several clinical laboratories in the United States, and a number of publications have been generated that demonstrate both feasibility and improved clinical outcome.[39-41] One of the most popular assays available tests for *S aureus* when gram-positive cocci in clusters are present in the blood cultures. A positive appears as multiple clusters of brightly fluorescent green cocci, signifying the presence of *S aureus*. Non–*S aureus* cocci in clusters are nonfluorescent. The sensitivity and specificity of this assay compared to culture were found to be 100% and 99.2%, respectively.[42] A newer version contains probes for both *S aureus* and coagulase-negative staphylococci, wherein the *S aureus* is fluorescent green and the CoNS is red. Traditional or molecular susceptibility testing is, however, required to determine if the isolate is an MRSA.

There is also a two-color PNA FISH assay that can be used to rapidly identify *Enterococcus faecalis*, which can be treated with ampicillin, and differentiated from other enterococci that are more likely to be resistant to this drug as well as to vancomycin.[43] The *Candida albicans* PNA FISH assay (Figure 3) was designed for similar reasons, since *C albicans* isolates are usually susceptible to fluconazole unless the patient has had significant exposure to this drug. Two additional yeast PNA assays have been approved for use by the FDA. There is a dual probe assay that detects both *C albicans*

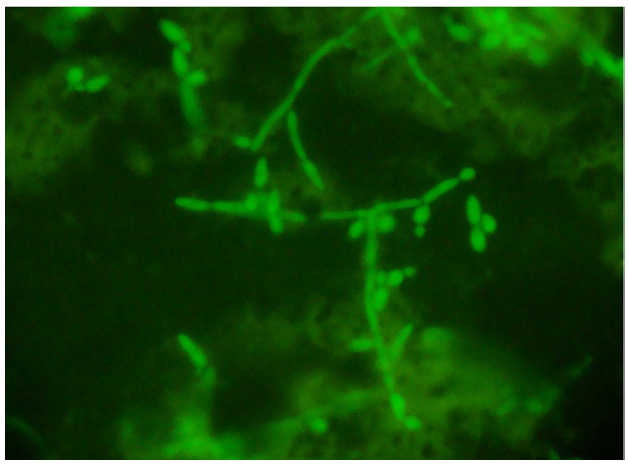

Figure 3. The *Candida albicans* in this positive blood culture demonstrates a green fluorescence following hybridization with the *C albicans* PNA FISH probe (AdvanDx, Wolburn, MA). Fluorescence microscopy; original magnification 500x.

(green) and *Candida glabrata* (red).[41,44] Interestingly, the most recently released assay does not definitely identify the yeasts, but rather highlights species that will likely be susceptible to fluconazole (green and yellow) and those that either may not be or are innately resistant (red).[45] In this assay, *C albicans* and *Candida parapsilosis* are highlighted green, *Candida tropicalis* is highlighted yellow, and *C glabrata* and *Candida krusei* are highlighted red. These assays produce rapid identification or differentiation that may be used to guide antifungal therapy.

MYCOBACTERIA, *NOCARDIA,* AND OTHER AEROBIC ACTINOMYCETES

Direct Detection

Immunocompromised patients are at increased risk for disease caused by mycobacteria, *Nocardia,* and aerobic actinomycetes. The rapid determination of the presence or absence of *M tuberculosis* from a respiratory specimen is critical from both clinical and public health perspectives. Traditional methods are time-consuming and labor-intensive. A modification of culture methods, the microscopic-observation drug-susceptibility (MODS) assay, was recently described: broth cultures are performed in multiple wells and viewed daily under light microscopy at 40x magnification.[46] The presence of cording is considered diagnostic for the presence of *M tuberculosis* in this assay. The sensitivity was 97.8% for MODS culture, 89% for automated mycobacterial culture, and 84% for culture using Lowenstein-Jensen medium. Importantly, the median times to culture positivity were 7 days, 13 days, and 26 days, respectively. When prospectively evaluated in Brazil and Honduras, this assay was proven to be suitable for these resource-limited areas that perform high-volume testing.[47] The method is, however, quite labor-intensive and time-consuming for laboratory personnel.

