Yi-Wei Tang · Charles W. Stratton
*Editors*

# Advanced Techniques in Diagnostic Microbiology

## Volume 2: Applications

*Third Edition*

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Yi-Wei Tang • Charles W. Stratton
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## Volume 2: Applications

Third Edition

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# Preface

Medical microbiology is a branch of medical science that deals with the prevention, diagnosis, and therapy of infectious diseases. A clinical microbiologist is a professional within the field of medical microbiology who is knowledgeable about the characteristics of microbial pathogens, including their modes of transmission as well as their mechanisms of infection and growth. Clinical microbiologists often practice in a clinical microbiology laboratory or a public health laboratory where they may direct these laboratories. Clinical microbiology laboratories are concerned mainly with the diagnostic aspects of the practice of medical microbiology, whereas public health laboratories are more concerned with the epidemiology of infectious diseases within given populations. There is, and must be, a strong link between clinical microbiology laboratories and public health laboratories. Clinical microbiology laboratories primarily determine whether pathogenic microorganisms are present in clinical specimens collected from individuals with suspected infections; if such microbial pathogens are found, these microorganisms are identified and susceptibility profiles, when indicated, are determined. Clinical microbiologists work closely with and serve as consultants with physicians who are caring for infected individuals. The importance of the field of medical microbiology can be appreciated by noting that hospitals in the United States annually report over 5 million cases of infectious disease-related illnesses.

Diagnostic microbiology within the clinical microbiology laboratory continues to undergo a quiet revolution that already has resulted in many benefits for microbiologists, clinicians, and most importantly patients. This revolution was initially made possible by the elucidation of the structure of DNA and the genetic code, which allowed scientific advances centered around hybridization probes, the polymerase chain reaction, genomics, transcriptomics, proteomics, and metabolomics. These technical advances in molecular microbiology over the first decade of the twenty-first century have profoundly altered every aspect of the clinical microbiology laboratory, including their staffing patterns, work flow, and turnaround time. More recently, fully automated sample processing systems with digital plate reading technology have emerged as a second wave of technical advances, and have enabled clinical microbiology laboratories to cope with large numbers of samples

with improved quality despite limited personnel and financial resources. Moreover, total laboratory automation in the clinical microbiology laboratory also provides superior and more rapid detection of microbial growth. The total laboratory automation system combined with molecular microbiology technical advances such as MALDI-TOF MS and rapid phenotypic susceptibility methods promises to markedly reduce the turnaround time and ultimately reduce the length of stay for hospitalized patients with infections.

The knowledge base required to stay current in the rapidly changing and advancing technology involved in molecular microbiology, as well as similar advances in total laboratory automation in the clinical microbiology laboratory, is enormous. In 2006 and 2013, the first and second editions of *Advanced Techniques in Diagnostic Microbiology* were published and were well received. According to its "Book Performance Report 2017," since its online publication on August 06, 2012, there has been a total of 145,240 chapter downloads for the second edition eBook by the end of 2017 on SpringerLink. This means the second edition has been one of the top 25% most downloaded eBooks in the relevant SpringerLink eBook Collection for 5 consecutive years. In order to continue to provide this kind of relevant and current information for microbiologists, the third edition of *Advanced Techniques in Diagnostic Microbiology* has been extensively revised and extended with a total of 55 chapters covering all current state-of-the art techniques and applications in the field of diagnostic microbiology. *Advanced Techniques in Diagnostic Microbiology* thus provides a comprehensive, well-referenced, and up-to-date description of these rapidly evolving advanced methods for the diagnosis of infectious diseases in the routine clinical microbiology laboratory.

The third edition is extended to two volumes. The first volume covers the principles and characteristics of important molecular techniques; these techniques include rapid antigen testing, advanced antibody detection, real-time/digital nucleic acid amplification techniques, state-of-the-art molecular typing techniques, and MALDI-TOF MS. New chapters on advanced techniques have been added; these include chapters on total laboratory automation systems, rapid phenotypic antimicrobial susceptibility methods, metabolic techniques, and transcriptomic methods. The second volume describes the application of these advanced techniques; new and evolving molecular applications such as molecular detection and characterization of carbapenem-resistant *Enterobacteriaceae*, advanced typing techniques applied to molecular epidemiology investigations, and multiplex approaches for syndromic testing are covered. Like the first two editions, a diverse team of authors provides authoritative, comprehensive, and well-referenced information on clinically relevant topics; these include sequence-based bacterial identification, blood and blood product screening, automated blood culture systems, molecular diagnosis of sexually transmitted diseases, advances in the molecular diagnosis of fungal/mycobacterial infections, metagenomic sequencing of microbiomes, and application of advanced techniques for antimicrobial stewardship.

We hope our readers like this technique- and application-based approach and their feedback is greatly appreciated. We want to again thank the authors who

devoted their time and effort to produce their chapters. We also thank the staff at Springer. Finally, we continue to appreciate the constant encouragement of our wives and family members throughout this long effort. They are, indeed, the "wind in our sails."

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# Bacterial Identification Based on Universal Gene Amplification and Sequencing

**Susanna K. P. Lau, Jade L. L. Teng, and Patrick C. Y. Woo**

## Introduction

Accurate identification of bacterial isolates is one of the fundamental tasks in clinical microbiology laboratories. This is crucial in providing microbiological diagnosis to infectious diseases, guiding appropriate antibiotic treatment and infection control measures. On the population scale, accurate bacterial identification is important for defining epidemiology of infectious diseases. Traditionally, identification of bacteria in clinical microbiology laboratories is performed using conventional phenotypic tests, including Gram smear, cultural requirements, growth characteristics, and biochemical tests. These tests are relatively inexpensive and accurate for most commonly encountered bacteria in clinical laboratories. However, in certain circumstances, these phenotypic tests may fail to work, and more sophisticated methods may be required. For example, accurate identification of anaerobic bacteria and mycobacteria may require special equipment and expertise such as gas chromatography-mass spectrometry. Moreover, phenotypic methods often fail to identify rare bacteria or bacteria which exhibit variable expression of certain traits and are associated with ambiguity in determining end point reactions. As phenotypic methods rely on the availability of pure culture for the study of growth characteristics and biochemical profiles, it also takes considerable time for slow-growing bacteria to be identified. Furthermore, these methods are not applicable for non-cultivable bacteria and in culture-negative infections.

As a result of the widespread use of PCR and DNA sequencing in the last two decades, amplification and sequencing of universal gene targets represent an advanced

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technology that theoretically provides solutions to these problems, yielding reproducible and unambiguous results even for rare or slow-growing bacteria within 1 or 2 days. Among the various studied gene targets, 16S rDNA gene has been the most widely used, having played a pivotal role in identification of bacteria in clinical microbiology laboratories. PCR and sequencing of 16S rDNA gene have been shown to be especially useful for identification of bacteria with unusual phenotypic profiles, rare bacteria, slow-growing bacteria, uncultivable bacteria, and culture-negative infections. Application of this advanced technique in diagnostic microbiology has not only provided etiological diagnosis to infectious diseases but also assisted the choice and duration of antibiotics and deployment of appropriate infection control procedures. In addition, it has also enabled better understanding of the epidemiology and pathogenicity of rarely encountered bacteria or those that are "unidentifiable" by conventional phenotypic tests, which has not been possible in the past.

Apart from bacterial identification, the use of 16S rDNA gene sequencing has also led to the discovery of a large diversity of previously undescribed, novel bacterial species. Many of them have been discovered from human specimens in the past decade [1]. The highest numbers of novel species discovered were of the genera *Mycobacterium* and *Nocardia*, whereas the oral cavity/dental-related specimens and the gastrointestinal tract were the most important sites for discovery and/or reservoirs of novel species [1]. Among the novel species, *Streptococcus sinensis*, *Laribacter hongkongensis*, *Clostridium hathewayi*, and *Borrelia spielmanii* have been more thoroughly characterized, with the reservoirs and routes of transmission documented, and *S. sinensis*, *L. hongkongensis*, and *C. hathewayi* have been found globally [2–42], although most *Nocardia* and *Mycobacterium* species were probably from the environment and most anaerobes were probably from the oral cavity and/or gastrointestinal tract. However, the focus of the current chapter is on bacterial identification, and the discovery of novel bacterial species by 16S rDNA gene sequencing will not be discussed in detail.

One of the limiting factors in putting 16S rDNA gene sequencing to routine use in diagnostic microbiology is the lack of full automation of the technology. Moreover, analysis and interpretation of 16S rDNA gene sequences is by no means a straightforward task to general technicians working in routine clinical microbiology laboratories. To solve the latter obstacle, several softwares with individualized databases have been developed to achieve automation of 16S rDNA gene sequence analysis for bacterial identification, which may make interpretation of results easier to users. Despite the well-recognized utility of 16S rDNA gene sequence in bacterial identification, there are limitations in using 16S rDNA gene sequence analysis for identification of certain groups of bacteria. In these situations, additional phenotypic or genotypic tests may be required for more accurate species identification. In this regard, a number of alternative gene targets have been explored for bacterial identification, especially those that cannot be readily identified to species level by 16S rDNA gene sequence analysis. New high-throughput technologies and availability of more complete bacterial genome sequences may allow the invention of improved methods for bacterial identification in diagnostic microbiology.

## 16S rDNA Gene Sequencing for Bacterial Identification

The discovery of conserved small rDNA gene sequences in the 1970s by Carl Woese and others has marked the beginning of the new era for the study of evolution and classification of living organisms. [43, 44] These rDNA gene sequences are, in general, highly conserved within living organisms of the same genus and species, but different in organisms of different genera and species. Phylogenetic studies using these rDNA gene sequences have also "rediscovered" three domains of life, *Archaea*, *Bacteria*, and *Eukarya*, in contrast to the traditional dogma of classifying living organisms into prokaryotes and eukaryotes only [45]. With the subsequent invention of PCR and automated DNA sequencing technology in the 1990s, 16S rDNA gene sequences have been widely used for phylogenetic studies and considered a new standard for bacterial classification and identification. As a result, a large amount of bacterial 16 rDNA gene sequence data has been generated. Numerous bacterial genera and species have been reclassified and renamed, and many novel bacterial genera and species have been discovered. Moreover, classification of uncultivable bacteria has been made possible. In the last decade, bacterial genome sequencing projects have allowed study of bacterial phylogeny using various genes and genomes, which confirmed the representativeness of 16S rDNA gene in comparison with complete genome [46]. With the increasing availability of PCR and DNA sequencing facilities, the use of 16S rDNA gene sequencing has not been limited to research purposes but also exploited in clinical microbiology laboratories for bacterial identification as well as other purposes such as direct identification of bacteria from clinical specimens or positive blood cultures [47–49].

The most common situations where 16S rDNA gene sequencing is employed for bacterial identification in the clinical laboratories are when a bacterium is "unidentified" by conventional phenotypic tests or commercial bacterial identification systems or the confidence level of identification by these methods is considered low, when the expected phenotypic profiles of a bacterium does not match its species identity, or when a rare bacterial species was encountered and technicians are uncertain about the accuracy of phenotypic identification. In these situations, 16S rDNA gene sequencing can be performed with the usual techniques for PCR and DNA sequencing. A pure bacterial culture will be used for DNA extraction, and PCR is usually performed using universal or degenerate primers which target the conserved regions of bacterial 16S rDNA genes. While single universal or degenerate primer sets for all bacterial species are available from automated systems such as the MicroSeq systems, "less" degenerate primers for specific groups of bacteria are often used, which may help minimize the risk of PCR contamination and false positive reactions. Once a PCR product is obtained and purified, it can be subject to DNA sequencing and sequence analysis by comparison to other 16S rDNA sequences from various sequence databases. To achieve maximum accuracy in identification, such sequence analysis results are best interpreted in light of conventional phenotypic test results.

### *Routine Bacterial Identification in Clinical Laboratories*

Since conventional phenotypic tests are usually capable of accurately identifying commonly encountered bacteria, "routine" use of 16S rDNA gene sequencing in clinical microbiology laboratories has been largely limited to situations where bacterial isolates are difficult to identify by phenotypic tests. Nevertheless, various studies have been conducted to evaluate the usefulness of 16S rDNA gene sequencing for identification of various medically important bacteria in comparison to conventional or commercial methods. Depending on the group of bacteria studied and the criteria used for species definition, the success rate of species identification by 16S rDNA gene sequencing ranged from 62% to 92% [50–56].

16S rDNA gene sequencing has been found to be particularly useful for identifying certain groups of bacteria. One notable example is anaerobic Gram-positive rods which are notoriously difficult to identify by conventional methods even to genus level. Using 16S rDNA gene sequencing, many previously undescribed or "rarely encountered" anaerobic Gram-positive rods have been characterized and found to contribute to cases of bacteraemia [57–61]. Thus, the prevalence and pathogenicity of these often ignored anaerobes can be better defined. For example, the genus *Eggerthella* was found to contribute to an unexpectedly high proportion of clinically significant bacteremia due to anaerobic, non-sporulating, Gram-positive rod, suggesting that this genus may be of high pathogenicity among this group of bacteria [57, 58]. Two novel *Eggerthella* species, now reclassified under the genus *Paraeggerthella*, were also discovered and may contribute to half of the cases of *Eggerthella* bacteremia [57, 62]. Using 16S rDNA gene sequencing, it was also found that patients with clinically significant clostridium bacteremia were associated with diseases in the gastrointestinal or hepatobiliary tract, which are also predictors of mortality [60]. 16S rDNA gene sequencing has been particularly useful in differentiating between *Actinomyces* and non-*Actinomyces* anaerobic Gram-positive bacilli, which may not be easily achieved by conventional phenotypic tests [63–65]. A definitive diagnosis or exclusion of actinomycosis is considered clinically important, because prolonged antibiotic treatment, in terms of weeks to months, is often recommended in actinomycosis to prevent relapse.

Another group of bacteria that are readily identified by 16S rDNA gene sequencing are the catalase-negative Gram-positive cocci. Application of this advanced technique has contributed to knowledge on the epidemiology and pathogenicity of the different *Streptococcus* and related bacterial species. For example, in the past, little was known about the relative importance of the four species of Lancefield group G beta-hemolytic streptococci in causing bacteremia. Using 16S rDNA gene sequencing, these cases were found to be almost exclusively caused by *S. dysgalactiae* subsp. *equisimilis* by 16S rDNA gene sequencing, except in dog owners where *S. canis* infections may be rarely reported. [66, 67] As for α-hemolytic streptococci, the relative importance of the three species of the "*Streptococcus milleri* group" in infective endocarditis was previously largely unknown. Using 16S rDNA gene sequencing, all six cases of "*Streptococcus milleri*" endocarditis in one study were found to be caused by *S. anginosus*, suggesting that this species may have the highest propensity to cause infective endocarditis among the three species of the

"*S. milleri* group." [68] The use of 16S rDNA gene sequencing in these clinically "unidentifiable" bacteria could be of clinical significance and carry treatment implications. For example, differentiation of *Enterococcus cecorum* from other *Enterococcus* species has allowed continuation of cefotaxime as treatment, as the organism is known to be susceptible to cefotaxime and ceftriaxone, unlike other *Enterococcus* species which are known to be resistant to cephalosporins. It was also found that the cephalosporin susceptibility of *E. cecorum* could well be explained by its unique phylogenetic position by 16S rDNA gene sequence, as it appeared to be the ancestor of other *Enterococcus* species and more closely related to *Streptococcus* species [69].

16s rDNA gene sequencing was also useful for identification of various Gram-negative bacteria. This technique has been found to be useful for accurate identification and understanding the epidemiology of infectious diseases caused by *Elizabethkingia* species [70]. The genus currently comprises three medically important species, *Elizabethkingia anophelis*, *Elizabethkingia meningoseptica*, and *Elizabethkingia miricola*, for which infections caused by *E. meningoseptica* were known to be difficult to treat and carry high mortalities. Therefore, accurate diagnosis is important to guide appropriate antibiotic regimens. Recently, we conducted a molecular epidemiology study of bacteremia caused by *Elizabethkingia* species in Hong Kong; it was found that *Elizabethkingia* bacteremia was predominantly caused by *E. anophelis* instead of *E. meningoseptica* in our locality. *E. anophelis* bacteremia was also shown to be associated with significant morbidity and mortality [70]. Although *Haemophilus* species are commonly isolated in the clinical laboratories, these organisms are often fastidious and may not be readily identified by conventional phenotypic tests. It has been shown that 16S rDNA gene sequencing can accurately identified the various *Haemophilus* species isolated from clinical specimens [71–74]. Using this technique, it was also found that *Haemophilus segnis* is an important cause of non-*Haemophilus influenzae* bacteraemia [71–73]. Examples of other commonly encountered Gram-negative bacteria which are sometimes "unidentifiable" by phenotypic tests but may benefit from 16S rDNA gene sequencing include the *Enterobacteriaceae*, *Acinetobacter*, and *Campylobacter* species [75–79]. Apart from establishing the correct microbiological diagnosis and guiding antibiotic treatment, accurate species identification could have important management and public health significance. For example, differentiating *Salmonella enterica* serotype Typhi from other members of the *Enterobacteriaceae* family is important to determine if cholecystectomy and eradication of carrier state is indicated. [77–79]

## *Identification of Rare Bacteria and Bacteria with Unusual Phenotypic Profiles*

Although microbiologists are usually facing common medically important bacteria most of the time in clinical laboratories, bacterial isolates that are rarely encountered or phenotypically aberrant are encountered from time to time. These are the times where mistakes in identification often occur. 16S rDNA gene sequencing is

most useful when these bacteria are suspected in clinical microbiology laboratories. The biochemical profiles of rarely encountered bacteria are often poorly studied or not included in the commercial biochemical identification system databases. There are times where a rare bacterium may be misidentified as a more commonly encountered bacterium. For example, *Francisella tularensis* subsp. *novicida* has been consistently misidentified twice by phenotypic methods as *Neisseria meningitidis* or *Actinobacillus actinomycetemcomitans* [80]. As for bacteria with unusual or atypical phenotypic profiles, the conventional tests are bound to fail. Unlike phenotypic characteristics which can be affected by deletion or mutation of various genes especially those encoding enzymes, 16S rDNA gene sequencing provides unambiguous identification of bacteria with atypical phenotypic characteristics. Therefore, in these situations, 16S rDNA gene sequencing is often the ultimate solution to diagnosing infections caused by these bacteria and guiding appropriate treatment [71, 81–91].

16S rDNA gene sequencing has been found to provide genus identification in >90% and species identification in 65 to 83% of these circumstances [92, 93]. Using the MicroSeq 500 16S rDNA-based identification system, 81% of clinically significant bacterial isolates with ambiguous biochemical profiles and 89.2% of unusual aerobic Gram-negative bacilli have been identified to the species level. [75, 94] The use of 16S rDNA gene sequencing on rare or unusual bacteria has led to better understand of the epidemiology and pathogenic role of these bacteria. For example, cases of invasive *Streptococcus iniae* infections in Asia have been diagnosed by 16S rDNA gene sequencing [95–98]. This rare aquatic bacterium has only been previously reported to cause human infection in North America [99]. Many other rarely encountered bacteria, which may have been unrecognized without using 16S rDNA gene sequencing, are now better defined in terms of their disease association and pathogenicity. Examples are *Bordetella*, *Arcobacter*, *Tsukamurella*, *L. hongkongensis*, and the *Streptococcus*-related Gram-positive cocci such as *Helcococcus*, *Gemella*, and the nutritionally deficient streptococci, *Granulicatella adiacens* and *Abiotrophia defectiva* [21, 75, 81, 82, 85–87, 100–102]. Using 16S rDNA gene sequencing, novel or rare clinical syndromes such as *Tsukamurella*-associated conjunctivitis and keratitis, prosthetic valve endocarditis due to *Streptobacillus moniliformis*, psoas abscess due to group A streptococcus, and continuous ambulatory peritoneal dialysis-related peritonitis due to *L. hongkongensis* can be recognized. [21, 100–104]

Using the technique, bacterial isolates with unusual biochemical profiles can now be identified unambiguously. For example, thermo-tolerant *Campylobacter fetus* strains have been identified as important causes of bacteremia in immunocompromised patients [105]. Melioidosis due to *Burkholderia pseudomallei* with ambiguous biochemical profile has been diagnosed. [106] Unusual strains of various Gram-positive and Gram-negative bacteria are also recognized. [77, 78, 107, 108] In summary, applications of 16S rDNA gene sequencing on rare or unusual bacteria can make significant impact on the decision whether to prescribe antibiotic treatment [77, 108, 109] and on the choice of specific antibiotic regimen [78, 104, 110], which could lead to improved clinical outcomes [75].

## *Identification of Slow-Growing and Uncultivable Bacteria*

16S rDNA gene sequencing and similar molecular identification methods have the additional advantage of shortening the time to identify slow-growing bacteria and being able to identify bacteria that are even not cultivable. It is well known that most *Mycobacterium* species, except the rapidly growing mycobacteria, usually take 6–8 weeks to grow in culture, and it often takes another few weeks to perform phenotypic tests using subcultures. Even for the "rapid growers," some biochemical reactions may take up to 28 days to complete. Moreover, whole-cell fatty acid analysis by gas chromatography, which is often required for definitive species identification, is not available in most routine clinical laboratories. 16S rDNA gene sequencing has been used for identification of *Mycobacterium* species, thereby speeding up clinical diagnosis and guiding prompt antibiotic treatment [109, 111]. Using the technique, a novel clinical syndrome, acupuncture mycobacteriosis, caused by relatively alcohol-resistant mycobacteria in patients receiving acupuncture has also been described. [112, 113] However, identification of mycobacteria by 16S rDNA gene sequencing is limited by the high sequence similarity among certain species, in which case alternative gene targets may be indicated.

Although bacterial culture plays a fundamental role in diagnosing bacterial infections in microbiology laboratories, some bacteria are known to be uncultivable even using modern techniques, which may make diagnosis difficult. Although direct microscopy and immunology-based assays have been used for such diagnosis, the sensitivities and specificities of these methods are often suboptimal and variable. The introduction of molecular diagnostics, in particular 16S rDNA gene sequencing, has enhanced our ability to diagnose these culture-negative infections. One of the most well-known examples of non-cultivable bacteria is *Mycobacterium leprae*, the causative agent of leprosy which can be difficult to diagnose. Application of molecular detection such as PCR and sequencing of 16S rDNA gene from skin biopsies of leprosy patients has provided an additional diagnostic tool. [114, 115] In addition, the first breakthrough in the understanding of Whipple's disease, after its initial description in 1907, was only made with the identification of *Tropheryma whippeli* as the causative agent by PCR and sequencing of its 16S rDNA gene [116] [91]. This *state-of-the-art* technique has also enabled the subsequent development of molecular diagnostic tests for this disease and accelerated research in to its pathophysiology [117–120]. Similar successes in applying 16S rDNA gene sequencing have also been seen in recognizing the etiological agents of bacillary angiomatosis (caused by *Bartonella henselae* and *Bartonella quintana*) [121, 122] and human ehrlichiosis (caused by bacteria in the genera *Ehrlichia* and *Anaplasma*) [123–125].

Using 16S rDNA gene sequencing, etiological agents can also be established in various culture-negative infections. For example, up to a third of cases of infective endocarditis can be culture-negative [126], which may be due to prior antibiotic therapy, inadequate microbiological techniques, or infection caused by fastidious or non-cultivable organisms. [127] It has been shown that 16S rDNA gene PCR

amplification and sequencing performed on DNA extracted from infected valves may provide a clue to the culprit organism [128–138]. Similar technique has also been used for diagnosis of culture-negative infections including meningitis [139–143], brain abscess [144], keratitis [145], urinary tract infections [146], empyema [147, 148], septic arthritis [149, 150], and septicaemia [127, 151, 152]. Although recent progress in these areas has been made through the use of broad-range real-time PCR design [142, 153, 154], the use of universal primer sets is sometimes associated with problems of false-positives due to PCR contamination and picking up sequences from colonizing bacteria. With continued improvements in technology design and performance, it is expected that 16S rDNA gene sequencing will play an increasing role in the diagnosis of culture-negative infections.

## *Guidelines for Interpretation of 16S rDNA Gene Sequence Results*

Despite its increasing use for bacterial identification in clinical microbiology laboratories, there are no widely accepted guidelines on the indications of using 16S rDNA gene sequencing as well as the interpretation of sequence data. In view of the limitations in identifying certain bacterial taxa, increasing taxonomic complexity, and large number of unvalidated 16S rDNA gene sequences in some databases, there have been recommendations on the use of 16S rDNA gene sequencing for bacterial identification [92, 155]. For indications of 16S rDNA sequencing, since certain bacterial taxa are known to present difficulties for identification by 16S rDNA gene sequence analysis, other gene targets should be considered for these bacteria [54, 106, 155–163]. As far as sequence analysis is concerned, it depends on the length and quality of sequences, the choice of appropriate programs and databases for analysis, and correct interpretation of similarity search results. It has been suggested that a minimum of 500–525 bp which covers the more variable 5'-region of the 16S rDNA gene may be adequate for identification, thus giving rise to the development of the MicroSeq databases. However, some recommended that full 16S rDNA gene sequences should be used whenever possible [92, 155]. This is particularly important for certain groups of bacteria such as *Campylobacter* species, where the 5'-region may not be sufficient for species differentiation [155]. A major difficulty and controversy in interpreting 16S rDNA gene sequence data is the lack of a universal threshold value or cutoff for species assignment, as different level sequence diversities are observed among different bacterial taxa which evolve at different rate. While a >97% similarity level has been proposed for bacterial speciation, a >0.5% difference may be indicative of a new species [164, 165]. Therefore, it may be necessary to use different cutoffs for different groups of bacteria [92]. For practical purposes, different cutoffs have also been used in different studies. For example, >99% and >97% sequence similarity has been used as the cutoffs for species and genus identification, respectively [92, 155].

Another commonly encountered problem when interpreting 16S rDNA gene sequence data is that when two different bacterial species share highly similar 16S rDNA gene sequence with <0.5–1% difference, it is not a straightforward job to decide whether the "first hit" or "closest match" is the real identity of a bacterial isolate, or this may lead to misidentification if the user is unaware of this problem. In view of this problem, studies have been carried out to systematically evaluate the usefulness of full and 527-bp 16S rDNA gene sequencing and the existing MicroSeq databases for identification of all medically important bacterial species listed in *Manual of Clinical Microbiology* [166–168]. Under the proposed guidelines, each medically important bacterial species was classified as [1] can be confidently identified by 16S rDNA gene sequencing, with >3% difference to other medically important bacteria; [2] cannot be confidently identified by 16S rDNA gene sequencing, with <2% difference to a closely related medically important bacterium; or [3] can only be doubtfully identified by 16S rDNA gene sequencing with 2–3% difference to a closely related medically important bacterium. If a bacterium belongs to [2] or [3], the bacterial species with similar 16S rDNA gene sequences will also be known, and additional/supplementary tests may be considered for differentiation among these closely related species. For MicroSeq database analysis, the reason for failure to identify the bacterium is also indicated. [167] Using this algorithm, it was found that full and 527-bp 16S rDNA gene sequencing are able to identify 52–63% of 130 anaerobic Gram-positive rods, 72–73% of 86 anaerobic Gram-negative rods, and 78% of 23 anaerobic cocci. Surprisingly, the MicroSeq databases were only able to identify 19–25% of 130 Gram-positive anaerobic rods, 38% of 86 Gram-negative anaerobic rods, and 39% of 23 anaerobic cocci. As for medically important aerobic Gram-positive bacteria, full and 527-bp 16S rDNA gene sequencing can identify 24 and 40% of Gram-positive cocci, and 21 and 34% of Gram-positive rods, whereas the full-MicroSeq and 500-MicroSeq databases can identify 15 and 34% GPC and 14 and 25% of GPR confidently to the species level. [169] These methods and databases are least useful for identification of staphylococci and nocardia but are most useful for identification of *Bacillus* and related taxa. A similar study performed on medically important aerobic Gram-negative bacteria showed that full and 527-bp 16S rDNA gene sequencing can identify 26.1% and 32.6% of these bacteria, whereas the full-MicroSeq and 500-MicroSeq databases can identify 15.2% and 26.1% confidently to the species level. [168] In particular, these methods or databases are least useful for identification of *Aeromonas*, *Bordetella*, and *Bartonella* species and are most useful for identification of members of *Pasteurellaceae* and *Legionellaceae* and *Campylobacter* species. Compared to results on anaerobic and Gram-positive bacteria, full and 527-bp 16S rDNA gene sequencing are able to confidently identify significantly more anaerobic Gram-positive and Gram-negative bacteria than aerobic Gram-positive and Gram-negative bacteria. In all three studies, the poor performance of the MicroSeq databases observed was mainly due to the absence of the sequences from the unidentified bacterial in their databases, suggesting that the MicroSeq databases can be much improved if they include more comprehensive and updated data sets. As such guidelines are still associated

with a number of limitations, it is preferable to interpret the results of 16S rDNA sequencing with preliminary phenotypic test results. Nevertheless, such guidelines and similar studies may help easier interpretation of sequence data by inexperienced users in the clinical microbiology laboratories and provide clues on the potential usefulness of 16S rDNA gene sequencing for selected bacterial isolates before they are chosen for such analysis.

### *Automation of 16S rDNA Gene Sequencing*

One of the major obstacles to put 16S rDNA gene sequencing into routine use in clinical microbiology laboratories is the lack of automation of the technology. At the moment, conventional phenotypic tests are still considered the routine and most user-friendly tests for bacterial identification in clinical laboratories. This is partly attributed to the availability of various automated commercial bacterial identification systems based on panels of biochemical tests. However, similar systems are currently not available for 16S rDNA gene sequencing, because most of the steps involved in DNA extraction, PCR, and sequencing have to be performed separately and manually. With high-throughput technologies being made more applicable, these steps may be incorporated into a robotic system for 16S rDNA gene sequencing platforms, making automation a possibility in future.

Another point of difficulty faced by technicians in using 16S rDNA gene sequencing in routine clinical microbiological laboratories is interpretation of sequencing results, which is often not straightforward to those not familiar with sequence and bioinformatics analysis. As a result, much effort has been put on development of automated 16S rDNA sequence analysis software or databases by various groups of scientists. This software usually contains database of 16S rDNA gene sequences from selected bacterial species, against which the input 16S rDNA gene sequence is matched to generate the output bacterial identity. The best-known software and databases include BLASTn against GenBank, the Ribosomal Database Project (RDP) [170–174], and MicroSeq. [55, 94, 175, 176] Newer software and databases have also been developed in recent years, including SmartGene Integrated Database Network System (SmartGene IDNS™) [177], SILVA ribosomal RNA database [178], and 16Spath DB database [179] (Table 1). Among the currently available databases, the GenBank contains the largest databases, with 15,489,568 16S rDNA gene sequences (searches were conducted using the keyword "16S rRNA" or "16S rDNA"). Although this comprehensive database is extremely useful to researchers in the field, it is also well known to contain unvalidated, inaccurate, and redundant sequences. As a result, it is often not easy for inexperienced technicians working in clinical microbiology laboratories to interpret results of BLASTn against the GenBank database. For example, the user may not be aware that the "first hit" may not represent the true identity of a bacterial isolate.

The other databases contain sequences from selected bacteria, with differences in their selection criteria of bacterial species, quality control of sequences, inclu-

**Table 1** Currently available 16S rDNA gene sequence databases

| Software | Partial/full 16S rDNA gene sequence included | Source of sequences | Database size | Quality control | Company/Organization | Year of first description | Website |
|---|---|---|---|---|---|---|---|
| The Ribosomal Database Project (RDP) | Partial and full | Genbank | 3,356,809 (release 11.5) | Partial | Michigan State University, USA | 1992 | http://rdp.cme.msu.edu/ |
| MicroSeq ID 16S rDNA 500 Library | Partial | Sequence 16S rDNA gene of one strain from each species | 2,100 (v2013) | All type strains from culture collections | Life Technologies Corporation | 1998 | NA |
| MicroSeq ID 16S rDNA Full Gene Library | Full | Sequence 16S rDNA gene of one strain from each species | 1,261(v2.0) | All type strains from culture collections | Life Technologies Corporation | 1998 | NA |
| SmartGene IDNS™ –Bacteria software | Partial and full | Genbank | 243,000 | Partial | SmartGene Services SARL, Switzerland | 2006 | http://www.smartgene.com/mod_bacteria.html |
| The SILVA ribosomal RNA database | Full | EMBL database | 552,377 (release 128) | Partial | Microbial Genomics Group, Max Planck Institute for Marine Microbiology | 2004 | http://www.arb-silva.de/ |
| 16SpathDB | Partial and full | Genbank | 1240 (v2.0) | All sequences manually selected from Genbank | Department of Microbiology, The University of Hong Kong, Hong Kong | 2010 | http://147.8.74.24/16SpathDB |

sion of partial or full 16S rDNA gene sequences, and cost. The databases of RDP and SmartGene IDNS™ contain selected sequences downloaded from GenBank and that of SILVA from EMBL, thus also with relatively large database sizes and associated with similar problems as GenBank. The databases of MicroSeq contain 16S rDNA gene sequences of selected bacterial strains from culture collections, thus with smaller database sizes. Although the sequence quality of these databases is better, their usefulness is limited by the choice of bacterial species. Since they do not possess a very comprehensive database of all medically important bacterial species, they are unable to identify those bacterial species that are not included in the database. The MicroSeq databases do not include a significant number of medically important bacteria that 16S rDNA gene sequencing is able to identify. For example, 98–108 (53.3–67.1%), 38–39 (22.7–37.3%), and 23–39 (19.8–41.9%) medically important anaerobic, aerobic Gram-positive, and aerobic Gram-negative bacteria, respectively, which can be confidently identified by 16S rDNA gene sequencing, are not included. [167, 169] Another problem when using these software packages, including BLASTn against GenBank, is that, when there is only minimal difference among the sequences of closely related bacterial species, the inexperienced user may not be aware that 16S rDNA gene sequence alone is unable to identify these bacterial species in such circumstances and may wrongly accept the "first hit" or "closest match" as the identity of the bacterium. To solve these problems, a database, 16SpathDB, was recently developed, which includes the most representative 16S rDNA gene sequences of all medically important bacteria listed in the 9th edition of the *Manual of Clinical Microbiology* [180], for identification of medically important bacteria using 16S rDNA gene sequencing in clinical microbiology laboratories [179]. All sequences were manually selected from GenBank, to ensure the quality of the sequences, and accurate identity and representativeness of the bacterial strains included. In contrast to RDP and SmartGene IDNS software, 16SpathDB includes only 16S rDNA gene sequences of medically important bacteria to minimize ambiguity during data interpretation, as the target users of 16SpathDB are technicians and clinical microbiologists who work on 16S rDNA gene sequencing for identification of clinical isolates. This database also uses an automated user-friendly platform that indicated the most likely identity of the 16S rDNA gene sequence of a medically important bacterium, as well as other medically important bacteria with similar 16S rDNA gene sequences that may be alternative identities, which the user should be aware of [179]. For example, the 16S rDNA gene sequences of *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus mitis*, and *Streptococcus oralis* are known to share more than 99% identity. In 16SpathDB, in addition to the species that shows the highest nucleotide identity to the query sequence, those species with 16S rDNA gene sequences having less than 1% difference from the species that showed the highest nucleotide identity to the query sequence will also be reported, and the user will be alerted that further tests may have to be carried out in order to distinguish between these species. Recently, the latest edition (10th) of the *Manual of Clinical Microbiology* was published, [181] in which 221 medically important bacterial species were newly added comparing to the number of species in the

previous edition. 16SpathDB was updated accordingly by adding the curated 16S rDNA gene sequences of these 221 bacterial species into its database. The identification algorithm was also improved. By June 2017, such improved and updated version of database, 16SpathDB 2.0, contained 1,240 16S rDNA sequences from 1,231 unique bacterial species [182]. We further evaluated 16SpathDB 2.0 using 689 16S rDNA sequences from 689 complete genomes of medically important bacteria, and the results showed that all 16S rDNA sequences were successfully identified. [182] While MicroSeq and SmartGene IDNS software are commercially available for purchase, RDP, SILVA, and 16SpathDB 2.0 are available for free via designated websites.

Various studies have also evaluated the usefulness of the different software for different groups of bacteria [50, 55, 75, 94, 175–177, 183–189]. However, these studies differ in study design, inclusion criteria for study strains, and interpretative criteria for "correct" identification, thus making direct comparison difficult [50, 175, 177, 184–187, 189]. As the intrinsic problems of the software may not be fully addressed, some of the stated accuracies of the software mentioned in the publications may be overestimated. The usefulness of 16SpathDB has also been evaluated using the 16S rDNA gene sequences of 91 nonduplicated medically important bacterial isolates, among which 71 (78%) were reported as a single bacterial species having >98.0% nucleotide identity with the query sequence, 19 (20.9%) as more than one bacterial species having >98.0% nucleotide identity with the query sequence, and 1 (1.1%) was reported as no match (*Gordonibacter pamelaeae* which has not been reported to cause human infection). With the development of more user-friendly software with high-quality and comprehensive databases, 16S rDNA gene sequencing can be more readily used for routine bacterial identification in clinical laboratories.

