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New Technologies for the Diagnosis of Infection

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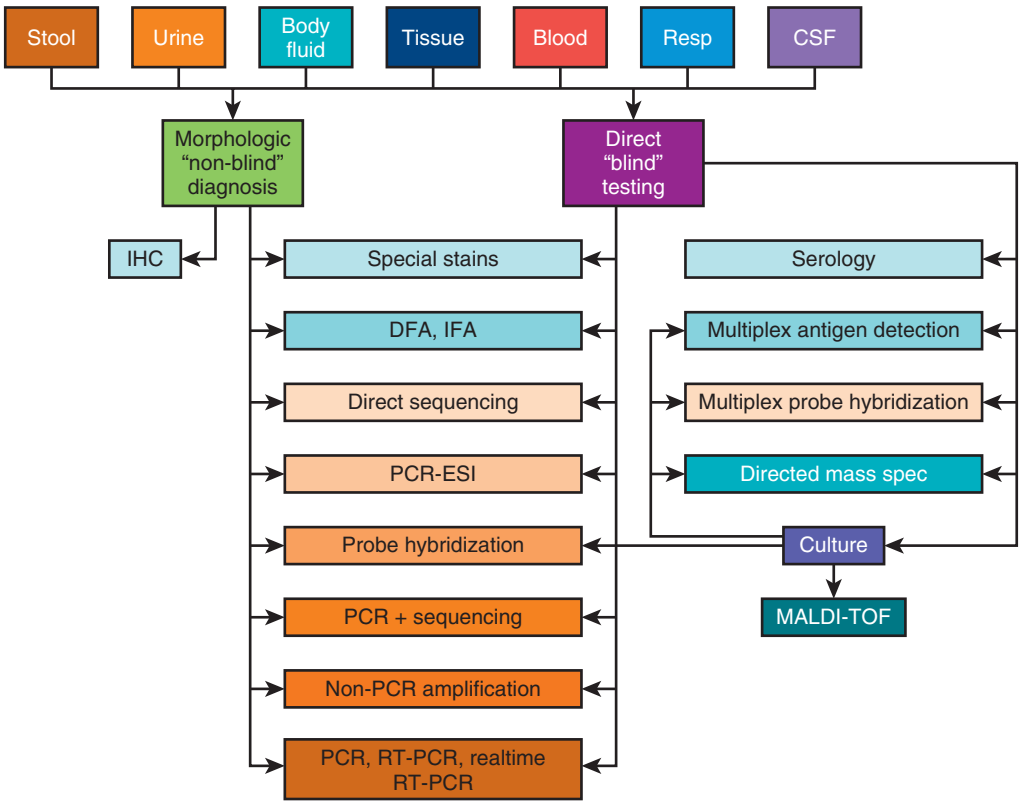
The clinical microbiology laboratory is constantly evolving and progressing. New technologies have revolutionized the characterization and diagnosis of pathogens. The purpose of this chapter is to review the features, benefits, and limitations of some of the key new methodologies in infectious disease diagnostics. The chapter will be organized by technology. New technologies have the most impact in the fields of bacteriology and virology, but there are also promising new developments in mycology and parasitology.

The mainstay of bacterial and mycologic diagnosis continues to be culture by traditional techniques, although increasingly these are automated (Fig. 6.1). After an organism is isolated, however, a host of newer diagnostics have enhanced, and in some cases replaced, the biochemical test algorithm that has historically defined clinical bacteriology. The advent of matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometry is one of the main forces behind this shift, allowing a cultured organism to be identified in minutes instead of hours to days or even weeks in the case of very slow-growing organisms, such as mycobacteria. Although still not in routine clinical use, the application of MALDI-TOF to direct patient samples, such as blood and urine, and applications such as antibiotic resistance are exciting developments. Beyond bacteria, MALDI-TOF is becoming established for the identification of yeasts and, with the development of more extensive and higher quality libraries, is expected to also do so for other fungi.

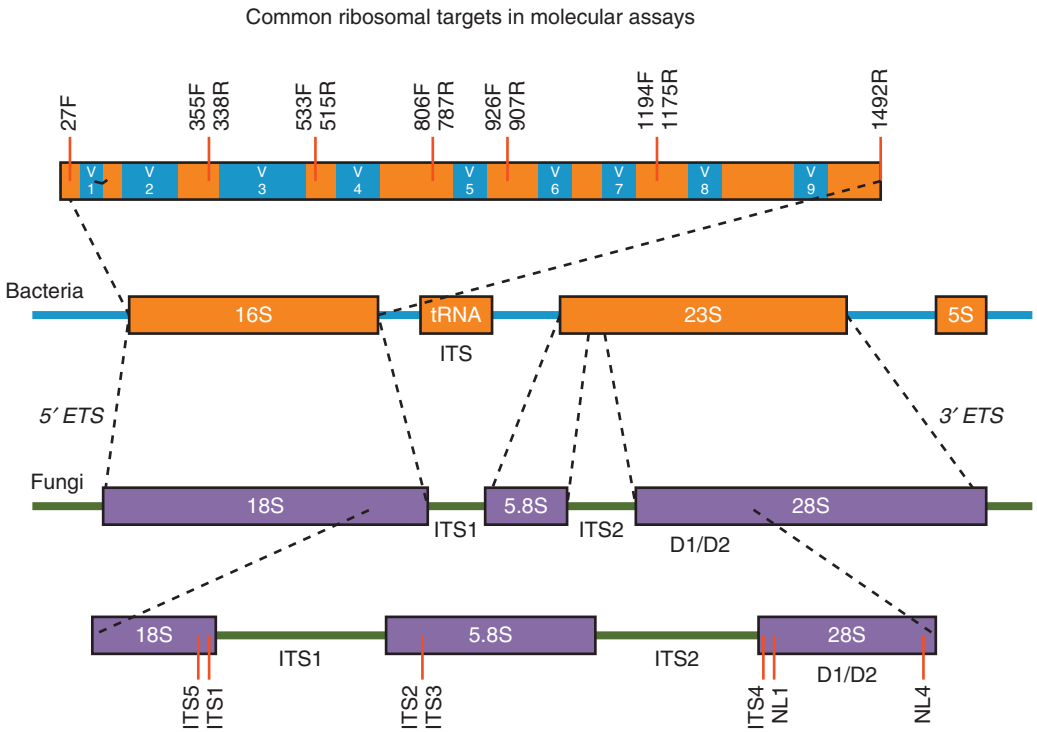
Nucleic acid–based technologies have also had a big impact. In particular, there are a growing number of test platforms that provide rapid diagnostics directly from primary specimens. Many of these are multiplexed to look at several pathogens at one time. The sequencing of the regions of ribosomal RNA (rRNA), such as the 16S rRNA gene in bacteria, already considered the gold standard for bacterial identification, is entering more and more into clinical diagnostics (Fig. 6.2). Similarly, the routine sequencing of the intergenic transcribed spacer (ITS) and 28S rRNA regions in fungi is a welcome addition to current laborious and morphology-heavy techniques for diagnosing fungal illnesses. The entry of next-generation sequencing (NGS) into clinical microbiology has also had a dramatic effect on both the profiling of individual specimens (i.e., members of a potential outbreak or highly drug resistant strains) as well as communities (i.e., microbiome analysis in health and disease). Finally, the emerging technology of polymerase chain reaction (PCR)-electrospray ionization (ESI) mass spectrometry holds great promise for all major phyla of pathogens.