There are two FDA-approved nucleic acid amplification tests available for the rapid detection of *M tuberculosis* in respiratory specimens: the Cobas Amplicor *M tuberculosis* assay (Amplicor; Roche Diagnostic Systems, Branchburg, NJ) and the Amplified *M tuberculosis* direct test (AMTD; bioMérieux, Gen-probe, Inc, San Diego, CA). Both of these tests are approved for use on smear-positive samples. An enhanced version of the AMTD test, the AMTD2, had been approved for use on smear-positive cases by the FDA in 1999. The Cobas Amplicor assay has a sensitivity for smear-positive specimens of 87.5%-100%; sensitivity is much lower for smear-negative specimens (17.2%-70.8%).[48] Specificity ranges from 91.3%-100%. Tuberculosis cannot be excluded by a negative test, but positive assays are useful.[49,50] Finally, multiple laboratory verified PCR assays have been described, which are used throughout the world.[51-53] A real-time PCR assay by Buggraf et al, for example, had excellent sensitivity (100%) and specificity (98.6%) compared to the Cobas Amplicor assay.[54] Clinicians should be aware that both FDA-approved and laboratory-verified assays are available, which can be used to rapidly detect *M tuberculosis*, particularly in patients with smear-positive respiratory specimens.

Identification of Mycobacteria From Culture

DNA probe technology is a rapid method that has been used for many years by clinical mycobacteriologists for the rapid identification of select *Mycobacterium* species. These widely used assays are commercially available (AccuProbe; Gen-Probe, San Diego, CA). Assays are suitable for the identification of *M tuberculosis complex, Mycobacterium avium complex, Mycobacterium intracellulare, Mycobacterium avium* and *Mycobacterium intracellularae* combined, *Mycobacterium kansasii,* and *Mycobacterium gordonae.* Although these assays are not new to the laboratory, they provide an easy-to use and highly reliable technique for the rapid identification of these medically important mycobacteria.

Reverse hybridization (such as line probe technology) has also been shown to be useful for the detection of common and less frequently encountered mycobacteria. In this method, biotinylated PCR products are afforded the opportunity to hybridize to corresponding species-specific probes that have been immobilized on a nitrocellulose strip. This reaction forms a banding pattern on the strip that is diagnostic of the *Mycobacterium* species present. Currently, there are two commercially available assays: the InnoLiPA Mycobacteria (Innogenetics NV, Ghent, Belgium) and the Genotype Mycobacterium (Hain Lifescience, Nehren, Germany). These assays are easy to perform and can rapidly and accurately detect as many as 16 mycobacterial species in a single strip. Their cost has limited the routine use of these otherwise excellent products in some laboratories.

DNA sequencing following PCR is currently the cornerstone for molecular-based identification of mycobacteria and related organisms. Where conventional methods of morphology and biochemical testing can require weeks and may still yield ambiguous results, DNA sequencing can provide rapid and more precise identification. This method has led to the discovery of numerous new mycobacterial species, many of which are difficult or impossible to differentiate using phenotypic methods alone. The most commonly used targets for amplification are the 16S rRNA , 65-kd heat shock protein, the 16S-23S rRNA internal transcribed spacer, and the *recA* gene.[55] The sequence obtained is compared to a database such as GenBank, the Ribosomal Differentiation of Medical Microsystems database (RIDOM), or others.

DNA sequencing has also become the preferred method for accurate identification of *Nocardia* species.