## Other Gene Targets for Bacterial Identification

Although 16S rDNA gene sequencing can achieve high discriminative power in identifying many groups of bacteria to species level, there are "blind spots" within some major genera. A well-known problem is that 16S rDNA gene sequences are sometimes not discriminative enough for differentiation between related species. In these circumstances, alternative targets, usually based on highly conserved proteins, have to be investigated (Table 2). For example, *groEL* (bacterial homologue of *hsp60* encoding house-keeping chaperon proteins that assist in proper protein folding) is useful for classification and identification of various bacteria. For example, *groEL* has been found useful in differentiating *B. pseudomallei* from *B. thailandensis*, of which the 16S rDNA gene sequences are indistinguishable [106, 162]. *groEL* is also useful for differentiating among the *Bartonella* species and in subtyping of *Bartonella henselae* [190]. Delineation of species within the genus *Acinetobacter* is often found to be problematic by phenotypic tests due to their catabolic diversity, and 16S rDNA gene sequences have failed to distinguish closely related genomic species due to its

**Table 2** Commonly used gene targets other than 16S rDNA gene for bacterial identification

| Gene target | Gene//protein function | Bacterial group | References |
|---|---|---|---|
| *groEL* | Heat shock protein | *Bartonella* species, *Burkholderia* species, rapidly growing mycobacteria, *Staphylococcus* species, *Tsukamurella* species | [106, 157, 160–162, 190, 202–203] |
| *gyrB* | Beta-subunit of DNA gyrase | *Campylobacter* species, slowly growing mycobacteria | [158, 194] |
| *gltA* | Citrate synthase | *Ehrlichia* species, *Rickettsia* species | [208, 209] |
| *dnaJ* | Heat shock protein | *Enterobacteriaceae*, *Mycobacterium* species | [192, 205] |
| ITS | 16S-23S rDNA gene internal transcribed spacer | *Campylobacter* species, slowly growing mycobacteria | [156, 195] |
| *recA* | Recombinase A | *Geobacillus* species, *Streptococcus mitis* group | [200, 202] |
| *rpoB* | Beta-subunit of RNA polymerase | *Acinetobacter* species, *Enterobacteriaceae*, *Geobacillus* species, non-tuberculous mycobacteria, *Staphylococcus* species, *Streptococcus* species | [159, 191, 193, 196, 221] |
| *sodA* | Superoxide dismutase | *Enterococcus* species, *Streptococcus* species | [199, 201] |
| *Tuf* | Elongation factor Tu | Coagulase-negative staphylococci | [54] |
| *secA* | Secretion ATPase | *Nocardia* species, *Gordonia* species | [222, 223] |
| *ssrA* | Stable small RNA | *Enterococcus* species, *Pseudomonas* species, *Enterobacteriaceae*, group B *streptococci* | [224, 225] |

extremely low polymorphism [191]. The gene *rpoB*, another commonly used target encoding the beta subunit of RNA polymerase, has been found to be able to separate different *Acinetobacter* species, with higher bootstrap support in phylogenetic trees than those obtained with 16S rDNA gene [191]. The family *Enterobacteriaceae* contains a large number of pathogenic and frequently encountered bacterial species, some of which may be difficult to identify by phenotypic methods. Since the phylogenetic relations among certain closely related species, e.g., *Salmonella* species, *Citrobacter freundii*, and *E. coli*, are not well defined by 16S rDNA sequences, other targets, such as *rpoB* and *dnaJ* (encoding HSP40), have been explored. [192, 193] For example, *dnaJ* has been found to be useful in phylogenetic study and identification at species level of the family, with more monophyletic groups obtained and greater degree of divergences than that obtained with 16S rDNA gene sequences [192] [169]. The high sequence similarity observed between members of the *Campylobacter* genus has also made differentiation between species such as *Campylobacter jejuni* and *C. coli* difficult based on 16S rDNA genes. Other gene targets, such as *gyrB* (encoding B subunit DNA gyrase protein) and 16S-23S rDNA internal transcribed spacer (ITS), have been found useful in this respect [194, 195].

As for Gram-positive bacteria, 16S rDNA gene sequencing has limited discriminatory power for closely related *Staphylococcus* species which are sometimes misidentified by phenotypic tests. Therefore, sequencing of the *groEL*, *tuf* (elongation

factor Tu), and *rpoB* genes has been proposed as more reliable methods for identification of staphylococci [54, 160, 161, 163, 196]. 16S rDNA gene sequences are also known to lack discriminatory power in distinguishing other aerobic Gram-positive cocci, e.g., among the *Streptococcus mitis* group and among *Enterococcus* species. Different gene targets, such as *rpoB*, *sodA* (manganese-dependent superoxide dismutase), and *recA* (recombinase subunit), have been found to constitute a more discriminative target [196–201]. *rpoB* and *recA* have also been found to be advantageous to 16S rDNA gene for identification of *Geobacillus* species [202]. The high sequence similarity shared between members of the *Tsukamurella* genus has also made differentiation between species difficult based on 16S rDNA genes. We recently evaluated several housekeeping genes, including 16S rDNA, *ssrA* (stable small RNA), *secA* (secretion ATPase), *rpoB*, and *groEL*, for species identification using all available type and reference strains of *Tsukamurella*. Among the five gene targets, only 16S rDNA and *groEL* gene sequences were able to show correct species assignment using the type and reference strains of *Tsukamurella* species. The usefulness of these two gene targets were further evaluated by determining their gene sequences of additional 34 clinical isolates, for which their species identities were confirmed previously by DNA-DNA hybridization. Interspecies similarities of 16S rDNA sequences of the tested isolates ranged from 96.7 to 99.9%, whereas those of *groEL* gene sequences ranged from 91.1 to 98.2%. It was also found that 16S rDNA failed to differentiate some distinct *Tsukamurella* species. Overall, the study showed that *groEL* gene was most useful for species identification of *Tsukamurella*, for which a threshold value of 98.2% based on the *groEL* gene sequence was proposed for species delineation [203, 204].

Although 16S rDNA gene sequencing can be used for identification of certain *Mycobacterium* species, a number of mycobacterial species are also known to be not distinguishable from one another by 16S rDNA gene sequencing, e.g., between *M. avium intracellulare* and *M. paratuberculosis*, between *M. chelonae* and *M. abscessus*, between *M. kansasii* and *M. gastri*, between *M. malmoense* and *M. szulgai*, between *M. marinum* and *M. ulcerans*, between *M. mucogenicum* and *M. phocaicum*, and among the *M. tuberculosis* complex. [109, 112, 113, 156, 205–207] Therefore, different gene targets, sometimes supplemented by phenotypic results, have to be used for differentiation of specific mycobacterial species, such as *hsp65* (the *hsp60* homologue in mycobacteria), *rpoB*, ITS, *gyrB*, and *dnaJ* [156–159, 205]. For example, in a study evaluating the use of *hsp65* sequencing for identification of rapidly growing mycobacterium, the technique unambiguously differentiated *M. chelonae* and *M. abscesses* [157]. On the other hand, slowly growing mycobacteria were found to display high sequence variation in their ITS, which can be used to distinguish between *M. kansasii* and *M. gastri* [156]. *dnaJ* is also found to constitute a higher discriminatory power with mean sequence similarity of 80.4% among the studied species compared to 16S rDNA, *rpoB*, and *hsp65* genes with 96.6%, 91.3%, and 91.1% mean sequence similarities, respectively, and is particularly useful for identifying the non-tuberculous *Mycobacterium* species [205].

As for the rare bacteria, *Ehrlichia*, although 16S rDNA and *groEL* were useful for taxonomic classification and differentiation of the various species, other gene

targets such as the citrate synthase gene, *gltA*, have also been explored to improve identification and diagnosis of ehrlichial diseases [208] [183]. *gltA* has also been used as a complementary approach to 16S rDNA gene sequencing for phylogenetic studies of the *Rickettsiaceae* [209]. The various alternative targets being explored to supplement 16S rDNA gene sequencing for identification of different groups of bacteria suggested that there is no single target that is superior to others. The limitations of 16S rDNA gene sequencing and other currently available gene targets also emphasize the importance of a "polyphasic" approach for accurate bacterial identification. By this "polyphasic" approach, it refers to the use of different methods, e.g., phenotypic tests plus 16S rDNA gene sequencing plus sequencing of other gene targets, to identify bacteria belonging to species which are known to be difficult to identify. With more bacterial complete genome sequences available in the near future from high-throughput sequencing technology, comparative genomic studies will also enable more comprehensive study of different gene targets for study of phylogeny and identification of bacteria [210].

## Future Developments and New Technologies for Bacterial Identification

16S rDNA gene sequencing has not only helped answer some of our most fundamental questions in biology, this technology has now developed beyond the research realm and matured into clinical applications. As 16S rDNA gene sequencing is associated with limitations for particular groups of bacteria, the development of alternative gene targets will continue to be important for identification of these bacteria by sequencing technology. Moreover, despite the wide range of software and databases available, automation of 16S rDNA gene sequencing is still not available and interpretation of results often difficult by inexperienced users. The development of more user-friendly guidelines and software with high-quality, comprehensive databases, as well as the integration of high-throughput technologies will make automation of universal gene amplification and sequencing a possibility in the near future, which may replace the use of conventional phenotypic methods for routine bacterial identification in clinical microbiological laboratories in one day.

Apart from universal gene amplification and sequencing, other advanced technologies coming up in the twenty-first century have emerged as new methods for bacterial identification in clinical laboratories, among which matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was shown to be the most promising technology. Although the MS technology has been established for over a century, it is only until recently that simple-to-use MALDI-TOF MS device has become available for the identification of pathogens in clinical microbiology laboratories. MALDI-TOF MS enables the analysis of biological molecules with no theoretical upper mass limit, and MS detects the mass-to-charge

ratio of a biological molecule. Using combination of these technologies, proteins and peptides are separated by their mass, giving an individual molecular fingerprint to each bacterium. In practice, a single bacterial colony or a centrifuged portion of a liquid culture is sufficient for MALDI-TOF MS analysis. A protein mass spectra database of known bacteria is then used to match the spectra of the bacterium under investigation. Theoretically, there is no limit to the identification ability of MALDI-TOF MS, as long as a suitable spectrum is present in the database. Researchers can also create their own library of bacterial mass spectra to increase its applicability. Different prospective studies have been carried out to examine the performance of MALDI-TOF MS identification using clinical samples [36, 42, 211–218]. A recent international study carried out in eight different laboratories also reported that this approach could achieve high interlaboratory reproducibility [219]. Overall, these studies showed that MALDI-TOF MS has become an efficient and reliable alternative method for bacterial identification, replacing the traditional biochemical techniques. However, for the bacterial species that are identified by MALDI-TOF MS with a low score, which may represent a bacterial species not included in the database or a novel bacterial species, retrieving the mass-to-charge ratio profile for a particular bacterial strain for manual interpretation is not possible. This is in contrast to gene sequencing which the manual interpretation of DNA sequence is allowed. In such circumstances, gene sequencing should be performed for final confirmation of identification. This is also the main reason that, despite the wide usage in many clinical microbiology laboratories, MALDI-TOF MS is still not considered the gold standard for identification of bacterial species. To date, MALDI-TOF MS identification still requires a growth step in order to obtain sufficient material for acquisition of mass spectra, and it is not able to identify all pathogens in mixed cultures. Nevertheless, along with further technical improvement, standardized work procedures, and the potential to create interlaboratory databases, MALDI-TOF MS technology will continuously play a significant role in diagnostic microbiology and probably use as a first-line epidemiological tool in the years to come.

With the advent of next-generation sequencing technology available at a much lower cost than traditional Sanger sequencing, genome sequencing represents a promising tool for rapid bacterial identification, particularly in cases where gene sequencing or a polyphasic approach involving both phenotypic and genotypic tests cannot give a confident species identity [220]. With further automation, lower sequencing costs and growing number of bacterial genome sequences, genome sequencing may emerge as a promising tool for bacterial identification in the field of diagnostic microbiology in future.

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# Molecular Techniques for Blood and Blood Product Screening

**Yuan Hu**

## Introduction

The Food and Drug Administration (FDA) is responsible for ensuring the safety of more than 15 million units of blood and blood components donated each year in the United States. "Blood banking has become a manufacturing industry, an industry that must conform to high standards and quality control requirements comparable to those of pharmaceutical companies or other regulated industries," said David A. Kessler, M.D., former FDA commissioner [1]. Screening donated blood for infectious diseases that can be transmitted through blood transfusion is a very important step in ensuring safety. The United States has the safest blood supply in the world [1], and the FDA is striving to keep it safe by decreasing the risk of infectious disease transmission. The regulatory agency is continuously updating its requirements and standards for collecting and processing blood. As mentioned earlier, an important step in ensuring safety is the screening of donated blood for infectious diseases [2, 3]. In the United States, tests for infectious diseases are routinely conducted on each unit of donated blood, and these tests are designed to comply with regulatory requirements (Table 1). The field of clinical microbiology and virology is now focusing on molecular technology. Currently, nucleic acid testing techniques have been developed to screen blood and plasma products for evidence of very recent viral infections that could be missed by conventional serologic tests [2]. It is time for all blood safety procedures to include molecular detection techniques [3]. This approach can significantly aid in blood safety to reduce the risk of transmission of serious disease by transfusion. This chapter will review and update the current antigen/antibody-based technology, molecular biological technology, and published regulatory policy data for blood safety.

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**Table 1** Licensed/approved clinical assays for infectious agents

| Trade name | Infectious agent | Format | Specimen | Use | Manufacturer | Approval date | STN |
|---|---|---|---|---|---|---|---|
| HBsAg assays (detect hepatitis B surface antigen) | | | | | | | |
| Abbott PRISM HBsAg; Abbott PRISM HBsAg Confirmatory | HBV | ChLIA ChLIA-specific antibody neutralization | Serum/plasma/ cadaveric serum | Donor screening: qualitative detection of HBsAgConfirmatory: to confirm the presence of HBsAg in samples found to be repeatedly reactive by the Abbott PRISM HBsAg assay | Abbott LaboratoriesAbbott Park, ILUS License 0043 | 7/18/2006 | BL103766 |
| Genetic Systems HBsAg EIA 3.0;Genetic Systems HBsAg Confirmatory Assay 3.0 | HBV | EIA | Serum/plasma/ cadaveric serum | In vitro diagnosticDonor screening: qualitative detection of HBsAg Confirmatory: to confirm the presence of HBsAg in reactive specimens | Bio-Rad LaboratoriesRedmond, WAUS License 1109 | 1/23/2003 | BL103590 |
| Anti-HBc assays (detect antibodies to hepatitis B virus core antigen) | | | | | | | |
| ORTHO HBc ELISA Test System | HBV | ELISA | Serum/plasma | In vitro diagnostic, Donor screening: qualitative detection of total antibody to hepatitis B core antigen | Ortho-Clinical Diagnostics, IncRaritan, NJ | 4/23/1998 | BL103062 |
| Abbott PRISM HBcore | HBV | ChLIA | Serum/plasma | Donor screening, qualitative detection of total antibody to hepatitis B core antigen | Abbott LaboratoriesAbbott Park, ILUS License 0043 | 10/13/2005 | BL103785 |

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|---|---|---|---|---|---|---|---|
| HBV nucleic acid assays (detect hepatitis B virus DNA); see also multiplex assays | | | | | | | |
| UltraQual™ HBV PCR Assay | HBV | PCR | Plasma | Source plasma donor screening: qualitative detection of HBV DNA. For in-house use only at NGI | National Genetics Institute Los Angeles, CA US License 1582 | 9/1/2011 | BL125193 |
| COBAS AmpliScreen HBV Test | HBV | PCR | Plasma/ cadaveric serum or plasma | Donor screening: qualitative detection of HBV DNA | Roche Molecular Systems, Inc.Pleasanton, CAUS License1636 | 4/21/2005 | BL125090 |
| Anti-HCV assays (detect antibodies to hepatitis C virus encoded antigen) | | | | | | | |
| Abbott PRISM HCV | HCV | ChLIA | Serum /plasma/ cadaveric serum | Donor screening: qualitative detection of antibodies to hepatitis C virus | Abbott LaboratoriesAbbott Park, IL US License 0043 | 7/11/2007 | BL103762 |
| Ortho HCV Version 3.0 ELISA Test System | HCV | EIA | Serum/plasma | Donor screening: qualitative detection of antibodies to hepatitis C virus | Ortho-Clinical Diagnostics, IncRaritan, NJUS License 1236 | 4/23/19982/18/2009 | BL103065 |
| HCV nucleic acid assays (detect hepatitis C virus RNA); see also multiplex assays | | | | | | | |
| Hepatitis C Virus RT PCR Assay | HCV | PCR | Plasma | Source plasma donor screening: qualitative detection of HCV RNA. For in-house use only by Baxter Healthcare International | BioLife Plasma Services, L.P.Deerfield, ILUS License 1640 | 2/9/2007 | BL125101 |
| UltraQual HCV RT-PCR Assay | HCV | PCR | Plasma | Source plasma donor screening: qualitative detection of HCV RNA. For in-house use only at NGI | National Genetics InstituteLos Angeles, CAUS License 1582 | 9/18/2001 | BL103868 |

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**Table 1** (continued)

| Trade name | Infectious agent | Format | Specimen | Use | Manufacturer | Approval date | STN |
|---|---|---|---|---|---|---|---|
| COBAS AmpliScreen HCV Test, ver 2.0 | HCV | PCR | Plasma/ cadaveric serum or plasma | Donor screening: qualitative detection of HCV RNA.Donor supplemental: to confirm HCV infection for specimens that are repeatedly reactive on a licensed donor screening test for antibodies to HCV and reactive on the COBAS® AmpliScreen HCV Test, v2.0 | Roche Molecular Systems, IncPleasanton, CAUS License 1636 | 12/3/2002 | BL125045 |
| Anti-HIV-1 assays (detect antibodies to human immunodeficiency virus type 1) | | | | | | | |
| Fluorognost HIV-1 IFA | HIV-1 | IFA | Serum/ plasmaDried blood spot | Diagnostic and donor supplemental: qualitative detection of antibodies to HIV-1 for use as an additional, more specific test in specimens found to be repeatedly reactive by screening proceduresDonor screening: only in special cases | Sanochemia Pharmazeutika AGVienna, AustriaUS License 1631 | 2/5/19925/14/1996 | BL103288BL103651 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Cambridge Biotech HIV-1 Western Blot Kit | HIV-1 | WB | Serum /plasma | Diagnostic and donor supplemental: qualitative detection of antibodies to HIV-1 for use as an additional, more specific test in specimens found to be repeatedly reactive by screening procedures | Maxim Biomedical, Inc. Rockville, MDUS License 1741 | 5/28/1998 | BL103843 |
| GS HIV-1 western blot | HIV-1 | WB | Serum/ plasmaDried blood spot | Diagnostic and donor supplemental: qualitative detection of antibodies to HIV-1 for use as an additional, more specific test in specimens found to be repeatedly reactive by screening procedures | Bio-Rad LaboratoriesRedmond, WAUS License 1109 | 11/13/1998 | BL103655 |
| Avioq HIV-1 Microelisa System | HIV-1 | EIA | Serum, plasma, dried blood spot, oral fluid | In vitro diagnostic: qualitative detection of antibodies to HIV-1 | Avioq Inc.,Rockville, MD | 9/21/2009 | BP090022 |
| Maxim (Calypte) HIV-1 Urine EIA | HIV-1 | EIA | Urine | In vitro diagnostic: qualitative detection of antibodies to HIV-1 | Maxim Biomedical, Inc. Rockville, MDUS License 1741 | 1/12/2001 | BP000009 |
| INSTI HIV-1/HIV-2 Antibody Test | HIV-1 and HIV-2 | Rapid immunoassay | Plasma /whole blood (venipuncture and finger stick) | In vitro diagnostic: qualitative detection of antibodies to HIV-1 | bioLytical Laboratories Inc.British Columbia, Canada | 1/28/2015 | BP090032/7 |

(continued)

**Table 1** (continued)

| Trade name | Infectious agent | Format | Specimen | Use | Manufacturer | Approval date | STN |
|---|---|---|---|---|---|---|---|
| Reveal Rapid HIV-1 Antibody Test | HIV-1 | Rapid immunoassay | Serum/plasma | In vitro diagnostic: qualitative detection of antibodies to HIV-1; point-of-care test | MedMira Laboratories, Inc.Halifax, Nova ScotiaCanada | 4/16/2003 | BP000023 |
| OraSure HIV-1 Western Blot Kit | HIV-1 | WB | Oral fluid | Diagnostic supplemental: qualitative detection of antibodies to HIV-1 for use as an additional, more specific test in oral fluid specimens found to be repeatedly reactive by the Oral Fluid Vironostika HIV-l Microelisa System | OraSure TechnologiesBethlehem, PA | 6/3/1996 | BP950004 |
| Cambridge Biotech HIV-1 Urine Western Blot Kit | HIV-1 | WB | Urine | Diagnostic supplemental: qualitative detection of antibodies to HIV-1 for use as an additional, more specific test in urine specimens found to be repeatedly reactive by Maxim HIV-1 Urine EIA | Maxim Biomedical, Inc. Rockville, MDUS License 1741 | 6/21/2001 | BP010009 |

| HIV-1 nucleic acid assays (detect human immunodeficiency virus type 1 RNA); see also multiplex assays | | | | | | | |
|---|---|---|---|---|---|---|---|
| Human Immunodeficiency Virus, Type 1 RT PCR Assay | HIV-1 | Qualitative PCR | Plasma | Source plasma donor screening: qualitative detection of HIV-1 RNA. For in-house use only by Baxter Healthcare International | BioLife Plasma Services, L.P.Deerfield, ILUS License 1640 | 1/31/2007 | BL125100 |
| UltraQual HIV-1 RT-PCR Assay | HIV-1 | Qualitative PCR | Plasma | Source plasma donor screening: qualitative detection of HIV-1 RNA. For in-house use only at NGI | >National Genetics InstituteLos Angeles, CAUS License 1582 | 9/18/2001 | BL103902 |
| COBAS AmpliScreen HIV-1 Test, ver 1.5 | HIV-1 | Qualitative PCR | Plasma/ cadaveric serum or plasma | Donor screening: qualitative detection of HIV-1 RNA | Roche Molecular Systems, Inc.Pleasanton, CAUS License 1636 | 12/20/2002 | BL125059 |
| APTIMA HIV-1 RNA Qualitative Assay | HIV-1 | TMA | Plasma/serum | In vitro diagnostic: qualitative detection of HIV-1 RNA | Gen-Probe, Inc.,San Diego, CAUS License 1592 | 10/4/2006 | BL103966 |
| COBAS HIV-1 | HIV-1 | Quantitative PCR | Plasma | Patient monitoring: quantitation of HIV-1 RNA in plasma of HIV-1 infected individuals | Roche Molecular Systems, Inc.Pleasanton, CAUS license 1636 | 12/18/2015 | BP150262 |
| Abbott RealTime HIV-1 | HIV-1 | Quantitative PCR | Plasma | Patient monitoring: quantitation of HIV-1 RNA in plasma of HIV-1 infected individuals | ABBOTT Molecular, Inc., Des Plaines, IL | 5/11/2007 | BP060002 |

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**Table 1** (continued)

| Trade name | Infectious agent | Format | Specimen | Use | Manufacturer | Approval date | STN |
|---|---|---|---|---|---|---|---|
| Amplicor HIV-1 Monitor Test | HIV-1 | Quantitative PCR | Plasma | Patient monitoring: quantitation of HIV-1 RNA in plasma of HIV-1 infected individuals. | Roche Molecular Systems, Inc.Pleasanton, CAUS License 1636 | 3/2/1999 | BP950005 |
| COBAS AmpliPrep/ COBAS TaqMan HIV-1 Test | HIV-1 | Quantitative PCR | Plasma | Patient monitoring: quantitation of HIV-1 RNA in plasma of HIV-1 infected individuals | Roche Molecular Systems, Inc.Pleasanton, CAUS License 1636 | 5/11/2007 | BP050069 |
| Versant HIV-1 RNA 3.0 (bDNA) | HIV-1 | Quantitative PCR | Plasma | Patient monitoring: quantification of HIV-1 RNA in plasma of HIV-1 infected individuals | Siemens Healthcare Diagnostics, Inc. | 9/11/2002 | BP000028 |
| ViroSeq HIV-1 Genotyping System | HIV-1 | Genotyping | Plasma | Patient monitoring: for detecting HIV genomic mutations (in the protease and part of the reverse transcriptase regions of HIV) that confer resistance to specific types of antiretroviral drugs, as an aid in monitoring and treating HIV infection | Celera DiagnosticsAlameda, CA | 6/11/20037/10/2008 | BK030033,BK080026 |

| Trugene HIV-1 Genotyping Kit and Open Gene DNA Sequencing System | HIV-1 | Genotyping | Plasma | Patient monitoring: for detecting HIV genomic mutations (in the protease and part of the reverse transcriptase regions of HIV) that confer resistance to specific types of antiretroviral drugs, as an aid in monitoring and treating HIV infection | Siemens Healthcare Diagnostics, Inc. | 4/24/20023/27/2012 | BK020005, BK120013 |
|---|---|---|---|---|---|---|---|
| Anti-HIV specimen collection devices, testing services, and home test kits | | | | | | | |
| Home Access HIV-1 Test System | HIV-1 | Dried blood spot collection device | Dried blood spot | In vitro diagnostic: self-use by people who wish to obtain anonymous HIV testing | Home Access Health Corp.,Hoffman Estates, IL | 7/22/1996 | BP950002 |
| OraSure HIV-1 Oral Specimen Collection Device | HIV-1 | Oral specimen collection device | Oral fluid | For use with HIV diagnostic assays that have been approved for use with this device | OraSure TechnologiesBethlehem, PA | 12/23/1994 | BP910001 |
| OraQuick In-Home HIV Test | HIV-1, HIV-2 | Immunoassay | Oral fluid | Over-the-counter (OTC) diagnostic home-use test. A positive result is preliminary, and follow-up confirmatory testing is needed | OraSure TechnologiesBethlehem, PA | 07/03/2012 | BP120001 |

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**Table 1** (continued)

| Trade name | Infectious agent | Format | Specimen | Use | Manufacturer | Approval date | STN |
|---|---|---|---|---|---|---|---|
| Anti-HIV-2 assays (detect antibodies to human immunodeficiency virus type 2) | | | | | | | |
| Genetic Systems HIV-2 EIA | HIV-2 | EIA | Serum/plasma | In vitro diagnostic, Donor screening: qualitative detection of antibodies to HIV-2 | Bio-Rad LaboratoriesRedmond, WAUS License 1109 | 4/25/1990 | BL103227 |
| Anti-HIV-1/2 assays (detect antibodies to human immunodeficiency virus types 1 and 2) | | | | | | | |
| Abbott PRISM HIV O Plus assay | HIV-1, HIV-2 | ChLIA | Plasma/serum/ cadaveric serum | In vitro diagnosticDonor screening: qualitative detection of antibodies to HIV-1 groups M and O and/or antibodies to HIV-2 | Abbott LaboratoriesAbbott Park, ILUS License 0043 | 9/18/2009 | BL125318 |
| Geenius HIV 1/2 Supplemental Assay | HIV-1, HIV-2 | HIV detection test | Blood | Used as specific test to confirm the presence of antibodies of HIV-1 and HIV-2 for the detection of specimens to be repeatedly reactive by screening procedures | Bio-Rad Laboratories, IncRedmond, WAUS License 1109 | 10/24/2014 | BP140120 |
| Genetic Systems HIV-1/HIV-2 Plus O EIA | HIV-1, HIV-2 | EIA | Serum/plasma/ cadaveric serum | In vitro diagnostic Donor screening: qualitative detection of antibodies to HIV-1 (groups M and O) and/ or HIV-2 | Bio-Rad LaboratoriesRedmond, WAUS License 1109 | 8/5/2003 | BL125030 |

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|---|---|---|---|---|---|---|---|
| ADVIA Centaur HIV 1/O/2 Rapid Test | HIV-1, HIV-2 | Microparticle chemi-luminometric immunoassay | Plasma/serum | In vitro diagnostic: qualitative determination of antibodies to HIV-1, including group O, and/or HIV-2 | Siemens Healthcare Diagnostics, Inc. | 5/18/2006 | BP050030 |
| VITROS HIV-1/ HIV-2 Reagent Pack and Calibrator | HIV-1, HIV-2 | Immunometric | Plasma/serum | In vitro diagnostic: qualitative detection of antibodies to HIV-1 and/or HIV-2 | Ortho-Clinical Diagnostics, IncRaritan, NJUS License 1236 | 3/27/2008 | BP050051 |
| Multispot HIV-1/ HIV-2 Rapid Test | HIV-1, HIV-2 | Rapid immunoassay | Plasma/serum | In vitro diagnostic: qualitative detection of antibodies to HIV-1 and/or HIV-2 | Bio-Rad LaboratoriesRedmond, WAUS License 1109 | 11/12/2004 | BP040046 |
| SURE CHECK HIV 1/2 ASSAY | HIV-1, HIV-2 | Rapid immunoassay | Finger stick and venous whole blood, serum, plasma | In vitro diagnostic: qualitative detection of antibodies to HIV-1 and/or HIV-2; point-of-care test | Chembio Diagnostic Systems, Inc.Medford, NY | 5/25/2006 | BP050009 |
| HIV 1/2 STAT-PAK ASSAY | HIV-1, HIV-2 | Rapid immunoassay | Finger stick and venous whole blood, serum, plasma | In vitro diagnostic: qualitative detection of antibodies to HIV-1 and/or HIV-2; point-of-care test | Chembio Diagnostic Systems, Inc.Medford, NY | 5/25/2006 | BP050010 |
| OraQuick ADVANCE Rapid HIV-1/2 Antibody Test | HIV-1, HIV-2 | Rapid immunoassay | Oral fluid, plasma, whole blood (venipuncture and finger stick) | In vitro diagnostic: qualitative detection of antibodies to HIV-1 and/or HIV-2; point-of-care test | OraSure TechnologiesBethlehem, PA | 11/7/20026/22/2004 | BP010047 |

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**Table 1** (continued)

| Trade name | Infectious agent | Format | Specimen | Use | Manufacturer | Approval date | STN |
|---|---|---|---|---|---|---|---|
| Chembio DPP® HIV 1/2 Assay | HIV-1, HIV-2 | Rapid immuno-chromatographic assay | Oral fluid, serum, plasma, Whole blood (venipuncture, finger stick) | In vitro diagnostic: qualitative detection of antibodies to HIV-1 and/or HIV-2; point-of-care test | Chembio Diagnostic Systems, Inc.Medford, NY | 12/19/2012 | BP120032 |
| Uni-Gold™ Recombigen® HIV-1/2 | HIV-1, HIV-2 | Rapid EIA | Serum, plasma, Whole blood (venipuncture, finger stick) | In vitro diagnostic: qualitative detection of antibodies to HIV-1 and/or HIV-2; point-of-care test | Trinity BiotechJamestown, NY | 12/23/200302/04/2013 | BP030025 |
| Anti-HIV-1/2 and HIV-1 antigen combo assays (detect HIV-1 antigen and antibodies to HIV types 1 and 2) | | | | | | | |
| BioPlex 2200 HIV Ag-Ab Assay | HIV-1 and HIV-2 | Multiplex flow immunoassay | Serum, plasma | In vitro diagnostic: simultaneous qualitative detection and differentiation of HIV p24 antigen and antibodies to HIV-1 (group M and group O) and/or HIV-2 | Bio-Rad LaboratoriesRedmond, WAUS License 1109 | 7/23/2015 | BP140111 |
| ADVIA Centaur HIV Ag/Ab Combo (CHIV) Assay | HIV-1, HIV-2 | Microparticle chemi-luminometric immunoassay | Serum | In vitro diagnostic: simultaneous qualitative detection and differentiation of HIV p24 antigen and antibodies to HIV-1 (group M and group O) and/or HIV-2 | Siemens Healthcare Diagnostics, Inc. | 6/8/2015 | BP140103 |

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|---|---|---|---|---|---|---|---|
| ARCHITECT HIV Ag/Ab Combo | HIV-1, HIV-2 | CMIA | Plasma/serum | In vitro diagnostic: simultaneous qualitative detection of HIV p24 antigen and antibodies to HIV-1 (group M and group O) and/or HIV-2 | Abbott LaboratoriesAbbott Park, ILUS License 0043 | 6/18/2010 | BP090080 |
| GS HIV Ag/Ab Combo EIA | HIV-1, HIV-2 | EIA | Plasma/serum | In vitro diagnostic: simultaneous qualitative detection of HIV p24 antigen and antibodies to HIV-1 (group M and group O) and/or HIV-2 | Bio-Rad LaboratoriesRedmond, WAUS License 1109 | 7/22/2011 | BP100064 |
| Alere DetermineTMHIV-1/2 Ag/Ab Combo | HIV-1, HIV-2 | Immunoassay | Serum, plasma, Whole blood (venipuncture, finger stick) | In vitro diagnostic: simultaneous qualitative detection of HIV p24 antigen and antibodies to HIV-1 and/or HIV-2; point-of-care test | Alere Scarborough, Inc. Scarborough, ME | 8/8/2013 | BP120037 |
| Anti-HTLV-I/II Assays (detect antibodies to Human T-Lymphotropic Virus types I and II) | | | | | | | |
| Avioq HTLV-I/II Microelisa System | HTLV-I, HTLV-II | EIA lysate | Serum/plasma | In vitro diagnostic, Donor screening: qualitative detection of antibodies to HTLV-I and HTLV-II | Avioq, Inc. Research Triangle Park, NC 27709US License 1856 | 03/26/2012 | BL125394 |
| Abbott PRISM HTLV-I/HTLV-II | HTLV-I, HTLV-II | ChLIA | Serum/plasma | Donor screening: qualitative detection of antibodies to HTLV-I and HTLV-II | Abbott LaboratoriesAbbott Park, ILUS License 0043 | 1/16/2008 | BL103761 |

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**Table 1** (continued)

| Trade name | Infectious agent | Format | Specimen | Use | Manufacturer | Approval date | STN |
|---|---|---|---|---|---|---|---|
| MP Diagnostic HTLV Blot 2.4 | HTLV-I, HTLV-II | WB | Serum/plasma | Donor supplemental: qualitative detection and differentiation of antibodies to HTLV-I and HTLV-II for use as an additional, more specific test in specimens found to be repeatedly reactive by screening procedures | MP Biomedicals Asia Pacific PTE. LTD | 12/11/2014 | BL125475 |
| Anti-*T. pallidum* assays (detect antibodies to *Treponema pallidum*) and other serological tests for syphilis | | | | | | | |
| CAPTIATM Syphilis (*T. Pallidum*)-G | *T. Pallidum* | EIA | Serum, plasma | Donor screening: qualitative detection of IgG antibodies to *Treponema pallidum* | Trinity BiotechJamestown, NY | 01/24/2002 | K014233 |
| ASI TPHA Test | *T. Pallidum* | Micro-hemagglutination | Serum | Donor screening: qualitative detection of IgG and IgM antibodies to *Treponema pallidum* | Arlington Scientific, IncSpringville, UT | 1/30/2003 | BK020031 |
| Olympus PK TP System | *T. Pallidum* | Micro-hemagglutination | Serum, plasma | Donor screening: qualitative detection of IgG and IgM antibodies to *Treponema pallidum* | Fujirebio Diagnostics Inc..Malvern, PA | 2/21/2003 | BK030007 |
| TPHA Screen | *T. Pallidum* | Hemagg-lutination | Serum, plasma | Donor screening: qualitative detection of IgG and IgM antibodies to *Treponema pallidum* | Immucor, Inc.Norcross, GA | 10/24/2012 | BK120021 |

| ASiManager-AT™ | *T. Pallidum* | Rapid plasma reagin (RPR) test | Serum, plasma | Donor screening: qualitative detection of reagin antibodies | Arlington Scientific, Inc. Springville, UT. | 2/24/2014 | BK 130001 |
|---|---|---|---|---|---|---|---|
| Anti-*T. cruzi* assays (detect antibodies to *Trypanosoma cruzi*) | | | | | | | |
| Abbott ESA Chagas | *T. Cruzi* | Multistep enzyme strip immunoassay | Serum, plasma | An additional, more specific test on human serum or plasma specimens found to be repeatedly reactive using a licensed screening test for antibodies to *T. cruzi* | Abbott LaboratoriesAbbott Park, ILUS License 0043 | 11/18/2011 | 125,361/23 |
| Abbott PRISM Chagas | *T. Cruzi* | EIA | Serum/plasma | Donor screening: qualitative detection of antibodies to *T. cruzi* | Abbott LaboratoriesAbbott Park, ILUS License 0043 | 4/30/2010 | BL125361 |
| ORTHO *T. cruzi* ELISA Test System | *T. Cruzi* | EIA | Serum/plasma/cadaveric | Donor screening: qualitative detection of antibodies to *T. cruzi* | Ortho-Clinical Diagnostics, Inc.Raritan, NJUS License 1236 | 12/13/2006 | BL125161 |
| WNV nucleic acid assays (detect *West Nile* virus RNA) | | | | | | | |
| Procleix *West Nile* Virus (WNV) Assay | WNV | Nucleic acid test (TMA) | Plasma/cadaveric plasma or serum | Donor screening: qualitative detection of West Nile virus (WNV) RNA. | Gen-Probe, Inc.,San Diego, CAUS License 1592 | 12/1/2005 | BL125121 |
| COBAS TaqScreen *West Nile* Virus Test | WNV | PCR | Plasma/cadaveric plasma or serum | Donor screening: qualitative detection of *West Nile* virus (WNV) RNA | Roche Molecular Systems, Inc.Pleasanton, CAUS License 1636 | 8/28/2007 | BL125245 |

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**Table 1** (continued)

| Trade name | Infectious agent | Format | Specimen | Use | Manufacturer | Approval date | STN |
|---|---|---|---|---|---|---|---|
| Anti-CMV assays (detect antibodies to *Cytomegalovirus*) | | | | | | | |
| Capture-CMV | CMV | Solid-phase red cell adherence | Serum, plasma | Donor screening: qualitative detection of IgG and IgM antibodies to CMV | Immucor, Inc.Norcross, GA | 12/22/1995 | BK950029 |
| Olympus PK CMV-PA System | CMV | Passive particle agglutination | Serum, plasma | Donor screening: qualitative detection of IgG and IgM antibodies to CMV | Fujirebio Diagnostics, Inc.Malvern, PA | 9/20/2007 | BK070030 |
| Multiplex assays | | | | | | | |
| COBAS TaqScreen MPX Test | HBV, HCV, HIV-1, HIV-2 | PCR | Plasma/ cadaveric plasma or serum | Donor screening: simultaneous qualitative detection of HBV DNA, HIV-1 group M and group O RNA, HIV-2 RNA, and HCV RNA | Roche Molecular Systems, Inc.Pleasanton, CAUS License 1636 | 12/30/2008 | BL125255 |
| COBAS TaqScreen MPX Test version 2.0 | HBV, HCV, HIV-1, HIV-2 | PCR | Plasma | Donor screening: simultaneous qualitative detection of HBV DNA, HIV-1 group M and group O RNA, HIV-2 RNA, and HCV RNA | Roche Molecular Systems, Inc.Pleasanton, CAUS License 1636 | 12/19/2014 | BL 125459 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Procleix Ultrio Assay | HBV, HCV, HIV-1 | Nucleic acid test (TMA | Plasma/serum/ cadaveric plasma or serum | Donor screening: simultaneous qualitative detection of HBV DNA, HCV RNA, and HIV-1 RNA | Gen-Probe, Inc.,San Diego, CAUS License 1592 | 10/3/2006 | BL125113 |
| Procleix Ultrio Plus Assay | HBV, HCV, HIV-1 | Nucleic acid test (TMA) | Plasma/serum/ cadaveric plasma or serum | Donor screening: simultaneous qualitative detection of HBV DNA, HCV RNA, and HIV-1 RNA | Gen-Probe, Inc.,San Diego, CAUS License 1592 | 5/25/2012 | BL125113 |

Source: Center for Biologics Evaluation and Research, US Food and Drug Administration

## Limitations for Current Technologies Used in Blood Safety

Direct detection of viral antigens and virus-specific antibodies has been a common tool for the diagnosis of virus infections in the past 40 years. There are some limitations. For direct detection of virus antigens, shortly after virus infection, only a few viruses release antigens in amounts sufficiently detectable in the body by an antibody-mediated assay. For indirect virus detection by virus-specific antibodies [e.g., an immunofluorescence assay or enzyme immunoassay (EIA), etc.], there is a problem in that shortly after infection by a pathogenic virus, and there is a window period in which antibody generation is insufficient for detection [4]. To reduce this window period of low detection, direct nucleic acid tests are needed.