Protein-Based Identification

Antigen-Based Identification uses rapid assessment for the presence of microbial antigens and provides a "point of care" solution for diseases for which immediate answers impact patient care decisions. The principle of all of these assays is that a microbial antigen is present at sufficient quantity for which an antibody has been generated for detection. Common platform types include lateral flow assays (antibodies bound to a matrix through which the patient sample containing antigen passes and results in a colorimetric readout; examples are BinaxNow for Malaria, Remel Xpect for *Clostridium difficile* assays, p24 antigen detection in human immunodeficiency virus [HIV]) and fluorescent immunoassays (similar to lateral flow concept but with automated detection and fluorescently labeled antibodies; examples are Sofia system for bacteria and viruses).[1,2] These assays may be available commercially to consumers or used in microbiology laboratories as inexpensive tools to screen for common diseases (e.g., seasonal influenza, Streptococcal antigens, diarrhea in acutely ill patients). In many laboratories, these rapid tests are confirmed by a second test which may include additional protein testing or nucleic acid



• **Figure 6.1** Algorithm of techniques for diagnosis of pathogens. *CSF,* Cerebrospinal fluid; *DFA,* direct fluorescent antibody; *ESI,* electrospray ionization; *IFA,* indirect fluorescent antibody; *IHC,* immunohistochemistry; *MALDI-TOF,* matrix-assisted laser desorption time of flight; *mass spec,* mass spectrometry; *PCR,* polymerase chain reaction; *resp,* respiratory; *RT,* reverse transcriptase; *RT-PCR,* real-time polymerase chain reaction.



• **Figure 6.2** Common ribosomal targets in molecular assays. *Red lines* indicate commonly used primer binding sites. *ETS,* External transcribed sequence; *ITS,* intergenic transcribed spacer. (Modified from Lafontaine DL, Tollervey D. The function and synthesis of ribosomes. *Nat Rev Mol Cell Biol*. 2001;2:514-20.)

testing. Immunohistochemistry (IHC) in anatomic pathology is based on the same principle as these rapid tests and has a wide range of uses for the detection and sometimes speciation of organisms in formalin-fixed, paraffin-embedded tissue (FFPE) sections. Organisms commonly identified this way include spirochetes, mycobacteria, DNA viruses, *Aspergillus, Candida,* and *Toxoplasma*, although special reference laboratories (e.g., The Infectious Disease Pathology Branch at the Centers for Disease Control and Prevention) have a wide range of antibodies for common to exotic pathogens for tissue confirmation.

MALDI-TOF, a mass spectrometry-based method, allows for a rapid (minutes) assessment of the characteristic complement of ribosomal proteins in an isolate, providing a species-level identification in many cases. After a microbial colony forms, it can be spotted onto a MALDI plate in matrix compound and loaded onto the mass spectrophotometer. This topic has been reviewed in great detail.[3-5] Briefly, a laser is focused onto the sample/matrix spot, causing sublimation, ionization, migration through a time of flight tube, and detection by a mass spectrometer (MS).The readout is a spectrum of peaks representing mass to charge ratios *(M/Z)* of different analytes. The mass range (approximately 2 to 20 kDa) is highly enriched for ribosomal proteins, which make up a large share of the typical MALDI spectra on clinical instruments. Spectra are compared with curated databases and the result is returned along with a metric describing the strength of the identification.

This technology has been widely adopted in Europe and is gradually entering clinical microbiology labs in the United States. There are currently two US Food and Drug Administration (FDA)-approved systems: the Vitek MS (BioMérieux, Durham, North Carolina) and the MALDI Biotyper (Bruker Daltonics Inc., Billerica, Massachusetts). Although the technology behind these two systems is essentially equivalent, they differ in their curated libraries, software, identification algorithms, and scoring systems.[5] For example, the Vitek MS reports isolates with a confidence value of 1% to 100%, whereas the Biotyper returns a score that can range from 0 to 3.000. For most applications a score of 2.000 or greater is necessary for a species level identification. Because of this, it can be difficult to directly compare results between the two systems. There have been several studies using both platforms back to back. One study on a large collection of 642 strains of bacteria, yeasts, and molds demonstrated comparable performance, although noting that the library coverage of the test cohort was greater with the Biotyper (572 strains) than the Vitek MS (406) and that the Vitek was more likely to output a misidentification for strains not represented in the library.[6]

For FDA-approved applications, both the Vitek MS and the Bruker may be used for a large library of aerobic gram-negative and gram-positive organisms, as well as several anaerobes and yeast. In general, performance has been reported to equal or exceed traditional biochemical systems, although it seems to be stronger for gram-negative than gram-positive organisms.[5,7] Lackluster performance, in particular, has been noted for several gram-positive rods. In a study with the Biotyper, the system was unable to identify several isolates of *Nocardia, Tsukamurella, Kocuria,* or *Gordonia*, although 89.7% of *Listeria* and 80% of *Rhodococcus* were correctly classified.[8] Members of *Corynebacteria, Lactobacillus,* and some *Listeria* also seem to present some difficulty, both in terms of difficulty discriminating between species and in requiring a lowered score cutoff (1.7) for species-level identification.[9] In similar studies using the Vitek platform, there were also reported problems in speciation of *Listeria*.[10] The misidentification of a *Kocuria* as a *Corynebacterium* has also been reported.[11] Among both gram-positive and gram-negative organisms, MALDI-TOF has several well-characterized difficulties discriminating certain species and complexes, including *Streptococcus pneumoniae/Streptococcus mitis, Escherichia coli/Shigella,* and members of the *Enterobacter cloacae* complex.[3] Finally, it is important to note that the Biotyper requires a Security-Relevant Library for the identification of such organisms as *Francisella tularensis, Burkholderia pseudomallei,* and *Brucella* spp.[12]

MALDI is rapidly becoming the method of choice for identifying anaerobic bacteria.[13] Several studies using large, diverse collections of anaerobes have demonstrated strong performances by both the BioTyper and the Vitek MS.[5] In one recent multicenter trial bringing together 651 anaerobic isolates for analysis by the Vitek MS 2.0, 91.2% of the isolates were correctly identified to species level.[14] The most notable difficulty was in identifying *Fusobacterium nucleatum* (42.9% identified correctly). The system also showed lower performance for certain species of *Bacteroides, Actinomyces,* and *Prevotella*. Similarly, in a study using the Biotyper platform to identify 197 anaerobes isolated from blood cultures, 86.8% of the strains were correctly identified to the species level (using a score of 2) and 94.9% to the genus level.[15] *Fusobacterium* spp. was again problematic, with only 23% being correctly identified to the species level. With formic acid/ethanol pretreatment, this number increased to 76.9%. In addition, 20.8% of the non-*fragilis Bacteroides* spp. was misidentified as other *Bacteroides*, with high confidence scores, and only 52.9% of the gram-positive anaerobic cocci were correctly identified to the species level. The latter was mostly a function of poor performance on several species of *Peptoniphilus*.[15] Interestingly, in a dedicated study looking at the performance of the Biotyper (2.0) on a collection of 277 isolates of *Bacteroides* spp., 97.5% were correctly identified to the species level.[16]