Many of these species, although genetically distinct, have similar physical and biochemical properties that make them difficult to distinguish by phenotype alone.[13] Additionally, other aerobic actinomycetes, such as *Rhodococcus, Gordonia,* and *Tsukamurella,* which are also challenging to identify using traditional methods, may be readily identified by DNA sequencing.[56]

In contrast to traditional Sanger sequencing, pyrosequencing is a novel method of DNA sequencing. This method is also known as sequencing by synthesis, since the sequence of DNA is determined based on the incorporation of each nucleotide as the strand of DNA is being synthesized. We and others have used this pyrosequencing to sequence select variable regions of the genome that contain signatures for the identification of mycobacteria, *Nocardia,* and select aerobic actinomycetes.[12,13,57]

VIRUSES

Respiratory Viruses

Respiratory viruses such as influenza A and B, respiratory syncytial virus (RSV), and parainfluenza 1, 2, and 3 cause significant morbidity and mortality among the very young, the elderly, and within the immunocompromised population.[58] Until recently, standard diagnostic methods consisted solely of culture and rapid immunochromatographic antigen and immunofluorescent tests. These are now slowly being replaced by molecular diagnostics.

Although direct immunofluorescent assays (DFA) and culture are highly sensitive and specific, they are laborious and time-consuming. Multiple studies have shown increased sensitivity of PCR over both DFA and culture.[59-62] Multiplex rapid cycle PCR detects multiple organisms simultaneously from a specimen in a single assay. The ProFlu-1 real-time assay (Prodesse, Waukesha, WI) was approved by the FDA in 2008 for the simultaneous detection of influenza A/B and RSV A/B from nasopharyngeal specimens. This one-step multiplex reverse transcription (RT)-PCR takes only 3 hours to perform.[63] In comparison to immunofluorescence testing and cell culture, the sensitivities of this assay for influenza A, influenza B, and RSV were 100%, 100%, and 97.8%, respectively.[63] Specificity was found to be 100% overall when discrepant results (PCR-positive, culture-negative) were resolved using laboratory-verified assays that target different genes than those targeted by the ProFlu-1 assay. Based on this analysis, conventional methods missed 8.7% of influenza A, 26.7% of influenza B, and 2.7% of RSV infections.

Another FDA-approved molecular test for respiratory viruses is the Luminex xTAG RVP Assay (Luminex Molecular Diagnostics, Toronto, Ontario, Canada). This assay detects 20 distinct respiratory viruses and subtypes for three of these viruses in a single reaction and has the ability to detect influenza A (H1, H3, and H5) and influenza B, RSV A and B, parainfluenza 1, 2, 3, and 4, rhinovirus, adenovirus, coronavirus (SARS, NL63, 229E, OC43, HKU), and human metapneumovirus; the SARS coronavirus and avian influenza, H5N1, are included in the assay distributed in Canada. By contrast, current culture and DFA have the ability to detect only six or seven conventional respiratory viruses. In a prospective study comparing the RVP assay and DFA/culture using 294 nasopharyngeal swab specimens, the RVP test detected 134 of 137 true positives (97.8% sensitivity), whereas the latter detected only 126 of 137 (91.9% sensitivity). In addition, the RVP assay detected a virus or viruses in 47 other specimens where a virus had not been detected by DFA and culture. These results showed greatly increased sensitivity of this multiplex test compared to conventional methods.[64]

Cytomegalovirus, Epstein-Barr Virus, and BK Virus

The monitoring of the cytomegalovirus (CMV) viral load in blood is essential in both treatment and preemptive therapy of CMV infection, especially in solid organ transplant recipients. Several quantitative real-time PCR assays have been developed, as well as a commercially available PCR-EIA assay by Roche Diagnostics, the Cobas Amplicor CMV Monitor. The newer rapid cycle PCR assays in general show increased sensitivity over the CMV antigenemia assay for early detection of CMV infection/reactivation, the benefit of stability of target DNA in blood specimens, better performance in neutropenic patients, greater accuracy and precision, and increased automation for testing of large number of specimens.[65] Viral load monitoring is rapidly becoming, or is already considered to be, standard of care for the monitoring of patients at risk for CMV disease.