## Application of Advanced Molecular Techniques in Blood Safety Applications

Through the application of molecular biology, biological and biochemical analyses have been revolutionized, and nucleic acid, gene-based techniques have been developed to screen blood and plasma donations for evidence of very recent and earlier viral infections that might otherwise be missed by conventional serologic testing. The nucleic acid tests can also provide evidence for genetic variation in viruses. Molecular methods include the use of nucleic acid probes as well as amplification-based and DNA sequence-based techniques. An increasing number of molecular diagnostic methods are now available commercially [2]. In comparison to classical methods, molecular biological methods are superior in terms of rapidness, specificity, and sensitivity. The current nucleic acid detection methods in the field may be grouped into two major classes: amplifying techniques such as PCR and non-amplifying techniques such as Southern blot hybridization. Amplifying techniques are more sensitive than non-amplifying techniques. There are two different types of amplifying methods [5], target amplification methods and signal amplification methods. Target amplifying techniques include PCR, nucleic acid sequence-based amplification (NASBA) [6, 7], self-sustaining sequence amplification (3SR), transcription-based amplification (TAS), transcription-mediated amplification (TMA), strand displacement amplification (SDA), and ligase chain reaction (LCR). Signal amplification methods include branched DNA signal amplification (bDNA) [8], cleavage-based signal amplification (cycling probe technologies and invader assay), Qß replicase, hybrid capture, cycling probe technologies (CPT), and rolling-circle amplification (RCA) [9]. To further insure the safety of blood products, it is of importance to further improve these and other types of nucleic acid testing [2–5].

## Major Different Generations of Nucleic Acid Detection Techniques

### *Southern Blot Hybridization (1970s)*

Southern blotting [10] was named after Edward M. Southern who developed this procedure at Edinburgh University in the 1970s. This technique is used to detect specific sequences within mixtures of DNA, which is size fractionated by gel electrophoresis and then transferred by capillary action to a suitable membrane. After blocking of non-specific binding sites, the nitrocellulose replica of the original gel electrophoresis experiment is then allowed to hybridize with an oligonucleotide probe representing the specific DNA sequence of interest. Should specific DNA be present on the blot, it will combine with the labeled probe and be detectable. By coelectrophoresing DNA fragments of known molecular weight, the size(s) of the hybridizing band(s) can then be determined. Southern blotting hybridization technology is one of the major tools that have already helped clinical staffs worldwide interpret genomic information. Other competing methodologies include in situ hybridization and solution hybridization. Important clinical examples of the use of this technology are DNA fingerprinting and the ability to detect DNA gene rearrangements.

### *Polymerase Chain Reaction (1980s)*

In 1983, Dr. Kary Mullis at Cetus Corporation conceived of polymerase chain reaction [11]. There is not a single technique that has had a greater impact on the practice of molecular biology than PCR. With this technique, we can detect infectious disease agents at an extremely low level. It is based on the ability of sense and antisense DNA primers to hybridize to a DNA of interest. Following extension from the primers on the DNA template by DNA polymerase, the reaction is heat denatured and allowed to anneal with the primers once again. Another round of extension leads to a multiplicative increase in DNA products. Therefore, a minute amount of DNA can be efficiently amplified in an exponential fashion to result in larger amounts of DNA that are more easily manipulated. By including critical controls, the technique can be made quantitative. The current level of the sensitivity and detection limit is as low as 10–50 copies per ml blood in HIV testing [1, 12, 13]. Important clinical examples of the use of PCR are detection of HIV and HCV [14–16]. PCR techniques have evolved into different branches. Some of them are now widely in use for virus detection in clinical diagnostics. These are real-time PCR by TaqMan (Roche), LightCycler (Roche), Smartcycler (Cepheid), in situ PCR, nested PCR, nested real-time PCR [17], broad-range PCR, multiplex PCR, RT-PCR, arbitrarily primer PCR, long PCR, and quantitative PCR. Real-time sequence technology will be coming soon for more detailed detection. In the past, identification of

viral serotypes was restricted to investigative methods using antibody detection and restriction fragment length polymorphism (RFLP). With real-time sequence technology, we will be able to detect a virus early as well as to obtain the viral sequence.

### *Microarrays (1990s)*

Microarrays were developed at Stanford University by Schena and co-workers in the early 1990s [18]. For medical applications, a microarray analysis offers a very accurate screening technology. It allows hundreds or thousands of nucleic acid hybridization reaction to be performed on a solid substrate. It promises to be a fast and accurate diagnostic tool in the field of clinical microbiology and virology. Applied to infection safety for blood and blood products, it will be able to screen for the presence of viral pathogens by matching genetic sequences. Compared with existing technologies, it allows for a wider variety of specific tests to be carried out simultaneously to determine the quality of the blood and will provide consumers with extra safety. With the use of molecular biology protocols, the microarray will permit the detection of lower concentrations of microorganisms in the blood and the accurate identification of many types of pathogenic contaminants. In the near future, progress can be expected in the application of microarray technology for screening of donated blood for infectious agents. It can provide vast information about the identity of blood-borne pathogens as well as their gene expression profiles [19].

## Screening of Donor Blood for Infectious Agents

To ensure a safe blood supply for those who may need a transfusion, an important step in ensuring safety is the screening of donated blood for infectious agents [20]. After donation, each unit of donated blood undergoes a series of tests for blood-borne agents such as human immunodeficiency virus (HIV)-1, HIV-2, hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell lymphotropic virus (HTLV)-1 and HTLV-II, *West Nile virus* (WNV), *Treponema pallidum* (syphilis), *Trypanosoma cruzi* (*T. cruzi*), and cytomegalovirus.

## Confirmatory Testing of Donor Blood for Infectious Agents

All of the above tests are referred to as screening tests and are designed to detect as many infectious agents as possible. Because these tests are so sensitive, some donors may have a false-positive result, even when the donor has never been exposed to the particular infection. In order to sort out true infections from such false-positive test results, screening tests that are reactive may be followed up with more specific tests

called confirmatory tests. Thus, confirmatory tests help determine whether a donor is truly infected. If any one of these tests fails, affected blood products are considered unsuitable for transfusion [20].

## Application of Nucleic Acid Testing for Infectious Agents

Nucleic acid testing (NAT) employs testing technology that directly detects the genomes of viruses. Because NAT detects a virus's genetic material instead of waiting for the body's response, the formation of antibodies, as with many current tests, it offers the opportunity to reduce the window period during which an infecting agent is undetectable by traditional tests [21], thus further improving blood safety. Nucleic acid testing is becoming the gold standard because of greater sensitivity compared to antibody tests [2].

Since 1999, NAT has been approved by the FDA and used to detect HIV-1 and HCV; this technology currently is under investigation for detecting other infectious disease agents. We know that for many viral infections, viral RNA appears very early in the infection, in 1 to 2 weeks, but the antibody doesn't appear until 10–12 weeks, e.g., HIV and HCV [21]. In order to virtually prevent infection by all the transfusion-associated viruses, we need to detect the viruses in their window period, and a NAT or gene-based testing method is needed. NAT also provides an opportunity for the viral, e.g., HIV or HCV, infected donor to seek early treatment. On the other hand, NAT is not only a sensitive method but also a rapid method, which is suitable for a blood bank laboratory because the turnaround time for maintaining blood donations is extremely critical.

### *Hepatitis B Virus*

The hepatitis B virus (HBV) is a highly infectious and often non-symptomatic virus that is transmitted primarily through blood and blood-derived fluids and is a leading cause of liver infection worldwide [22]. The World Health Organization (WHO) estimates that 2 billion people worldwide have been infected with HBV and 350,000,000 people are chronically infected. Chronic infection results in a high risk for liver cancer and cirrhosis of the liver, which cause about 1000,000 deaths each year. Each year up to 200,000 people become newly infected in the United States alone. Since screening for HBV began in 1969, the rate of infection through blood transfusions has greatly decreased. However, as of 2000, HBV is still transmitted through blood transfusions in 1 out of 137,000 units of blood. One reason for this is that currently available blood screening technologies detect core antibodies or surface antigens, which appear up to 8 weeks after infection. Serologic tests for hepatitis B virus include hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (HBcAb).

## *Hepatitis B Surface Antigen*

HBV, which mainly infects the liver, has an inner core and an outer envelope (the surface). The HBsAg test detects the outer envelope, identifying an individual infected with the hepatitis B virus. This virus can cause inflammation of the liver, and in the earliest stage of the disease, infected people may feel ill or even have yellow discoloration of the skin or eyes, a condition known as jaundice. Fortunately, most patients recover completely and test negative for HBsAg within a few months after the illness. A small percentage of people become chronic carriers of the virus, and in these cases, the test may remain positive for years. Chronically infected people can develop severe liver disease as time passes and need to be followed carefully by an experienced physician. To reduce the occurrence of posttransfusion hepatitis, it is essential to screen all blood donations for hepatitis B surface antigen by the most sensitive and specific assays. Blood donations that are found to be reactive in the HBsAg test are automatically confirmed by the HBsAg confirmatory assay. If the specimen is neutralizable in the confirmatory test, the specimen is considered positive for HBsAg. Hepatitis B surface antigen testing of donated blood has begun in 1975 (Table 1).

Currently, all blood donors are screened for HBsAg, but occasional transmission of HBV still occurs due to the inclusion of window period donations (i.e., blood from recently infected donors who are antibody negative but still viremic). Detection of early HBV infection of blood donors is still a major problem of blood transfusion. The current third-generation licensed HBsAg tests (mostly radioimmunoassay and enzyme immunoassays) cannot detect HBV in the window period for HBV infection. This is a strong motivation for introducing molecular detection techniques to the field [23]. There are some commercially available test methods for detecting HBV DNA in the market now, such as Chiron's Quantiplex HBV DNA [24], Digene's Hybrid Capture, Abbott's HBV DNA assay, and Roche's Amplicor HBV Monitor. Using these commercial hybridization or PCR-based assays, HBV DNA can be detected 1–3 weeks before the appearance of HBsAg [25]. Some chronically infected patients who have lost their HBsAg remain HBV DNA positive but are disqualified as potential blood donors. Molecular detection of HBV DNA is more sensitive than current methods employed for HBsAg screening [22–25].

## *Antibodies to the Hepatitis B Core Antigen (Anti-HBc)*

Determination of anti-HBc (total) is also used to monitor the progress of the hepatitis B viral infection. Determination of anti-HBc (IgM) is employed to distinguish an acute hepatitis B infection from a chronic infection. The anti-HBc test developed in 1987 detects an antibody to the hepatitis B virus that is produced during and after infection. If an individual has a positive anti-HBc test, but the HBsAg test is negative, it may mean that the person once had hepatitis B but has recovered from the

infection. Of the individuals with a positive test for anti-HBc, many have not been exposed to the hepatitis B virus; thus, there is a frequent problem of false positives. Although the individual may be permanently deferred from donating blood, it is unlikely that the person's health will be negatively affected. (Note: This antibody is not produced following vaccination against hepatitis B [26].

## *Hepatitis C Virus*

The hepatitis C virus (HCV) is a member of the *Flaviviridae* family of viruses, which are associated with both human and animal diseases [27]. Hepatitis caused by HCV is the most common chronic blood-borne infection in the United States. Over 4 million Americans are believed to be infected. HCV can also be transmitted through blood transfusion. HCV causes inflammation of the liver, and up to 80% of those exposed to the virus develop a chronic infection, which can lead to liver inflammation, cirrhosis, cancer, and death. Eventually, up to 20% of people with HCV may develop cirrhosis of the liver or other severe liver diseases. As in other forms of hepatitis, individuals may be infected with the virus, but may not realize they are carriers since they do not have any symptoms. Because of the risk of serious illness, people with HCV need to be followed closely by a physician with experience evaluating this infection. Since the full-length HCV cDNA was first cloned in 1989, significant progress has been made in characterizing its molecular biology [13]. But, the natural history of HCV infection is still evolving, and current treatment options for patients are either limited or expensive [27]. There is no vaccine for HCV, and the current treatment includes a combination of alpha interferon and ribavirin as well as the combination of the nucleotide polymerase inhibitor sofosbuvir and the NS5A inhibitor velpatasvir [28]. Although the former is efficacious in only a minority of patients [29], the latter has been shown to be effective in a broad range of patients [28]. The life cycle of the HCV continues to be poorly understood due to the lack of an efficient cell culture system [30]. There is an urgent need to develop a highly sensitive detection method for studying possible extrahepatic sites for the replication of hepatitis C virus. We recently established a cell culture system for the replication of HCV by using human T and B leukemia cell lines [31]. This model should represent a valuable tool for the detailed study of the initial steps of the HCV replication cycle and for the evaluation of evolving antiviral molecules. Currently, appropriate vaccine strategies for HCV have not been developed. Early detection and prevention of HCV infection are most important for blood safety.

It is a formidable task to design primers and probes for sensitive nucleic acid level diagnostic assays throughout the open reading frame of the HCV genome because of a high mutation rate in this genomic region. However, the untranslated region of about 341 nucleotides contains highly conserved domains which allows for stable primer design for qualitative and quantitative diagnostic tests which have equivalent sensitivity against the known six various genotypes of HCV.

## *Antibodies to the Hepatitis C Virus (Anti-HCV)*

In 1990, the first specific test for hepatitis C virus, the major cause of "non-A, non-B" hepatitis, was introduced. Now, a third-generation ELISA kit is available to detect antibodies to HCV, and screening blood for HCV antibodies is recommended. These assays are based on detection of serum antibody to various HCV antigens because these antibodies are nearly universally present in patients who are chronically infected with HCV [32]. The HCV screening tests are known to have significant limitations, and positive samples should be further tested by HCV confirmatory tests.

## *HCV Confirmatory Tests*

Guidelines provided by the CDC recommend that HCV antibody screening test-positive samples should be confirmed with serologic or nucleic acid supplemental testing. HCV confirmatory tests include the recombinant immunoblot assay in which several recombinant peptide antigens are applied on a strip that is then probed with the patient's serum. In this way, the response to individual antigens can be recognized, and some false-positive ELISA results can be eliminated (e.g., RIBA, Chiron HCV 3.0, and PCR assay) (e.g., Roche COBAS Amplicor HCV test, version 2.0). Laboratories can choose to perform this testing on all positive specimens or based on screening test-positive (signal to cutoff) ratios. The positive predictive values (s/co) can vary depending on the prevalence of infection in the population being screened.

HCV antibodies are not generally detectable for at least 6 weeks and may not appear for several months. Acute HCV infections are relatively rare among blood donors, but the antibody tests often fail to detect these patients in the window period between the time of infection and the time of appearance of antibody detectable by the above assays. High-sensitivity detection of HCV during the window period is a long-term technical challenge in the field. Tests for HCV RNA genome detection based on the PCR or other highly sensitive RNA detection systems have been used for the diagnosis of acute hepatitis [26]. Sensitive detection of HCV RNA based on RT-PCR or other nucleic acid amplification techniques can be readily accomplished with kits that are now available commercially. For example, in 1999 the FDA-approved Roche's Amplicor HIV-1 Monitor ultrasensitive quantitative assay. It can measure HIV levels at as few as 50 copies/ml, and another commercial kit, the LCx HIV RNA quantitative assay from Abbott Laboratories, also has a detection limit at 50 copies/ml. Some studies even showed a sensitivity limit at one copy [33]. In fact, a qualitative assay should be much more sensitive than a quantitative assay for HIV/HCV screening. A sensitive qualitative HCV molecular detection assay will possibly interdict and virtually prevent all transfusion-associated HIV/HCV. The current sensitivity standard for clinical diagnostics is 100 copies per ml, but since there has been an improvement in technology, this would be the time to change sensitivity standard to 50 copies per ml.

## Human Retroviruses

### *Antibodies to Human Immunodeficiency Virus: Types 1 and 2 (Anti-HIV-1, -2)*

HIV-1 and/or HIV-2 virus cause acquired immunodeficiency syndrome or AIDS. The test is designed to detect antibodies directed against antigens of the HIV-1 or HIV-2 viruses. HIV-1 is much more common in the United States, whereas HIV-2 is prevalent in Western Africa. Donors are tested for both viruses because both are transmitted by infected blood, and a few cases of HIV-2 have been identified in US residents. In 1985, the first blood screening EIA test to detect HIV was licensed and quickly implemented by blood banks to protect the blood supply. In 1992, testing of donor blood for both HIV-1 and HIV-2 antibodies (anti-HIV-1 and anti-HIV-2) was implemented. In 1996, HIV p24 antigen testing of donated blood was mandated. Now, the p24 antigen testing is going to be compared with a PCR-based test for their ability to detect HIV in the window period.

### *Antibodies to Human T-Lymphotropic Virus: Types I and II (Anti-HTLV-I, Anti-HTLV-I-II)*

HTLV retroviruses are endemic in Japan and the Caribbean but relatively uncommon in the United States [34]. They cause adult T-cell leukemia/lymphoma and a neurological disorder similar to multiple sclerosis. The infection can persist for a lifetime but rarely causes major illnesses in most people who are infected. In rare instances, the virus may, after many years of infection, cause nervous system disease or an unusual type of leukemia. HTLV-II infections are usually associated with intravenous drug usage, especially among people who share needles or syringes. Disease associations with HTLV-II have been hard to confirm, but the virus may cause subtle abnormalities of immunity that lead to frequent infections or rare cases of neurological disease.

In 1989, human T-lymphotropic virus antibody testing of donated blood was begun. Blood is now routinely screened for antibodies to HTLV-I/II. These test screens for antibodies directed against epitopes of the HTLV-I and HTLV-II viruses. Several commercial assays based on the enzyme-linked immunosorbent assay (ELISA) or particle agglutination formats are used for screening of HTLV antibodies, followed by confirmatory assays using Western blotting. In some infected individuals, the serologic response to HTLV infection is very low. These problems have been solved by the application of PCR amplification of specific sequences in the virus genome. PCR can be used to detect HTLV-I/II proviruses and is now the method of choice for detection of HTLV DNA directly from blood and many other tissues. Commercial PCR kits for HTLV are available [34].

## West Nile Virus

The *West Nile virus* (WNV) is a single-stranded RNA virus of the *Flaviviridae* family and is one of the most recent emerging infectious disease threats to public health and, potentially, to the safety of our blood supply [35–37]. In 2002, WNV was identified as transfusion transmissible. It is transmitted by mosquitoes to birds and other animals through a mosquito bite. The virus can infect people, horses, many types of birds, and some other animals. WNV was shown in 2002 to be transmissible by blood [35], with an estimated mean risk of 2/10,000–5/10,000 in outbreak regions in the United States. The most common symptoms of transfusion-transmitted cases of WNV were fever and headache. Detection of WNV includes either a measurement of WNV antibodies or of WNV nucleic acid (detecting genetic material from the virus itself). There are two types of WNV antibody testing: IgM and IgG. In most individuals, IgM antibodies will be present within 8 days after the initial exposure to WNV, followed by IgG production several weeks later. But, the antibodies tested to detect WNV are not expedient for donor blood screening. Nucleic acid testing involves amplifying and measuring the *West Nile virus*'s genetic material to detect the presence of the virus in the blood or tissue. WNV NAT will be negative in the blood once clinical illness has occurred. In this situation, both NAT and IgM antibody testing may be needed. Nucleic acid tests to screen blood for WNV are commercially available and in current use. But, the viral yield for WNV infection is much lower than other viruses. Consequently, a more sensitive WNV NAT system for donor blood screening will be required, which could further reduce the risks of transfusion-transmitted WNV.

## *Syphilis*

Serum samples from all blood units should be subjected to either the VDRL (venereal disease research laboratory) test or a treponemal test, such as the *Treponema pallidum* hemagglutination (TPHA) test before transfusion. Any unit found positive should be discarded as per standard safety procedures. This test is done to detect evidence of infection with the spirochete that causes syphilis. Blood centers began testing for this shortly after World War II, when syphilis rates in the general population were much higher. The risk of transmitting syphilis through a blood transfusion is exceedingly small (no cases have been recognized in this country for many years) because the infection is very rare in blood donors and because the spirochete is fragile and unlikely to survive blood storage conditions. Sensitivity and specificity of serologic tests vary depending on the type of test performed and the stage of the disease. If the donor has spirochetemia, their serologic tests are usually negative, and if the donors are antibody positive, their blood is not infectious. Syphilis serological tests for donors have less clinical

significance. A nucleic acid test for accurately detecting syphilis is needed. It can be used to determine whether a blood donor is currently or has recently been infected with the spirochete.

## Other Concerns

### *Hepatitis Viruses*

In recent years, numerous infectious agents found worldwide have been identified as potential threats to the blood supply, and among these are several newly discovered hepatitis viruses that present unique challenges in assessing possible risks. Even if the hepatitis virus test is negative for all known A-E hepatitis agents, there are some unidentified hepatitis viruses, called non-A-E hepatitis viruses that can still be transmitted by blood transfusion. In the future, advances in NAT may allow rapid discovery of the unknown hepatitis viruses.

### *Hepatitis Delta Virus*

Hepatitis delta virus (HDV) is a small RNA virus that can infect only individuals who have HBV; worldwide more than 15 million people are coinfected [38]. HDV is clinically important because it generally makes HBV infections more damaging to the liver. Increased understanding of the molecular virology of HDV will identify novel therapeutic targets for this most severe form of chronic viral hepatitis. PCR and real-time PCR methods are available for HDV RNA detection [39].

### *TT Virus*

TT virus (TTV) [40], named for the patient from whom it was first isolated with non-A-E and G posttransfusion hepatitis in Japan in 1997, is a newly discovered transfusion-transmitted, single-stranded and circular DNA virus [41]. TTV is non-enveloped, and its entire sequence of ~3.9 kb has been determined. It is also often interpreted as a transfusion-transmitted virus [42]. At least 16 genotypes have been identified, and TTV is now found all over the world. TTV infection was sought by detection of TTV DNA in serum by polymerase chain reaction using primers generated from a conserved region of the TTV genome, e.g., the UTR region [42]. Donor blood and blood product can be screened for TTV DNA by using PCR or real-time PCR. The significance of positive findings is still unclear, because high-level TTV carriers in healthy populations are currently found [42–44]. Whether TTV actually causes hepatitis remains to be determined.

## *Cytomegalovirus*

Cytomegalovirus (CMV) is a virus belonging to the herpes group that is rarely transmitted by blood transfusion. Donor blood is not routinely tested for CMV, and the prevalence of CMV antibody ranges from 50 to 80% of the population. But, blood contaminated with CMV can cause problems in neonates or immunocompromised patients. It also remains a major pathogen for solid-organ transplant recipients causing febrile syndromes, hepatitis, pneumonitis, retinitis, and colitis. Potential problems in selected patient populations can be prevented by transfusing CMV negative blood or frozen, deglycerolized red blood cells. Serologic tests for antibody to CMV are useful for determining whether a patient had CMV infection in the past, a determination of great clinical importance for organ and blood donors and in the pretransplant evaluation of prospective transplant recipients [45]. Commercial NAT kits are available for CMV [5], and these include the Amplicor PCR CMV Monitor Test and Hybrid Capture system CMV DNA test.

## *Trypanosoma Cruzi*

Chagas disease is caused by the blood-borne parasite, *Trypanosoma cruzi*, which is transmitted to humans through insects. In the United States, Chagas disease is considered one of the neglected parasitic infections, a group of five parasitic diseases that have been targeted by CDC for public health action [46]. Commercial anti-*T. cruzi* assay kits are available for the qualitative detection of antibodies, *Trypanosoma cruzi* (*T. cruzi*), the causative agent of Chagas disease in human serum and plasma specimens by Abbott diagnostics [47].

## *Malaria*

Sensitive screening tests for malaria are neither commercially available nor officially approved yet. The most effective way of screening donors is to take a proper history of malaria or of fever that could be due to malaria [48]. Donor selection criteria should be designed to exclude potentially infectious individuals from donating red blood cells for transfusion. Because there are no practical laboratory tests available to test donor blood, donors traveling to high-risk malaria areas are excluded from donating blood for 6 months. However, there is a need to develop suitable screening tests, especially for use in an endemic area. A number of clinical research approaches have been developed for the extraction, amplification, and detection of malaria parasite DNA from blood products [49].

## *Variant Creutzfeldt-Jakob Disease*

Variant Creutzfeldt-Jakob disease [50] (vCJD, a rare but fatal brain infection) was first described in 1996 in the United Kingdom. vCJD is strongly linked with exposure to the bovine spongiform encephalopathy (BSE) agent. BSE is a transmissible spongiform encephalopathy (TSE) affecting cattle and was first reported in the United Kingdom in 1986. It has different clinical and pathologic characteristics from classic CJD. Each disease also has a particular genetic profile of the prion protein gene [51]. In recent years, questions have been raised concerning the potential risk of variant Creutzfeldt-Jakob disease for recipients of plasma-derived clotting factors, including the United States licensed factor VIII (pdFVIII), factor IX (pdFIX), and other plasma-derived products such as immune globulins and albumin. In the past 10 years, there have been some reported cases of probable variant Creutzfeldt-Jakob disease (vCJD) transmission by red blood cell transfusions in the United Kingdom [52]. Prion infections are associated with long and clinically silent incubations [50, 51]. The number of asymptomatic individuals with vCJD prion infection is unknown, posing risk to others through blood transfusion, blood products, organ or tissue grafts, and contaminated medical instruments. In order to decrease the risk, there is a need to establish a blood-based molecular assay for detection of vCJD prion infection [52]. Recently research papers have shown that sensitivity detection methods are available for vCJD prion [53–55]. However, commercial detection kits are not yet available.

## *Dengue Viruses*

The dengue virus (DENV) is a member of the virus family *Flaviviridae* and is transmitted to people through the bite of an infected mosquito [56]. The dengue virus has been shown to have four subtypes. These subtypes are different strains of dengue virus that have 60–80% homology between each other. Dengue has emerged as a worldwide problem only since the 1950s. With more than one-third of the world's population living in areas at risk for transmission, dengue infection is a leading cause of illness and death in the tropics and subtropics. According to CDC, as many as 100 million people are infected yearly [57]. Dengue is caused by any one of the four related viruses transmitted by mosquitoes. There are not yet any vaccines to prevent DENV infection, and the most effective protective measure is to avoid mosquito bites. There have been healthcare-related transmissions, including transmission by blood products [58]. Dengue infection has a viremic phase that lasts 4–8 days, and blood collected during this phase may be infective when transfused into susceptible hosts [58]. There are currently no tests for direct detection of dengue virus, but there are, however, commercial ELISA tests to detect antibodies of the dengue virus in blood samples from patients [59]. Recently, research papers have shown that PCR detection methods are available for any dengue virus strain [57, 60].

## **Babesia *Species***

*Babesia* is a protozoan parasite of the blood that causes a hemolytic disease known as babesiosis [61]. Babesiosis is a malaria-like parasitic disease [62], and there are over 100 species of *Babesia* identified [63]. In the United States, *Babesia microti* is the agent most commonly reported to cause human infection. Clinical confusion between human babesiosis and malaria is often reported in literature [62]. *Babesia* infection can also be acquired by blood transfusion [64, 65]. In fact, there have been many cases of transfusion-induced babesiosis documented [64, 65]. Risk of developing this clinical infection is increased for elderly, asplenic, or immunosuppressed patients. Current standards issued by the American Association of Blood Banks (AABB) require the indefinite deferral of a blood donor with a history of babesiosis [65]. There is a need to develop methods for identification *Babesia microti* in order to reduce the risk of transmission of babesiosis by transfusion. Diagnosis depends upon finding parasites on blood film examination which can be detected 2–4 weeks after a tick bite. Hamster inoculation and serology have also been used for diagnosis. The indirect fluorescent antibody test (IFAT) is available for *B. microti* and is the most useful serological test for early diagnosis [66]. Also, the PCR screen tests for babesiosis are technically available in the field [67].

## ***Chagas' Disease***

Chagas disease is named after the Brazilian physician Carlos Chagas, who discovered the disease in 1909 [68]. Chagas disease is spread mainly by blood-sucking insects infected with *Trypanosoma cruzi*. Chagas disease can also be spread through blood transfusion, organ transplants, and from a mother to an unborn child. National screening of the blood supply [69] was instituted in early 2007 by FDA, and more than 1000 donors with *T. cruzi* infection have been identified within the past 3 years of testing. "Screening for T. cruzi is an important safety measure to help protect our blood supply and to help prevent the spread of Chagas disease," says Karen Midthun, M.D., acting director of the FDA's Center for Biologics Evaluation and Research. Currently, serological ELISA tests are available to diagnose chronic Chagas disease [70]. PCR test is not a tool for diagnosis of chronic Chagas disease in clinical practice yet, although some research results have showed that PCR is a very sensitive parasitological test for Chagas disease in active transmission regions [71]. More studies are needed for the development of this molecular method.

## *Severe Acute Respiratory Syndrome*

Coronavirus is an RNA virus known to be associated with respiratory disease. Severe acute respiratory syndrome (SARS) is a newly recognized coronavirus whose genome sequence does not belong to any of the known coronavirus groups and which quickly spread all over the world from Asia in 2003. There has been no evidence that this infection is transmitted from blood donors to transfusion recipients, but the virus associated with SARS is present in the blood of people who are sick, and it is possible that the virus could be present in the blood immediately before a person gets sick, so that an individual with infection but no symptoms possibly could transmit SARS through a blood donation. To help determine whether or not an individual might be infected with SARS, a blood collection facility will ask a potential donor orally or in writing about any travel to a SARS-affected country or a history of SARS or possible exposure to SARS. Enzyme-linked immunoassays for detection of specific IgG and IgM antibodies and RT-PCR for detection of SARS coronavirus-specific RNA in the SARS patients have been developed. Rapid, sensitive, and specific identification of SARS and other novel coronaviruses by molecular methods will be very important in the future.

## *Ebola Virus*

Ebola virus disease (EVD) is a rare and deadly disease caused by infection with one of the *Ebolavirus* species [72]. The recent outbreak in 2014–2015 is the largest Ebola outbreak since the Ebola virus was first discovered in 1976, first in Yambuku, Democratic Republic of Congo, and then in Nzara, South Sudan.

The virus family *Filoviridae* includes three genera: *Cuevavirus*, *Marburgvirus*, and *Ebolavirus*. There are five species that have been identified: *Zaire*, *Bundibugyo*, *Sudan*, *Reston*, and *Taï Forest*. The first three, *Bundibugyo ebolavirus*, *Zaire ebolavirus*, and *Sudan ebolavirus*, have been associated with large outbreaks in Africa. The virus responsible for causing the 2014 West African outbreak belongs to the *Zaire* species (WHO) [72].

Samples from patients are an extreme biohazard risk. Currently, a number of approaches have been developed and are available for diagnoses of Ebola virus disease: (1) antibody-capture enzyme-linked immunosorbent assay (ELISA), (2) antigen-capture detection tests, (3) serum neutralization test, (4) reverse transcriptase polymerase chain reaction (RT-PCR) assay, (5) electron microscopy, and (6) virus isolation by cell culture tests.

The WHO and FDA are working to help expedite the development and availability of medical products – such as treatments, vaccines, diagnostic tests, and personal protective equipment – with the potential to help bring the Ebola epidemic in West Africa under control as quickly as possible (FDA) [73].

## Discovery of Unrecognized and Uncharacterized Viral Agents

Based on past history, it is not just a hypothetical risk that many people have been infected with unrecognized viruses, for example, many patients with symptoms of non-A-E, G, and TTV posttransfusion hepatitis. It is still possible that unexplained cases of posttransfusion hepatitis may be caused by a new, undiscovered pathogen. In recent years, numerous new infectious agents found worldwide have been identified through time-consuming procedures. By the time a new virus, such as HCV, HIV, and SARS, is found, many people are infected, and there could be a large number of fatalities. There is an urgent need to develop methods for rapid identification and characterization of previously unknown pathogenic viruses. The most recent technologies for detecting and identifying previously unrecognized pathogens are expression library screening, representational difference analysis (RDA), and broad-range polymerase chain reaction (BR-PCR). But they are all time-consuming approaches. The new unrecognized and uncharacterized viral agents can be rapidly identified by some of the new molecular approaches, e.g., subtraction hybridization [74] and DNA microarray.

## Conclusion

Ensuring the safety and efficacy of blood and blood products is a critical regulatory challenge. The high safety level of the blood supply is the result of continued improvements in blood donor screening and testing. It will be achieved by introducing more updated nucleic acid tests to the field of blood safety [2, 3]. Nucleic acid testing is a method of testing blood that is more sensitive and specific than conventional tests that require the presence of antibodies to trigger a positive test result. Also, NAT works by detecting the low levels of viral genetic material present when an infection occurs but before the body develops an immune response to a virus. This improved sensitivity should enable us to significantly decrease the infection window period, allowing for earlier detection of the infection and diminishing the chances for transmission of the agent via transfusion. We are not only to protect the blood supply from known pathogens but also the emergence of new and unrecognized and uncharacterized infectious agents [4, 36, 37, 75]. The NAT methods are more sensitive and specific compared with non-NAT. In the future, NAT technology, such as PCR, may allow routine screening of donors for all the known and unknown pathogens of concern to blood safety.

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# Molecular Diagnostics of Sexually Transmitted Diseases: Bacterial, Trichomonas, and Herpes Simplex Virus Infections

**Kenneth L. Muldrew**

## Introduction

Molecular techniques for identifying and detecting microorganisms have been proven readily adaptable for use in the clinical diagnostic laboratory. Sexually transmitted diseases (STDs) constitute the most common infectious diseases globally and bear significant consequences for the individual as well as the public health of the community. The Centers for Disease Control and Prevention (CDC) estimates there are 19 million new STD cases each year. Nearly half of cases occur in individuals between the ages of 15 and 24 years. In particular, cases of chlamydia and gonorrhea exceeded 2.0 million reported in 2015, and some estimates suggest that over half of new chlamydia and gonorrhea infections remain undiagnosed. [1] Globally, an estimated one million new cases of bacterial sexually transmitted infections occur each day, and half a million babies die in sub-Saharan Africa alone each year due to congenital syphilis [1, 2]. Antibiotic therapy for bacterial, *Trichomonas vaginalis*, and Herpes simplex virus STDs is essential to resolve the initial infection and severe complications; treatment is outlined in Table 1. In the last decade, rapid development of new technologies has shifted the paradigm of laboratory diagnosis from traditional serologic and phenotypic microbiology testing to molecular methods for detection and identification of the major agents of sexually transmitted infections.

A milestone in biotechnology that heralded the beginning of molecular diagnostics was the development of the polymerase chain reaction (PCR) by Mullis and colleagues [3]. Since then, numerous molecular detection techniques have been

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**Table 1** Standard treatment for bacterial, *Trichomonas vaginalis*, and Herpes simplex virus infections [101]

| Microorganisms | Primary antibiotics | Secondary antibiotics |
|---|---|---|
| *Chlamydia trachomatis* | Azithromycin or doxycycline | Erythromycin |
| *Neisseria gonorrhoeae* | Ceftriaxone | Azithromycin (high dose) |
| *Treponema pallidum* | Penicillin G | Doxycycline or ceftriaxone |
| *Haemophilus ducreyi* | Ceftriaxone or azithromycin | Erythromycin or ciprofloxacin |
| *Mycoplasma* species | Azithromycin | Moxifloxacin |
| *Ureaplasma* species | Azithromycin or ciprofloxacin | Doxycycline |
| *Trichomonas vaginalis* | Metronidazole (single high dose) | Tinidazole (single high dose) |
| Herpes simplex virus | Acyclovir | Valacyclovir or famciclovir |

designed to detect specific nucleic acids without relying on the ability to culture or directly observe intact organisms. With the addition of automation in molecular diagnostics, we have seen a dramatic paradigm shift in our ability to rapidly detect and identify these pathogens. Silent, non-cultivatable pathogens, such as HPV (chapter "Multiplex PCR for Detection and Identification of Microbial Pathogens"), can now be detected and typed using molecular techniques, providing the ability to determine the oncogenic potential and prognostic outcome in positive patients [4, 5]. These powerful molecular techniques have had a marked impact on public health programs designed for the control and prevention of STDs worldwide.