The identification of yeasts is an FDA-approved application for both the Biotyper and Vitek MS. Both systems have demonstrated strong performance in multiple studies, reviewed by Cassagne et al.[17] One recent study with the Biotyper, for example, used a library of 303 clinical isolates representing a diverse array of yeasts and found an agreement with standard methods in 84.8%.[18] In an additional 26 isolates the Biotyper returned a result that was confirmed as true by sequencing in 21. The Biotyper failed to make identification in an additional 20 isolates. Bader et al. used an even larger cohort of clinical yeast isolates (1192) to compare the performance of traditional methods with the Bruker Biotyper and the Vitek SARAMIS (research use only database). They found an overall agreement between the methods of 95.1% and noted not only that both MALDI methods were able to discriminate species that traditional methods could not, but that the savings in time and money were substantial.[19] More recently, Chao et al. analyzed a collection of 200 clinical yeast isolates with both the Biotyper and the Vitek MS.[20] They found that the Biotyper slightly outperformed the Vitek MS (92.5% vs 79.5% correctly identified to the species or complex level). This compared with rates of 89% and 74% for two common phenotypic methods (Phoenix 100 YBC and the Vitek 2 Yeast ID, respectively). This trend was also observed by Mancini et al., who used a collection of 197 clinical yeast isolates, which included 157 *Candida* or related species and 40 non-*Candida* species. They found that the Biotyper identified 89.8% of isolates, whereas the Vitek MS identified 84.3% using the standard commercially available database. Importantly, the Vitek MS had a much higher rate of misidentification (12.1% vs 1%).[21] In general, it has been found that a formic acid/ethanol

extraction method and lowered identity cutoffs (i.e., 1.7 on the Biotyper) are optimal for the analysis of yeasts.[22]

Although not an FDA-approved application, MALDI has tremendous promise for the identification of mycobacteria and is already the preferred identification method of choice for these organisms in some clinical laboratories.[5] Caveats include the need for enhanced sample preparation methods for efficient cell breakage for biosafety reasons and to ensure high-quality spectra.[3,23,24] Several studies have shown promising results. Using the Biotyper equipped with the mycobacterial library version 1.0 and the Vitek MS, 84.7% to 93.8% of isolates were identified correctly to the species level.[24,25] Bruker has subsequently released a new version of the mycobacterial library, version 3.0, containing 149 different species. It reportedly demonstrated high performance (>95% correct identification to species level) in a group of 1045 clinical samples.[26] Version 3.0 of the Biotyper mycobacterial library was also described by Rodriguez-Sanchez et al., who found 91.7% identification to species level on a group of 109 non-tuberculosis mycobacteria.[27]

There is a great deal of interest in the identification of fungi other than yeast by MALDI, also not an FDA-approved application. Concerns with this group include not only efficient cell lysis but also the need for higher-quality reference databases.[17] Few studies have assessed the Biotyper or Vitek MS platforms on fungal isolates using only the commercially available databases. Iriart et al. analyzed a group of 236 clinical isolates with the Vitek MS (192 yeast and 44 *Aspergillus*) and found that 93.2% of the isolates were correctly identified, including 81.8% of the *Aspergillus*.[28] This compared with 94.1% and 88.6%, respectively, by routine laboratory methods. When the authors limited the study to only those species that were present in the database, 100% of the *Aspergillus* isolates were correctly identified.[28] In another study the Vitek MS was able to correctly discriminate closely related *Aspergillus* species, *Aspergillus fumigatus* and *Aspergillus lentulus*.[29] Schulthess et al. did a prospective study on 200 isolates using the Biotyper/Filamentous Fungi Library 1.0. They achieved a 79% species and 83.5% genus level identification. Particularly poor performance was noted for *Mucor* and *Scopulariposis*.[30] Chen et al. evaluated 50 clinical mold isolates with the Biotyper and found that several were not identified or identified with a low score (<1.7).[31] Twenty-eight isolates of *Penicillium marneffei* were not identified due to the lack of reference spectra in the database; however, the rate of identification went up to 85.7% after a single *P. marneffei* spectrum was added to the database. This result is consistent with a number of studies indicating high performance of MALDI on fungal isolates using custom databases.[32-37]

Although the use of MALDI-TOF in the microbiology lab has been confined, for the most part, to cultured organisms, there have been several studies exploring the feasibility of its use in primary samples. For example, the use of MALDI directly on positive blood cultures is attractive for both its rapidity and the breadth of detection possible with an open system. Sample processing directly from blood is not trivial and not yet FDA approved, but each major platform has a dedicated process and the results are promising with high clinical accuracy (80% to 90%).[3,38,39] Other promising applications include direct analysis of urine and cerebrospinal fluid (CSF), although, as with blood, additional screening and processing steps are required.[3,40-43]

In summary, MALDI-TOF in the clinical microbiology lab has been developed to complement and in many cases replace the traditional biochemical diagnosis of bacteria and yeasts and can shorten turnaround time (TAT) by hours to days (depending on the organism in question).[5,7] Other benefits to MALDI in routine clinical microbiology include low costs of reagents and less production of waste materials (reduced consumables). There are a few shortcomings of this technology. For the most part, MALDI is limited to cultured isolates, so although it offers tremendous TAT advantages it is inherently limited by the time to growth of the organism. Sparse database coverage over certain phyla (e.g., filamentous fungi, some anaerobes) presents an issue in some cases, although commercially available libraries continue to be expanded. Another major caveat of MALDI is that it is not yet a practical method for assessing antibiotic resistance. Direct detection of resistance-conferring enzymes is not practical within the assay's limits of detection; however, some promising studies have been published looking at the ability of MALDI to detect breakdown products after incubation in the presence of antibiotic.[3] However, resistance testing is unlikely to be incorporated into a standard MALDI microbial identification platform in the near future.

Mass spectroscopy has been applied to human tissues, including FFPE, for a variety of purposes, but, to date, there is not a commercially available or routine method for using MS in the detection of organisms in FFPE.[44,45] With the advent of rapid and inexpensive nucleic acid methods for FFPE, this field largely lies dormant, although incredible potential for detection and other data extraction from FFPE through MS may be possible in the future.