Similarly, quantitative assays are commonly used for Epstein-Barr virus (EBV) and the BK polyoma virus. Quantitative EBV is used to monitor the load of this virus in the blood of patients at risk for EBV-associated post-transplant lymphoproliferative disorder. Quantitative BK viral load assays are monitored in renal allograft recipients who are known to be infected with the BK virus, in an effort to avoid BK nephropathy. These assays, although not currently FDA-approved, will continue to be used to periodically assess patients at risk for EBV and BK disease. Likely FDA-approved products will be available for testing within the next 1-2 years.

Enterovirus

Enteroviruses are the most common cause of aseptic meningitis in both children and adults. Similar to the GeneXpert platform for rapidly testing *S aureus* described earlier, the same manufacturer offers a fully

automated real-time multiplex RT-PCR assay for enteroviruses. This FDA-approved assay affords the qualitative detection of enterovirus RNA from cerobrospinal fluid-specimens. In a prospective 1-year study by Seme et al, the GeneXpert Dx system (Cepheid, Sunnyvale, CA) showed excellent agreement (98.1%) with two other PCR-based assays for detection of enterovirus RNA from 162 CSF samples.[66]

FUNGI

Yeasts and Filamentous Fungi

Some of the methods to rapidly identify yeasts in clinical specimens, particularly blood cultures, have been described above. Both these fungi and filamentous fungi that are difficult to identify may be characterized by broad range PCR and DNA sequencing. This type of testing is available in research settings and at large reference laboratories, but significant limitations exist, one of the main being the reliability of the existing databases. While significant work needs to be done in database construction, DNA sequencing will likely continue to be employed and potentially used more frequently for the identification of fungi that are difficult to identify by traditional methods.

There are also numerous opportunities for advanced molecular diagnostics for the detection of opportunistic fungal infections in the immunocompromised host. Currently, antigen-based assays and nucleic acid-based tests are being explored. The antigen-based assays, which are better standardized and commercially available, include the galactomannan and the beta-glucan assay. These tests allow the clinician to follow antigenic trends in the blood of patients at risk for invasive fungal infections. Similarly, it has been postulated that fungal DNA loads, the quantified resultant product of a broad-range fungal PCR, could be similarly monitored to follow patients at risk for opportunistic fungal infections. A potential advantage of this approach is that post-amplification analysis may be used to discern the identity of the infecting fungus, with obvious therapeutic implications. Time and well-controlled studies will help to determine which of these methods will be most useful to monitor immunocompromised hosts for early evidence of an opportunistic fungal infection.

Pneumocystis jiroveci

Pneumonia caused by *Pneumocystis jiroveci* (PCP) is an important disease in immunocompromised hosts. The current standard of diagnosis is direct identification using direct microscopy since PCP cannot be cultured in the routine laboratory.[67] A number of staining techniques have been used, each with strengths and limitations.[68] Real-time PCR for the detection of *P jiroveci* has many potential advantages over morphologic detection by direct microscopy: rapid turnaround, greater sensitivity, and accuracy. The quantitative aspects of rapid cycle PCR also afford the setting of thresholds for detection and the monitoring of patients undergoing therapy. Several laboratory verified real-time PCR assays have been described and have been shown to be highly sensitive and specific compared to DFA.[4,69,70] In our laboratory, PCR has been shown to be superior to toluidine blue staining and comparable to toluidine blue staining, cytopathology, and surgical pathology combined.[71]

SUMMARY

The past decade has witnessed advances in the rapid detection, identification, and characterization of infectious microorganisms. Although there have been some improvements in culture-based assays such as chromogenic media for *S aureus* and the MODS assay for *M tuberculosis*, the majority of the vanguard work has been related to molecular diagnostic methods. Many of these assays, which were first performed in sophisticated university or reference laboratories only, have been made user-friendly and are now utilized routinely in many clinical laboratories worldwide. Complex molecular diagnostics that use DNA sequencing and microarrays are available, but there are also significant efforts to make such assays easier to use and more reproducible so that they too may be implemented as diagnostic tools in more laboratories. The more widespread implementation of these and similar technologies will undoubtedly improve the laboratory-based diagnosis of infectious diseases.

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