An estimated 50% of STDs occur asymptomatically, and this forms a major reservoir of infectious source that persists in the community. The more sensitive molecular assays are required for detecting asymptomatic individuals with low microbial load [6]. Currently available molecular techniques such as nucleic acid amplification and hybridization can now offer high sensitivity in screening for these infections and disrupt the transmission chains within the community leading to a decrease in the public health burden of these infections.

This chapter describes molecular diagnostic techniques for detection of bacterial (*Chlamydia trachomatis* (CT), *Neisseria gonorrhea* (NG), *Treponema pallidum*, *Haemophilus ducreyi*, *M. genitalium*, and *U. urealyticum*), *Trichomonas vaginalis*, and Herpes simplex virus (HSV) STD infections. Human immunodeficiency virus (HIV) and human papillomavirus (HPV) sexually transmitted infections will be discussed in chapters "Laboratory Technical Advances in the Diagnosis of *Clostridium difficile*" and "Multiplex PCR for Detection and Identification of Microbial Pathogens", respectively.

## *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

*C. trachomatis* and *N. gonorrhoeae* cause the two of the most prevalent sexually transmitted diseases worldwide, and in the USA, 2,066,868 cases (CT: 1,598,354 and NG: 468,514) were reported to the CDC in 2016 [1]. The rate of *C. trachomatis*

infections in the USA has been steadily increasing since 2000; women are almost twice as likely to be infected compared to men (Fig. 1) [1]. *C. trachomatis* is a non-motile bacterium with two cellular forms (reticulate and elementary bodies) and is an obligate intracellular pathogen. It has a unique two-stage life cycle and requires the host cell for replication. Its genome is 1.04 mega base pairs (mbp) in size, contains a GC content of 40%, and has 894 coding sequences [7, 8]. *C. trachomatis* is a causative agent of nongonococcal urethritis, epididymitis, proctitis, cervicitis (Fig. 2), pelvic inflammatory disease, and eye infections. Asymptomatic infections are common in men and women, and routine screening for *C. trachomatis* is recommended by the CDC for sexually active teenagers and adults of ≤24 years [1].

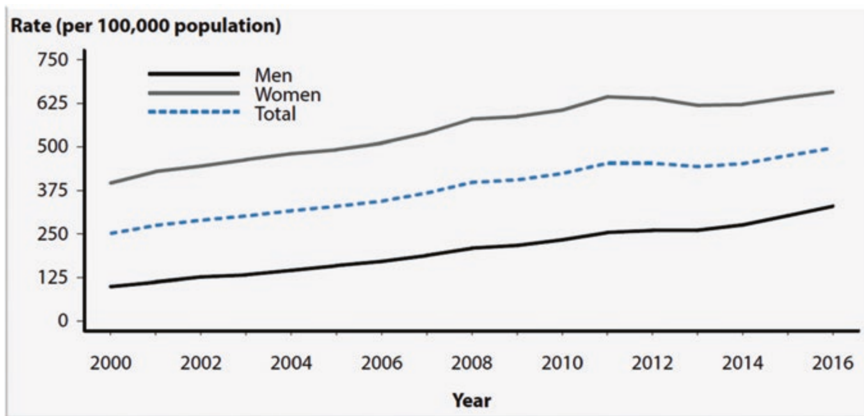


**Fig. 1** Chlamydia – rates of reported cases by sex, United States, 2000–2016. (Adapted from Ref. [1])

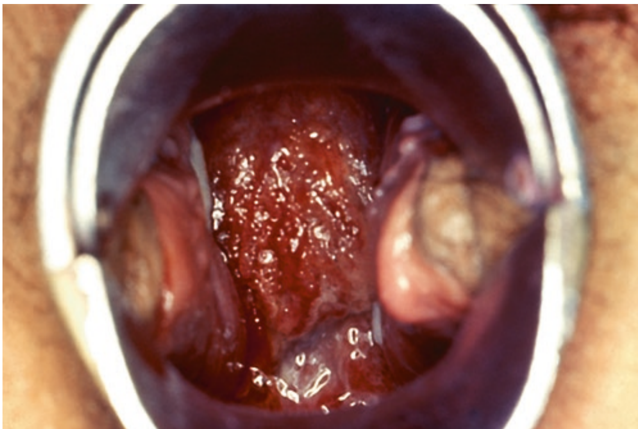

**Fig. 2** Nongonococcal cervicitis. Examination of the cervix reveals purulent exudate, erythema, and erosion in this case of cervicitis due to *C. trachomatis*. (Source: CDC Public Health Image Library, L. Fraw and J. Pledger)

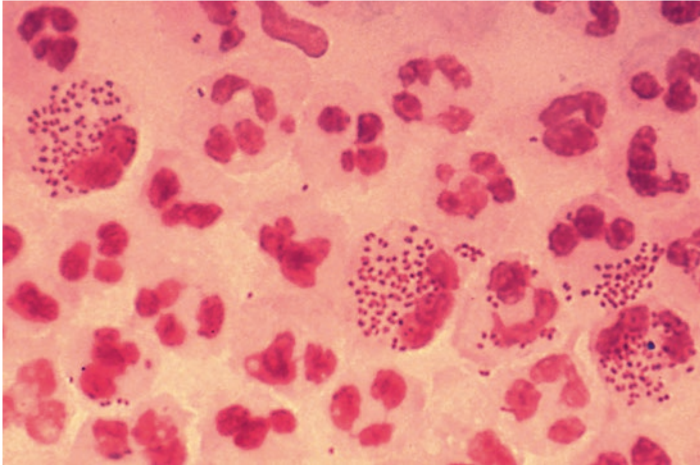

**Fig. 3** Gram Stain, urethral exudate. Both intracellular and extracellular gram-negative, "kidney bean" shaped bacteria are present in this patient with urethritis caused by *N. gonorrhoeae*. (Source: CDC Public Health Image Library, J. Millar)

*N. gonorrhoeae* is the etiologic agent of gonorrhea. It is a nonmotile, oxidase-positive, catalase-positive, gram-negative bacterium that exhibits diplococcal "kidney bean-shaped" morphology in smears of exudate from mucocutaneous lesions (Fig. 3). Similar to *H. ducreyi*, *N. gonorrhoeae* can be morphologically and biochemically identified by traditional culture and several manual (e.g., Rapid ID NH system, Remel; API NH, bioMérieux) and automated (e.g., Vitek 2, bioMérieux) systems. [9, 10] The genome is 2.04 mbp in size, contains 2069 genes, and has a GC content of 54.0% [10, 11].

In the USA, gonorrhea is the second most commonly reported notifiable condition. It causes cervicitis and urethritis and serious outcomes such as tubal infertility, ectopic pregnancy, and chronic pelvic pain in women and ophthalmia neonatorum in neonates (Fig. 4) [1]. Infection rates for both men and women have remained relatively constant from 2007 to 2012, ranging from 110 to 120 infections per 100,000 population [1]. From 2012 to 2016, there has been a significant increase in the infection rate for men up to 170 cases per 100,000 population in 2016, while the rate for women has remained relatively constant (Fig. 5) [1]. Since coinfections of *C. trachomatis* and *N. gonorrhoeae* are common, many diagnostic test platforms or systems have been designed for simultaneous detection of both microorganisms. Originally in 2002, the CDC recommended supplemental/confirmatory testing for nucleic acid detection tests, but this is no longer recommended in the assessment of patients that are at risk [12].

Several FDA-approved commercial assays that are currently available for *C. trachomatis* and *N. gonorrhoeae* include signal amplification, polymerase chain reaction (PCR), strand displacement amplification (SDA), and transcription-mediated amplification (TMA). For further details on these methods, the reader is referred to

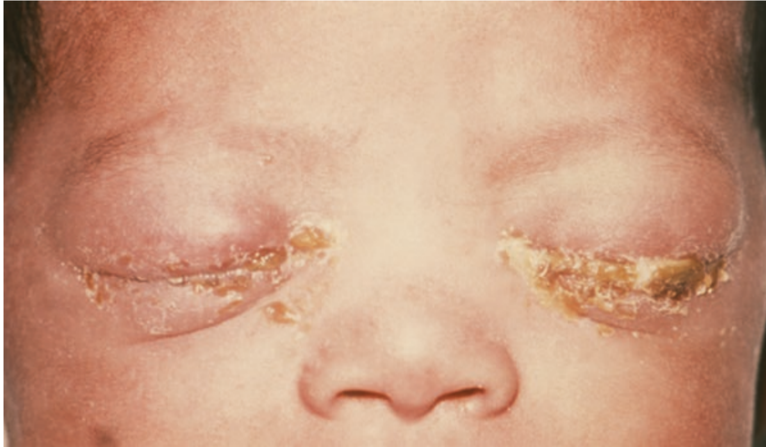

**Fig. 4** Ophthalmia neonatorum due to *N. gonorrhoeae.* This infection is acquired during birth and infects the corneal epithelium causing microbial keratitis and ulceration and, if untreated, perforation and blindness. (Source: CDC Public Health Image Library, J. Pledger)

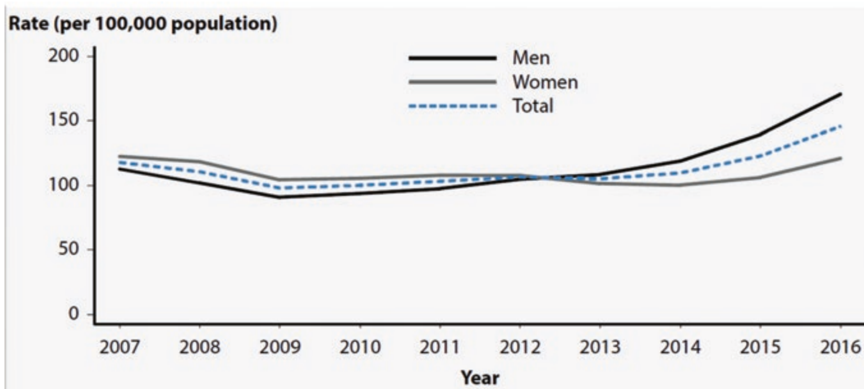


**Fig. 5** Gonorrhea – rates of reported cases by sex, United States, 2007–2016. (Adapted from Ref. [1])

chapters "PCR and Its Variations" and "Real-Time and Digital PCR for Nucleic Acid Quantification" in Vol. I for PCR and chapter "Non-PCR Amplification Techniques" in Vol. I for SDA and TMA.

## *Signal Amplification*

The Hybrid Capture II (HC2) (Digene Corporation, Gaithersburg, MD) is a signal amplification test targeting both genomic DNA and cryptic plasmid DNA sequences of *C. trachomatis* and *N. gonorrhoeae* using probe hybridization and signal

amplification using chemiluminescence [13, 14]. Single-stranded RNA probes are added to the sample which bind to the target DNA. The RNA-DNA hybrids are captured by hybrid-specific antibodies in microtiter plates and alkaline phosphatase-labeled antibodies targeting the bound RNA-DNA hybrids that are added to the microtiter plate well. The hybrids are detected in luminometer by adding chemiluminescent substrate. The sensitivity of the HC2 CT/NG assay was 95% for detection of *C. trachomatis* and *N. gonorrhoeae* in endocervical specimens, while the specificity was found to be greater than 98% compared with *N. gonorrhoeae* culture [13, 14].

### *Strand Displacement Amplification (SDA)*

Strand displacement amplification (SDA) is a multiplex isothermal DNA target amplification method. The BD ProbeTec ET *C. trachomatis* and *N. gonorrhoeae* (CT/NG) amplification assays and ProbeTec GC $Q^X$ assay (Becton Dickinson, Franklin Lakes, NJ) utilize SDA technology and fluorescent resonance energy transfer probes targeting DNA sequence homologous to the cryptic plasmid in CT and different regions within the multicopy pilin gene-inverting protein homologue [15–22]. The assays can be performed using either a semiautomated BD ProbeTec ET system or the automated BD VIPER system. The sensitivity and specificity of the BD ProbeTec assay using vaginal or endocervical and urine samples ranges from 95.2% to 100% and 92.6% to 100% for *C. trachomatis* and from 84.9% to 98.5% and 92.5% to 98.6% for *N. gonorrhoeae*, respectively [15–24].

### *Transcription–Mediated Amplification (TMA)*

The APTIMA Combo 2 sssay (Gen-Probe, San Diego, CA) is a target amplification nucleic acid probe test combining target capture, transcription-mediated amplification (TMA), and dual kinetic assay (DKA) technologies [25–30]. This assay replicates a specific region of the 23S rRNA from CT and a specific region of the 16S rRNA from GC using unique sets of primers. The target rRNA molecules are isolated by the use of capture oligomers and separated with magnetic particles. The rRNA amplification products are detected by hybridization with chemiluminescent single-strand DNA probes and an enzyme labeled DNA-RNA duplex antibody. Sensitivity and specificity for the CT assay ranges from 94.2% to 100% and 97.6 to 100% in swab and urine, while the corresponding values for GC were ranged from 91.3% to 99.2% and 98.7% to 99.3% respectively [23, 24, 28, 31, 32]. The assay has been evaluated for use on non-indicated sample types including the eye with good sensitivity and specificity [25, 27, 30, 33]. However, in one study, the assay had a lower sensitivity and specificity when testing rectal and glans swab specimens and is not likely to perform well in asymptomatic or low-risk populations [19].

## *Polymerase Chain Reaction (PCR)*

Several laboratory-developed tests (LDTs) targeting different regions of *C. trachomatis* and *N. gonorrhoeae* (NG) genome have been reported. The targets, methods, primer/probe sequences, and references are shown in Table 2. The target regions for *C. trachomatis* detection include the cryptic plasmid [34], major outer membrane

**Table 2** Laboratory-developed tests: PCR assays of *C. trachomatis* and *N. gonorrhoeae*

| Organisms | Target regions | Primer and probe sequences (5′–3′) | References |
|---|---|---|---|
| *C. trachomatis* | Cryptic plasmid (PCR) | CT2A: CGCATGCAAGATATCGAGTATGCGTTGTTAGG<br>CT2B: GACCGGCCTCTAGCGCTGCG | [34] |
| *C. trachomatis* | Cryptic plasmid (377-bp deletion) (RT-PCR) | Forward swCT: TCCGGATAGTGAATTATAGAGACTATTTAATC<br>Reverse swCT: GGTGTTTTACTAGAGGACTTACCTCTTC<br>Probe: swCT: FAM-GGATCCGTTTGTTCTGG-MGB | [42] |
| *C. trachomatis* | Cysteine-rich protein (PCR) | Forward: CAAACTCATCAGACGAG<br>Reverse: CCTTCTTTAAGAGGTTTTACCC | [37] |
| *C. trachomatis* | Major outer membrane protein (MOMP) (PCR) | Forward: GACTTTGTTTTCGACCGTGTT<br>Reverse: ACATAATACATCAAATCGATCCCA | [35, 36] |
| *C. trachomatis* | Phospholipase D endonuclease (CT157) (PCR) | P1: TCTTTTTAAACCTCCGGAACCCACTT<br>P2: GGATGGCATCGCATAGCATTCTTTG | [38] |
| *C. trachomatis* | 16 s RNA (PCR) | Forward: AGCAATTGTTTCGGCAATTG<br>Reverse: CACATAGACTCTCCCTTAAC | [39, 40] |
| *N. gonorrhoeae* | Outer membrane protein III (*ompIII*) (PCR) | Forward: CGTCGGCATCGCTTTTG<br>Reverse: CAGGCTGTTCATGCGGTAGTC | [31, 43] |
|  | *cppB* gene (PCR) | Forward: GCTASCGCATACCCGCGTTGC<br>Reverse: CGAAGACCTTCGAGCAGACA | [31, 43–45] |
| *N. gonorrhoeae* | *opa* gene (RT-PCR) | papTM-F: CAGCATTCAATTTGTTCCGAGTC-3′<br>papTM-R: GAACTGGTTTCATCTGATTACTTTCCA<br>papTM-P: FAM-CGCCTATACGCCTGCTACTTTCACGC-BHQ1 | [44] |
| *N. gonorrhoeae* | *porA* pseudogene (RT-PCR) | GcopaF: TTGAAACACCGCCCGGAA<br>GcopaR: TTTCGGCTCCTTATTCGGTTTAA | [44] |
|  |  | GcopaP: FAM-CCGATATAATCGCTCCTTCAACATCAG-TAMRA |  |

protein (MOMP) [34–36], cysteine-rich protein [37], a protein from the phospholipase D endonuclease superfamily (CT157) [38], and 16S rRNA genes [39, 40]. A new variant of *C. trachomatis* with a 377-bp deletion in the cryptic plasmid has been reported in Halland Country, Sweden [41], and a new real-time PCR assay was developed to detect this new variant [42]. The gene encoding outer membrane protein III (*ompIII*) [31, 43], the *cppB* gene [44, 45], the *opa* gene [44, 46], and the *porA* pseudogene [47] of *N. gonorrhoeae* are the target regions used in several other PCR assays. The specificity and sensitivity of the *ompIII* assay were 96.4% and 78.6%, respectively [43]. No false-positive or false-negative results have been described in *ompIII* PCR assays [44]. Using a coded panel of 500 DNA samples, the *cppB* gene assay identified only 94% of NG strains, and therefore the *cppB* gene appears to be an unsuitable target [44]. The *opa* and *porA* pseudogene assays have been validated as suitable confirmatory test for positive nucleic acid amplification tests [47, 48].

The Cobas Amplicor CT/NG assay (Roche Molecular Systems, Branchburg, NJ) is a FDA-approved nucleic acid amplification test for detecting *C. trachomatis* and *N. gonorrhoeae* [38, 48–51]. The targets for the assay are a 207 bp sequence within a cryptic plasmid in *C. trachomatis* and a 210 bp sequence in the cytosine methyltransferase gene of *N. gonorrhoeae*. Internal control target DNA is used for co-amplification in each reaction to detect the presence of inhibitors. The sensitivity and specificity of this assay ranges from 94.2 to 98.1% and 98.4 to 100%, respectively [51, 52]. However, it has been reported that the Cobas Amplicor CT/NG assay for *N. gonorrhoeae* cross-reacts with certain strains of nonpathogenic *Neisseria* species, such as *N. subflava*, *N. lactamica*, and *N. cinerea.* [53] Consequently, supplementary confirmatory testing or *N. gonorrhoeae* nucleic acid amplification test is now widely used. [54, 55] In 2006, a new variant of *C. trachomatis* (nvCT) with 377 bp deletion in the cryptic plasmid was discovered in Sweden that was not detectable with the Cobas Amplicor test. [41, 56] The proportion of nvCT was found to be 20–64% of the detected chlamydia cases thus limiting the utility of this assay in the Swedish population [57].

The Cobas 4800 CT/NG assay (Roche Molecular Systems, Branchburg, NJ) is a real-time PCR assay run on the fully automated Cobas 4800 system for the detection of *C. trachomatis* and *N. gonorrhoeae* [58–64]. The multiplex real-time PCR assay has incorporated a dual-target strategy for detecting *C. trachomatis*, including a 206 bp conserved sequence in cryptic plasmid of *C. trachomatis* and 182 bp sequence of the major outer membrane protein gene, to ensure accurate and reliable detection of chlamydia, including the variant strain found in Sweden [58]. The *N. gonorrhoeae* assay was newly designed to target a direct repeat region called DR-9. This target region has two highly conserved sequence variations that are repeated three times on the genome. The sensitivity and specificity of this assay on urine and swab samples ranges from 92.0% to 94.5% and 99.5% to 100%, respectively, for *C. trachomatis*, and 92.9% to 100% and 99.4% to 100%, respectively, for *N. gonorrhoeae* [58]. Some data suggests that the optimal sample type for maximal recovery is the vaginal swab regardless of whether it is clinician

collected or self-collected. [64] The newer Cobas CT/NG test is run on the newer Roche platform (Cobas 6800/8800) and was shown to be equivalent to the Cobas 4800 CT/NG assay run on the older Cobas 4800 with an overall agreement of greater than 98.5% [65].

Another commercially available real-time PCR assay for detection of *C. trachomatis* and *N. gonorrhoeae* is the Abbott Real-Time CT/NG assay run on the m2000 platform (Abbott Molecular, Des Plaines, IL) [50, 66, 67]. The assay contains two sets of primers and probes targeting 122 bp conserved sequence in the *C. trachomatis* cryptic plasmid and a 140 bp sequence located outside the deleted region of nvCT. The target region for *N. gonorrhoeae* is a 122 bp conserved sequence in the *N. gonorrhoeae* opacity (Opa) gene. The sensitivity and specificity of this assay ranges from 92.4% to 99.6% and 99.2% to 99.7% for *C. trachomatis* and from 96.9% to 100% and 99.7% to 99.8% for *N. gonorrhoeae*, respectively [50, 68, 69].

Attractive alternatives [GeneXpert and the io(R) single module system] to the traditional automated CT/NG assays have been developed to reduce the turnaround time and improve simplicity of the testing, which is an important step toward point-of-care diagnostics leading to rapid office or emergency room visit diagnosis and immediate therapeutic decisions.

The GeneXpert is a random-access, real-time PCR system in which all reagents and steps of the testing process (controls, extraction, purification, and real-time PCR reagents) are contained within a cartridge that is loaded on the PCR instrument. Throughput is lower compared to more automated systems, but the turnaround time is shorter for all available assays. The GeneXpert CT/NG assay provides a result within 90 minutes and exhibits comparable sensitivity and specificity to more traditional automated assays [25, 27, 70–74].

A new, rapid, point-of-care device, the cartridge-based io(R) single module system (Atlas Genetics, Ltd.), has an investigational assay, the io® MSTI (Multi-STI), which detects *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis* and provides a result within 30 minutes [75].

Biofire Diagnostics, LLC, has developed a promising research-use-only multiplex two-step real-time PCR STD panel using the FilmArray technology in which all processes from sample extraction to multiplex PCR are contained within a single pouch (one sample per pouch). In addition to *C. trachomatis* and *N. gonorrhoeae*, this assay detects seven other STDs including *T. pallidum*, *H. ducreyi*, *Mycoplasma*, *Ureaplasma*, *T. vaginalis*, HSV-1, and HSV-2 [76]. The assay can provide a result in about an hour, but the system is a moderate to low throughput technology. In their evaluation, 39 of 280 specimens were positive by the FilmArray for *C. trachomatis* and had a high correlation with the standard molecular test (kappa = 0.98; 95% CI = 0.95–1.0) [76]. Nineteen of the 280 specimens were positive by the FilmArray for *N. gonorrhoeae* and also had a high correlation with the standard molecular test (kappa = 0.97; 95% CI = 0.92–1.0) [76]. It is not known if this assay has been or will be submitted for FDA clearance.

## *Treponema Pallidum*

Syphilis is a chronic and multistage sexually transmitted infection caused by the spirochete *Treponema pallidum* subspecies *pallidum*, which continues to be a worldwide public health problem with over 12 million new cases per year [77]. In the USA, over 88,000 new cases were reported to the CDC for 2016 [1]. From 1999 to 2016, rates have continued to increase, with a striking increase in the male to female ratio from 1:5 to 8:1 (Fig. 6) [1]. Syphilis has three main stages: primary, secondary, and tertiary, with secondary and tertiary stages exhibiting more severe sequelae such as multiple ulcerative mucocutaneous and genital lesions (Fig. 7a) and necrotizing granulomatous inflammation involving the bone, skin, soft tissues, brain, cardiovascular system, and other organs [78–80]. *T. pallidum* is a spiral-shaped obligate intracellular bacterium with periplasmic flagella measuring 0.2 μm by 6–20 μm that is not identified by gram stain but tissues can show the organism by silver staining (Fig. 6b) [78–80]. The genome of *T. pallidum* is 1.14 mbp in length, has a GC content of 53%, and has 1090 genes – several of which are prime targets for molecular identification [78–80]. Owing to the slow generation time and the inability to survive ex vivo, repeated attempts over the years to develop in vitro culture techniques for *T. pallidum* have been largely unsuccessful, and in order to propagate the organism for research studies, rabbit infectivity testing (RIT) is utilized. [78–80] Dark-field microscopic examination, immunoperoxidase and immunofluorescent stains of lesion exudate and tissue, and serology are definitive methods for diagnosing early syphilis. The presumptive diagnosis of syphilis is possible by using nontreponemal serological tests, such as rapid plasma regain (RPR) and venereal disease research laboratory (VDRL), and treponemal tests, such as fluorescent treponemal antibody absorption (FTA) and *T. pallidum* hemagglutination assay (TPHA) [80]. However, these tests may be problematic in the

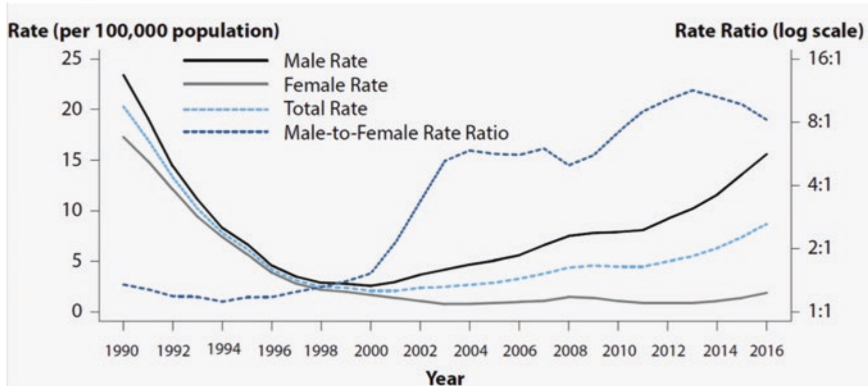


**Fig. 6** Rates of reported cases of syphilis by sex and male to female ratio, United States, 1990–2016. (Adapted from Ref. [1])

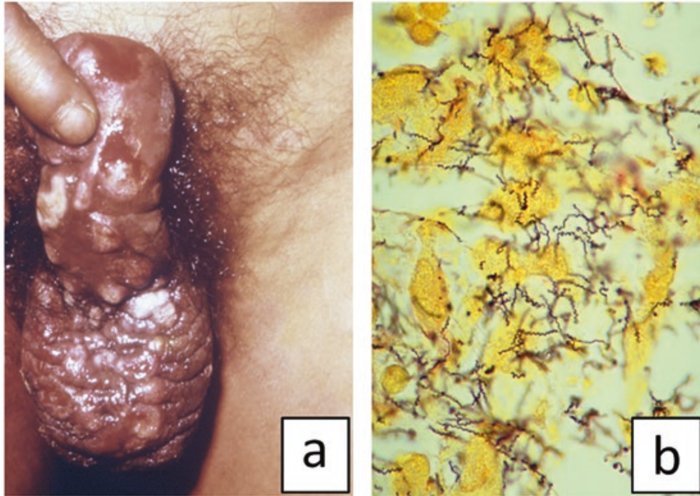


**Fig. 7** (**a**) Secondary syphilis. Multiple purulent, erythematous ulcers due to T. pallidum are present on the penis and scrotum of this patient. (**b**) Biopsy material positive for the spiral-shaped T. pallidum *bacteria* using the Steiner silver stain. (Source: CDC Public Health Image Library, S. Lindsleyand E.P. Ewing, Jr)

early stages of primary syphilis, as both serological tests and microscopic examinations are limited by low degrees of sensitivity and specificity [1, 78–81]. Molecular assays for *T. pallidum* have demonstrated clinical benefit and can be used with a variety of sample types. Recent assays using loop-mediated isothermal amplification (LAMP) (see chapter "Non-PCR Amplification Techniques" in Vol. I) have been applied to the detection of *T. pallidum* but are not readily available, and therefore PCR is the current method widely employed [82–84]. Despite the fact that no FDA-approved/FDA-cleared *T. pallidum* molecular test is available, numerous laboratory-developed tests (LDTs) using PCR have been utilized in patients suspected of having syphilis.

## *Polymerase Chain Reaction (PCR)*

Polymerase chain reaction (PCR) provides a fast and reliable alternative for the diagnosis and rapid identification of the disease (primary, secondary, and tertiary syphilis), specifically in settings in which dark-field microscopy cannot be performed. [85–88] Several PCR-based tests of *T. pallidum* have been developed on the basis of membrane lipoproteins [89], *TmpA* and *4D* genes [90], 16S rRNA [91], *tp47* gene [92], and DNA polymerase I (*polA*) gene [86, 93, 94]. Table 3 lists the genomic targets, methods employed, primer sequences, and associated

**Table 3** Laboratory-developed tests: molecular assays for *Treponema pallidum*

| Target regions | Methods | Primer and probe sequences (5′–3′) | References |
|---|---|---|---|
| Membrane lipoproteins | Traditional PCR | Forward: GACAATGCTCACTGAGGATAGT Reverse: ACGCACAGAACCGAATTCCTTG | [89] |
| tmpA and 4D genes | Traditional PCR | Forward: CAGGTAACGGATGCTGAAGT Reverse: AACGCCTCCATCGTCAGACC | [90] |
| 16S rRNA | Traditional PCR | Forward: CTCTTTTGGACGTAGGTCTTTGAG Reverse: TTACGTGTTACCGCGGCGTGG | [91] |
| tpp47 gene | Traditional PCR | Forward: CGTGTGGTATCAACTATGG Reverse: TCAACCTGTACTCAGTGC | [92] |
| DNA polymerase I (polA) gene | Traditional PCR | Forward: AGACGGCTGCACATCTTCTCCA Reverse: AGCAGACGTTACATCGAGCGGA | [93] |
| DNA polymerase I (polA) gene | TaqMan real-time PCR | SyphTF: AGGATCGCCCATATGTCCAA SyphTR: GTGAGCGTCTCATCATTCCAAA SyphTP: FAM-ATGCACCAGCTTCGA-MGB | [96] |
| DNA polymerase I (polA) gene | TaqMan real-time PCR | TP FP: AGGTCATTATCGTGGTGTTAC | [94] |
| | | TP RP: CAATCCATCCGTTTCACAATC | |
| | | TP probe: ROX-CACACCATTCGCACACG-eclipse | |

references. The levels of detection of these assays ranged between 1 organism by real-time PCR and 10–50 organisms by amplifying the gene fragment encoding the 47 kDa membrane lipoprotein [89]. A multiplex PCR assay for simultaneous detection of *H. ducreyi*, *Treponema pallidum*, and HSV (HSV) type 1 and 2 was developed to give three diagnoses from one assay in a single genital ulcer swab specimen [89].

Two simple real-time PCR assays using TaqMan probes targeting the polA gene of *T. pallidum* have been developed [95, 96](Table 3) which are robust, sensitive, and specific and have a short turnaround time and ease of performance. Compared with serology, one real-time PCR assay showed 95% agreement with a sensitivity of 80.4% and a specificity of 98.4% [96]. Another real-time PCR assay reported high sensitivities and specificities using the Rotor-Gene (QIAGEN Inc., Valencia, CA) and iCycler (Bio-Rad Laboratories, Hercules, CA) platforms; however, it had a disappointingly low sensitivity (43%) for the detection of secondary syphilis, and it had no added value for the clinical diagnosis of secondary syphilis, even though the specificity was high (98%) [95, 97].

As stated previously, Biofire Diagnostics, LLC, has developed a promising research-use-only rapid, multiplex two-step real-time PCR STD panel using the FilmArray technology that detects *T. pallidum* and eight other STD pathogens [76]. In their evaluation of a total of 190 patients, nine cases were positive for *T. pallidum* by the FilmArray assay in which three were primary syphilis, four were secondary syphilis, and two were asymptomatic "early" syphilis; all nine were confirmed as positive by serology [76]. Because of the sample size of syphilis-positive cases, no accurate determination could be made as to sensitivity and specificity.

## *Haemophilus Ducreyi*

Chancroid is a genital ulcer caused by *H. ducreyi* and is prevalent mainly in developing countries, such as Africa, Asia, and Latin America [98]. In the USA, *H. ducreyi* infections have dramatically decreased from a peak of 9515 cases per 100,000 population in 1947 to 7 per 100,000 population in 2016, likely due to the widespread use of antibiotics and improved public health initiatives [1]. Genital ulceration has a strong association with an increased risk of transmission HIV infection, and thus effective diagnosis and treatment of genital ulcer disease has become increasingly important [1, 99–101]. Several studies have shown that the accuracy of a clinical diagnosis for chancroid ranges from 33% to 80% [102, 103].

*H. ducreyi* is a facultative anaerobe that is oxidase positive and catalase negative and has a growth requirement for X factor (hemin) but not V factor (NAD) [9, 104, 105]. The organism appears as gram-negative bacilli in pairs or rows ("school of fish") in gram stains of lesion material and grows on chocolate agar [9, 104, 105]. Like *N. gonorrhoeae*, *H. ducreyi* can be morphologically and biochemically identified by traditional culture and several manual (e.g., Rapid ID NH system, Remel; API NH, bioMérieux) and automated (e.g., Vitek 2, bioMérieux) systems [9, 10]. It is a fastidious organism requiring complex media and growth conditions for culture; therefore, the sensitivity of culture is only between 50 and 90% in experienced and well-equipped laboratories [9, 104]. The genome of *H. ducreyi* is 1.70 mbp in length, has 1717 genes, and has a low GC content of 38% which can make primer design a challenge in certain situations [104, 105]. Several DNA amplification and probe-based DNA hybridization LDTs (see chapter "Nonamplified Probe-based Microbial Detection and Identification" in Vol. I for DNA hybridization) that detect *H. ducreyi* in patient samples have been developed of which PCR is the most commonly employed in clinical laboratories. It is likely that because there is such a small number of cases annually, no commercial assays have been submitted and approved by the FDA [1]. These assays have significantly improved the sensitivity of diagnostics over culture and other traditional laboratory tests for chancroid. LAMP (see chapter "Non-PCR Amplification Techniques" in Vol. I) has been described for detection of *H. ducreyi*; however, this assay is not widely available [83].

### Nucleic Acid Hybridization

Two probe hybridization assays for detection of *H. ducreyi* have been reported. [106, 107] In one assay, three ^{32}P-labeled DNA probes demonstrated to react strongly with *H. ducreyi* DNA in both bacterial suspensions and in infected rabbit lesion material blotted onto nitrocellulose membranes [106]. The sensitivity of this probe hybridization assay was around $10^3$–$10^4$ CFU of *H. ducreyi* in both pure and mixed cultures. For clinical laboratories, the obvious drawback to this assay is the complexity and the use of radioactive $^{32}$P-labeled DNA probes. The other probe hybridization assay was based on the development of specific rRNA-derived oligonucleotide probes for *H. ducreyi* [107]. Hybridization probes were chemically synthesized on eight oligonucleotide sequences complementary to different regions in the 16S and 23S rRNA molecules. This DNA-RNA hybridization assay demonstrated high specificity with culture isolates. No complete evaluation was reported on the usefulness of these DNA or RNA probe hybridization techniques in the diagnosis of chancroid by *H. ducreyi* detection in clinical specimens.

### Polymerase Chain Reaction (PCR)

Several PCR assays have been developed to improve the sensitivity of the laboratory diagnosis of chancroid [89, 106, 108–110]. Target regions of the primers of these assays include 16S rRNA gene [89, 108], the *rrs* (16S)-*rrl*(23S) ribosomal intergenic spacer region [109], an anonymous fragment of cloned DNA [110], and the *groEL* gene encoding the GroEL heat shock protein [106]. As previously mentioned in the discussion of *T. pallidum*, a multiplex PCR assay with colorimetric detection and a real-time PCR assay were developed for simultaneous detection of *H. ducreyi*, *T. pallidum*, and HSV (HSV) type 1 and 2 [89, 111]. The sensitivity and specificity of the multiplex PCR detection of *H. ducreyi* was 98.4% and 99.6%, respectively, as compared to 74.2% and 100% for culture.

The previously described Biofire Diagnostics rapid, real-time PCR STD panel using the FilmArray technology detects *H. ducreyi* and eight other STD pathogens [76]. In their evaluation of a total of 190 patients and 295 samples, *H. ducreyi* was not detected by the assay or its comparator [76]. Further studies are needed to assess the ability of the FilmArray STD assay to accurately identify patients with *H. ducreyi* infection.

### Mycoplasma and Ureaplasma

Urethritis is one of the most common sexually transmitted diseases among heterosexual men and categorized etiologically as gonococcal or nongonococcal urethritis (NGU). *C. trachomatis* is a cause of acute NGU and accounts for 30–50% NGU

cases in men [112, 113]. For non-chlamydial NGU, there is evidence that mycoplasmas and ureaplasmas are associated with persistent and recurrent NGU cases [114, 115]. *M. hominis*, *M. genitalium*, *Ureaplasma parvum*, and *U. urealyticum* are important etiological agents of cervicitis, postpartum fever, infertility, and pelvic inflammatory diseases [116–118]. Genital mycoplasmas can be cultured in diploid cell lines, and ureaplasmas are cultivated in special selective broth and agar media [118]. These time-consuming procedure requires 2–5 days for *Ureaplasma* spp. and *M. hominis* and up to 8 weeks for *M. genitalium* [118].

*Mycoplasma* species are pleomorphic ovoid or twisted rods measuring 300–800 nm in diameter, lack a cell wall, and, instead, have a lipid membrane surrounding the cytoplasm [118]. They do not stain well with the gram stain but will stain with acridine orange. For certain species, they have a colony morphology that is "fried egg" in appearance [118, 119]. The genome of *M. genitalium* is 580,070 bp and has a GC content of 37% with 525 genes [118, 119].