Nucleic Acid–Based Techniques

For more than 30 years, a wide spectrum of assays based on the detection of nucleic acids has been developed for the diagnostics of infectious disease. They range from simple probes to qualitative and quantitative target amplification to sequencing. For example, the first nucleic acid probe-based assay was launched in 1985 to test for legionnaires' disease (Gen-Probe). Real-time platforms, such as the Smartcycler and the Lightcycler, have an established place in both detection and quantification of pathogens, although the major impact has mostly been in virology, where the nucleic acid–based techniques were quickly adopted to overcome the difficulties inherent in viral culture. For example, hepatitis C virus (HCV) and HIV viral loads are routine applications of these technologies. There have been a number of newer nucleic acid–based technologies that have had a tremendous impact on microbial diagnostics, many of which are also geared towards bacterial pathogens. These include highly sensitive probes for use in direct specimens, to alternative amplification methods, rapid assays of single targets, and multiplexed systems that allow for the detection of many organisms in one assay. Sequencing assays are becoming more commonplace, with targets ranging from 16S rRNA to whole genomes and even metagenomic studies looking at the make-up of complex microbial ecosystems within the human host. In this section the technologies behind several of the most important new assays will be briefly introduced, followed by a discussion of their application to clinical syndromes, such as respiratory and gastrointestinal (GI) disease, bloodstream infections, meningitis, and reproductive health/sexually transmitted disease.

Probe-Based Assays

Probe hybridization assays were one of the earliest diagnostic techniques using nucleic acids as a target. The most commonly used probes are marketed by Hologic Gen-Probe Inc. (Marlborough,

Massachusetts) and consist of single-stranded DNA probes tagged with acridinium esters that hybridize to rRNA. Tests are read in a luminometer. Because there is no amplification, probe-based assays generally suffer low sensitivity and are reserved for culture confirmation, where organisms are present in high numbers. AdvanDX (Woburn, Massachusetts) has produced a series of probe-based assays using peptide nucleic acid (PNA) probes tagged with fluorescent markers.[46] PNA probes have several advantages over traditional DNA probes, such as increased stability and ability to penetrate the bacterial cell wall. Fluorescent tags have the added advantage of allowing for multicolor detection and the creation of limited panels. These are rapid assays (less than 1 hour) and are mostly targeted towards rapid diagnostics of positive blood culture bottles. The AdvanDX systems (PNA fluorescence in situ hybridization [FISH] and QuickFISH) offer a variety of testing options, such as *Staphylococcus* (*Staphylococcus aureus* [SA] vs coagulase-negative staphylococci [CNS]), *Enterococcus* (*Enterococcus faecalis* vs *Enterococcus faecium* or other enterococci), and gram negatives (*E. coli* vs *Klebsiella pneumoniae*, vs *Pseudomonas aeruginosa*) that can be chosen based upon the Gram stain results.[47-50] PNA probes have been used for rapid identification of organisms, such as fungi, bacteria, and mycobacteria, in human tissues sections for fresh, frozen, and FFPE.[51-54] In situ hybridization (ISH) of Epstein Barr encoding region for confirmation of Epstein-Barr virus infection on FFPE is routinely used in cancer diagnostics, but few, if any, other common viral infections are confirmed this way, with IHC being preferred. Application of RNA-ISH to *Aspergillus* and *Candida* in FFPE showed less sensitivity than real-time PCR with sequencing (gold standard), although some FISH-positive, PCR-negative cases with obvious fungal elements were seen, suggesting refinements of this technique may be valuable for rapid identification of these common organisms, especially if mucormycosis is in the differential.[55]

A unique probe-based approached to nucleic acid detection is the RNA hybridization and digital counting technology offered by NanoString (Seattle, Washington). This method uses a capture probe (usually a specific 50-mer) and a reporter probe (a second, adjacent, specific 50-mer attached to a unique molecular color barcode) to bind up individual molecules of RNA in a sample, capture them on a solid state matrix, and use digital imaging to count the barcodes in the sample. The current version can measure up to 500 independent capture:reporter pairs. The technology can be applied to any RNA target (human or microbial), and the great advantage in infectious disease is the extremely low input quantities that can be detected without interference from other RNA. Because of the counting algorithm, with proper probe selection in a given mixture, accurate quantification of RNA is possible without amplification. One pitfall to this technology is that the presence of any given target in a disproportionate ratio to other targets will flood the digital analysis (i.e., consider hemoglobin relative to cytokines in peripheral blood). Relative to sequencing, the technology remains expensive, but the benefit of quantification of extremely limited sample with minimal processing makes this an attractive target for future test development. For microorganisms, broad panels using signature genes and/or focused panels using genes in antibiotic metabolism pathways can be created. Studies in a range of diseases have shown that RNA from any source, including FFPE, is sufficient for NanoString analysis,[56-58] In one study, quantitative expression of *Plasmodium falciparum* schizonts from human tissue (both frozen and FFPE) were measured and, using imputation, the entire expression profile for the organisms determined.[59]

Singleplex or Limited Target Assays

There are a variety of focused assays in the clinical microbiology lab and anatomic pathology samples that query one or a handful of genes by amplification of the target. The majority of these relies on the PCR and includes both qualitative and quantitative assays, such as those run on real-time PCR platforms (i.e., the Lightcycler [Roche Diagnostics, Indianapolis, Indiana] and the Smartcycler [Cepheid, Sunnyvale, California]). Target detection is typically by fluorescent probes, such as TaqMan.[60] Single-target PCR has been applied to fresh, frozen, and FFPE in anatomic pathology for a large range of microorganisms but largely through home-brew or in-house assays and/or performance by large specialty reference labs, due to the challenges of control tissue, validation, and complexity of interpretation.

Recent developments in this area are based on the same technology but are innovative in their convenience, detection method, and, in some instances, sensitivity. For example, using standard real-time PCR technology, FDA-approved assays, such as the Prodesse kits (Hologic Gen-Probe), provide convenient detection of small sets of respiratory viruses. The Xpert system (Cepheid) has had a large impact on the clinical lab due to its ease of use (sample-to-result platform), excellent performance, and rapid TAT (approximately 1 hour). Detection is by melt curve analysis rather than fluorescence. This platform offers a variety of cartridges (e.g., *C. difficile*, MRSA, MTB/RIF) focused on quick answers to major clinical decision points.[61-64] Newer assays from Cepheid are in development for FFPE, which will include microbiologic applications.

For novel detection methods and increased sensitivity, the T2MR platform (T2 Biosystems, Lexington, Massachusetts) is one of the most innovative technologies. It is a complete sample-to-result platform that relies on detection of pathogen-specific amplicons by hybridization with tagged superparamagnetic particles.[65] The T2Candida Panel (T2 Biosystems) is the only FDA-approved system that directly analyzes blood (without culturing) and has demonstrated impressive results in clinical trials (91% sensitivity with a 4.2-hour TAT).[66] T2 Biosystems is currently developing a bacterial panel as well, which is reported to target *Acinetobacter baumannii, E. faecium, K. pneumoniae, P. aeruginosa,* and SA.[67]

Although PCR remains the most common means of nucleic acid amplification, other technologies have been developed that address the presence of single targets. Transcription-mediated amplification (TMA) has been applied to mycobacteria, HCV, *E. coli*, and *Listeria*, where RNA is bound by a target oligo and then acted upon, in sequence, by reverse transcriptase (RT) (to produce cDNA), RNase H (to degrade RNA template), RT again to produce a double-stranded DNA promoter sequence, and then RNA polymerase to produce many copies of single-stranded RNA—then the process repeats itself. Another isothermal reaction occurs in loop-mediated isothermal amplification, which uses a group of four to six primers and *Bst* DNA polymerase, with the creation of complimentary sites to generate exponentially amplified loop structures. These assays have been developed for a wide range of targets and produce larger quantities of DNA than traditional PCR in a matter of minutes to hours. This technique has been applied to viruses, bacteria, protists, fungi, and mycobacteria in a point of care manner.[68-72] LAMP techniques are limited by the challenges of complex primer design, limited ability to multiplex, and common inhibitors of the enzymes in certain human samples, including blood. A last isothermal amplification is

accomplished by the introduction of helicase, which unwinds DNA, and single-stranded DNA binding proteins, which keep the templates separated. This is followed by primer hybridization and amplification to produce more single-stranded DNA and thus a continuous reaction. Assays to measure herpes simplex virus (HSV)-1 and HSV-2 using helicase have been developed with similar performance to traditional molecular methods and assays are available for *Bordetella*, group A *Streptococcus*, *Trichomonas*, and malaria.[73] Commonly used platforms that use these technologies include the Tigris and Panther systems by Gen-Probe, which are based on TMA, SmMIT-LAMP based on LAMP, and Ampli-Vue based on helicase. Use of these assays in FFPE tissue has shown some promise with improved performance over PCR for some organisms (such as HCV in TMA), equivalent performance to PCR (such as human papillomavirus [HPV] in head and neck cancers with LAMP), and detection of limited signatures (such as microcarcinomas in small biopsies with helicase assays).[74-76]

Multiplexed Systems

The ability to detect multiple organisms in primary samples using PCR-based panels has both provided clinicians with tremendous diagnostic opportunities and presented new challenges.[77] Major platforms include FilmArray (BioMérieux), Verigene (Nanosphere), xTAG/xMAP (Luminex, Austin, Texas), with technologies that range from real-time PCR to solid and liquid phase arrays. For a comprehensive listing of platforms see www.captodayonline.com/productguides. For an in-depth discussion of the major technologies see the articles included in the reference list.[78,79] These systems are generally geared towards syndromic testing, with panels directed towards respiratory, GI, and sexually transmitted diseases, as well as meningitis and bloodstream infections. After discussing the specific challenges of each of the major syndromes, this section will focus on commonly used FDA-approved panels that are currently available from a number of manufacturers and that scan for a wide array of microbes, encompassing viral, bacterial, and fungal organisms.

Panels aimed at respiratory pathogens are focused most heavily on viruses but also incorporate several bacterial agents.[80,81] Commonly covered organisms include *Mycoplasma pneumoniae, Chlamydia pneumoniae,* and *Bordetella* species. *M. pneumoniae and C. pneumoniae*, both very common causes of community-acquired pneumonia, are particularly well-suited to nucleic acid amplification tests because they are difficult to culture.[82] Although less common, *Bordetella pertussis* is considered to be the most common vaccine-preventable disease in the United States, is increasing in incidence, and is also difficult to culture.[83] The "bread and butter" of respiratory panels (RPs) focus on viruses with influenza A (and B), adenovirus, and respiratory syncytial virus (RSV) being always including, along with a mixture of other coronavirus, enterovirus, metapneumovirus, and the parainfluenza group. These panels are extremely powerful from the clinician's point of view but also provide a larger range of diagnostics for respiratory viruses than can be found in most anatomic pathology labs, which are largely limited to the DNA viruses. Although not all of these viral diagnoses are chemotherapeutically actionable, confirmation of a viral cause is valuable for prognosis and resource allocation.

Diarrheal diseases are a global public health concern. Although these diseases are a common cause of illness and hospitalization in the United States, they impose an enormous toll in the developing world, particularly on children. The most common bacterial pathogens include *E. coli* and *Shigella, Salmonella, Campylobacter,* and *Clostridium perfringens*. Typical viral pathogens included in these panels are adenovirus, norovirus, and rotavirus, representing the three most common symptomatic viral diarrheas. Diagnosis is moving more and more towards multiplex panels that cover major bacterial pathogens, as well as some parasitic and viral agents.[84-86] In addition to standard agents of infectious diarrhea, there are special considerations when testing for disease caused by *C. difficile*. These panels are, again, extremely valuable because the tools to diagnose these disease in anatomic pathology are often very limited and too invasive for most patient situations.

Parasitic enteric infections represent a major challenge in developing countries but remain rare in countries in which most new technologies are being developed. Despite this disparate situation, efforts have been made to approach parasitic infections, and thus the inherent challenges have become apparent. Extraction from stool for suspected parasite infections requires that protists (ciliates, ameoba, flagellates, coccidia), helminth larvae (nematodes), and helminth eggs (nematode, trematode, and cestode) be lysed sufficiently to release DNA efficiently. This challenge has led to eight or more different extraction protocols with a range of preparation requirements, including spin columns, automated magnetic bead assays, and "bead beating" methods. Molecular targets for subsequent detection assays as specific single targets across this range of organisms has included 18S rDNA, ITS, COX 1, pOV-46, 5.8S rDNA, Segment A, COWP, actin, HDP2, DL1, and 28S rDNA.[87] In commercially available, multiplex PCR assays, the majority of the platforms target only *Cryptosporidium, Giardia, Entamoeba histolytica,* and, rarely, *Cyclospora*. Because these first three represent 80% to 99% of parasites isolated by all methods from stool in developed nations, additions of other much less common pathogens is not common. Inclusion of nonpathogenic ameba is common in these assays but of questionable value.

Genitourinary samples (fluid from cervical samples, urethral swabs) are another sample that benefits from multiplex testing as public health screening of sexually transmitted diseases are extremely valuable for individual patient diagnosis, as well as epidemiologic survey, especially with the inclusion of HIV. *Neisseria gonorrhea, Chlamydia trachomatis,* and HPV in the Hologic Panther system can be performed on a single sample, and this system also can test for *Trichomonas*. The FilmArray from BioFire (Salt Lake City, Utah) detects *Chlamydia, Neisseria,* syphilis, *Trichomonas, Mycoplasma/Ureaplasma*, herpes simplex, and chancroid in a single sample.[88]

Although relatively uncommon, meningitis and encephalitis (both nosocomial and community-acquired) carry high morbidity and mortality.[89-91] Time is of the essence when diagnosing a causative agent, particularly when trying to determine whether it is a bacterial pathogen that needs immediate antibiotic coverage along with herpes simplex, which needs immediate antivirals. Common bacterial pathogens include *S. pneumoniae, Streptococcus agalactiae, Neisseria meningitidis, Haemophilus influenzae,* and *Listeria monocytogenes*, although there are also several other, less frequently isolated, pathogens. Viral pathogens in these panels include the primary actionable viruses (*Cytomegalovirus*, herpes simplex, varicella zoster virus), as well as other common viruses (*Enterovirus, Parechovirus*, and human herpesvirus (HHV)-6). Platforms also may include *Cryptococcus* species of fungi, which is very valuable in immunosuppressed populations.[92] Current diagnostics are heavily focused on CSF biochemical profile (glucose, protein, lactate), cell count, and Gram stain. The latter is the most specific, but the limit of detection is approximately 10(4)

organisms per milliliter.[90] There is a great need for rapid molecular diagnostics in this area. In anatomic pathology, sample with culture and/or morphology with special stains and/or IHC can resolve many of these infections; however, optimal patient care and best outcomes occur in those patients who can be diagnosed early with these panels and not require an invasive biopsy.