*U. urealyticum* is the most frequent of the *Ureaplasma* species to cause urogenital tract STDs. The cells are coccoid in morphology and measure 500 nm in size and also has a "fried egg" colony appearance [119, 120]. The genome of *U. urealyticum* is 751,719 bp in size and has a GC content of 26% with 652 genes [118–120]. The extremely low GC content of this organism can make it difficult to design primers and probe sequences in certain regions of the genome. These bacteria can be detected in less than 8 hours by nucleic acid amplification or hybridization methods. As yet, no FDA-approved assays are available for clinical use to detect the *Mycoplasma* and *Ureaplasma* species that are causes of nongonococcal urethritis and genital infections [121].

## *Nucleic Acid Hybridization*

Probe sequences based on species-specific regions of 16S rRNA gene has been widely utilized for synthesis of specific probes for hybridization. Several rRNA probe hybridization assays have been reported for detecting mycoplasmas, even though many of the rRNA probes were designed for the purpose of detecting contamination in tissue cell cultures [122, 123]. Specific probes designed from genomic libraries of *M. pneumonia* and *M. genitalium* have also been reported. [124] Dot-blot hybridization methods with ^{32}P-labeled, digoxigenin or biotin-labeled probes for detection of mycoplasmas have been described [125]; however, the detection limit of these assays is approximately 1 ng of specific mycoplasma DNA or $10^4$–$10^5$ CFU, which is not sufficiently sensitive for use in clinical laboratory [123, 126]. A rapid PCR-microtiter plate hybridization assay reported in the literature detects *M. genitalium*, *M. hominis*, *U. parvum*, and *U. urealyticum* in genitourinary samples [115]. In this assay, four species-specific capture probes were used to detect the targets by PCR amplification of a part of the 16S rRNA gene followed by 96-well microtiter plate hybridization. The sensitivity of this assay was ten copies of the 16S rRNA gene of each of the four species without cross-reactions with other human mycoplasmas or ureaplasmas [115].

### *Transcription-Mediated Amplification (TMA)*

The CE marked non-FDA-approved Gen-Probe Aptima *M. genitalium* assay (Gen-Probe, San Diego, CA) is a TMA assay for the detection of *M. vaginalis*-specific 16S rRNA to aid in the diagnosis *M. genitalium* urogenital tract disease [127]. A recent European study evaluated the assay compared to alternative nucleic acid tests polymerase chain reaction (PCR) in 5269 patients, and the sensitivity was 99.1%, and specificity was 100% [127]. To correlate with therapy (Table 1), the authors tested for molecular resistance to azithromycin and moxifloxacin and found the prevalence of resistance mutations was 41.4% and 6.6%, respectively [127].

### *Polymerase Chain Reaction*

Relevant mycoplasmas and ureaplasmas are easily and efficiently detected by PCR methods [128–131]. Specific primers have been designed for different target regions including 16S rRNA genes and other repetitive sequences, such as the MgPa adhesion gene of *M. genitalium* and the urease genes of *Ureaplasma* species [132–134]. Because *Mycoplasma* and *Ureaplasma* species are difficult to culture – requiring special media and incubation conditions – PCR has become a very attractive method for detecting these organisms, such as *M. genitalium*, where only small amount of bacterial DNA is required. This has improved turnaround time and throughput. Several different real-time PCR assays for quantifying *M. genitalium* have been validated for clinical use and utilize detection of a fragment of the MgPa adhesion gene and 16S rRNA gene [131, 135–137]. Certain specimens such as amniotic fluids and endotracheal aspirates of newborns are more difficult to culture, and the use of PCR technology in these situations enhances detection of these bacteria. A multiplex PCR assay has been developed to simultaneously detect six sexually transmitted pathogens, *C. trachomatis*, *N. gonorrhoeae*, *M. hominis*, *M. genitalium*, *T. vaginalis*, and genital *Ureaplasma* (*U. urealyticum* and *U. parvum*) [138]. The development of this multiplex PCR test has been useful in improving our understanding of the epidemiology of these important sexually transmitted diseases in areas where these are endemic. Denaturing gradient gel electrophoresis (DGGE) fingerprinting of the 16S rRNA gene of 32 *Mycoplasma* species has also been developed for rapid identification of *Mycoplasma* species of human origin [139]. It represents a significant improvement in the traditional diagnosis of *Mycoplasma* infection by detecting the organism directly from clinical samples in less than 24 h. Several other real-time PCR assays have been designed that target different gene sequences in *U. urealyticum* [140–142]. One of these assays targets the urease gene and detects *U. urealyticum* and *U. parvum* by TaqMan real-time PCR [141]. When evaluating patient samples with comparison to culture, the sensitivity was 96%, 95%, and 89% for female urethral swabs, cervical swabs, and male urethral swabs, respectively. The specificity for the three sample types was 100%, 87%, and 99%, respectively.

The Biofire Diagnostics research-use-only, rapid, real-time PCR STD multiplex panel using the FilmArray technology detects the *Mycoplasma* and *Ureaplasma* species implicated in urethritis and genital infections [76]. In their evaluation of a total of 190 patients and 295 samples, 12% were positive for *U. urealyticum*, and 3% were positive for *M. genitalium* [76]. Further studies are needed with larger sample sizes to adequately determine the performance characteristics of this assay to detect these pathogens.

## *Trichomonas Vaginalis*

Trichomoniasis is one of the most common sexually transmitted infections worldwide and is caused by a parasitic protozoan, *T. vaginalis*. The infection in women has been linked to increased risk of HIV transmission and complications including premature labor, low infant birth weight, and post-abortion or post-hysterectomy infection [1, 143, 144]. Over 180 million cases of trichomoniasis are reported annually worldwide, while an estimated 5 million women and 1 million men in the USA are infected annually [145]. However, the actual figures are expected to be higher than these estimates because (i) the infection can be asymptomatic, particular in men; (ii) trichomoniasis is not a reportable disease in the USA and other countries; and (iii) the sensitivities of different diagnostic tests vary between different laboratories, due to different methods. Direct microscopic examination of vaginal secretions and urine samples can be used. Detection of *T. vaginalis* by culture has been the "gold standard" for the diagnosis of this infection, but the sensitivities of both direct microscopic examination and culture are low (40–70%) [146]. Molecular assays have improved the diagnosis of trichomoniasis and are routine practice.

Several molecular detection methods are available for detection of *T. vaginalis*, which include nucleic acid hybridization, helicase-dependent amplification (HDA) (see chapter "PCR and Its Variations" in Vol. I), TMA, and PCR assays. These assays have been devised to target *T. vaginalis*-specific 16S rRNA and other regions or genes of the genome including 2.3 kb *T. vaginalis* fragment [147], the ferredoxin gene [148], beta-tubulin gene [149], highly repeated DNA sequences [150] and 18S rRNA genes [151].

## *Nucleic Acid Hybridization*

BD Affirm VPIII assay (Becton Dickson, Sparks, MD) is a commercially available kit that is an FDA-cleared RNA probe-based diagnostic test to detect *Gardnerella vaginalis*, *T. vaginalis*, and *Candida* species [152, 153]. Compared to microscopy and culture, the sensitivities of the Affirm VPIII assay for detection of *T. vaginalis* were 100% and 80%, respectively. In this evaluation, there were no false positives and three false negatives for the Affirm VP test. [152, 153]

### Strand Displacement Amplification (SDA)

Strand displacement amplification (SDA) is an isothermal DNA target amplification method. The BD ProbeTec *Trichomonas vaginalis* (TV) Q$^x$ assay performed on the BD Viper system utilizes SDA technology and fluorescent resonance energy transfer probes targeting DNA sequence within *T. vaginalis*. According to FDA submission data, the sensitivity and specificity of the BD ProbeTec assay was excellent using vaginal swabs (983.% and 99.0%), endocervical swabs (96.7% and 99.4%), and urine samples (95.5% and 98.7%) compared to the Aptima *Trichomonas* assay [121]. Further clinical studies have confirmed its utility and performance characteristics [154–156].

### Helicase–Dependent Amplification (HDA)

A new isothermal DNA-based amplification test, the Solana *Trichomonas* assay, uses HDA technology with a turnaround time of 35 minutes [157]. Biotinylated primers are used that target multicopy sequence of DNA that is separated by a helicase enzyme. Labeled DNA probes (FITC) bind to the single-stranded biotinylated amplicons and bind to streptavidin-coated read latex beads. As the beads migrate, immobilized anti-FITC antibodies in the test line bind to the FITC-labeled sequences, and the beads are detected. [158] Two sample types are approved – urine and genital swabs. According to FDA submission data, the assay is 95.0% sensitive and 98.2% specific in urine samples and 99.2% sensitive and 98.7% specific in genital swab samples [121].

### Transcription-Mediated Amplification (TMA)

The Gen-Probe Aptima *Trichomonas vaginalis* (ATV) assay (Gen-Probe, San Diego, CA) is a TMA assay for the detection of *T. vaginalis*-specific 16S rRNA to aid in the diagnosis of trichomoniasis using the TIGRIS DTS System [159]. This assay combines the technologies of target capture, TMA, and the hybridization protection assay (HPA). Compared with another commercially available molecular assay, BD Affirm VPIII (Becton Dickson, Sparks, MD), the ATV assay was more sensitive (100% versus 63.4%, $p < 0.0001$), identifying 36.6% more positive patients [159]. In multiple studies, the Aptima assay performed well against other comparator methods [160–162].

### *Polymerase Chain Reaction (PCR)*

Several PCR assays have been reported to detect *T. vaginalis* from clinical samples. A PCR assay with eight specific primer pairs targeting the unique sequences of the genome of *T. vaginalis* and one primer pair amplifies a 102 bp genomic fragment termed the A6p sequence, which appears highly selective for a broad range of *T. vaginalis* isolates [148]. The beta-tubulin gene of *T. vaginalis* is a well-conserved region and specific for the organism and, as such, has been used as a target in PCR assays [163]. In this report, the sensitivity and specificity of the beta-tubulin assay was 97% and 98%, respectively, while the sensitivities of culture and wet preparation were only 70% and 36%, respectively [163]. Another target region of *T. vaginalis* that has been used for PCR amplification is a 2000 bp repeat region in which two sets of highly specific primers were used in an assay that exhibited good sensitivity and specificity compared to traditional methods [150]. A PCR assay targeting a specific region of 18S rRNA gene of *T. vaginalis* utilized target amplification in which the product was subsequently confirmed by enzyme digestion with *HaeIII*. The overall sensitivity and specificity of the 18S rRNA PCR assay on vaginal swab samples was 100% and 98%, respectively [151]. A multiplex PCR for direct simultaneous detection of six sexually transmitted pathogens, including *T. vaginalis*, in clinical specimens, has been developed as reported previously [138].

Real-time PCR assays for detection of *T. vaginalis* have used TaqMan or fluorescence resonance energy transfer (FRET)-based probes, and several have shown a high level of agreement between compared to traditional culture [149, 164, 165]. As previously discussed, the GeneXpert is a random-access, real-time PCR system in which all reagents and steps of the testing process (controls, extraction, purification, and real-time PCR reagents) are contained within a cartridge that is loaded on the PCR instrument. The Xpert TV is a TaqMan real-time PCR *Trichomonas vaginalis* assay run on the GeneXpert provides a result within 65 minutes and exhibited comparable sensitivity and specificity (95.0% and 100.0%, respectively) to an alternative PCR assay [166]. By saving hands-on time and labor, these real-time PCR methods are rapid and allow for large-scale screening of patients at risk for *T. vaginalis* infection. As previously mentioned, a new, rapid, point-of-care device, the cartridge-based io(R) single module system (Atlas Genetics, Ltd.), has a CE marked, non-FDA-approved CT assay [75]. Future development by Atlas Genetics includes an assay [io® MSTI (Multi-STI)] to detect *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis* that will provide a result within 30 minutes [75].

## Herpes Simplex Virus

HSV is a significant cause of STDs and exhibits a wide range of clinical manifestations including genital and dermal ulcers, proctitis, urethritis, encephalitis, and meningitis [167, 168]. The primary modes of transmission for HSV-1 are via oral

secretions and non-genital contact, while infection with HSV-2 is usually via sexual transmission, although both viruses can be detected in genital and non-genital samples. During a primary infection, HSV-2 virions migrate into the sacral nerves and ganglion and establish latency [167, 168]. When immunosuppression occurs, skin lesions on the buttocks and genitalia can indicate reactivation. Many infections are asymptomatic, especially in women; viral shedding is significant during these times, and the patient can unknowingly transmit the virus to sexual partners [167, 168].

Herpes simplex virus [HSV type 1 (HSV-1) and type 2 (HSV-2)] is an enveloped, protein-spiked, icosahedral-encapsulated, double-stranded DNA virus that is around 200 nm in size [167, 168]. The genome is large relative to other pathogenic viruses (152 kbp and 155 kbp for types 1 and 2, respectively) [167, 168]. Both types share 83% homology in their protein-encoding regions, and the GC content is 68% for type 1 and 70% for type 2 [167, 168]. The replication of the virus starts with the attachment of the viral glycoproteins to type-specific receptors on the host cell membrane [167, 168]. Upon internalization, the capsid is translocated to the nucleus with release of the genome for replication, expression, and protein synthesis; replicated DNA is packaged into capsids within the nucleus and then are extruded from the nuclear membrane which becomes the envelope of the virus [167, 168]. Finally the virus is transported outside the cell to infect other cells leading to cell lysis and cell death. Four hundred and seventeen million people in the world between 15 and 49 years old are infected with HSV-2 with a global prevalence of 11.3%, the prevalence highest among women (almost twofold greater than men). For the same age range, the seroprevalence in the USA is around 15% [167].

Vesicular lesions can be prominent, and a presumptive diagnosis can be made using the Tzank smear technique which includes smearing lesion material on a slide, staining, and examining under a light microscope (Fig. 8a). Historically, the preferred method for diagnosis of herpes infection was virus isolation in tissue culture followed by type-specific immunofluorescence detection (Fig. 8b) [167, 168]. Shell vial culture – a variant of this technique – has improved turnaround time and simplicity, but nucleic acid amplification methods are superior to culture-based methods [167, 168]. Nucleic acid amplification techniques including SDA, LAMP, and PCR are used for detection of HSV. Eleven FDA-approved molecular tests for HSV-2 are available in the USA for in vitro diagnostic use [121]. Molecular techniques are now widely recognized as the reference standard method for the sensitive and specific diagnosis of CNS, genital, and other infections caused by HSV [169–171].

### *Loop–Mediated Isothermal Amplification (LAMP)*

A newly FDA-cleared LAMP assay is available (illumigene HSV-1 and HSV-2 DNA amplification assay) which uses specialized primers for isothermal amplification, and as a result of amplification, magnesium pyrophosphate precipitate is generated, leaving a turbid solution. Changes in turbidity are monitored by the illumipro-10 incubator/reader and, if detected, indicate the presence of HSV DNA

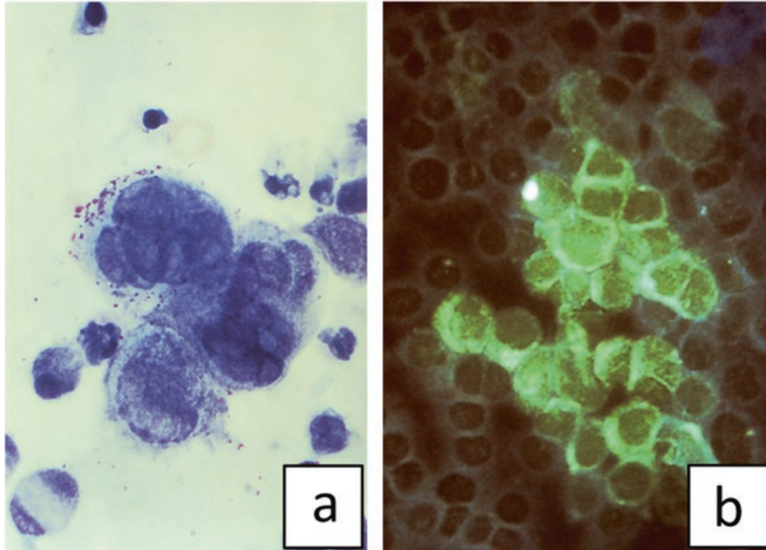


**Fig. 8** (**a**) Herpes simplex virus, Tzank smear. Several multinucleated giant cells from a lesion are present. (**b**) Multiple cells containing HSV are detected using HSV-specific immunofluorescence. (Source: CDC Public Health Image Library, S. CDC/ Dr. Craig Lyerla)

target in the solution [172, 173]. The only report in the literature to date showed good sensitivity and specificity data for genital mucocutaneous samples (HSV-1, 93.0% and 95.7%; HSV-2, 99.2% and 94.5%). For the genital cutaneous samples, the assay also performed well at classifying HSV-2 status (HSV-2, 100.0% and 88.9%). The specificity of the assay for HSV-1 on genital cutaneous samples was 98.4%; however, the sensitivity was significantly (75.0%) lower which was most likely due sample size, as a low frequency (8/72) of HSV-1 was found in the genital cutaneous samples [173].

### *Strand Displacement Amplification (SDA)*

The BD ProbeTec herpes simplex virus (HSV-1 and HSV-2) Qx Amplified DNA assay (Becton Dickson, Sparks, MD) was the first fully automated, FDA-cleared molecular assay for detection of HSV-1 and HSV-2 [174, 175]. This assay uses SDA technology to qualitatively detect (glycoprotein G gene) and differentiate HSV-1 and HSV-2 DNA for anogenital specimens as an aid in the diagnosis of herpes infection. The advantages of this fully automated assay include a greatly reduced turnaround time by reading up to 98 results in two and a half hours. It is currently performed on the BD Viper system for which the company also offers tests for *C. trachomatis* and *N. gonorrhoeae* [174, 175]. It has a good sensitivity and specificity for detecting HSV-1 and HSV-2 in anogenital swabs (Table 2) [175].

**Table 4** FDA-approved/FDA-cleared molecular diagnostic tests for HSV-1 and HSV-2

| Test name | Genomic target | Methods | Sample type | (HSV-1) Sensitivity/ specificity | (HSV-2) Sensitivity/ specificity | Comparator methods | References |
|---|---|---|---|---|---|---|---|
| MultiCode®-RTx HSV-1 and HSV-2 kit | Glycoprotein B | RT-PCR | Genital swab | 92.4%/98.3% | 95.2%/93.6% | Cell culture-based ELVIS® HSV-ID/typing test system | [186] |
| ARIES® HSV-1 and HSV-2 | Unknown | RT-PCR | Cutaneous swab<br>Mucocutaneous swab | 91.1%/94.2<br>97.0%/95.4% | 95%/88.8%<br>98.5%/93.2% | Cell culture-based ELVIS® HSV-ID/D3 typing test system | [187, 188] |
| BD ProbeTec™ (HSV-1 and HSV-2) Qx (BD Viper system) | Glycoprotein G | SDA | Anogenital swab, UVT media<br>Anogenital swab, diluent | 96.8%/97.6%<br>96.7%/95.8% | 98.4%/83.7%<br>98.4%/80.6% | Cell culture-based ELVIS® HSV-ID/D3 typing test system | [175, 189] |
| Illumigene HSV-1 and HSV-2 | Glycoprotein G | LAMP | Cutaneous genital swab<br>Mucocutaneous genital swab | 94.1%/97.2%<br>95.0%/94.9% | 100%/97.2%<br>98.6%/95.6% | Cell culture-based ELVIS® HSV-ID/D3 typing test system | [172] |
| Roche Cobas HSV-1 and HSV-2 | Thymidine kinase and glycoprotein B | TaqMan RT-PCR | Anogenital swab | 92.9%/98.8% | 97.0%/94.6% | Cell culture-based ELVIS® HSV-ID/D3 typing test system | [190, 191] |

### *Polymerase Chain Reaction (PCR)*

PCR has become the mainstay laboratory diagnostic method for HSV encephalitis over the past decade and has been considered as the most sensitive method for direct detection of HSV [171, 176].

The major features of three FDA-approved PCR assays are described in Table 4 [121]; all provide high sensitivity and specificity for detecting HSV-1 and HSV-2 in anogenital swabs.

A variety of LDTs have been reported in the literature. A multiplex LDT PCR assay has been reported to detect *T. pallidum*, *H. ducreyi*, HSV-1, and HSV-2 [89]. Other assays include PCR for HSV-1, varicella-zoster (VZV), and enteroviruses [177];multiplex PCR for HSV-1, HSV-2, VZV, human cytomegalovirus (CMV), and Epstein-Barr virus (EBV) [178]; and multiplex herpesvirus PCR for CMV, EBV, VZV, HSV, and human herpesvirus 6 (HHV-6) [169, 179–181]. Several gene targets have been selected for the detection of HSV by real-time PCR, including gene coding for the DNA polymerase; glycoproteins B, C, D, and G; thymidine kinase; and DNA-binding protein [179–181]. A real-time PCR assay simultaneously detects five human herpesviruses (CMV, EBV, HSV-1, HSV-2, and VZV) in a single LightCycler assay [182]. The artus HSV-1/2 PCR kit, SmartCycler Non-typing and SmartCycler Typing ASR kits, showed improved sensitivity (100%, 98%, and 99%, respectively) compared to culture (37%), and all real-time PCR assays were highly specific (100%) [182, 183]. The Biofire Diagnostics research-use-only, rapid, real-time PCR STD panel using the FilmArray technology detects HSV in addition to eight other STD pathogens [76]. In their evaluation of a total of 190 patients and 295 samples, only a small number of samples were positive for HSV-1 (2%) and HSV-2 (2%); therefore, no conclusions could be made as to the sensitivity and specificity of the assay at detecting HSV in anogenital samples [76].

## Conclusion

The diagnosis of the sexually transmitted infections (STI) has dramatically changed in the past 10 years. Molecular detection methods have now been demonstrated to be a powerful tool for research and diagnosis of sexually transmitted diseases (STDs) and have become an important part of infectious disease control and prevention [184, 185]. From an epidemiological perspective, accurate delineation of sexual networks and disease transmission patterns within populations can be constructed and understood by molecular typing methods. With the experiences gained in the different molecular techniques and approaches to several STDs including *N. gonorrhoeae*, *C. trachomatis*, *T. pallidum*, *H. ducreyi*, *Mycoplasma and Ureaplasma*, *T. vaginalis*, and HSV, the advantages of molecular detection of STDs are readily apparent compared with traditional methodologies, such as direct examination, culture, and serology.

The major advantages of molecular approaches to STD diagnosis over traditional methods are increased sensitivity, improved specificity, and reduced turnaround time of test results. Real-time PCR, TMA, and SDA can be used for both qualitative and quantitative analysis, as well as for genotyping. By using a closed-tube format without post-amplification processing, real-time PCR minimizes the potential of cross-contamination problems with specimens containing minimal organism nucleic acid. The ability to simultaneously identify the most prevalent STD pathogens (viral and bacterial) by multiplex real-time PCR assays is very useful owing to the fact that clinical presentation can be similar with different STD pathogens. It provides a rapid and more cost-effective diagnostic tool than traditional tests that look for single pathogens. This benefits the patient by reducing the time from presentation to treatment and reducing the likelihood of complications such as extensive pelvic inflammatory disease.

In recent years, the extensive development of array technology has shown promise for highly multiplexed detection and screening for all STD pathogens. Over the coming years, increasing automation, miniaturization, and point-of-care technologies for molecular diagnostics will improve treatment and will enhance public health measures that focus on prevention and control of STDs.

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# Advances in the Diagnosis of *Mycobacterium tuberculosis* Infection

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## Introduction

Tuberculosis (TB) is an airborne infectious disease caused by organisms of the *Mycobacterium tuberculosis* (MTB) complex, which is composed of several closely related *Mycobacterium* species, such as *M. tuberculosis*, *M. canettii*, *M. africanum*, *M. microti*, *M. bovis*, *M. caprae*, and *M. pinnipedii* [1]. Tuberculosis is an ancient disease that, for the most part, affects poor, vulnerable, and marginalized populations, living primarily in low- and middle-income countries [2]. For centuries, these countries have had the greatest burden of disease. TB is a neglected disease; although it is preventable and curable, it still causes high rates of morbidity and mortality worldwide and kills more people than any other infectious disease [3].

There are many risk factors for TB infection, disease activation, treatment failure, and relapse. Although HIV-positive people represent only 0.5% of the world's population, HIV coinfection represents the most important risk factor for TB. People who are infected with HIV are 20 to 30 times more likely to develop active TB. Other important risk factors for TB are host characteristics (age, gender, immune status, malnutrition, comorbidities such as diabetes mellitus, and others), socioeconomic factors (homeless, inmates), behavioral factors (alcoholism, smoking), demographic and ethnic factors, and indoor air pollution, among others [3, 4].

Based on clinical and public health criteria, patients with TB can be classified in three groups. The first group is represented by carriers, or individuals with latent TB infection (LTBI); these individuals are asymptomatic and are not able to transmit the disease. The second group is that of individuals with active TB, which is the

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transmissible form of the TB disease; it has specific symptoms, such as fever, fatigue, lack of appetite and weight loss, persistent cough, and hemoptysis. However, some patients with active, culture-positive TB may be asymptomatic and are separated in a third group as cases of subclinical TB. Active TB can be detected clinically and also through laboratory exams, usually based in mycobacterial culture but also molecular methods [2, 5].

Although TB is completely curable with the appropriate course of antibiotics, anti-TB therapy has several challenges in order to achieve disease cure and prevent relapse. To accomplish these goals, anti-TB therapy needs to kill actively dividing bacilli, control semidormant/dormant bacilli, prevent relapse, and prevent the emergence of bacilli that are resistance to the antibiotic used [6]. For the treatment of drug-susceptible TB, the World Health Organization (WHO) recommends a standard regimen of 2 months of treatment with all four of the most effective first-line oral drugs (isoniazid, rifampicin, ethambutol, and pyrazinamide) followed by 4 months of treatment with only rifampicin and isoniazid. To ensure the maximum success rate during treatment, it is important that the health worker responsible for the patient constantly seeks information from the patient and provides him/her with supervision and support [3, 6].

Currently, one of the major challenges for TB treatment is the increase in the incidence of drug-resistant strains. According to the WHO, drug resistance by *M. tuberculosis* can be categorized in several classes as follows: (i) mono-resistance, when the strain of *M. tuberculosis* is resistant to only one first-line drug; (ii) poly-resistance, when the strain is resistant to more than one first-line drug, with the exception of rifampicin and isoniazid; (iii) rifampicin resistance (RR), when the strain is resistant to rifampicin, with or without resistance to other drugs; (iv) multidrug resistance (MDR), when the strain is resistant to at least both isoniazid and rifampicin, with or without resistance to other first-line drugs; and (v) extensive drug resistance (XDR), when the strain is resistant to both isoniazid and rifampicin, with or without resistance to other first-line drugs, and is resistant to any fluoroquinolone and at least one of three second-line injectable drugs (capreomycin, kanamycin, and amikacin) [3]. MDR- and XDR-TB cases represent a major hurdle for TB control, since the strains are resistant to some of the most important antibiotics used during TB treatment. *M. tuberculosis* acquires resistance after the random appearance of spontaneous chromosomal mutations. Drug-resistant mutants can be selected for by incorrect drug prescription by healthcare providers, poor patient adherence to the treatment regimen, and poor supply or quality of drugs, as well as other factors such as differences in metabolism and nutrition. After the acquired resistance develops, treatment is compromised, the number of resistant cells increases, and resistant organisms can be transmitted to others, leading to primary drug resistance, which will result in the patient failing to respond to standard therapy [3, 6, 7].

## *Mechanism of* Mycobacterium tuberculosis *Infection*

Several studies have shown that the majority of TB cases are attributed to *M. tuberculosis* or a closely related organism, such as *Mycobacterium africanum. M. tuberculosis* has no known environmental sources, and humans are its only known reservoir. Thus, MTB is considered by some experts as both a pathogen and a symbiont, which has implications for our understanding of the interactions between the human body and this pathogen [2, 5]. MTB is an intracellular pathogen that is able to establish an infection throughout the entire lifetime of the host. The infection is mainly caused by the inhalation of infectious particles containing bacilli that are suspended in the air. These droplets are spread by the cough, speech, or sneezing of a patient with pulmonary tuberculosis (active tuberculosis) [2, 5, 8]. MTB infection begins with the inhalation of such aerosol droplets containing infectious particles, which cross the upper airways, overcome barriers along the way, reach the pulmonary alveoli, and are phagocytosed by resident alveolar macrophages [2, 5, 8]. Contrary to what happens with most other bacteria when phagocytosed by macrophages, evolution has equipped *M. tuberculosis* with the ability to evade and/or tolerate some of the antimicrobial mechanisms of these cells. If the macrophages kill MTB, the infection is controlled, but if this first line of defense fails to eliminate the bacteria, MTB will multiply in this niche, and the infection will spread, invading the lung interstitial tissue [2, 5, 8, 9]. If the initial infection with *M. tuberculosis* is not controlled, immune cells will transport MTB to pulmonary lymph nodes for T cell priming, culminating in the recruitment of other immune cells, both B and T cells, to the lung parenchyma to form a granuloma [2, 5, 8–10]. The replication of the bacteria inside the granuloma leads to its progressive increase in size. If bacterial growth becomes uncontrolled, the granuloma will fail to contain the infection and bacteria will disseminate to other body sites. During this stage of infection, bacteria can reach the bloodstream and establish niches in other organs. For instance, bacteria can be transported to the respiratory tract, from where they will be released to the environment, creating a host that is now infectious and symptomatic, an active case of TB disease [2, 5, 9].

More evidence indicates that MTB has evolved multiple mechanisms to manipulate their cellular niches for their own advantage. They modulate the trafficking and maturation of the phagosomes in which they reside, allowing them to evade lysosome mechanisms of restriction. Additionally, *M. tuberculosis* uses several virulence mechanisms to optimize their spread from cell to cell, such as the ESX1 type VII secretion system, for instance [11]. Additionally, MTB possesses multiple mechanisms for inhibiting host cell apoptosis, inducing the prolonged survival of infected cells and allowing for a larger number of bacteria to accumulate in a given cell before they are released by cell death [10].

## *Epidemiology of Tuberculosis*

Tuberculosis is one of the oldest diseases on record, and its history intertwines with the history of mankind. Although the efforts for its control and eradication have been a constant global battle, it remains in the top ten biggest causes of death worldwide. In fact, in 2014, for the first time, TB surpassed HIV as the number one cause of infectious disease-related deaths [3, 12]. Throughout modern history, there have been thousands of records on tuberculosis, many studying its ability to cause disease and death. Yet, diagnosing it can still be challenging and many of the hurdles dealt with nowadays have been present for decades or centuries. Although much effort has been made over the years, tuberculosis control strategies require a concerted effort, as it continues to infect millions of people every year worldwide [3, 13]. In 2015, 1.4 million people died of tuberculosis. Of these TB deaths, 0.4 million were among HIV-positive people [3]. Gaps in the diagnosis and treatment of TB continue to persist, although 39 million deaths were prevented between 2000 and 2015 [3]. In 2015, according to the WHO annual report, it was estimated that 10.4 million cases of TB occurred, of which 1.2 million were among HIV-positive people. There were an estimated 6.4 million (range, 5.7 million to 7.2 million) cases of TB among males, of which 5.9 million (range, 5.3 million to 6.7 million) were adults and 0.47 million (range, 0.42 million to 0.53 million) were children. There were 4.0 million (range, 3.1 million to 4.9 million) cases of TB among females, of which 3.5 million (range, 2.7 million to 4.4 million) were adults and 0.48 million (range, 0.41 million to 0.56 million) were children [3]. Regarding active cases, the incidence is twofold higher in men when compared to women. Additionally, it is estimated that 10% of all new cases occur in children [2, 5]. The number of notified TB cases increased from 2013 to 2015, mostly due to a 34% increase in notifications in India. Globally, it has been estimated that there is a 4.3 million people gap between incident and notified cases, with India, Indonesia, and Nigeria accounting for almost half of this gap, which reflects a mixture of underreporting and underdiagnosis of TB cases [3]. The burden of TB (pulmonary and extrapulmonary) is heterogeneously distributed. For instance, high-income countries have the lowest rates of active disease, with less than 10 cases per 100.000 people per year. In contrast, in most of the 30 high-burden countries, the rates of TB are between 150–300 cases per 100.000 people per year [2, 3, 5].

The year 2017 marks exactly 135 years of the discovery of the tuberculosis bacillus, by the German doctor Robert Koch, on March 24, 1882, when he surprised the scientific community by announcing that he had discovered the cause of tuberculosis. At the time of Robert Koch's announcement in Berlin, tuberculosis raged across Europe and the Americas, killing one in seven people [2, 3, 5]. This day was declared by the Berlin Physiological Society as the World TB Day. The combination of previous scientific knowledge, such as the idea advocated by the doctor Jean-Antoine Villemin that tuberculosis was a communicable disease, and two innovations introduced in the diagnosis of this disease, one in the staining procedure that allowed Koch to consistently observe the new microorganism in tuberculous lesions and the use of a solid medium, based on serum instead of broths for the culture, were two great contributions to the discovery of the microorganism that causes TB [14].

The discovery of the causative agent of TB and the instrumental and technological innovations that arise each year allow us to best diagnose one of the deadliest diseases in human history. The discovery of different antibiotics allowed for a rigorous diagnosis, treatment, and surveillance of TB. The disease continues to receive attention from governments and healthcare institutions worldwide. MTB infects one-third of the world's population, and in 1993 the World Health Organization declared TB a global emergency, largely because of the emergence of multidrug-resistant MTB strains, along with the AIDS pandemic in the 1980s. TB was part of one of the United Nations Millennium Development Goals (MDGs), adopted by 191 countries since its declaration on September 8, 2000, and appears again among the goals of sustainable development, the "Strategy for the Elimination of TB," which was approved by the World Health Assembly in 2014 and predicts a 90% reduction in TB mortality and 80% decrease in TB incidence by 2030, compared to 2015 [3].

Epidemiological data show a trend toward a decline in the number of people with TB, with the mortality rate falling by 22% between 2000 and 2015, with the fastest rate of decline in the mortality rate in the Eastern Mediterranean and European regions (6.5% and 6.2% per year), respectively, and slower in the African region (2.2% per year). Although TB occurs in all parts of the world, in 2015, 30 countries accounted for 87% of new TB cases. The majority of TB cases are concentrated in Asia (61%) and Africa (26%) [3].

## *Epidemiology of Drug–Resistant TB*

The mechanisms and genes involved in the resistance of *M. tuberculosis* to the various antibiotics available for the treatment of TB are well described in the literature; in addition, new mechanisms and genes involved in resistance are continuously being described [15].

Several types of drug-resistant strains of *M. tuberculosis* have been well described. Two forms of drug-resistant TB are well defined: multidrug-resistant tuberculosis (MDR-TB), which is represented by strains that are resistant to two of the four standard, and first-line antibiotics for TB treatment, namely, isoniazid and rifampicin. Patients with MDR-TB undergo a treatment regimen that may last for more than 2 years, involving fluoroquinolones and injectable aminoglycosides to compensate for the loss of two potent medicinal products (isoniazid and rifampicin). The acquisition of resistance to fluoroquinolones and injectable aminoglycosides will result in a change of classification of the strain, from MDR-TB to the extensively drug-resistant tuberculosis (XDR-TB) [16–18]. Treatment for XDR-TB requires an even longer treatment period than infections caused by MDR strains, with more expensive, less effective, and more toxic antibiotics [18]. An estimated 480,000 cases of MBR-TB occurred in 2015. Of these, 9.5% are expected to be XDR-TB cases. However, in May of 2016, the WHO issued a report proposing that people with TB resistant to rifampicin, with or without resistance to other drugs, should be treated as MDR-TB, thereby including cases of rifampicin-resistant TB

(RR-TB) in the same treatment guidelines as MDR-TB [3]. The estimated number of RR-TB cases for the same year was 100,000. This number reflects the increasing use of the Xpert MTB/RIF assay, which we described in more detail later in this chapter. Together, MDR- and RR-TB were responsible for 580,000 estimated cases in 2015. Effectively treating MDR-TB is difficult, requiring a long time of treatment and complex medication combinations that are expensive and toxic. This complexity is even more prevalent in low- and middle-income countries [3, 15].

Drug-resistant strains of *M. tuberculosis* pose a serious obstacle to the progress of TB control strategies [16]. While the overall trend is for a decrease in the incidence of new antibiotic-sensitive TB cases, there is an opposite trend toward new cases of antibiotic-resistant TB [17]. Globally, in 2015, an estimated 580,000 people developed MDR-TB, and among these several hundred cases were of XDR-TB [16]. The countries with the highest numbers of MDR-TB are China, India, and Russia, which had an increase of more than 20% in 2015 [3, 15].

Access to adequate diagnosis is particularly troublesome in patients with MDR-TB and in cases of TB in children, leading to underestimated rates of MDR-TB [19]. In 2015, 30% of the 3.4 million new bacteriologically confirmed and previously treated TB cases notified globally were reported to have had drug susceptibility testing (DST) for rifampicin performed, with coverage of 24% for new TB patients and 53% for previously treated TB patients. Globally, 132,120 cases of MDR-TB or rifampicin-resistant TB (MDR-/RR-TB) were detected and notified in 2015, and 124,990 were enrolled on treatment programs [3]. By the end of 2015, XDR-TB had been reported by 117 WHO Member States. Of these, 88 countries and 5 territories reported data from continuous surveillance regarding the proportion of MDR-TB cases that were XDR-TB. On average, the proportion of MDR-TB cases that were XDR-TB was 9.5%, similar to estimates from previous years (9.7% in 2014 and 9.0% in 2013) [3].

Currently, more than 20 antibiotics are used in the treatment of TB. Tuberculosis caused by strains susceptible to antibiotics is effectively treated with first-line antibiotics: isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin. More than 12 anti-TB antibiotics are used in the second-line treatment regimen [6]. The current treatment regimen for antibiotic-sensitive tuberculosis includes 2 months of rifampicin, isoniazid, pyrazinamide, and ethambutol, followed by 4 months of rifampin and isoniazid. Effective treatment and cure of MDR-TB requires prolonged time (usually 2 years) and a combination of antibiotics, including second-line drugs that are, to a large extent, less potent than first-line agents and more toxic. Unfortunately, TB treatment with second-line drugs is not insensitive to the emergence of resistance; if the factors that produce MDR-TB remain in place, MDR-TB will likely evolve to XDR-TB [7].