Sepsis continues to be a tremendous cause of mortality, ranking as one of the top 10 causes of death in the United States.[93] Organisms commonly associated with bloodstream infections include SA, *E. coli, Enterococcus* spp., *K. pneumoniae,* CNS, *P. aeruginosa, Candida albicans, E. cloacae,* and *Serratia marcescens.*[94] The current gold standard for diagnosis is culture, with several automated systems (such as the BacT/Alert [BioMérieux Inc.]) available to clinical laboratories.[95] Although these systems are quite sensitive, approximately only one-third of patients with sepsis have positive cultures.[96] Some of this lack of sensitivity may be due to pretreatment with antibiotics, and some of it may be due to the low numbers of organisms present in many clinically significant bacteremias.[97] In addition to sensitivity, speed is also a major issue. The rapid diagnosis of bloodstream infections is critical for patient outcomes, with estimates of a 7.6% decrease in survival for every hour that appropriate antimicrobials are delayed after the onset of hypotension.[98] The need for sensitive, fast detection, ideally accompanied by some assessment of antibiotic resistance determinants, has inspired a number of new technologies geared towards improved speed and sensitivity of blood culture diagnostics. Several of these have been reviewed.[99-101]

Platforms and Technologies

FilmArray (BioFire/BioMérieux)

The FilmArray system consists of nested PCR followed by high-resolution melt curve analysis.[102] All steps of the assay, from cell lysis to the final analysis, take place within a pouch containing freeze-dried reagents that can be stored at room temperature. FilmArray has a short TAT of approximately 1 hour. Disadvantages of the system include the relatively high price of the pouches and restriction of the platform to one test at a time. Laboratories must purchase multiple FilmArray platforms if they desire to run tests in parallel.

The FilmArray panel was the first FDA-approved RP to include bacterial pathogens, covering *B. pertussis, C. pneumoniae,* and *M. pneumoniae*, along with 18 common respiratory viruses.[102] For GI testing, the FilmArray is the most comprehensive of the current FDA-approved panels, covering an array of 22 bacteria, viral, and parasitic targets, including the common agents listed above, as well as *Plesiomonas shigelloides, Yersinia enterocolitica*, and several species of *Vibrio.* It can also differentiate between enteroaggregative, enteropathogenic, enterotoxigenic, Shiga toxin-producing, and enteroinvasive *E. coli* (EIEC). Studies have shown a sensitivity of 95.9% to 100% and a specificity of 96.6% to 100% for bacterial pathogens.[103,104] In many cases the FilmArray detected pathogens in samples that were negative and was far more likely to diagnose mixed infections than standard techniques.[104,105] For viral pathogens the FilmArray GI panel has shown value in the younger age groups (patients younger than 12 years) for most tested pathogens (sensitivity: 95.5% to 100%; specificity: 99.1% to 99.9%), whereas Norovirus appears to be valuable across all age groups (sensitivity: 94.5%; specificity: 98.8%).[103] Performance for parasitic pathogens in this panel is equally high for *Cryptosporidium, Cyclospora,* and *Giardia* (sensitivity: 100%; specificity: 99.5% to 100%), but, as has been common with many panels and individual tests, laboratories have difficulty obtaining natural clinical cases of *E. histolytica*.[103]

Currently, the only FDA-approved multiplex assay for agents of meningitis and encephalitis is the FilmArray meningitis panel. It covers 14 pathogens, including the following bacteria: *E. coli K1, H. influenza, L. monocytogenes, N. meningitides, S. agalactiae,* and *S. pneumoniae.* Although the panel was only recently approved by the FDA (October 2015), there are a few reports of its performance. The pre-FDA evaluation was conducted both on archived samples and prospectively on a multicenter collection of 1560 samples of CSF. Among 235 archived samples (32 with bacteria), the percent positive and negative agreement was 100% for bacterial targets. Among the 1560 prospective samples, there were only eight with bacterial pathogens (none with *L. monocytogenes* or *N. meningitides*). Of those that were present, the FilmArray ME panel did not identify the only *S. agalactiae*. The archived arm of the evaluation included two *S. agalactiae* samples, both of which were correctly identified.[106] Since FDA approval, one US study has been published on the performance of the panel in several Texas medical centers. In that study of 48 patients with community-acquired meningitis and a negative Gram stain, the FilmArray detected two samples with bacterial pathogens, both *S. pneumoniae*. Culture detected only one of these, although the other one was positive for streptococcal urinary antigen.[107]

Finally, the FilmArray blood culture identification (BCID) panel tests for an array of 19 bacterial targets, including: *Enterococcus, L. monocytogenes,* SA, *Streptococcus* (multiple), *A. baumannii, P. aeruginosa, E. coli,* and *K. pneumoniae*. It also tests for five species of *Candida* and three bacterial resistance genes: *mecA, vanA/B,* and *kpc*. It has performed well on both monomicrobial and polymicrobial specimens in several clinical studies.[108,109]

BD Max (BD Diagnostics)

The BD Max is a sample-to-result real-time PCR–based platform. Amplicons are detected via fluorescent probes. A distinguishing feature is the capability to run laboratory-developed tests alongside FDA-approved panels. Current FDA-approved assays include GBS (for group B Streptococci), CT/GC/TV (for vaginitis), MRSA XT (for extended MRSA coverage), Cdiff (for C. difficile), and StaffSR (for Staphylococcus aureas and MRSA). The bacterial panel includes *Salmonella* spp., *Campylobacter* spp. (*jejuni*/*coli*), Shigellosis disease-causing agents (*Shigella* spp. and EIEC), as well as Shiga-toxin producing *E. coli*. It has demonstrated high sensitivity for the organisms in its library in several studies.[110-112] The parasite panel includes the three most common protist parasites in humans in developed countries, including *Giardia lamblia, Cryptosporidium,* and *E. histolytica*, but has the difficult challenge of very low population prevalence relative to the bacterial assays, making cost a challenge over other methods for uptake.[113]

Luminex

Verigene

Verigene is another widely used multiplex PCR platform incorporating PCR of multiple viral and bacterial targets followed by hybridization to a solid-phase microarray. Detection occurs via sandwich hybridization with another analyte-specific probe bound to a gold nanoparticle. The signal is amplified via deposition of colloidal silver that is then detected by light diffraction. Verigene

has a TAT of approximately 2 hours and has the advantage of offering flexible pricing by which the laboratory is charged only for the tests they request, instead of the entire panel. Disadvantages include the need to select the appropriate cartridge based on Gram stain results for bloodstream diagnostics. Respiratory, GI, and bloodstream panels are available.

The Verigene platform can detect 13 respiratory viruses with high sensitivity (including multiple types of influenza, parainfluenza) and three *Bordetella* species (*B. pertussis, Bordetella parapertussis/Bordetella bronchiseptica,* and *Bordetella holmesii*),[114,115] although one advantage of this system is the flexibility to test limited panels based on a given population. The GI panel is somewhat more limited than the FilmArray, covering 22 targets, including 13 bacteria, 5 viruses, and 4 parasites. Bacteria include the *Campylobacter* group, *Salmonella* spp., *Shigella* spp., *Vibrio* group, and *Y. enterocolitica*, as well as Shiga toxin. In one study of 725 stool samples the Verigene demonstrated a 98.5% agreement rate with culture.[116] For the diagnosis of bloodstream infections, the Verigene system has separate cartridges for gram-positive and gram-negative organisms, necessitating an accurate Gram stain to pick the appropriate test and making polymicrobial testing a bit more complicated. Each panel assesses a few extra organisms than the FilmArray single panel. In addition, the gram-negative panel queries several more resistance targets than the FilmArray, including CTX-M, IMP, NDM, OXA, and VIM.[117] Verigene in RPs has excellent performance for the influenza viruses (A and B), including the ability to subtype viruses with perfect agreement with gold standard methods (molecular); however, mixed infections with non-influenza viruses dampen performance.[118] Head-to-head comparisons of the limited Verigene panel (influenza A and B, RSV) with FilmArray RP showed equivalent detection of the three major pathogens at a slightly reduced cost for the Verigene; thus analysis of the clinical population and the desires of the local clinical team for reported virus and actionable diagnoses may help in choosing between these technologies.[114]

xTAG

The xTAG assay system from Luminex is a liquid array based on multiplex PCR followed by amplicon extension using a target-specific primer. The extension step incorporates a hybridization tag onto the amplicon, along with a biotin label. Elongated, tagged amplicons are then incubated with polystyrene microbeads of various fluorescent profiles, each coated with a specific anti-tag sequence. Bead/amplicon pairs are then stained with another streptavidin-linked fluorophore. The beads are analyzed by a system of two lasers on the xMAP instrument. The first determines which beads are bound to amplicons by way of the streptavidin-fluorophore. The second laser indicates which specific target was amplified by way of the unique fluorescence of that bound bead.[119] Although the Luminex platforms generally have the potential to detect the greatest number of targets, drawbacks include the need for a separate nucleic acid extraction step and longer TAT (4 hours or longer) then the other multiplex platforms.

There are two xTAG platforms focused on respiratory testing: the original xTAG platform, restricted to viral pathogens, and the next-generation system (NxTAG), which included several bacterial pathogens as well, including *Legionella pneumophila*.[120] The xTAG Gastrointestinal Pathogens Panel (GPP) tests for nine bacterial, three viral, and three parasitic targets. Among bacterial loci, the Luminex xTAG covers *Campylobacter*, *C. difficile* toxin A/B, *E. coli* O157, enterotoxigenic *E. coli* (ETEC) LT/ST, Shiga toxin-producing *E. coli* (STEC) stx1/stx2, *Salmonella, Shigella, Vibrio cholerae,* and *Y. enterocolitica*. In one study comparing the xTAG with the FilmArray and conventional techniques, the two platforms behaved comparably, with the xTAG slightly trailing the FilmArray in the diagnosis of mixed infections (27% for FilmArray vs 14.1% for xTAG vs 8.3% for routine testing).[104] The performance of xTAG for viral pathogens when compared with realtime RT-PCR and multiplex realtime RT-PCR, showed a wide variation in sensitivity across viruses for xTAG (20% to 100%), with performance for RSV-A, RSV-B, H1N1, and influenza B being 13.3%, 47.3%, 54.2%, and 20%, respectively.[121] In head-to-head comparisons of FilmArray, eSensor RVP, and xTAG, the three methods were essentially equivalent, with xTAG having the best performance for influenza A.[122]

Some of the caveats of the xTAG system are addressed with newer Luminex platforms, such as the Aries, which is a sample-to-result platform for testing primary clinical specimens. The assay consists of real-time PCR using the Luminex MultiCode technology. This system uses primers that incorporate isobases, isoC and isoG, which, when incorporated, can pair only with each other. One isobase is tagged with a fluorescent reporter, and the other is tagged with a quencher. Upon the successful generation of product, both isobases are incorporated and fluorescence decreases. A subsequent melt curve analysis is performed, and fluorescence is restored. Unlike the xTAG technology, MultiCode can be run on either commercially available real-time PCR systems or within the sample-to-result Aries platform from BD. There is only one FDA-approved test for this panel, an HSV-1 and HSV-2 panel for use in testing cutaneous and mucocutaneous lesions.

Sequencing of Ribosomal RNA for Pathogen Identification

Although all of the nucleic acid–based systems described above demonstrate high levels of sensitivity and specificity, they are all inherently limited to a select group of organisms based on the probe/primer sets they contain. Open assays using broad-range primers that target nearly universal sequences are nearly all based on the 1977 discovery by Carl Woese that the 16S rRNA subunit could be used to determine the phylogenetic structure of the domains of life.[123] Although this idea represented a fundamental shift in bacteriology, it took many years for it to be widely accepted, and even longer for the use of 16S rRNA sequencing (16Sseq) to be used as a standard means for identifying bacteria in the clinical laboratory.[124] However, since 2000, 16Sseq has become the gold standard for determining bacterial species, and its analysis has led to a great deal of restructuring and renaming among phyla.[125] Briefly, the 16S gene encodes a portion of the small ribosomal (30S) subunit and is composed of nine variable regions alternating with nine conserved regions (Fig. 6.2). The gene is sequenced using primers that bind to the highly conserved regions and then compared with a reference database to identify an isolate.

The choice of database is typically the most challenging part of the assay. There are a number of databases that can be used to identify the sequence, ranging from large, public, uncurated or partially curated databases, such as Genbank and the Ribosome Database Project (RDP, University of Michigan) to commercial, highly curated collections with a much more limited scope. Identification algorithms include Ripseq (Pathogenomix, Santa Cruz, California), the Integrated Database Network System

(IDNS) (SmartGene, Raleigh, North Carolina), and MicroSEQ (Applied Biosystems, Thermo-Fisher, Waltham, Massachusetts). The first two products are solely bioinformatic reference solutions. The last includes both reagents and access to a curated database. None of these products are FDA approved. The Clinical and Laboratory Standards Institute (CLSI) has published guidelines for the identification of organisms by nucleic acid sequencing in which the recommended percentage identity to make a match and guidance for organisms requiring additional targets (e.g., *rpoB, hsp65*) for accurate speciation are laid forth.[126]