Treating MDR-TB and XDR-TB is much more expensive, when compared to drug-sensitive TB. Treatment for MDR- and XDR-TB strains can cost up to 25 times as much as drug-sensitive infections, and the treatment usually takes three times as long as for drug-susceptible TB. The cost per patient treated is usually in the range of 100–1000 US dollars for drug-susceptible TB and 2000–20,000 US dollars for MDR-TB [3, 17]. In countries with a high tuberculosis rate, the expenditure for treatment per patient is much higher than previously mentioned, and much of this cost is related to drug-resistant TB (MDR- and XDR-TB) [20].

For an effective response to the global TB epidemic, it is imperative that the End TB Strategy milestones for 2020 are achieved. The estimates of the funding required to implement the Stop TB Partnership's Global plan to End TB, 2016–2020, involving TB prevention, diagnostic and treatment interventions rose from almost 9.5 billion US Dollars in 2016 to 14 billion dollars in 2020. Most of these funds (75%) are directed toward diagnosis and treatment of drug-susceptible TB, growing from 7.4 billion dollars in 2016 (6.4 billion in low- and middle-income countries) to 9.7 billion dollars in 2020. However, the amount for drug-resistant TB doubled from 1.8 billion in 2016 to 3.6 billion dollars in 2020. The remaining funds will be directed for TB/HIV interventions [3].

## *Epidemiology of Tuberculosis and HIV Coinfection*

Among the major known risk factors for TB, HIV infection is the strongest one. It was estimated that, in 2015, 0.8% of adults aged 14–59 were living with HIV. Of all new active TB cases, 12% occurred in HIV-positive individuals. Additionally, 25% of all TB-related deaths occurred in HIV-positive individuals, and the majority (75%) of HIV-associated active cases and deaths occurred in the WHO African Region, and exceeded 50% in parts of Southern Africa [2, 3, 5, 12, 21]. With the advent of XDR-TB in KwaZulu-Natal, Southern Africa, the transmission of resistant strains to HIV-infected individuals was catastrophic. HIV-infected individuals exposed to drug-susceptible or drug-resistant strains of *M. tuberculosis* progress rapidly to active TB disease and were more likely to die from TB if active TB developed [7]. In Ethiopia, the prevalence of *M. tuberculosis* infection among HIV-positive patients is even higher than in South Africa, with a prevalence of 63,2% [12, 22]. TB remains as the leading cause of death among HIV-infected persons. HIV substantially increases the risk of progression from latent TB infection (LTBI) to active disease [12]. The risk for TB disease among individuals with LTBI is 26-fold higher among people living with HIV, when compared to those without the virus [12].

Globally, there were an estimated 200,000 TB deaths among HIV-positive men, 140,000 among HIV-positive women, and 40,000 among HIV-positive children in 2015, with the WHO African Region accounting for 75% of these deaths [3]. Tuberculosis and HIV/AIDS have a high synergism, each increasing the magnitude of morbidity and mortality caused by the other. Many studies indicate that the presence of HIV infection affects the progression of extrapulmonary tuberculosis infection. This is an intuitive observation, since the immune deficiency caused by HIV favors the dissemination of bacilli from the primary site of infection, the lung, to other body parts. This is due to the fact that little to no granuloma formation occurs during TB-HIV coinfection and the functional disruption of the local immune response within the granuloma favors dissemination [22]. Triggering of the HIV-associated immunosuppression markedly increases susceptibility to TB, which in turn accelerates HIV-associated morbidity and mortality [12].

The annual risk of developing TB among people living with HIV is 5–15% and is significantly higher than the lifetime risk of 5–10% of HIV-negative persons. HIV-positive people can develop TB following a new infection or as a reactivation of LTBI [21]. The higher risk of TB infection or reactivation commences almost immediately after HIV infection, even when CD4 cell counts are still high [12]. The same study shows that this risk doubles within the first year following serum conversion among those newly infected with HIV compared to those who remained HIV-negative [12].

## *New Compounds in Development*

According to the latest WHO global report on tuberculosis, in August 2016, the status of the pipeline for new anti-TB drugs showed that there are currently seven compounds in preclinical development (TBI-166, CPZEN-45, SQ609, 1599, BTZ-043, TBA-7371, and GSK-070) and nine new or repurposed drugs in phase I, II, or III trials for treatment of drug-susceptible, MDR-TB, or LBTI. Six of these are new compounds (bedaquiline, delamanid, PBTZ169, pretomanid, Q203, and sutezolid), and three are drugs that have already been approved or have repurposed and are undergoing further testing (linezolid, rifampicin, and rifapentine) [3].

Bedaquiline is a newly approved antimycobacterial agent used in pulmonary, multidrug-resistant TB, in combination with other antituberculosis agents. Its use is not approved in LTBI, drug-sensitive TB, or in atypical mycobacterial infections [23]. Although most countries have not yet used this antimycobacterial drug in their treatment regimens, its use was approved by the United States Food and Drug Administrations in December of 2012, and the WHO has recommended its use in June of 2013 [3]. Bedaquiline shows activity against multidrug-resistant *M. tuberculosis* both in vitro and in vivo. Cohort studies of patients with MDR-TB and XDR-TB receiving bedaquiline in combination with optimized background regimens have shown success rates of 62–96% [23].

Delamanid is a nitro-dihydro-imidazole derivate that inhibits mycolic acid biosynthesis. It has been used for treatment of pulmonary MDR-TB in adult patients. Although the full metabolic profile of delamanid is still unknown, it is predominantly metabolized in plasma by albumin. It is not recommended for use during pregnancy and does not require dosage adjustment in cases of hepatic and renal comorbidities. Delamanid is approved by the European Medicines Agency but is not approved in the United States [3, 15].

Two benzothiazinones, PTBZ-169 and BTZ-043, are in the final stages of clinical development. The results obtained to date show that they have a potent activity against drug-susceptible and drug-resistant TB. These drugs use a novel mechanism of action that inhibits the enzyme decaprenylphosphoryl-β-D-ribose 2-epimerase (DprE1) in *M. tuberculosis*, preventing the synthesis of decaprenylphosphoryl arabinose, a key precursor for the biosynthesis of cell wall arabinans [24, 25]. PBTZ169 is compatible with all other TB drugs and appears to have synergism with bedaqui-

line and clofazimine. A phase I trial of PBTZ169 was completed in Russia in July of 2016, and a second phase I trial will be undertaken in Switzerland in 2017. A phase IIa trial is expected to start toward the end of 2016 in Russia [3, 25].

Pretomanid is a nitroimidazole developed by the Global Alliance for TB Drug Development (TB Alliance). Pretomanid comprises a bicyclic nitromidazofuran that is presently undergoing stage III clinical assessments. Research shows that it can be used for 6-month therapy for pulmonary XDR-TB, for non-reacting or unaccepting MDR-TB individuals and for LTBI. Pretomanid has bactericidal action toward mycobacteria through two mechanisms, namely, by functioning as a donor of nitric oxide with subsequent intracellular ATP exhaustion and by restricting mycolic acid biosynthesis of cell walls [3, 26].

Imidazopyridine amide (Q-203) is a new class of drugs that inhibit mycobacterial growth by blocking the respiratory cytochrome bc1 complex, which is essential to maintain the membrane proton gradient and the subsequent ATP synthesis and homeostasis. It is active against MDR- and XDR-TB isolates of *M. tuberculosis* from humans, and, in mouse models, it shows a 100–1000-fold reduction of colony-forming units and blockage of granuloma formation [3, 25].

Sutezolid is a linezolid analog; it has bactericidal activity through limiting protein synthesis. In contrast to linezolid, in vitro and in vivo research showed that sutezolid has a greater antimycobacterial action, leading to a significant reduction in the number of colony-forming units [26].

## Cell- and Culture-Based Methods of TB Diagnosis

### *Bacilloscopy*

Smear microscopy is a technique based on the staining and observation under optical microscopy of a biological sample. Although it is a very basic and old methodology, it remains as a widely used and recommended technique for the detection of mycobacteria in clinical samples. The main advantages are the fact that this technique is quick, easy to perform, and inexpensive and can be easily introduced in a microbiology laboratory, even those with limited resources. However, one caveat of this method is that smear microscopy is incapable of differentiating species of mycobacteria. Additionally, it is incapable of detecting the viability of bacilli. Also, a few other organisms can be stained even when using staining methods specific to mycobacteria, such as *Nocardia* spp. [3, 27]. Ziehl-Neelsen staining is one of the most classic staining methods used in smear microscopy. This methodology is based on the staining of clinical material previously fixed by heat on a slide. The sample is then covered with phenolic fuchsin and heated to facilitate the internalization of the dye. Then, the material undergoes an alcohol-acid discoloration step, which permits dye retention only by the alcohol-acid-resistant bacilli, while any other cells present in the slide will be discolored. The slide is then covered with methylene

blue, which will serve as a counterstain for the cells that had previously been discolored [27, 28]. Smear microscopy using the Ziehl-Neelsen staining can be used as the initial diagnosis of tuberculosis. However, to do this it is necessary to perform this technique in two clinical samples collected on consecutive days. However, due to its low sensitivity (detection of the bacillus in the smear microscopy requires the presence of approximately 5000 bacilli in the clinical sample), it is recommended that this technique be performed in parallel with culturing in specific media, which, in addition to improving sensitivity of the diagnosis, allows the consequent identification of the *Mycobacterium* species that is causing the infection [29].

## *In Vitro Culturing*

With its use dating back to the original description of *M. tuberculosis* by Robert Koch, in vitro culturing remains as one of the main methods of detecting mycobacteria, not only due to its high sensitivity but also for its ability to ascertain the viability of the bacilli present in the clinical sample, being essential for the evaluation of the therapeutic regimen to be employed. Usually, culturing of mycobacteria can be performed using solid or liquid culture media, with the choice depending on the characteristics of each media. For example, one of the advantages of the use of solid media is the possibility of evaluating the morphological characteristics of the colonies formed, which may help in the identification of the *Mycobacterium* species being cultivated. On the other hand, the use of liquid media allows a much faster growth, by virtue of the greater ease of obtaining the nutrients present in the medium [29].

Due to the slow growth of mycobacteria in culture medium, it is essential that the clinical sample be previously decontaminated to prevent other microorganisms from growing, which would deplete the medium from nutrients and prevent mycobacteria from growing. Several methods of decontamination are known (Petroff, Ogawa-Kudow, NALC, among others), all having as principle the alcohol-acid resistance characteristic of mycobacteria. Among the main advantages of using in vitro culturing as a method of detecting mycobacteria is the high sensitivity compared to other methods, as well as the possibility of distinguishing colonies of *M. tuberculosis* from other species of mycobacteria. On the other hand, the main disadvantage is the delay in obtaining a positive result, even when using liquid culture medium, which can lead to a delay in treatment decisions by physicians.

## *BACTEC MGIT960 System*

Once *M. tuberculosis* is detected in the clinical sample, another critical step to determine the appropriate therapeutic regimen is to determine the resistance of the strain isolated to the available antibiotics. Drug susceptibility testing will then consist

of evaluating bacterial growth in the presence of each antibiotic when compared to growth in conditions without antibiotics. The drug susceptibility assay based on the BD BACTEC MGIT 960 semiautomated commercial system is one of the most widely used today. This system uses real-time measurements of oxygen consumption for the evaluation of bacterial growth. For this, the bacterial isolate is placed in liquid culture medium in a tube bearing a silicone ring with ruthenium salts in its bottom. This ring has the property of emitting fluorescence, and this fluorescence is inhibited by the presence of oxygen. Once the bacterial growth and consequently the oxygen consumption occur, the fluorescence rises and is detected by the system in an automated manner, serving as a proxy measure for bacterial growth. The main advantage of this system is the use of liquid medium (Middlebrook 7H9 medium), which reduces the time to obtain a result. In addition, the automated detection of growth circumvents the need for laboratory staff to periodically evaluate mycobacterial growth in each test tube. As a potential disadvantage, one could point out that extreme care must be taken in the event of a contamination of the culture tube, since it may lead to a false result.

## Molecular Methods

### *NAAT (Nucleic Acid Amplification Test)*

A critical step to control the spread of tuberculosis is the utilization and development of more sensitive and rapid diagnostic techniques, especially in cases of HIV coinfection, negative results on sputum smear microscopy, and multidrug-resistant infections. The employment of such techniques can lead to earlier treatment initiation and a reduction in person-to-person transmission and consequently a decrease in TB-related morbidity and mortality rates. These tests usually rely on molecular methods, since these are most often faster and more sensitive than cell-based assays. One such diagnostic method is the nucleic acid amplification test (NAAT) for TB, which represents an important tool in the diagnosis of TB, in view of the fact that the growth of *M. tuberculosis* requires several weeks [30]. NAAT allows rapid and accurate diagnosis of TB and can also predict drug resistance. Generally, NAAT uses PCR to amplify and detect mycobacterial RNA or DNA directly from a clinical sample (sputum, cerebrospinal fluid, lymph node aspirates, etc.) [13]. According to the US Centers for Disease Control and Prevention (CDC), NAAT should be performed on patients with symptoms of pulmonary TB but who do not yet have a confirmed diagnosis of tuberculosis. However, it is important to note that this test should not replace acid-fast bacilli staining and culture [31]. There are many NAAT assays that are used in reference and intermediate laboratories; however, not all are approved by the Food and Drug Administration (FDA). Some of the commercially available NAAT assays are described below and in Table 1.

**Table 1** Summary of the molecular assays used for TB diagnosis and their characteristics

| Assay | Sensitivity and specificity | Comments | References |
|---|---|---|---|
| Xpert MTB/RIF | TB detection 53–95% sens. 96% spec. RIF resistance 96–99% sens. 96% spec. | Automated, real-time, hemi-nested PCR that detects MTB complex as well as resistance to rifampicin through mutations in *rpoB* | [32, 33] |
| Cobas TaqMan MTB test | 75.8–85% sens. 98.1–99.4% spec. | Automated, real-time PCR based on the 16S rRNA coding region for rapid detection of *M. tuberculosis* from clinical specimens | [31, 34, 35] |
| Abbott RealTime MTB automated assay | 81–97% sens. 97% spec. | Automated, real-time PCR test for the detection of MTB complex based on the protein antigen b and the IS6110 regions | [31, 36, 37] |
| BD ProbeTec ET TB system | 81.8–100% sens. 96.2–98.3% spec. | Semi-automated, real-time PCR test based on IS6110 and 16S rRNA genes | [31, 38–41] |
| Gen-probe amplified *Mycobacterium tuberculosis* direct test | 85.7–97.8% sens. 97.6–100% spec. | Amplified *M. tuberculosis* direct test based on the isothermal transcription-mediated amplification of 16S rRNA | [31, 42, 43] |
| Multiplex allele-specific PCR (MAS-PCR) | RIF resistance 91.3–100% sens. 100% spec. ISO resistance 82.3% sens. 100% spec. | Multiplex assay that can simultaneously detect *M. tuberculosis* and mutations associated with resistance to first- and second-line antituberculosis drugs | [44–47] |
| PCR-reverse blot hybridization assay (REBA) | 97.5–100% sens. 85.4–97.5% spec. | Reverse blot hybridization for the rapid identification of mycobacterial species from liquid cultures using 25 probes that detect mutations in genes associated with drug resistance | [48–50] |
| Line probe assay | 74.6–93.0% sens. 96.9–99.4% spec. | Simultaneously detection of MTB complex and drug resistance through mutations in *rpoB*, *katG*, and *inhA* | [3, 51, 52] |

(continued)

**Table 1** (continued)

| Assay | Sensitivity and specificity | Comments | References |
|---|---|---|---|
| GenoType MTBDRplus | RIF resistance 98.1% sens. 98.7% spec. ISO resistance 84.3% sens. 99.5% spec. | DNA amplification followed by reverse hybridization using a membrane with immobilized probes for *rpoB*, *katG,* and *inhA* for the detection of MDR isolates | [27, 53] |
| Nipro NTM + MDRTB detection kit 2 | 85.2% sens. 99.1% spec. | Contains the same mutation probes for *rpoB*, *katG,* and *inhA* used in the GenoType MTBDRplus and also identifies species of the *M. tuberculosis* complex, *M. avium*, *M. intracellulare,* and *M. kansasii* | [3, 54] |
| GenoType MTBDRsl® V 1.0 and V 2.0 | 82.2–99.1% sens. 76.5–98.5% spec. | Simultaneous identification of the *M. tuberculosis* complex and XDR-TB, based on mutations in *gyrA, gyrB*, *rrs*, and *embB* | [55–57] |
| GenoQuick MTB VER 1.0 | 85.4% sens. 92.8% spec. | Line probe assay for identification of the *M. tuberculosis* complex based on the IS6110 region | [58] |
| GenoType MTBC VER 1.X | 93–100% sens. 100% spec. | Line probe assay for differentiation of the major species of the *M. tuberculosis* complex by a multiplex PCR targeting 23S rRNA, *gyrB*, and RD1 deletion | [59] |
| IS6110-based restriction fragment length polymorphism (RFLP) | 94.7% sens. 100% spec. | Genotyping method based on IS6110 polymorphisms | [60–62] |
| Spacer oligonucleotide typing (Spoligotyping) | 83% Sens. 40% spec. | Genotyping tool that identifies genetic polymorphisms based on 43 known spacer sequences present at the direct repeat (DR) locus, a particular genomic region of *M. tuberculosis* complex strains | [63, 64] |
| Mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR) | 52% sens. 56% spec. | Combines the analysis of multiplex PCRs based on 24 loci of variable number tandem repeat (VNTR) interspersed throughout the mycobacterial genome for molecular epidemiology typing of MTBC, increasing the chances of detecting mixed infections | [64–67] |
| Loop-mediated isothermal amplification PCR (LAMP) | 55.6–98.2% sens. 93% spec. | Replacement test for AFB smear microscopy for the diagnosis of pulmonary TB in adults with signs and symptoms; based on an isothermal amplification using six different DNA sequences as targets | [2, 3, 5, 30, 68–70] |

(continued)

**Table 1** (continued)

| Assay | Sensitivity and specificity | Comments | References |
|---|---|---|---|
| Next-generation sequencing | – | NGS can provide a complete genome of a strain of *M. tuberculosis* in a matter of hours, allowing the identification of mutations that could cause resistance; can be used as an epidemiological survey of tuberculosis in real time | [30, 71, 72] |
| Whole genome sequencing | 90% sens. 100% spec. | WGS can aid in the identification and detection of genetic markers, which in cases of outbreaks can help identify the chain of transmission and allow a quicker view of the genotype of the organism, providing the identification of all known resistances within the 25 resistance-related genes known | [73, 74] |

## *Xpert MTB/RIF*

Xpert MTB/RIF (CEPHEID) is an automated assay based on a real-time hemi-nested PCR capable of detecting MTB complex as well as resistance to rifampicin in approximately 2 hours. For this, the clinical sample is processed in an automated way in a single cartridge, where all necessary steps for molecular detection are performed (bacterial lysis, DNA extraction, PCR, and fluorescence detection). The assay detects and analyzes the *rpoB* gene (whose mutations are associated with rifampicin resistance) by measuring the hybridization of five specific probes. As a control, the Xpert MTB/RIF uses *Bacillus globigii*, a spore-forming soil organism, as a control of the functioning of the cartridges [32, 33].

Despite being an automated test, the Xpert MTB/RIF still depends on some manual handling for some of its steps, such as the pretreatment of the clinical sample with a reagent to liquefy and inactivate the bacteria, and the transfer of the liquefied material to the cartridge, which acts as a “Lab on a Chip” and runs on the GeneXpert platform [33]. The main advantages of this test are the fact that it is an extremely simple test to perform and it has high sensitivity and high specificity, its usefulness with pulmonary and extrapulmonary material, and its ability to detect not only the presence of *M. tuberculosis* but also resistance to rifampicin. These combined advantages allow an early diagnosis of tuberculosis (2 hours), which is much faster than the conventional methods used for the detection of *M. tuberculosis* and for the microbiological evaluation of resistance to rifampicin [75, 76]. The main disadvantages are the fact that the test is able to detect only strains of the MTB complex and is not useful for the detection of non-tuberculous mycobacteria (NTM) infections. Additionally, the assay cannot be used in treatment follow-up, since it is based on

the exclusive analysis of the presence of bacterial DNA and does not assess bacterial viability. Another disadvantage is the fact that, since the only resistance evaluated is resistance to rifampicin and because of the high incidence of isoniazid mono-resistance, the results from this assay can only be used as a suggestive diagnosis of MDR-TB, and other tests are required to diagnose resistance to other antibiotics [75].

Thus, according to the WHO, the Xpert MTB/RIF should be used under some recommendations, such as the use as an initial diagnostic test but with the understanding that smear microscopy, culture and susceptibility testing are still required for treatment follow-up and for the identification of resistance to other antibiotics used in the treatment of tuberculosis [77]. More recently, the WHO has recommended the use of a new TB detection cartridge based on the GeneXpert platform (Xpert MTB/RIF Ultra), whose main modification is its ability to detect fewer bacilli (16 bacilli per mL of sputum compared to 131 bacilli per mL for the Xpert MTB/RIF) [78].

### *Cobas TaqMan MTB Test*

To replace the Cobas AMPLICOR MTB assay, Roche Diagnostics has introduced the new system, Cobas TaqMan MTB test, based on real-time PCR technology for rapid detection of *M. tuberculosis* from clinical specimens (including expectorated and induced sputum and bronchial alveolar lavages). The assay uses specific primers to bind a highly conserved region of the *Mycobacterium* genome, containing the gene coding for 16S rRNA [31, 34, 35]. According to the manufacturer, this technique is based on monitoring the emission intensity of fluorescent reporter dyes released during the amplification process, where MTB and *Mycobacterium* internal control probes are labeled with different fluorescent reporter dyes. When the probes labeled with the two fluorescent dyes are intact, the fluorescence is suppressed by the quencher dye. During PCR, the probe hybridizes to a target sequence and is cleaved by a thermostable DNA polymerase. When the reporter and quencher dyes are separated, the fluorescence of the reporter dye increases. The test utilizes the AMPLICOR® Respiratory Specimen Preparation Kit for manual specimen preparation and the Cobas® TaqMan® 48 Analyzer for automated amplification and detection (Cobas® TaqMan® MTB Test 48 AMPLICOR® Respiratory Specimen Preparation Kit) [34].

### *Abbott RealTime MTB Automated Assay*

The Abbott RealTime MTB assay is an automated qualitative real-time PCR test for the detection of MTB complex in smear-positive or smear-negative specimens (sputum or bronchial alveolar lavages) collected from individuals suspected of

having tuberculosis. The assay is based on the amplification of two specific gene regions, the protein antigen b and the insertion sequence 6110 (IS6110), in a single reaction, which increases the diagnostic sensitivity and specificity of the assay due to the presence of multiple copies of the IS6110 gene in some strains [31, 36, 37]. According to the manufacturer, the DNA can be extracted manually or automatically, allowing the processing of a maximum of 96 specimens in a single batch by the m2000sp technology and real-time PCR analysis using the m2000rt technology (Abbott RealTime MTB Amplification Reagent Kit). Abbott Molecular has also developed the Abbott RealTi*me* MTB RIF/INH Resistance assay, which allows the qualitative detection of rifampicin and isoniazid resistance in *M. tuberculosis*-positive samples, through the targeting of *rpoB*, *katG*, and *inhA* promoter regions (Abbott RealTime MTB RIF/INH Resistance Amplification Reagent Kit).

### *BD ProbeTec ET TB System*

The BD ProbeTec ET (DTB), developed by Becton Dickinson (Sparks, USA), is a semiautomated real-time molecular technique for the detection of MTB complex in pulmonary samples such as bronchoalveolar lavages and sputum, through the amplification of the *Mycobacterium*-specific insertion sequence IS6110 and 16S rRNA genes, using specific primers and fluorescently tagged probes, which give an accurate result for smear-positive samples but are very variable for smear-negative ones. This system is not yet approved by the FDA [31, 38–41].

### *Gen-Probe Amplified* Mycobacterium tuberculosis *Direct Test*

Amplified *Mycobacterium tuberculosis* Direct Test (AMTDT) (Gen-Probe, San Diego, USA) was the first NAAT authorized by the Food and Drug Administration (FDA), in 1998, to be used on smear-positive or smear-negative samples, presenting high sensitivity and specificity. AMTDT is a test based on the isothermal (42 °C) transcription-mediated amplification of mycobacterial specific rRNA targets (mycobacterial 16S rRNA), followed by transcription of acridinium ester-labeled DNA templates (intermediate), which are detected by chemiluminescence. Although studies have shown high sensitivity and specificity for some extrapulmonary samples, such as cerebrospinal fluid (CSF), lymph nodes, and gastric washings, the manufacturer does not recommend the use of this test for non-respiratory samples [31, 42, 43].

## *Multiplex Allele-Specific PCR (MAS-PCR)*

Multiplex allele-specific PCR (MAS-PCR) is a simple, fast, and inexpensive alternative for the screening of MDR-TB. This assay can simultaneously detect *M. tuberculosis* and genetic mutations associated with resistance to first- (isoniazid, rifampicin, and ethambutol) and second-line (fluoroquinolone, aminoglycosides) antituberculosis drugs, in smear- and culture-positive specimens. This technique was described by Mokrousov in 2008 and is based on PCR combining three different primers, resulting in the amplification of different fragments of specific alleles [44]. In sensitive strains, three bands are revealed after electrophoretic separation on agarose gels, whereas in mutated strains no bands are revealed after electrophoretic separation [45]. Several mutations in specific genes are used to identify resistance to first-line drugs. For instance, mutations in the *rpoB* gene (codons 531, 526, 516), *the katG* gene (codon 315), the *mab-inhA* promoter region (c-15 t), and the *embB* gene (codon 306), which are associated with rifampicin, isoniazid, and ethambutol resistance, respectively, can be detected by this method [46]. With regard to second-line drugs, mutations in the *gyrA* gene (codon 94 and 90) and *rrs* gene (codons 1401 and 1484), associated with fluoroquinolone and aminoglycoside resistance, respectively, can be detected [79]. As with most molecular assays aimed at the detection of mutations involved in drug resistance, the specificity and sensitivity of MAS-PCR varies according to the geographical area where the strains were isolated. Therefore, in order to obtain more reliable results, more targets are constantly added, so that the specificity and sensitivity of the methodology can be improved [47, 80].

## *PCR–Reverse Blot Hybridization Assay (REBA)*

REBA (REBA MycoID®, M&D Inc., Wonju, Korea) is a method based on the principle of reverse blot hybridization for the rapid identification of mycobacterial species from liquid cultures for subsequent adequate therapy aimed at the causative agent of the disease [48, 49]. According to the manufacturer, PCR-REBA has a short turnaround time, usually taking no longer than 4 hours, in which multiple oligonucleotide probes are immobilized on nitrocellulose strips and hybridized with biotin-labeled PCR products [49]. Recently, REBA MTB-XDR (REBA-XDR) was developed to detect ofloxacin, kanamycin, and streptomycin resistance in AFB smear-positive sputum specimens using molecular line probe assay technology. Briefly, this assay consists of 25 probes that detect mutations in genes associated with drug resistance, namely, the *gyrA* gene, related to ofloxacin resistance; the *rrs* gene, related to kanamycin resistance; and the *rpsL* gene, which is related to streptomycin resistance [50].

## *Line Probe Assay*

The line probe assay (LPA) is a rapid molecular diagnostic with high sensitivity and specificity that simultaneously detects MTB complex and drug resistance for rifampicin and isoniazid. The assay works through the detection of previously described genetic mutations in the *rpoB* gene, associated with rifampicin resistance, as well as the *katG* and *inhA* genes, associated with resistance to isoniazid. LPA consists of DNA extraction from strains (indirect test) or clinical specimens (direct test), polymerase chain reaction with amplification of the wild-type region or the determined region of resistance, hybridization with oligonucleotide probes immobilized on a strip, and colorimetric detection that facilitates the identification of probe hybridization for the *M. tuberculosis* complex and for resistance-associated mutations or the wild-type genetic sequences. Mutations are detected by the absence of binding in the wild-type probes but binding in the probes specific to the mutated regions [51]. Although this assay takes longer to perform (about 12 hours) than the Xpert MTB/RIF assay, it can detect resistance to two drugs, whereas the form can only detect rifampicin resistance [52, 81, 82]. LPAs were approved by the WHO, in 2008, to diagnose rifampicin resistance in smear-positive samples, and represented a revolution in the molecular diagnosis of tuberculosis [51, 52]. The higher reproducibility of commercial assays when compared to in-house assays made the WHO recommend the use of commercial assays for the diagnosis of TB as well as drug resistance [3, 51]. According to the WHO, two LPAs are currently available to identify MDR-TB, the GenoType MTBDRplus assay (Hain Lifescience, Hain Version 2 assay) and the Nipro NTM + MDRTB detection kit 2 (Nipro Corporation, Japan), and two other LPAs to identify resistance to second-line antituberculosis drugs are also available: the GenoType MTBDRsl VER. 1.0 (Hain Lifescience) and GenoType MTBDRsl VER. 2.0 (Hain Lifescience). Two other LPAs are marketed by Hain Lifescience, the GenoQuick® MTB VER 1.0 and the GenoType MTBC VER 1.X.

## *Genotype MTBDRplus*

The Genotype MTBDRplus version 1.0 and the more recent Genotype MTBDRplus version 2.0 (Hain Life Sciences, Nehran, Germany) were both approved by WHO in 2011 for the detection of MDR isolates. The assay uses DNA amplification followed by reverse hybridization using a membrane with immobilized probes covering the wild-type sequences of *rpoB*, *katG*, and *inhA* and also probes for the detection of mutations in the *rpoB* gene (codons 81, 516, 526, and 531), which determine rifampicin resistance; mutations in the *katG* gene (S315 T), responsible for low-level resistance to isoniazid; and mutations in the promoter region of the *inhA* gene (C15T, A16G, and T8A), involved in high-level resistance to isoniazid

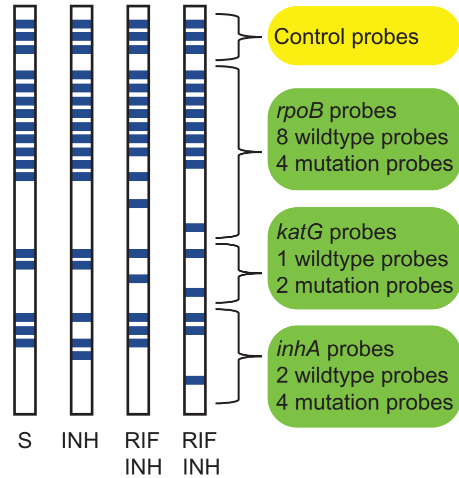


**Fig. 1** Schematic of the GenoType MTBDRplus format. The assay evaluates the presence of mutations in *rpoB*, *katG*, and *inhA*. The results obtained when testing a susceptible strain (S), a strain resistant only to isoniazid (INH), and two strains resistant to both isoniazid (INH) and rifampicin (RIF) are shown

[27]. Each membrane consists of 27 reaction zones (bands), including control bands for validation of the assay (Fig. 1). This assay can be performed in clinical isolates of pulmonary tuberculosis patients as well as in cultured material. Results can be obtained in as little as 5 hours, which is significantly faster when compared to the conventional methods that take about 1–2 months. This quick diagnosis allows clinical staff to choose the appropriate treatment regimen, reducing transmission and spread of drug-resistant TB [3]. Sensitivity and specificity were 98.1% and 98.7%, respectively, for detection of resistance to rifampicin. For isoniazid resistance, on the other hand, sensitivity and specificity were 84.3% and 99.5%, respectively. Although highly useful, this is an assay that requires high-level technical infrastructure and qualified staff [53].

## *Nipro NTM + MDRTB Detection Kit 2*

The Nipro NTM + MDRTB detection kit 2 (Nipro Corporation, Japan) contains the same mutation probes for *rpoB*, *katG*, and *inhA* used in the GenoType MTBDRplus VER. 2.0 assay, although there are some variations for the wild-type probes used in each of these assays [54]. This assay detects rifampicin and isoniazid resistance in only 1 day and identifies species of the *M. tuberculosis* complex, *M. avium*, *M. intracellulare, and M. kansasii*. It has a specificity of 98.5% and a sensitivity of 92% for the detection of rifampicin resistance and 100% specificity and 85% sensitivity for the detection of isoniazid resistance. When analyzed together, the sensitivity for MDR is 85.2% and the specificity is 99.1%. Results can be obtained from clinical specimens and culture materials [3].

## *Genotype MTBDRsl® V 1.0 and V 2.0*

Genotype MTBDRsl® has two commercial versions produced by Hain Lifescience (Hain Lifescience, Nehren, Germany) that allow the simultaneous identification of the *M. tuberculosis* complex and XDR-TB. Version 1.0, developed in 2009 and approved by WHO in 2011, is the first-line probe assay developed for the rapid detection of mutations associated with resistance to fluoroquinolones (ofloxacin, moxifloxacin, levofloxacin), in the *gyrA* gene; aminoglycosides (kanamycin, amikacin) and cyclic peptides (capreomycin), in the *rrs* gene; and ethambutol, in the *embB* gene [55, 56]. It can be performed on sputum samples or culture material from samples with positive microscopy for mycobacteria. It shows sensitivity of 82.2% and 85.6% to detect resistance to fluoroquinolones when performed in sputum samples and cultured isolates, respectively, and specificity of 98.6% and 98.5% also for sputum and cultured isolates, respectively. For the detection of resistance to injectable drugs, its specificity and sensitivity for positive sputum samples were 99.5% and 87%, respectively. For cultured isolates, on the other hand, its specificity and sensitivity were 99.1% and 76.5%, respectively [57]. Genotype MTBDRsl® version 2.0 was developed in 2015 and approved by WHO in 2016, after several studies proved its reliability in detecting drug resistance. The two versions have few differences; version 2.0 uses probes for the identification of mutations in the *gyrA* and *gyrB* genes, which increase its accuracy in detecting resistance to fluoroquinolones, as well as probes for mutations in the *rrs* gene and also in the *eis* promoter region, for the detection of resistance to aminoglycosides and cyclic peptides [3, 51]. Unlike in version 1.0, in version 2.0 the probes used for the detection of ethambutol (*embB*) resistance were removed, since this is a drug used in first-line treatment (Fig. 2). Also, version 2.0 is more sensitive and can be performed on sputum that produced a negative microscopy result. However, the meta-analysis performed by the WHO to determine its sensitivity and specificity showed very heterogeneous results [3, 51]. Both versions provide a result in 24 hours; however, the second version cannot identify individual resistance within the classes of fluoroquinolones. Mutations in *gyrA* and *gyrB* genes are more related to resistance to ofloxacin and levofloxacin, and the correlation with resistance to moxifloxacin and gatifloxacin is not very well determined. Therefore, the inclusion of these drugs in the MDR-TB treatment regimen should be assessed by conventional methods for resistance profile determination [3, 51].

## *GenoQuick® MTB VER 1.0*

GenoQuick® MTB VER 1.0 is a line probe assay used as a rapid test for identification of the *M. tuberculosis* complex. It can be used with clinical specimens that produced positive or negative results in microscopy and also with cultured strains. It consists of DNA extraction, PCR targeting the IS6110 region, hybridization of the

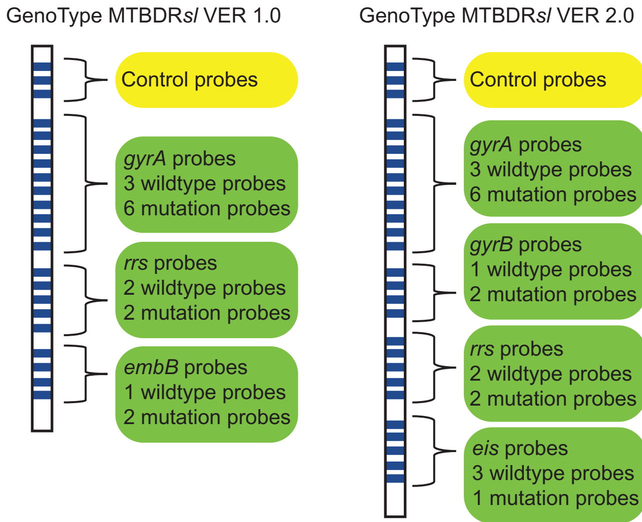


**Fig. 2** Schematic of the Genotype MTBDR*sl*® assay format. The assay evaluates the presence of mutations in *gyrA*, *rrs*, and *embB* for version 1.0 and *gyrA*, *gyrB*, *rrs*, and *eis* for version 2.0

amplicons, and easy determination of the result by a colorimetric readout, as specified by the manufacturer. The results can be released within 3 hours, and the test shows high sensitivity and specificity by using a specific target region of the *M. tuberculosis* complex genome and internal controls that increase its reliability [58].

## *GenoType MTBC VER 1.X*

Genotype MTBC is a commercial line probe assay developed by Hain Lifescience GmbH (Nehren, Germany) and used to differentiate the major species that comprise the *M. tuberculosis* complex (*M. africanum*, *M. bovis BCG*, *M. bovis* ssp. *bovis*, *M. bovis* ssp. *caprae*, *M. microti*, *M. tuberculosis*/*M. canettii*) in both solid and liquid cultures. It uses a multiplex PCR with biotinylated primers for the amplification of DNA encoding 23S rRNA, fragments specific for all bacteria of the complex, polymorphisms of the gyrase B gene (*gyrB*) for species differentiation, and region of differentiation 1 (RD1) deletion of *M. bovis BCG*. Based on the principle of reverse hybridization, the assay has a membrane strip with 13 oligonucleotide probes specific for each complementary fragment. The development of the probes is mediated by biotin-streptavidin, producing six possible different patterns, as shown in Fig. 3 [59]. The species are identified according to the interpretation chart provided by the manufacturer. The results can be obtained in 5 hours, representing a major advance from the traditional biochemical and phenotypic tests that take weeks to produce results.