The use of 16Sseq in the clinical microbiology laboratory has been shown to be particularly useful in such applications as the identification of unusual and/or slow-growing organisms and the identification of organisms from specimens in which culture is not possible or has failed, such as in specimens from antibiotic-treated patients and fixed specimens.[127-130] For example, the identification of mycobacterial species identified by acid-fast staining of FFPE is a commonly used application of 16S sequencing, although additional targets, such as IS6110 and *hsp65*, are often needed to differentiate species.[131,132] Specimen types most successfully analyzed by direct 16Sseq include heart valves, CSF, and synovial fluid.[133-138]

Fungal pathogens are also identified using rRNA sequencing, although it is a less established practice than it is for bacterial targets. The fungal rRNA 18S corresponds to bacterial 16S, although it has fewer hypervariable regions and is generally not as useful for isolate discrimination.[139] The most common regions for fungal identification are the ITS regions 1 and 2, as well as the D1 and D2 hypervariable regions of the 28S rRNA large subunit component (Fig. 6.2).[139-141] When using these targets to identify an isolate, criteria required for percentage identity and percentage difference between competing matches are not as clear for fungi as they are for bacteria.[126]

The lack of high-quality databases has been a major difficulty in fungal sequencing, with estimates of up to 20% of the entries in GenBank being incorrectly identified down to species level.[142] To address this, several of the private databases that are available for bacterial sequencing also include curated or partially curated fungal databases (IDNS and RipSeq).[143] The MicroSEQ system also includes a fungal component, but it is limited to the D2 region of 28S. Recently, several public databases have become available with high-quality, curated content. Databases that are most focused on species of medical importance include the International Society for Human and Animal Mycology (ISHAM) ITS database contains 3200 sequences representing 524 human/animal pathogenic fungal species and is a major resource for clinical laboratories.[144] It can be searched via a web-based platform (its.mycologylab.org). The National Center for Biotechnology Information RefSeq ITS database contains 3060 sequences representing 270 families from 39 classes. The smaller 28S RefSeq database contains approximately 500, representing greater than 100 families from 21 classes.[145] This technique is particularly promising for identifying fungi from fixed tissue.[55,146,147]

Important caveats of rRNA sequencing include high cost and complexity, issues with uncurated databases (described above), and the potential for false positives. This is particularly the case when broad-range sequencing is applied to primary specimens to "fish" for an infectious agent in the absence of any corroborating culture or gram stain. Furthermore, rRNA should not be used to determine whether an infection has "cleared," due to the potential for lingering nucleic acid even in a successfully treated infection. Analysis should be restricted to normally sterile sites for tests using Sanger sequencing, which is generally unable to assess polymicrobial infections, although some analysis software packages have algorithms to deconvolute traces of 16Sseq from two or three different organisms.[148]

Next-Generation Sequencing

NGS is a high-throughput method that generally refers to a massively parallel process that generates tremendous quantities of raw sequence reads. There are a number of commercially available platforms that vary in the quantity, quality, and length of the reads that they produce. The majority of these include a step for clonal amplification of the template and produce short (50 to 400 bp) reads. For excellent reviews see articles provided in the reference list.[149,150] The advent of NGS was a groundbreaking event in clinical medicine and is a key component of initiatives for "precision medicine" for cancer. Beyond human genomic analysis, NGS has also had a tremendous influence on clinical microbiology that extends from infectious disease testing and epidemiology to analysis of the human microbiome in health, disease, and intervention.[151-153]

Within the diagnostic clinical microbiology laboratory, NGS of microbial whole genomes has been applied as an alternative to traditional methods, such as pulsed-field gel electrophoresis, for clonality analysis in outbreak tracking.[154] The refinement made possible with total chromosomal single-nucleotide polymorphism analysis, for example, allowed one group to assess the spread of variants of a colonizing population of carbapenem-resistant *K. pneumoniae* from one patient to several others in multiple transmission events. These variants were differentiated from each other by fewer than a dozen nucleotides.[155] Other applications that are becoming more and more routine include the characterization of unusual pathogens, the tracking of antibiotic resistance genes, and even the characterization of the plasmids that carry them through horizontal gene transfer.[156-159] Although not yet routine, NGS has been used to diagnose infections directly from primary samples in cases in which all conventional techniques have failed.[160] In one report, after 4 months of progressive neurologic symptoms and a thorough but unrevealing infectious disease workup, NGS was finally able to diagnose a case of neuroleptospirosis directly from the CSF of a 14-year-old boy.[161]

In addition to the detection and characterization of pathogens, NGS has also proven to be an invaluable tool for the characterization of the human microbiome. The influence of the microbiome on human health and disease has become progressively more established. Examples are many and include the development of obesity, the metabolism of therapeutic drugs, and the development of inflammatory bowel disease.[162-164] The two most common ways of assessing the microbiome are by 16S rRNA or shotgun metagenomics.

NGS 16S analysis entails sequencing one portion of 16S rRNA from a mixed community (e.g., stool sample) with subsequent clustering of sequences either against a reference dataset or into operational taxonomic units (OTUs). It gives a picture of taxonomic diversity in a sample and allows for the analysis of changing taxonomic composition in the face of various perturbations and interventions, such as antibiotic administration. Shotgun metagenomics refers to the sequencing of total DNA from a mixed community. This technique allows for more refined organism identification and an analysis of biochemical pathways, resistance genes, etc. but requires a greater amount of raw sequence.[165,166]

In FFPE in which an organism has been seen but not identified (such as bacteria, fungal elements, viral cytopathic or necrotic effect, or unidentified parasitic elements), NGS has enormous potential to rectify theses diagnoses but is currently limited by cost per sample and the enormous amounts of raw human data in comparison with the relatively small amount of the organism's sequence. As costs fall for sequencing and rapid bioinformatic methods for elucidating causative sequence emerge, NGS will become a primary tool for confirmation of microbial infections.

One last technology to consider is the hybrid system, PLEX-ID by Abbott, which combines a PCR step to amplify through a set of primers (PLEX-ID Viral IC, PLEX-ID BAC Spectrum BC, or PLEX-ID Flu) a broad range of organisms and then detects the PCR products through mass spectroscopy. This approach combines amplification of many sample types, including body fluids, tissue, FFPE, culture isolates, and blood, with a highly sensitive detection system to make subtle species determinations across organism types, including bacterial, fungal, viral, and some parasitic.[167-170] As the database of spectra improves and the multiplex primers improve, this system will gain popularity, although it is still limited by initial organism burden and procedure time (up to 8 hours).

Conclusion

The microbiology laboratory remains the most important companion for anatomic pathologists who have a potentially infectious diagnosis under the microscope. Anecdotal and small studies have demonstrated that the yield from molecular tests that are performed blindly (without visualization) versus with pathologist's review is miniscule and extremely expensive. Therefore anatomic pathologists and their microbiologic colleagues must have a constant line of communication from the moment the specimen is collected until final reporting to ensure interpretation of all modalities is maximally beneficial to patient care. This dialogue should also include discussions of molecular modalities that are applied to clinical and anatomic pathology specimens and changes, advantages, pitfalls, and benefits of those modalities in an ever-evolving manner. As with any approach to medicine, evidence-based use of the most efficient and inexpensive method that produces the highest-impact result for the patient is the constant goal.

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