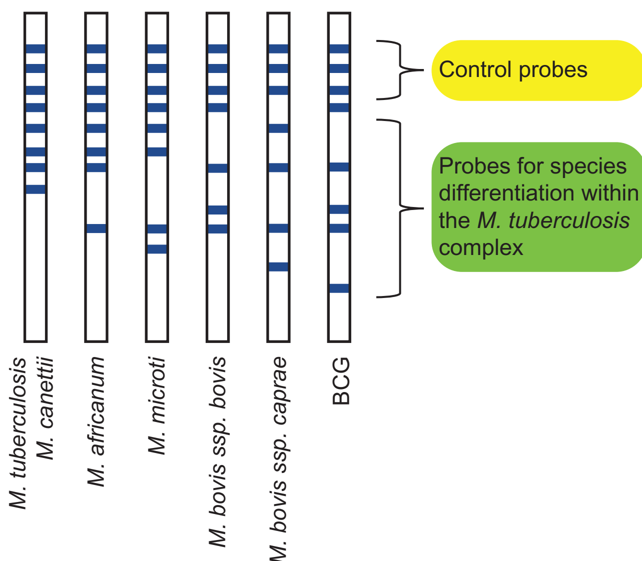


**Fig. 3** Schematic of the GenoType MTBC VER 1.X assay format. The assay can differentiate some of the species and subspecies within the *M. tuberculosis* complex, namely *M. tuberculosis*/*M. canettii*, *M. africanum*, *M. microti*, *M. bovis* ssp. *bovis*, *M. bovis* ssp. *caprae*, and BCG

## *IS6110–Based Restriction Fragment Length Polymorphism (RFLP)*

Restriction fragment length polymorphism (RFLP) of the insertion sequence 6110 (IS6110) is a genotyping method that is widely applied in molecular epidemiological studies of *M. tuberculosis* and that contributes to TB control. The assay employs PCR amplification of IS6110 using previously established primers and procedures and labeling using a DIG-High Prime Nucleic Acid Labeling and Detection Kit (Roche) [60, 61]. This technique is based on the analysis of IS6110 copy numbers and their locations within the MTB genome and represents the gold standard method for typing MTB strains [60]. Briefly, the method includes digestion of DNA with the PvuII restriction enzyme, which cleaves the IS6110 sequence, producing diverse DNA fragments that are separated through gel electrophoresis. These fragments are then transferred to a membrane and hybridized with a peroxidase-labeled probe that is complementary to part of the IS6110 sequence [83, 84].

## *Spacer Oligonucleotide Typing (Spoligotyping)*

Spoligotyping is a valuable genotyping tool based on a polymerase chain reaction (PCR) used to simultaneously detect and differentiate strains of MTB, which assists researchers in molecular epidemiology and evolutionary studies of

MTB in TB-endemic countries [60, 84]. This method identifies genetic polymorphism based on 43 known spacer sequences which are present at one particular genomic region of *M. tuberculosis* complex strains called direct repeat (DR) locus [63]. Spoligotyping is a highly reproducible, simple, and fast method. As a consequence, many studies around the world have been developed to determine the association of drug resistance with spoligotype defined lineages [85]. In this assay, DNA is extracted from *M. tuberculosis*, primers DRa and DRb are used to amplify the DR region by PCR, and the amplified products are hybridized and detected by chemiluminescence through biotin labeling of the PCR products and a streptavidin-peroxidase conjugate system and then visualized by autoradiography. Spoligotyping results are accurate and are obtained in up to 2 days [63, 85].

### *Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats (MIRU-VNTR)*

The diagnosis and treatment of mixed infections with multiple *M. tuberculosis* strains is a major problem in surveillance and control of TB. However, several molecular genotyping methods are available and can detect the phenomenon of mixed infections during tuberculosis disease. MIRU-VNTR is a technique applied in molecular epidemiology typing in MTBC, increasing the chances to detect mixed infections [65]. This technique is based on PCR amplification using primers specific for the flanking regions of the variable number tandem repeats (VNTRs) and on the determination of the sizes of the amplicons, after electrophoretic migration. The method combines the analysis of multiplex PCRs for the target loci on a fluorescence-based DNA analyzer with computerized automation of the genotyping [65]. The latest version of MIRU-VNTR is based on 24 loci of variable number tandem repeat (VNTR) interspersed throughout the mycobacterial genome, which includes a subset of 15 highly discriminatory loci [66, 67]. MIRU-VNTR typing generates numerical results that can be easily analyzed, with high efficiency and limited labor, which allows individual strain identification based on large reference databases. Due to this, this assay has been implemented as the routine and gold standard method for *M. tuberculosis* genotyping in many countries around the world [86]. The MIRU-VNTRplus web server was created using a collection of 186 strains that represent the major MTBC lineages. For each strain, species, lineage, and epidemiologic information was stored, together with copy numbers of 24 MIRU loci, spoligotyping patterns, regions of difference (RD) profiles, single nucleotide polymorphisms (SNPs), susceptibility data, and IS6110 RFLP fingerprint images [87].

### *Loop-Mediated Isothermal Amplification PCR (LAMP)*

The loop-mediated isothermal amplification (LAMP) method is an alternative NAAT developed by Notomi et al. (2000) and is based on an isothermal amplification requiring only a heat block, using four primers to target six different DNA sequences with high specificity and efficiency in less than 2 hours and with minimal equipment [30, 68–70]. In 2011 a commercial assay, Eiken's Loopamp™ MTB kit (Eiken Chemical Company Ltd., Tokyo, Japan), was developed based on LAMP technology for the detection of *M. tuberculosis* complex. This is a manual assay that requires less than 1 hour to perform, minimal laboratory infrastructure and biosafety requirements, and can be visually read under ultraviolet light by the addition of fluorescent intercalating dyes such as SYBR Green and turbidity measurements [13]. According to the WHO, LAMP may be used as a replacement test for AFB smear microscopy for the diagnosis of pulmonary TB in adults with signs and symptoms consistent with TB and may be considered as a follow-on test to smear microscopy, especially when further testing of sputum smear-negative specimens is required [2, 3, 5].

### *Next-Generation Sequencing*

Next-generation sequencing (NGS) is a relatively new nucleic acid sequencing technology, which has surpassed the conventional Sanger method in terms of its decreased cost and increased data production. NGS can provide a complete genome of a strain of *M. tuberculosis* in a matter of hours, allowing the identification of mutations that could cause resistance as well as potentially novel mutations [30, 71, 72]. There are a few different NGS platforms available, which can provide short or long sequence reads. For long reads, some of the platforms used are the PacBio single-molecule, real-time sequencing (Pacific Biosciences, USA) and the MinION nanopore sequencing, which can provide long reads in a device of size comparable to a memory stick (Oxford Nanopore Technologies, UK) [88]. To generate short reads, some of the technologies used are sequencing by synthesis (Illumina Inc., USA) and Ion Torrent semiconductor sequencing (ThermoFisher Scientific, USA) [71, 89]. The most widely used platform in mycobacterial research is PacBio, which provides longer reads [30]. NGS for TB diagnosis can be used on isolates of MTB following culture, or directly from clinical samples, which presents a great potential, by decreasing diagnostic time (from weeks to hours), besides determining the genetic potential for drug resistance of an isolate. Additionally, it can identify multiple coinfecting strains, as well as if the patient has been infected with a new strain or whether the original infection was not fully cured by the initial treatment. Furthermore, NGS can aid in the study of chains of transmission and epidemiological surveys of tuberculosis in real time. As such, NGS technologies have the potential to effectively change global health and the management of TB and could also provide a rapid and comprehensive

analysis of drug resistance profiles, facilitating the patient's treatment, improving treatment outcomes, and reducing the spread of drug resistance [30, 71].

## *Whole Genome Sequencing*

Over the past few years, the quality of nucleic acid sequencing methods has improved considerably, making the identification of mutations in a genomic scale much more reliable. The whole genome sequencing (WGS) of *M. tuberculosis* provided a broader and quicker view of the genotype of the organism, allowing the identification of all known resistances within the 25 resistance-related genes, making it possible to reduce the need for phenotypic tests to determine drug susceptibility. WGS can also aid in the identification and detection of genetic markers, which in cases of outbreaks can help identify the chain of transmission [73, 74, 90]. WGS was first used to understand anti-TB drug resistance profiles and consequently to identify new resistance-causing mutations. Taking into account the slow diagnostic time (1–2 months) of conventional culture-based methods, WGS offers several benefits: reduced diagnostic time, rapid outbreak control, and decreased empirical treatment of the patient, providing susceptibility results for first- and second-line drugs in a matter of hours [91]. WGS can be divided into the following steps: extraction and purification of genomic DNA; DNA fragmentation in sequences of 100–500 base pairs (bps); sequencing of these fragments, generating "reads"; and bioinformatics data analyses. Currently, several platforms are intended for WGS; if the goal is to identify new species, it is recommended that platforms that provide longer "reads," such as the PacBio RS (Pacific Biosciences, Menlo Park, CA, USA), be used. On the other hand, if the goal is to determine drug resistance, it is advisable to use platforms that provide shorter "reads," such as the Illumina MiSeq (Illumina, San Diego, CA, USA) or the Ion Torrent PGM (ThermoFisher Scientific, Waltham, MA, USA) [92]. Bioinformatics analysis is also critical to the quality of the result produced and can be divided into the following steps: alignment of DNA sequences obtained with a reference genome, usually the *M. tuberculosis* strain H37Rv; determining the probability that a single nucleotide polymorphism (SNP), that is, a difference in a single base in the genome compared to the reference, is not due to a random error of the sequencing reaction; and resistance prediction based on the SNPs found. Several bioinformatics tools are available in the market, but all have their strengths and weaknesses, and the choice usually relies on personal preference [74]. Despite the great advantages of diagnosing tuberculosis through WGS, it presents a high cost when compared to traditional tests and requires specialized infrastructure and staff. Additionally, it requires prior enrichment through culture, extending the time required for diagnosis. Previous studies have used liquid culturing using mycobacterial growth indicator tubes (MGIT) and were able to shorten the time of diagnosis to 2 weeks, but this is still considered a long time and may represent an undesired opportunity for infection transmission [73, 91, 92]. Nevertheless, WGS is not a

stationary technology, and several platforms and handheld devices are currently being developed. In the future, costs will be reduced, allowing the implementation of WGS in the diagnosis of TB in low-income laboratories [74, 91].

## Immunological Methods

Immunological assays for the diagnosis of tuberculosis measure the patient's immune response to the infection and are widely used to diagnose both latent (LTBI) and active TB. Some of the methods currently in use are discussed below and in Table 2.

### *Tuberculin Skin Test (TST)*

The tuberculin skin test is based on the intradermal inoculation of a small amount of TB-purified protein derivative (PPD) in the forearm. Within 48–72 hours, a trained worker must read the reaction on the arm by observing and measuring the induration at the site of injection (palpable, raised, hardened area, or swelling). If the response to the antigen injection in the region produced an induration halo larger than or equal to 10 mm, the result is considered positive [93]. Even though TST is widely applied for diagnosing LTBI and active TB, especially in high-burden areas, false-positive results are common in individuals who have been vaccinated with BCG, and immunocompromised people such as HIV/AIDS patients may not react to the test, since their immune systems are impaired [13]. Thus, because of these potential inaccuracies, it is necessary to perform additional exams in case of a positive result, in order to confirm the active disease, since a positive TST result is indicative of the presence of *M. tuberculosis* but does not determine whether the patient has an active infection [13].

### *Interferon–Gamma Release Assay*

The interferon-gamma (IFN-γ) release assay (IGRA) was introduced as an alternative to TST for the diagnosis of LTBI [31]. IGRA measures an ex vivo response of IFN-γ-producing T cells against ESAT-6 and CFP-10 antigens, which are not present in most environmental mycobacteria and the vaccine strain, bacillus Calmette-Guérin (BCG). Thus, there is no cross-reaction in people vaccinated with BCG [94]. Even though IGRA was designed to identify LTBI, it can also detect active TB, although it is not efficient in differentiating LTBI from active TB. In 2011, the FDA approved the QuantiFERON-TB Gold in tube test (QFT-GIT, Cellestis, Australia) and the T-Spot TB test (T-Spot, Oxford Immunotec, UK) as indirect and adjunct

**Table 2** Summary of the immunological assays used for TB diagnosis and their characteristics

| Assay | Sensitivity and specificity | Comments | References |
|---|---|---|---|
| Tuberculin skin test (TST) | 87–98% sens. 74–96% spec. | Test based on the intradermal inoculation of a small amount of TB-purified protein derivative, diagnosing LTBI, and active TB; WHO issued a negative recommendation since 2011 | [13, 93] |
| Interferon-gamma release assay | 75–84% sens. 75–91% spec. | Introduced as an alternative to TST for the diagnosis of LTBI; it measures an ex vivo response of IFN-γ-producing T cells against ESAT-6 and CFP-10 antigens | [94] |
| Urine lipoarabinomannan rapid test | 39–66.7% sens. 98% spec. | Used for the diagnosis and screening of active TB in HIV-positive individuals that are severely ill or with low CD4 counts | [29, 95] |
| Nanodisk-MS platform | 75–92.3% sens. 87.1–100% spec. | Rapid and specific blood-based assay that uses antibody-conjugated nanodisks to diagnose active TB; clinical utility for HIV-positive and HIV-negative individuals | [96] |

tests for TB diagnosis [97]. The QFT-GIT test uses fresh blood, which is incubated with controls and a mixture of synthetic peptides representing ESAT-6, CFP-10, and TB7.7. An enzyme-linked immunosorbent assay (ELISA) is used to determine the concentration of IFN-γ released in the plasma separated from blood. For the T-Spot test, peripheral blood mononuclear cells (PBMCs) are incubated with control and two mixtures of peptides representing ESAT-6 and CFP-10. Then, the number of IFN-γ-producing cells (spots) is determined using an automated ELISPOT reader, which counts spot-forming units (SFUs) [98, 99]. Although IGRAs were widely used in high-burden countries for the diagnosis of LTBI, the WHO issued a negative recommendation on the use of these tests in low- and middle-income countries, due to their inaccuracy in high TB- and/or HIV-burden settings [97, 100, 101].

## *Urine Lipoarabinomannan Rapid Test*

The lipoarabinomannan rapid test (lateral flow LAM, LF-LAM) is used for the diagnosis and screening of active tuberculosis in HIV-positive individuals [95]. The lipoarabinomannan (LAM) antigen is a lipopolysaccharide present in the mycobacterial cell wall and can be found in urine from patients with active tuberculosis; it is released by metabolic activity or by cellular degeneration. As urine samples are easy to collect and store, urine-based tests show advantages over other assays, especially when compared to sputum, once it lacks the risk of infection associated with sputum collection [2, 5, 95]. Urine-based tests are an alternative to detection *M. tuberculosis* bacilli or DNA, which requires more complex laboratory facilities.

The LF-LAM assay is a commercially available test performed manually by applying 60 μL of urine to the test strip, followed by an incubation step of about 25–35 minutes at room temperature. Then, the strip is visually inspected in order to check for any visible band. The intensity of the band is compared to the intensities of the bands from the reference scale supplied by the manufacturer [95, 102]. Although the LF-LAM assay has advantages compared to direct sputum analyses, it lacks sensitivity for the diagnosis of TB in patients that are not HIV-infected, and it is recommended by the WHO only for HIV-associated TB diagnosis in patients with low CD4 counts (<100 cells/μL), or severely ill HIV patients [29]. Besides, LF-LAM does not permit the differentiation of *M. tuberculosis* infection from infections caused by other mycobacterial species. Nevertheless, in TB-endemic regions, a positive result is sufficient to initiate treatment, since LF-LAM shows a positive predictive value significantly higher in these countries. A negative test, however, is not enough to preclude the diagnosis of TB, even in high-burden countries. Regardless of the limited applicability of this test, LAM results may reduce early mortality among HIV patients suspected to have TB, since a positive test can lead to rapid onset of treatment within this group [102].

## *Nanodisk–MS Platform*

The Nanodisk-MS platform was designed as a blood-based assay for rapid diagnosis of active TB infections and showed evidence of clinical utility in a case-control study with HIV-positive and negative patients [96]. According to the authors of the study, the Nanodisk-MS is a rapid and specific test, detecting cases of active TB infection with high sensitivity. Using antibody-conjugated nanodisks, it was possible to enrich complex solutions for *M. tuberculosis* antigenic peptides CFP-10 and ESAT-6 found in trypsin-digested serum samples from the Houston Tuberculosis Initiative (HTI), a population-based TB surveillance study. Therefore, since CFP-10 and ESAT-6 are secreted by virulent *M. tuberculosis* strains, their serum concentration can be used to diagnose active disease [103].

The development of antibody-conjugated nanodisks considerably increased target peptide enrichment for matrix-assisted laser desorption/ionization (MALDI) of bound peptides to optimize the detection by high-throughput time-of-flight mass spectrometry (MALDI-TOF MS) [96]. For this purpose, human serum samples are mixed with $NH_4HCO_3$ and trypsin, placed in a water bath, and irradiated in a microwave oven at 20% power for 20 minutes. Then, trifluoroacetic acid is added at a 0.1% final concentration, and the serum sample is incubated at 37 °C for 12 hours for an overnight trypsin digestion [96]. To immobilize antibodies, nanodisk suspensions are pelleted, vacuum-dried, and suspended in phosphate buffer saline (PBS) containing synthetic antibodies (anti-1593.75 and anti-1900.95) (GL Biochem). The suspension is mixed for 2 hours at 25 °C, pelleted and incubated for 30 minutes with Tris-NaCl buffer, washed three times with PBS, pelleted and suspended again, and stored at 4 °C until use. In order to quantify *M. tuberculosis* antigens in human

serum samples, healthy donor serum is spiked with 0–100 nM of recombinant CFP-10 or ESAT-6 to generate standard curves. Serum is submitted to a microwave-assisted digestion, spiked with internal standard peptides, and mixed with nanodisks. Samples are evaluated by MALDI-TOF MS, and the MS signal intensity of each target peptide and internal standards are analyzed. MS intensity ratios of clinical samples are converted to absolute molar concentrations through substitution into the calibration curve [96].

Nanodisk-MS enables multiplex detection of serum *M. tuberculosis* antigen concentrations for the identification of active disease. It requires a low volume of blood, being preferred over invasive methods such as biopsies. Although MALDI-TOF MS is an expensive technique, many hospitals and microbiology laboratories currently employ it for other purposes, such as microbial identification [104].

## Microscopy Methods

In order to diagnose tuberculosis in people presenting symptoms, microscopy is the primary method of examination. Some techniques have been developed to improve the detection of acid-fast bacilli in clinical samples from active disease cases.

### *Automated Microscopic System, TBDx*

TBDx is an automated smear microscopy system that performs all microscope functions automatically. It loads slides (1–200) onto the microscope and focuses and captures images. Smears are categorized as positive or negative by a computerized algorithm system. TBDx processes digital microscopic images in order to detect acid-fast bacilli, and since it uses an algorithm as part of the diagnostic decision, the reliability of the results is improved. For this method, sputum samples need to be digested and decontaminated by the N-acetyl-L-cysteine-NaOH method. Slides are prepared the same way as for ZN staining [105]. After slides are completely air-dried, they are fixed for 2 hours on an electric slide warmer. Then slides are stained using the auramine-O method, decolorized with an acid-alcohol solution, and counterstained with potassium permanganate. Microscopy analyses are performed using a mercury vapor microscope with a system of objective and eyepiece that gives a 400x visual magnification. Data is downloaded to a computer, which uses algorithms to detect and count AFBs on the digitalized fields. Smear results are quantified according to WHO recommendations [106].

This novel technique has the advantage of relying on digital analyses of high-throughput microscopy images, and therefore it does not require an experienced technician. Besides, a study showed that the TBDx system, used as a screening method prior to Xpert MTB/RIF (GXP), detected 90% of patients with GXP-positive TB. Therefore, the number of GXP tests required was considerably

reduced [107]. Thus, the automated microscopy system can improve the diagnosis of TB by reducing the volume of expensive confirmatory tests, and the necessity of trained microscopists, especially in regions with limited resources.

### *Sputum Smear Light–Emitting Diode Fluorescent Microscopy (LED–FM)*

In 2011, the WHO recommended the use of LED-FM as an alternative to light microscopes and mercury vapor FMs in regions with limited resources [108]. The performance of fluorescent microscopy is significantly better than Ziehl-Neelsen staining. On average, LED-FM enhances the sensitivity of AFB detection by 10% when compared to conventional light microscopy. Besides, LED fluorescent microscopes are inexpensive, use affordable light bulbs, can run on batteries, and do not demand a dark room [109, 110]. With respect to reading time, studies showed that LED-FM presents a 2–4 times faster examination time per slide [111–113]. Another advantage over light microscopy is the higher sensitivity of FM for paucibacillary patients, a relevant group for tuberculosis diagnosis in HIV-infected people.

In order to perform LED-FM, slides are stained using the auramine-O method. Fluorescent smears are ready at 400× magnification and classified according to the WHO guidelines for fluorescent microscopy [114]. Even though the sensitivity of LED-FM shows a wide variation among different studies (56–80%), its specificity is around 92–98% (both compared to culturing), and the WHO endorsed the use of LED-FM for the detection of AFB. Besides, compared to ZN, the observation time is reduced by 50–70%. Regarding HIV coinfected patients, there is no reduction in the reading performance. However, it was demonstrated that a brief orientation for technicians is insufficient to achieve appropriate performance of the new method, and this could interfere in the results generated [115]. Therefore, adequate training in the use of LED-FM must be emphasized.

## Concluding Remarks

As can been seen throughout this chapter, methods for the diagnosis of tuberculosis as well as *M. tuberculosis* drug resistance are far from scarce. However, each of these methods has its strengths and weaknesses, and therefore the development of new assays must continue. The final goal should be a diagnostic method that is highly robust, being specific, sensitive, cost-effective, and requiring minimal to no infrastructure and trained human resources. It is important to keep in mind that many of the TB-afflicted areas of the world have little to no resources, and, therefore, a perfect diagnostic tool will be one that could function with robustness under such conditions.

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# Advanced Microbial Strain Subtyping Techniques for Molecular Epidemiology Investigations

**Lee W. Riley**

## Introduction

Chapter "Molecular Typing Techniques: State of the Art" in Vol. I, described microbial strain subtyping methods based on state-of-the-art molecular microbiology techniques. Today, these subtyping methods are applied in a variety of disciplines, including taxonomy, phylogenetics, molecular evolution, clinical microbiology, and molecular epidemiology of infectious diseases. This chapter will discuss how these new techniques are applied to conduct infectious disease investigations.

Molecular epidemiology is an established discipline in epidemiology that has evolved with advancements made in molecular microbiology techniques. The discipline of molecular epidemiology shares the same molecular microbiology tools with taxonomy, phylogenetics, molecular evolution, and clinical microbiology, but each discipline uses these tools for its distinct goal. In taxonomy, phylogenetics, and molecular evolution, microbial strain subtyping data are compared to each other; the main goal of these disciplines is to infer evolutionary relationships of microbes based on their genetic characteristics. In clinical microbiology, these tools are used for infectious disease diagnosis, and thus the goal of this discipline is to demonstrate relationships of microbes to disease states of individual hosts. In molecular epidemiology, the subtyped microbes are compared to each other as well as to a group of hosts (population) from which these microbes are isolated in an environmental setting. Thus, the main goal of molecular epidemiology is to investigate determinants of infectious diseases in populations in a defined environment. Because of these differences in goals, the application of molecular microbiology strain typing methods to each of these disciplines is driven by a distinct set of premises

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and pedagogical principles. This chapter will focus on how advancements made in molecular biology typing tools have contributed to our new knowledge of infectious disease epidemiology and how they can be effectively incorporated into public health investigations.

## Definition of Molecular Epidemiology

"Epidemiology" is usually defined as "the study of the distribution and determinants of diseases and injuries in human populations" [1]. Thus, including veterinary diseases, a simple extension of the definition of "molecular epidemiology" would be "the study of the distribution and determinants of diseases and injuries in human and nonhuman animal populations using molecular microbiology methods" [2].

One major activity of infectious disease epidemiologic investigations is the determination of mechanisms and modes of pathogen transmission. Every communicable infectious agent has its own programmed strategy to transmit itself to other human or animal hosts. For example, tuberculosis transmission can only occur from an index patient with symptomatic lung infection, whereas most enteric pathogens are best transmitted to other hosts from an asymptomatic carrier who continues to shed the pathogen after recovering from a diarrheal illness. These transmission mechanisms are genetically determined. Therefore, the definition of molecular epidemiology can be extended to include studies that examine genetics of microbes that underlie their mechanisms and modes of transmission.

Interestingly, when biochemical tests or immunological tests are used to subtype infectious agents to conduct epidemiologic investigations, we do not refer to such studies as "biochemical epidemiology" or "immunological epidemiology." It is therefore odd that epidemiologic studies that use molecular microbiology techniques have come to be termed "molecular epidemiology." Nevertheless, the term "molecular epidemiology" has become established to describe a branch of epidemiology and that it should not be confused with the other disciplines that use the same strain typing tests based on molecular microbiology techniques.

One way to distinguish the discipline of molecular epidemiology from the other disciplines that utilize molecular microbiology typing tools is to understand the target of analysis. As mentioned above, in molecular epidemiology, the genotyped microbes are compared to each other, and this information is linked to a group of hosts from which these microbes are isolated. These hosts comprise a transmission pathway in which microbes disseminate. In taxonomy, phylogenetics, and molecular evolution, microbes are the main target of analysis. In clinical microbiology, an individual host is the main target of analysis. In molecular epidemiology, a population of hosts is the target of analysis. Both molecular epidemiology and clinical microbiology are motivated by an opportunity for disease prevention and intervention against infectious diseases. Thus, molecular epidemiology is a component of public health.

## Genotyping Techniques Used to Conduct Epidemiologic Investigations

Microbes can be subtyped according to phenotypic or genotypic characteristics. Phenotype subtyping tests commonly include serogrouping, serotyping, and antimicrobial or antiviral drug susceptibility profiling. When a serotype needs to be further subtyped, a genotyping test is usually required. Thus, nucleic acid contents serve as targets of analyses in molecular epidemiology, and these analyses will vary by the class of microbes analyzed—bacteria, viruses, fungi, protozoa, or helminths. Despite the wide variety of genotyping tests used, they are all variants of three basic methods—(1) electrophoresis of nucleic acid fragments, (2) nucleic acid hybridization matrix, and (3) nucleic acid sequencing. The large repertoire of genotyping tests used today is reviewed in Vol. I, Chapter "Molecular Typing Techniques: State of the Art". In this chapter, the most common genotyping tests currently used to conduct epidemiologic investigations of bacterial infections will be reviewed. With a few exceptions and, of course, differences in genetic targets, many of the basic concepts of molecular epidemiology applied to bacteria will apply to other categories of infectious agents.

Genotyping tests for bacteria (as well as fungi, protozoa, and helminths) can be classified into those based on analysis of parts of a genome (microdiversity) or the whole genome (macrodiversity). Of course, the whole-genome sequence (WGS) data provide the most discriminating information. Until recently, due to technical and cost constraints of WGS, most bacterial genotyping tests have relied on microdiversity analysis. However, the higher speed and decreasing cost of next-generation sequencing (NGS) technology have made macrodiversity analysis increasingly accessible. In fact, information derived from WGS has facilitated the validation of established microdiversity genotyping tests as well as the development of new and more precise strain microdiversity genotyping tests.

This chapter will focus on genotyping tests that are currently in wide use in epidemiologic investigations. With advancements made in nucleic acid sequencing technology, many of the early-generation genotyping tests are less frequently used. These tests include phage typing and electrophoretic DNA fragment pattern analyses such as plasmid profile analysis, arbitrarily primed PCR (AP-PCR), randomly amplified polymorphic DNA (RAPD) [3–7], restriction fragment length polymorphism (RFLP) analysis (including RFLP of large PCR-amplified DNA products) [8, 9], amplified ribosomal DNA restriction analysis (ARDRA) [10, 11], restriction-site-specific (RSS) PCR [12], and amplified fragment length polymorphism (AFLP) genomic analysis [13–15]. Some of them, while still highly useful, have become typing tests of historical interest (e.g., phage typing, plasmid profile analysis). They will not be further discussed in this chapter.

Strain typing tests that are currently in wide use in epidemiologic investigations are listed in Table 1. They include tests based on electrophoresis of PCR-amplified products or bacterial genome digested with endonucleases and sequence-based tests. Despite the increased use of sequence-based tests, some of the PCR-based

**Table 1** Bacterial genotyping tests currently used to conduct epidemiologic investigations

| Test | Microbes targeted | Features | |
|---|---|---|---|
| **Broad-spectrum PCR-based tests targeting repeat DNA elements** | | **Advantages** | **Disadvantages** |
| Repetitive palindromic element PCR (REP-PCR) | Across multiple phyla | Simple and rapid, can be used to screen a large number of strains | Limited intra- and interlaboratory reproducibility, contamination problems |
| Enterobacterial repetitive intergenic consensus (ERIC) PCR | Usually members of *Enterobacteriaceae* spp. | Simple and rapid, can be used to screen a large number of strains | Limited intra- and interlaboratory reproducibility, contamination problems; ERIC sequence must be present in target strain |
| BOX-PCR | Usually Gram-positive bacteria (e.g., *Streptococcus*) | Simple and rapid, can be used to screen a large number of strains | Limited intra- and interlaboratory reproducibility, contamination problems; BOX elements must be present in target strain |
| **Species-specific PCR-based tests targeting repeat DNA elements** | | | |
| Variable number tandem repeats (VNTRs) (e.g., *spa* typing) | Across multiple phyla (e.g., *Staphylococcus aureus*) | Simple and rapid, good reproducibility and interlaboratory comparability | Costly, resolution dependent on number of targeted repeat sequences |
| Multilocus VNTR analysis (MLVA) | Across multiple phyla | Simple and rapid, high reproducibility and interlaboratory compatibility, can be used for surveillance | Costly, resolution dependent on number of targeted repeat sequences |
| Direct repeat (DR)-PCR or CRISPR typing (e.g., spoligotyping) | Across multiple phyla (e.g., *Mycobacterium tuberculosis*) | Simple and rapid, good screening test | Low discriminatory power, requires hybridization step, limited epidemiologic study applications |

(continued)

**Table 1** (continued)

| Test | Microbes targeted | Features | |
|---|---|---|---|
| **Electrophoresis-based tests** | | | |
| Pulsed-field gel electrophoresis (PFGE) | Across multiple phyla | Highly discriminatory and reproducible, does not require prior knowledge of target sequences, used for surveillance systems | Requires expertise to compare band patterns, interlaboratory portability difficult, opaque genotype nomenclature |
| *IS*6110 restriction fragment length polymorphism (RFLP) | *Mycobacterium tuberculosis* | Highly discriminatory and reproducible, reference standard for *M. tuberculosis* | Labor intensive, requires a hybridization step, slow |
| **Sequence-based tests** | | | |
| Multilocus sequence typing (MLST) | Across multiple phyla | Highly discriminatory and reproducible, simple genotype nomenclature system, used for surveillance | Costly, requires intermediate-level skill and tools to analyze sequences |
| Whole-genome sequencing (WGS) (see Table 2) | Across multiple phyla | Most discriminatory and reproducible test | Costly, requires advanced computational skills |

genotyping tests for bacteria remain widely used because of their simplicity, speed, versatility, high throughput, and low cost. They are often used in outbreak settings to rapidly identify risks, trace vehicles or sources of such outbreaks, and assess magnitude of the outbreaks. They are also used to screen a large number of isolates to help reduce the number of isolates that need to be tested by more discriminating but labor-intensive or expensive procedures. These tests are described below.

## *Repetitive Element PCR Genotyping Tests*

The genomes of prokaryotic and eukaryotic organisms contain stretches of repetitive nucleic acid sequences called repetitive DNA elements. They can be exploited for typing strains. Bacterial genomes contain three main types of such repetitive elements—interspersed repetitive sequences comprised of short, noncoding oligonucleotide sequences containing about 15 to several hundred base pairs, ribosomal RNA genes (rRNA), and insertion sequences (IS) randomly distributed in a genome [16, 17]. The latter two repetitive elements tend to be large coding sequences that occur in low copy numbers and are generally species restricted. Similar interspersed repetitive elements can be found across multiple bacterial species, while others are restricted to a limited set of species. Those that are shared across multiple species are considered broad-spectrum repetitive DNA elements. Species-specific

interspersed repetitive elements (e.g., variable number of tandem repeats or VNTR, clustered regularly interspaced short palindromic repeats or CRISPR) are targeted for species-specific genotyping tests.

## Broad–Spectrum Repetitive Element PCR Genotyping Tests

Subtyping tests based on broad-spectrum interspersed repetitive elements are designed to amplify sequences located between these elements, and such tests are called repetitive element PCR or rep-PCR. The flanking regions of these repeat sequences serve as annealing sites for outward-oriented complementary oligonucleotide primers. Thus, an identical pair of primers will amplify sequences across all spaces located between these repeats that will generate multiple PCR products. The PCR-amplified products are resolved by agarose gel electrophoresis, which generates band patterns with each pattern representing a single bacterial strain. The genetic relationship of the strains is inferred from similarity analysis of the band patterns that can be performed by image analysis software tools that are either commercially or freely available (e.g., BioNumerics, PyELph, QuantityOne).

Bacterial interspersed repetitive elements commonly used as targets for genotyping include repetitive palindromic elements (REPs), enterobacterial repetitive intergenic consensus (ERIC) sequences, and BOX sequences [16–22] (Table 1). REP sequences are distributed throughout the bacterial phyla [18, 22, 23]. ERIC sequences comprised of stretches of 124–127 bp are most frequently found in enteric Gram-negative bacterial species, including *E. coli*, which has 30–50 copies, and *S. typhimurium*, which can have up to 150 copies [21]. BOX sequences comprise sets of mosaic repetitive elements first described in Gram-positive bacteria *Streptococcus pneumoniae*, which has about 25 *box* repeat units [78]. They form clusters comprised of different combinations of three subunit repetitive sequences, which are called boxA, boxB, and boxC [24]. One study showed *E. coli* and *S.* Typhimurium to contain boxA-like sequences but not boxB or boxC [25]. Genotyping tests based on these repetitive elements are called REP-PCR, ERIC-PCR, and Box-PCR, respectively (Table 1).

In general, ERIC-PCR is used to genotype enteric Gram-negative bacteria, and BOX-PCR is used to genotype Gram-positive bacteria. They are particularly useful during investigations of clusters of an illness to confirm them as an outbreak or, if confirmed, to rapidly link a suspected source or a contaminated vehicle to the outbreak. They are also useful for screening a large collection of bacterial isolates that need to be genotyped rapidly [26–29].

As described above, REP, ERIC, and BOX elements are conserved across several bacterial species and even genera. Interestingly, recent studies based on WGS analyses of different prokaryotic species have shown that a large proportion of bacterial species carry distinct species-specific repetitive elements. Up to 97% of 613 prokaryotic species studied by Koressaar et al. contained at least one such species-specific repetitive element [30]. Thus, it is now possible to develop species-specific

rep-PCR tests for a wide variety of bacterial species. A computational tool to identify such repeats in prokaryotes and design a rep-PR test for them has been developed [31] (http://bioinfo.ut.ee/multimprimer3/).

### Limitations of Broad-Spectrum Rep-PCR Genotyping Tests

Rep-PCR tests are all based on electrophoretic band-pattern analysis of PCR-amplified products. As such, these tests have several limitations inherent in many PCR-based tests. The reproducibility of PCR product band patterns can be affected by technical factors, including the type of thermocycler used, as well as the DNA template concentration, primer composition and concentration, annealing temperature, quality of polymerase, buffer salt composition, and other conditions. These factors affect electrophoretic band intensity as well as numbers, which can vary from one lane to another or across different gels. Variation can occur from one day to the next even when the procedure is performed by the same individual in the same laboratory following the same protocol. DNA contamination is also a common problem in PCR assays. PCR assays that target repetitive elements could also non-specifically amplify segments outside of the targeted repeat region. Thus, these rep-PCR genotyping tests must take into consideration these limitations and strictly adhere to protocols designed to minimize sources of electrophoretic band-pattern misinterpretation [32, 33](http://www.protocol-online.org/biology-forums-2/electrophoresis.html).

### Species–Specific Repetitive Element PCR Genotyping Tests

One widely used PCR-based genotyping test that relies on species-specific repetitive elements is called multilocus VNTR analysis or MLVA [34]. VNTRs are clusters of repeat DNA sequences that occur at multiple loci in intergenic regions of prokaryotic and eukaryotic organisms. The length of each VNTR locus in different strains of a bacterial species can vary by copy number of tandem repeats per locus. In *Mycobacterium tuberculosis*, the agent of tuberculosis, the VNTR loci are called mycobacterial interspersed repetitive units (MIRUs), which are comprised of 40–100-bp repeat elements dispersed in the chromosome [35, 36]. *M. tuberculosis* has 41 distinct MIRU loci, and they serve as basis for a genotyping test called MIRU-VNTR analysis, which is a type of MLVA applied to *M. tuberculosis* [35, 37].

Here, instead of amplifying spaces between the repetitive elements, each MIRU locus is separately amplified by distinct pairs of primers. The size (MW) of amplified products resolved by agarose gel electrophoresis depicts copy numbers of the repeat units of each MIRU locus. The differences in copy numbers of the repeat units at each corresponding locus across different *M. tuberculosis* strains serve as the basis for strain discrimination. Thus, in VNTR analysis, it is not the electrophoretic

band patterns but the copy numbers of the repeat units at each locus that are compared. Each genotype, therefore, is given a numerical designation, which facilitates portability and exchange of the genotype data across laboratories.

The discriminatory power of the MIRU-VNTR test depends on the number of these distinct loci that is targeted for PCR amplification. A 15-locus MIRU-VNTR was proposed for the routine epidemiologic studies of tuberculosis, and a 24-locus test was proposed as the standard for high-resolution phylogenetic analyses of *M. tuberculosis* [37, 38]. The predictive value of both 15- and 24-locus MIRU-VNTR tests were shown to be comparable to that of the *IS*6110 RFLP test that had been considered the "gold standard" for genotyping *M. tuberculosis* for studying tuberculosis transmission [39].

Because VNTR loci are widely distributed across prokaryotic and eukaryotic species, MLVA has been applied to genotype a variety of bacterial species [40–47]. Unlike the rep-PCR tests described above, the high reproducibility of MLVA has allowed it to be used in infectious disease surveillance systems. It is used by public health laboratories of many countries together with pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) as part of a surveillance system for enteric pathogens [48, 49].

There are other species-specific repetitive element PCR tests frequently used to genotype strains belonging to a bacterial species (Table 1). For example, *M. tuberculosis* harbors a direct repeat (DR) stretch, which happens to be a clustered regularly interspaced short palindromic repeats (CRISPR) locus [50–52]. CRISPR systems are used by bacteria to recognize and eliminate foreign DNA such as phages and plasmid DNA [51, 53]. In *M. tuberculosis*, this locus has been targeted for a genotyping test called spacer oligonucleotide typing or spoligotyping.

Here, instead of comparing electrophoretic band patterns, spoligotyping compares patterns in a hybridization array comprised of synthetic oligonucleotides representing 43 spacers of the CRISPR locus of a reference *M. tuberculosis* strain (H37Rv) and *M. bovis* BCG covalently linked to an activated membrane [54]. Extracted *M. tuberculosis* DNA is subjected to PCR designed to amplify spacers between the DR sequences. One of the primers that anneals to the DR is linked to biotin, and the amplified products hybridize to the oligonucleotides in the membrane. The membrane is next incubated with peroxidase conjugated to streptavidin, which catalyzes a reaction with a substrate (e.g., chemiluminescence detection system) that yields a hybridization pattern visible as an array of parallel lines [54]. Discrimination of the strains is based on differences in the number and sequences of the spacers. The oligonucleotide membrane is commercially available.

Each spoligotype is assigned a numerical designation [55], which is then archived in international publicly accessible databases [56–58]. Currently, a database called SITVIT WEB maintains these data as spoligotype international types (SITs) (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/). An algorithm called SpotCluster can be used online (http://tbinsight.cs.rpi.edu/run_spotclust.html) to assign the lineage of a newly genotyped strain. Globally distributed spoligotype clades include East African-Indian (EAI), Beijing, Haarlem, Latin American and

Mediterranean (LAM), Central Asian (CAS), European clade of IS*6110* low banders (T), and X clades [58]. Other clades are referred to as orphan clades.

The main advantage of spoligotyping is its simplicity. However, it lacks the resolution of IS*6110*-RFLP (for *M. tuberculosis* strains with >2 copies of *IS*6110) or the 24-locus MIRU-VNTR genotyping tests mentioned above. While differences in global distribution of spoligotype clades have been widely described, spoligotyping does not have the resolution to characterize tuberculosis epidemiology in detail, as described for other more discriminating tests below.

## *Pulsed-Field Gel Electrophoresis (PFGE)*

Unlike the repetitive element-based genotyping tests described above, PFGE is a type of macrodiversity genotyping test that does not require prior knowledge of target nucleic acid sequences ( [59, 60]). It is a type of analysis designed to generate and compare large pieces of DNA fragments from a whole genome (restriction fragment length polymorphism or RFLP). Thus, it can be applied to a wide variety of microbes with a large genome. The technique is described in detail in Vol. I, Chapter "Molecular Typing Techniques: State of the Art". PFGE compares fingerprints in a gel of large pieces of DNA fragments generated from a genome digested with an endonuclease. The differences in fingerprints result from changes that occur at endonuclease recognition sites, such as a point mutation, insertion, or deletion. These differences can be used to infer strain relatedness.

Guidelines have been proposed by Tenover et al. to interpret PFGE patterns (Tenover's criteria) [61, 62] based on analyses of a large number of bacterial isolates from epidemiologically well-characterized outbreak investigations [62]. Strains are considered genetically "indistinguishable" if their PFGE patterns show the same number and size of DNA fragments. Strains are considered "closely related" if their PFGE patterns differ from a reference strain (e.g., outbreak strain) by changes consistent with a single genetic event (a point mutation, insertion, or deletion). Such patterns will differ from the reference strain pattern by 2–3 bands. If they differ by 4–6 bands, they are considered to have undergone two independent genetic events and therefore "possibly related." If the strains have >6 band differences, they are considered "different" and genetically unrelated [61, 62]. This guideline applies only to electrophoretic band patterns generated by PFGE and not to other electrophoresis patterns generated by the other genotyping tests described earlier.

It is important to note that, for Tenover's criteria to be meaningfully applied, the newly generated PFGE patterns must be compared to a reference strain pattern [63]. If the sources of the test isolates are unknown or if they are obtained from geographically distant or different time periods, this guideline cannot be reliably used to infer strain relatedness. Therefore, strains used as reference should include a well-characterized epidemic or outbreak strain or a well-characterized dominant

strain circulating in an institutional (e.g., hospital), geographic (e.g, community), or temporal settings identified through a reliable surveillance system.

Because of its high discriminatory power and reproducibility, PFGE was adopted as the first genotyping test for a national foodborne disease surveillance system in the United States called PulseNet USA. It was launched in 1996 as collaboration among Centers for Disease Control and Prevention (CDC), Association of Public Health Laboratories (APHL), and the food regulatory agencies, including the United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA) (https://www.cdc.gov/pulsenet/). PulseNet genotypes *Salmonella* spp., Shigatoxin-producing *E. coli* (STEC), *Listeria monocytogenes*, *Campylobacter jejuni*, *Cronobacter*, *Shigella* spp., *Vibrio cholerae,* and *Vibrio parahaemolyticus*. PulseNet has been expanded internationally (PulseNet international, http://www.pulsenetinternational.org/), which currently includes 83 countries in Africa, Asia Pacific, Canada, Europe, Latin America, the Caribbean, and the Middle East.

One major drawback of PFGE is the portability of the electrophoresis pattern data it generates. Although patterns can be reproducibly generated by strict adherence to a standard protocol, the comparison of the patterns across laboratories is not straightforward. The patterns generated from different laboratories need to be analyzed by trained personnel skilled in the use of an image analysis software tool. Because of this limitation and because of the increasing accessibility of the WGS technology, PulseNet USA will phase out PFGE and replace it with WGS. In fact, in June 2017, *Eurosurveillance* proposed to adopt the WGS technology in place of PFGE in PulseNet international and recommended standardizing a subtyping schema based on whole-genome multilocus sequence typing (wgMLST) for the surveillance of foodborne enteric illnesses [64].

## *Multilocus Sequence Typing (MLST)*

MLST is currently one of the most widely used nucleic acid sequence-based genotyping tests [65–67]. It compares sequence polymorphism within short segments (400–600 bp) of a set of bacterial "housekeeping" genes. These segments are amplified by PCR, and the PCR products are sequenced. Different alleles of these genes that exist across different bacterial strains are the basis for strain discrimination. For *Staphylococcus aureus,* for example, the genes that are targeted include *arcC, aroE, glpF, gmk, pta, tpi,* and *yqiL*. The sequences from all seven loci can be concatenated to represent a *S. aureus* genotype, which is designated as a sequence type (ST). Each ST is given a numerical designation (e.g., ST300, the most common community-associated methicillin-resistant *S. aureus* in the United States). Each of these gene loci could have 18–33 alleles, so the combinations of these sequences could theoretically generate more than 3 billion possible STs just for *S. aureus*. It is highly discriminatory and because it is a sequence-based test, information is readily portable and exchangeable, which makes it amenable as a reliable genotyping tool for long-term infectious disease surveillance systems.

The most important feature of MLST is its simple nomenclature system, which has phylogenetic significance and validity. All *E. coli* strains designated ST131, for example, no matter where they are isolated, are phylogenetically related and are distinct from strains that are given other ST designations. When the bacterial strains' MLST concatenated sequences are depicted as a dendrogram, the phylogenetic classification of the strains based on WGS of the same set of strains matches closely with the MLST consensus phylogenetic tree. Salipante et al. showed that a phylogenetic tree based on WGS analysis of extraintestinal pathogenic *E. coli* (ExPEC) isolates closely paralleled their classification by MLST [68].

As with other databases created to archive genotype data, several curated MLST databases have been established. PubMLST is hosted by Oxford University (http://pubmlst.org/). Another site called MLST (http://www.mlst.net/) is hosted by Imperial College, and EnteroBase (http://enterobase.warwick.ac.uk/) is maintained by Warwick Medical School that curates data for *Enterobacteriaceae* members *E. coli/Shigella, Salmonella, Yersinia*, and *Moraxella*.

Of course, MLST does not have to be restricted to just seven "housekeeping genes." The WGS technology has facilitated in silico analyses of WGS databases to create a variety of MLST schema, which are called whole-genome MLST or wgMLST. wgMLST could be based on the seven "housekeeping" genes used in the curated schemas described above, a set of recognized virulence genes, all conserved genes (core genome MLST or cgMLST) in a genome, or any combination of these genes [69–72]. Most national infectious disease surveillance systems are most likely to adopt WGS-based genotyping tests in the very near future.

## *Whole-Genome Sequence (WGS)-Based Genotyping Tests*

Next-generation sequencing (NGS) platforms used to generate WGS are constantly evolving as new platforms are introduced to maximize DNA fragment read lengths to sequence, increase speed and the number of reads per run, and reduce cost [73]. While the first-generation sequencing technology (e.g., Sanger sequencing) is still frequently used, the currently most widely used NGS platforms, referred to as high-throughput or second-generation sequencing, involve fragmentation of the genomic DNA, labeling the fragments with adapters, immobilizing the fragments onto a solid surface (e.g., flow cell, beads), amplifying these fragments by PCR, and subjecting these amplified products to massively parallel sequencing by synthesis (Table 2). The main difference in these platforms is in the way nucleotide incorporation signal is detected during synthesis [74, 75]. The maximum read lengths of these platforms vary from 150–400 bases, which can be rapidly and massively sequenced in parallel. The disadvantage of these short-read sequences, however, is that they require data-intensive computational analyses to produce reliable WGS data.

The more recently developed platforms (third generation) do not require PCR amplification. Instead, single DNA strands are sequenced in real time one base at a time (Table 2). This single-molecule real-time (SMRT) technology has the

**Table 2** Whole-genome sequencing technology currently being used to conduct infectious disease epidemiologic studies

| Generation of WGS technology platforms[a] | Chemistry behind sequencing | Nucleotide incorporation signal detection | Features |
|---|---|---|---|
| **First generation** | | | |
| Sanger sequencing | Sequencing by synthesis | Sensor detects fluorescence of dye-labeled nucleotide terminator | Useful for sequencing small genomes (viruses) or filling in gaps in HTS-generated sequences |
| **Second generation (high-throughput sequencing, HTS)** | | | |
| Illumina platforms | Bridge amplification, sequencing by synthesis | Sensor detects fluorescence of reversible dye-labeled nucleotide terminator | ~4 billion reads/run, ~125 bp/read, computationally demanding to assemble |
| Ion Torrent (Thermo-Fisher) | Emulsion PCR, sequencing by synthesis | Microchip detects hydrogen ion released during nucleotide incorporation | ~50 million reads/run ~200–400 bp/read, computationally demanding to assemble |
| **Third generation (single-molecule real-time sequencing, SMRT)** | | | |
| PacBio platforms (Pacific Biosciences) | Single-molecule sequencing in real time | Zero-mode waveguide chip detects fluorescence released from dye-labeled nucleotides | ~47,000 bp/run Average read length ~10,000 bp Assembly faster but error prone |
| Nanopore platforms (Oxford Nanopore Technologies) | Single-molecule sequencing in real time | Sensor detects change in current as nucleotide transits protein nanopores embedded in electrically resistant polymer | Read length: up to 150 kb/read High error rate |

[a]Earlier generation HTS platforms such as 454 pyrosequencing (Roche) or SoLiD sequencing by ligation (Applied Biosystems) are not included here as they are no longer produced

advantage of being able to sequence long read lengths, but the error rates remain higher than those of early-generation platforms. Their high error rates limit their application to phylogenetics or molecular epidemiology.

One major limitation of the NGS technology is the organization and analyses of the complex sequence data, which are substantially more computationally challenging than any of the other genotyping techniques discussed above. The analyses of billions of raw sequence reads require a systematic workflow based on algorithms or pipelines designed to clean up the raw sequence data (quality check), assemble the sequences (de novo or reference mapping), annotate the sequences, align the

sequences for phylogenomic comparison, and identify subsequences of interest (e.g., virulence genes, drug-resistant genes, plasmids, phages) [74–76]. These pipelines can be accessed from commercial (e.g., BioNumerics, Geneious, CLC Genomics Workbench) and open web-based (e.g., Galaxy, https://usegalaxy.org/; Bioinformatics Software and Tools, http://bioinformaticssoftwareandtools.co.in/ngs.php) sources. Such computational workflow application requires trained and skilled individuals.

Finally, to apply WGS to epidemiologic investigations, a simple nomenclature system to assign each WGS into an operational taxonomic unit (OTU) needs to be established. As discussed above, with MLST, the ability to describe a genotype by a sequence type number (e.g., *E. coli* ST131) makes it possible to create curated databases and establish public health surveillance systems. Without a nomenclature system for the OTUs based on WGS, portability and exchangeability of WGS data across laboratories will become complex.

wgMLST is one way to assign OTUs for WGSs. As mentioned above, wgMLST could be established in a variety of ways—based on the same seven housekeeping genes targeted in the standard MLST schema, core genome sequences, a set of virulence genes, or any combination of these gene sequences. Indeed, in 2015 in the United States, the Centers for Disease Control and Prevention (CDC) has launched a program to help build WGS capabilities in state health departments to establish wgMLST databases for major foodborne pathogens—*Campylobacter, Vibrio, Shigella* spp., *Salmonella*, Shiga toxin-producing *Escherichia coli*, and other *E. coli* pathotypes [76]. According to Eurosurveillance, PulseNet International has proposed to do the same in Europe [64].

As will be emphasized under the section below on applications of genotyping tests to conduct epidemiologic investigations, WGS-based genotyping tests must be applied with what is known about the intrinsic mutation rates of bacterial strains in question, the clinical manifestations associated with the infectious agent, and epidemiology of that infection. Salipante et al. compared WGS and PFGE patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from 17 different hospital outbreaks [77]. They compared Tenover's criteria for PFGE and 3 or fewer pairwise SNP differences in two pairs of isolates by WGS to infer strain relatedness [62]. Among 20 MRSA isolates deemed not distinguishable by PFGE, they found 5 that were considered clonal by WGS. Thirty-five strains considered unrelated by PFGE (>6 band differences) were all considered unrelated by WGS. Of 23 isolates shown to be closely related by PFGE (2–3 band differences), all were considered unrelated or non-clonal by WGS. Of 58 isolates which had 4–6 band differences by PFGE (possibly related), all were non-clonal by WGS [77]. Thus, when the PFGE patterns show strains to be unrelated, concordance with WGS is 100%, but when the PFGE patterns are indistinguishable or possibly related, concordance decreases. When the tests were performed on *Acinetobacter baumannii* isolates, the PFGE patterns that were closely or possibly related were found to be 73% and 85% concordant with WGS, respectively [77]. Thus, the comparison of concordance or discriminatory power of a genotyping test cannot be generalized across different bacterial species.

## Applications of Genotyping Tests in Epidemiologic Investigations

At the beginning of this chapter, molecular epidemiology was defined as "the study of the distribution and determinants of diseases and injuries in human and nonhuman animal populations using molecular microbiology methods." A large proportion of the published reports on molecular epidemiology of infectious diseases focus on the "distribution" part of the definition. These reports describe distribution of genotypes in various geographic settings and changes over time. Most of these reports, thus, are descriptive studies that do not address the other more important and interesting component of the definition—"determinants of diseases and injuries…." Descriptive studies are useful as background source of information, but from the perspective of public health, molecular epidemiology studies need to provide opportunities for intervention. That is, they need to be able to identify risk factors for transmission and quantitate the magnitude of these risks, characterize potential mechanisms of disease spread, detect biologic determinants of pathogens that newly emerge or reemerge, and provide opportunities to design analytical epidemiologic investigations [2]. These types of studies address "determinants" of disease manifestation and transmission. Below, examples of such analytical studies that create opportunities for public health interventions are discussed.

### *Foodborne Diseases*

The CDC estimates that in the United States, approximately 48 million foodborne illnesses occur each year with about 128,000 hospital admissions and 3000 deaths (https://www.cdc.gov/foodborneburden/index.html). The most common bacterial agent of foodborne disease is non-typhoidal *Salmonella* spp.; they are estimated to be responsible for nearly 1 million cases of foodborne illness each year in the United States [78]. Under ideal circumstances, if cases are identified as part of a recognized outbreak, they will get reported to the local or state health departments and ultimately to the CDC. However, less than 10% of foodborne illnesses are recognized to be part of an outbreak and therefore, most of the cases never get reported [79, 80]. That is, most of the cases of foodborne illnesses are recognized as sporadic infections. In recognized outbreaks, investigations can be initiated to identify contaminated vehicles or risk factors for the illness. It is extremely difficult to identify vehicles or risk factors for sporadic infections. This means that in the United States, the vehicles of most foodborne illnesses go undetermined and hence no opportunities exist for any targeted intervention.

Several past studies using early-generation genotyping tests have suggested that a large proportion of what appear to be sporadic *Salmonella* infections are actually part of multiple unrecognized outbreaks [81, 82]. That is, sporadic foodborne infections may be comprised of multiple small outbreaks unrecognized as such because

only bacterial species or serotype information were available. In 1994, the Minnesota Department of Health initiated a program to genotype by PFGE all *S.* Typhimurium isolates submitted from clinical laboratories in the state [83]. PFGE detected six foodborne outbreaks in the state based on isolates that were genotyped between 1994 and 1998. Of 958 such isolates, 79 (8%) clustered over a 15-week period in 1998, and 32 of them were found to be indistinguishable. This information allowed public health investigators to conduct more targeted food intake interviews. They eventually found that this cluster of cases was part of an outbreak associated with a commercial microwavable chicken product. The Minnesota Department of Health recalled the product and made changes in product labeling policy. Here, PFGE data were used to make a public health intervention and change policy.

Similar applications of PFGE are now routinely performed by the national PulseNet surveillance system coordinated through the CDC. The foodborne pathogen isolates are obtained through another surveillance system FoodNet (https://www.cdc.gov/foodnet/index.html), which is an active sentinel surveillance system established in ten states, designed to identify *Campylobacter, Cryptosporidium, Cyclospora, Listeria, Salmonella,* Shiga toxin-producing *Escherichia coli* (STEC) O157 and non-O157, *Shigella, Vibrio,* and *Yersinia* from patients. Because it is an active surveillance system, it is designed to detect sporadic cases as well as outbreaks of foodborne illness. This enables PulseNet USA to identify about 1500 clusters of foodborne disease each year, many of which are outbreaks. Its ability to rapidly detect contaminated food products enables regulatory agencies to institute product recall before the products are consumed by the public. CDC estimates that more than 1 billion pounds of contaminated food have been recalled since this PFGE-based surveillance system was established (https://www.cdc.gov/pulsenet/). An economic impact analysis of PulseNet USA suggested that each year, 270,000 illnesses from Salmonella, 9500 illnesses from *E. coli*, and 60 illnesses due to *Listeria monocytogenes* are averted, which translates to annual medical and productivity cost savings of $507 million [84]. It costs American taxpayers $7.3 million each year to support the PulseNet surveillance system [84].

As mentioned earlier, PFGE used by PulseNet will soon be replaced by WGS. In 2013, in preparation for this change and to assess feasibility of incorporating WGS to national and regional foodborne disease surveillance systems, the CDC initiated a project called Listeria Whole Genome-Sequencing Project in collaboration with the FDA, USDA, NIH, National Center for Biotechnology Information (NCBI), and state and local health departments (http://www.cdc.gov/listeria/). Here, WGS is performed along with PFGE on all clinical isolates of *Listeria monocytogenes* from food and environmental sources in the United States. In the first 2 years, the project was able to show that WGS can detect clusters of listeriosis earlier than PFGE, which would prevent more illnesses and even deaths [85]. In one of these investigations, WGS identified two genotypes of *Listeria* that were traced to caramel apples made from apples from a single supplier [85]. While PFGE may also have been able to trace the same strains to the same source, it may have taken much longer.

The application of WGS to track sources of infection, trace chain of transmission, and establish a long-term surveillance system requires additional considerations.

As mentioned above, the comparison of WGS of multiple bacterial strains requires a protocol to arrange their WGS into OTUs based on a set of criteria. Such a protocol needs to include parameters that can be used to assess relatedness of the OTUs.

This was attempted for a foodborne pathogen *Salmonella enterica* serovar Typhimurium. In a study of multiple *Salmonella* Typhimurium DT170 outbreaks in Australia during 2006–2012, investigators examined WGS of *S.* Typhimurium isolates and attempted to determine cutoff values for the number of single-nucleotide polymorphism (SNP) differences in the strains needed to decide which cases should be included or excluded from an outbreak. Based on reported mutation rates of *S.* Typhimurium (lowest rate of $1.9 \times 10^{-7}$ [86], to the intermediate rate of $3.4 \times 10^{-7}$ [87], to the highest rate of $12 \times 10^{-7}$ substitutions per site per year [88]), they determined that from the moment of food contamination (ex vivo) to human infection (in vivo) that lasted less than 1 month, the maximum number of SNP differences was two or four at the lowest or highest mutation rate, respectively. For ex vivo/in vivo time of up to 3 months, the SNP differences were three to nine at the lowest or highest mutation rate, respectively [89]. Using these SNP cutoff values, the investigators linked additional cases of *S.* Typhimurium infections that were not recognized to be part of the outbreaks and exclude others that were initially included in these outbreaks. Thus, they developed a set of criteria based on the number of SNP differences to show relatedness of the WGS data for *S.* Typhimurium DT170, validated by the outbreaks they investigated.

Here, it should be noted that SNPs in repeat regions, insertion sequences, or prophage sequences in the WGS database were excluded from the analysis [89]. That is, the investigators used mutation frequency in core WGS to develop a set of criteria to infer strain relatedness, which had to be validated by linking the database to epidemiologic information—their knowledge that the isolates did or did not belong to recognized outbreaks. As shown below, such a set of criteria developed for one bacterial species may not apply to other bacterial species associated with other types of clinical manifestations.

## *Tuberculosis*

In 2016, 1.4 million new cases of tuberculosis were estimated to have occurred worldwide [90]. Tuberculosis remains the most common infectious cause of death in adults from a single pathogen. Molecular epidemiology studies of tuberculosis have been applied to not only examine geographic and temporal distributions of key *M. tuberculosis* lineages (e.g., Beijing clade, [91]) but also confirm or unmask outbreaks; distinguish new tuberculosis cases due to recent transmission infection (primary or rapidly progressive tuberculosis) from cases resulting from transmission that occurred in the remote past (reactivation disease); differentiate exogenous reinfection from relapse infection after completion of treatment; identify transmission dynamics to track sources and chain of transmission; characterize bacterial genetic

determinants of transmission, virulence factors, and drug resistance; and evaluate effect of an intervention on a specific set of genotypes.

For public health tuberculosis control, one of the most important information to obtain is the relative proportion of incidence of primary vs reactivation tuberculosis in a community. If a large proportion of new tuberculosis cases in a community is due to recent transmission, this would indicate that the current tuberculosis control program is not adequate. If most of the new cases are due to reactivation disease, the control program in the past was not adequate, but it is currently working well to prevent new transmissions. One of the first genotyping tests applied to make this differentiation was *IS*6110-RFLP, which is based on RFLP of *M. tuberculosis* genome followed by a DNA hybridization step designed to target insertion sequence *IS*6110 [39] (Table 1). Here, an assumption is made that it is highly unlikely that two individuals residing in a same community would develop tuberculosis caused by a strain of *M. tuberculosis* belonging to an identical genotype if the infections had occurred many years earlier. In urban settings with highly mobile populations, clusters of new tuberculosis cases caused by strains belonging to identical *IS*6110-RFLP are considered to be due to recent transmission, and cases infected with an unique pattern strain are considered to be due to reactivation disease from an infection acquired many years earlier. Using this rationale, several studies in the 1990s attempted to estimate the relative proportion of primary vs reactivation tuberculosis in different communities in the world [92–101].

More than 20 years have passed since the above studies were conducted. The disturbing revelation is that more than 20 years later, many of the genotypes from that period that were thought to represent recent transmission strains are still circulating in the same regions. Thus, these strains cannot be said to be “recent transmission strains.” What we do know is that strains that dominated in the past dominate now. So the past assumptions made to distinguish tuberculosis resulting from recent transmission vs past transmission based on *IS*6110 RFLP came to be questioned.

Today, WGS is mostly used to genotype *M. tuberculosis* to conduct molecular epidemiology studies. Can WGS be used to differentiate tuberculosis due to recent vs past transmission? In the above discussion on salmonellosis, a cutoff threshold of SNP differences in determining strain relatedness was proposed. As noted above, SNP cutoff thresholds to assess relatedness of strains determined for one bacterial species may not necessarily apply to other species, especially when the clinical manifestations of the disease they cause and their modes of transmission differ. Tuberculosis, for example, is an airborne, person-to-person transmitted chronic infectious disease in which public health investigators seek to determine the direction of transmission as part of their tuberculosis control program. *M. tuberculosis* can latently reside in a single host for many years and may not undergo frequent replication during such a state of infection. Thus, its mutation rates may be lower than those of bacterial species that cause acute infectious diseases.

Walker et al. conducted a longitudinal WGS analysis of *M. tuberculosis* isolates from 30 patients with tuberculosis and 25 families and estimated that the rate of mutation in *M. tuberculosis* was 0.5 SNPs per genome per year [102]. Then, based

on WGS analyses of isolates from multiple community clusters of tuberculosis in United Kingdom, they proposed 12 SNP differences as the upper threshold to determine plausible transmissions [102, 103]. They suggested that isolates with five or fewer SNP differences are likely to have resulted from recent transmission [102].

In a later study of a single large tuberculosis outbreak in London, Casali et al. analyzed WGS 344 *M. tuberculosis* isolates that belonged to an identical 24-locus MIRU-VNTR type, isolated from tuberculosis patients over 14 years [104]. WGS showed that 96 (38%) were indistinguishable. The WGS analysis was able to demonstrate the direction of transmission in only 16 (4.7%) of 344 of cases [104]. They found that multiple transmission events can occur with no changes in SNP, and therefore even completely identical isolate pairs could not be concluded to have resulted from a direct transmission event. Such data, therefore, could not be used to distinguish cases of tuberculosis that result from recent transmission vs reactivation tuberculosis. Furthermore, they found that multiple *M. tuberculosis* isolates from one patient could have up to six SNP differences [104]. The investigators concluded that, in assessing tuberculosis transmission, WGS is useful in ruling out direct transmission when isolates are found to be separated by a large number of SNPs, but in ruling in transmission, supporting epidemiological evidence needs to be provided [104].

Thus, as the above examples illustrate, it is important to emphasize that WGS SNP cutoff values to infer timing or direction of transmission of an infectious agent cannot be generalized across all bacterial species. They need to be empirically determined based on isolates from well-characterized outbreaks and surveillance systems as well as what is known about the clinical manifestations of an infectious disease, modes of transmission, and intrinsic rate of acquisition of mutation over time of an implicated genotype a species.

## *Healthcare-Associated Infections*

One of the first applications of molecular microbiology methods to epidemiology was made to investigate hospital infections. Phage typing was used to investigate nosocomial outbreaks of *Staphylococcus aureus* infections. In the early 1950s–1960s, most hospital outbreaks of *S. aureus* infections were caused by a single phage group complex (52/52A/80/81) [105]. These early hospital outbreaks were what led to the establishment of modern nosocomial infection control practices [106].

Today, *S. aureus* genotyping tests include staphylococcus protein A (*spa*) typing, staphylococcus cassette *mec* (SCC*mec*) typing, PFGE, MLST, and WGS (Table 1). These are applied in both healthcare settings as well as in community-onset infections. *spa* typing is actually based on a VNTR locus containing highly polymorphic *S. aureus* protein A gene (*spa*) composed of multiple 24-bp repeats [107]. This target is amplified by PCR and then sequenced. This is not a rep-PCR test described earlier since it does not compare electrophoretic band patterns [108]. SCC*mec* typing is based on mobile DNA elements SCC*mec* that carry a gene (*mecA*) encoding

resistance to methicillin [109, 110]. The organization of these elements is highly diverse, which serves as the basis for genotyping. A combination of *S. aureus* MLST, *spa* typing, and SCC*mec* typing is frequently used to conduct molecular epidemiology studies of *S. aureus* infections.

One important epidemiological issue that often arises in hospital settings is whether infections caused by common hospital-associated bacterial species such as *S. aureus* or *Klebsiella pneumoniae* represent outbreaks caused by a limited number of bacterial lineages or sporadic infections caused by multiple unrelated bacterial lineages. In a study of 152 bloodstream isolates of *S. aureus* collected in 2015 from a Minnesota hospital, *spa* typing identified 66 *spa* types and 10 singletons, while wgMLST found 31 STs [111]. By core genome MLST (cgMLST), only 2 of the 152 isolates were found to differ by 9 SNPS, and all others had more than 40 allelic differences [111]. Although these two cases of bacteremia occurred 55 days apart, the patients shared the same service and the same nurse practitioner. Thus, the more discriminating cgMLST was able to precisely identify a specific episode of transmission [111]. In this study, then, only one outbreak was detected, while the other infections appeared to have been caused by strains that were not genetically related as revealed by cgMLST.

cgWGS was applied to investigate a cluster of neonatal MRSA ST22 infections in a neonatal intensive care unit at Cambridge University Hospitals in the United Kingdom [112]. It revealed a distinct cluster that distinguished strains in this cluster from other ST22 strains. Furthermore, it detected a transmission episode that was previously missed between two patients with bacteremia who were not part of the outbreak.

In a separate study at the same hospital, 12 infants in a special care baby unit (SCBU) were found to be colonized with MRSA in a 6-month period in 2011, and therefore, an outbreak was suspected. The MRSA isolates all belonged to ST22 by conventional MLST. By WGS, the investigators found 26 related MRSA carriage isolates, which were involved in transmission in the SCBU, between mothers on a postnatal ward and in the community [113]. Here, WGS was applied to carriage isolates of MRSA to document that, even during a period when no invasive disease cases were identified, an outbreak of MRSA was sustained by transmissions that resulted in colonization. A healthcare staff was suggested to be the source of some of these transmissions [113].

WGS was used to investigate a prolonged hospital outbreak caused by carbapenem-resistant strains of *K. pneumoniae* ST258 at the US National Institutes of Health Clinical Center in 2011 [114]. This outbreak involved 18 patients diagnosed over a period of 6 months, and 11 of them died. Despite early implementation of infection control, the outbreak persisted. Single-nucleotide variance (SNV) analysis based on WGS of the *K. pneumoniae* isolates together with epidemiologic analysis revealed three independent chains of transmission triggered by a single patient [114]. Interestingly, a ventilator was found to be contaminated with a strain of *K. pneumoniae* ST258, but it itself was found to be in a chain of transmission and did not serve as a source for subsequent transmissions. Without the WGS data, such an instrument may have been considered a source and would have been subjected to a decontamination procedure that would not have interrupted the outbreak.

## Other Applications of NGS Technology in Epidemiologic Investigations

The above examples discussed the use of these new molecular biology strain typing tools to track geographic and temporal distribution of infectious disease agent, distinguish epidemic from endemic infectious disease occurrence, stratify data to refine study designs, study epidemiology of healthcare- or institution-associated infectious diseases, conduct surveillance, and identify direction and chain of transmission of an infectious agent. Other applications include distinguishing pathogenic variants (pathovars) of a bacterial species from commensal or saprophytic variants (non-pathovars), such as intestinal pathogenic *E. coli* and extraintestinal pathogenic *E. coli* vs commensal *E. coli* [115]; identifying new modes of transmission of an infectious disease [26, 116]; identifying hidden social networks to unmask infection transmission links [117]; and identifying genetic determinants of infectious agent transmission [118]. As WGS-based tests get established in surveillance systems, opportunities to conduct these and new types of molecular epidemiology investigations will greatly expand.

## Next-Generation Molecular Epidemiology

The new genotyping tools are clearly advancing our understanding of the epidemiology of many important infectious diseases and creating opportunities for new and focused public health interventions. However, with tools such as WGS and other NGS applications, new algorithms and criteria for epidemiologic relatedness specific for each microbial species still need to be developed. Opportunities to develop new epidemiologic interventions will largely depend on increased accessibility and simplification of the computational tools to analyze the vast amount of data generated by the NGS platforms. No matter what new tools and algorithms are developed, one must keep in mind that, ultimately, these advancements must be validated by basic epidemiologic principles. When they do, molecular epidemiology of infectious disease will undergo yet another major evolutionary transformation.

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# Molecular Detection and Characterization of Carbapenem-Resistant *Enterobacteriaceae*

**Siqiang Niu and Liang Chen**

## Introduction

Carbapenem-resistant *Enterobacteriaceae* (CRE) have emerged as a major class of bacterial pathogens which pose a significant threat to global public health. CRE are usually resistant to all β-lactam antibiotics and frequently carry additional resistance mechanisms against other antimicrobial agents, resulting in limited treatment options. One current clinical dilemma is that CRE infections are associated with high mortality (~30–70%) in immunocompromised hosts, while identification of CRE by culture typically takes 2–3 days, leading to delays in appropriate therapy.

CRE are generally defined as *Enterobacteriaceae* that are non-susceptible (i.e., intermediate or resistant) to a carbapenem [1]. Resistance to carbapenems can arise from multiple mechanisms, including alterations in outer membrane permeability mediated by the loss of porins, the upregulation of efflux systems along with hyperproduction of AmpC β-lactamases or extended-spectrum β-lactamases (ESBLs), or, more commonly, the production of carbapenemases [2]. In its 2015 Update CRE Toolkit [1], CDC updated the definition of CRE to include *Enterobacteriaceae* that are (i) resistant to any carbapenem antimicrobials (i.e., minimum inhibitory concentrations (MICs) of ≥2 μg/ml against ertapenem or ≥4 μg/ml against doripenem, meropenem, or imipenem) or (ii) documented to produce carbapenemase through a phenotypic or molecular assay, regardless of in vitro carbapenem susceptibility.

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Of these two categories, carbapenemase-producing CRE (CP-CRE) have received more attention, as they are more widely disseminated in comparison with non-carbapenemase-producing CRE. This is primarily due to the fact that carbapenemase genes are frequently harbored by mobile elements found on large conjugative plasmids, thereby facilitating horizontal transfer of resistance into different bacterial strains and species. In addition, plasmids harbored by CP-CRE often carry additional resistance elements and thus have the potential to increase resistance to multiple drug classes.

Carbapenemase can be divided into different Ambler classes [3], primarily Ambler A, B, and D. Ambler classes A (e.g., KPC carbapenemases) and D (e.g., OXA-48-like carbapenemases) contain active serine sites, whereas Ambler class B (metallo-β-lactamases, or MBLs)—including IMP (active on imipenem), VIM (Verona integron-encoded MBL), and NDM (New Delhi metallo-β-lactamase)—requires zinc ions in their active sites. Among the aforementioned, KPC, NDM, and OXA-48 carbapenemases are the most common. KPCs are most frequently identified in *Klebsiella pneumoniae* in the USA, China, Colombia, Israel, Greece, and Italy, with NDMs primarily found in *K. pneumoniae* and *Escherichia coli* from the Indian subcontinent and OXA-48-like carbapenemases frequently seen in *K. pneumoniae* and *E. coli* from North Africa and Turkey [4]. In addition, KPC producers have been mostly identified among nosocomial isolates, whereas NDM and OXA-48 producers are associated with both nosocomial and community-acquired pathogens [4].

CP-CRE are currently disseminated throughout most global regions, wherein they are frequently associated with high mortality and morbidity due to their unprecedented multi- or pan-drug resistance, in addition to the absence of standardized, clinically effective detection methods for early identification [5]. Consequently, there is an urgent need for rapid and accurate detection of carbapenem resistance in clinical laboratories, as it is imperative for patient treatment, infection control, and epidemiological studies aimed at limiting further spread of CRE. In actual practice, however, clinical laboratories commonly struggle with how best to detect CRE and especially how to detect carbapenemase-producing isolates [6].

Molecular detection of CP-CRE, in comparison with conventional culture-based phenotypic tests, offers several advantages, including the rapid turnaround time, the definitive identification of specific carbapenemase types, and, in some cases, the ability to test directly from clinical specimens without the need for culture (Table 1) [9]. Molecular detection of carbapenemase genes is often regarded as the gold standard for studies evaluating detection methods for CP-CRE. In this chapter, we will discuss the progress achieved to date on molecular detection methods for CRE and particularly CP-CRE.

Based on the detection targets involved, molecular detection of CP-CRE can generally be divided into nucleic acid-based assays and non-nucleic acid-based assays, with the former being the most commonly used.

**Table 1** Main characteristics of phenotypic and molecular carbapenemase detection methods [7, 8]

| Method | Turnaround time | Targets | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Cost | Expertise required | Limitation |
|---|---|---|---|---|---|---|---|---|---|
| **Phenotypic detection** | | | | | | | | | |
| Modified Hodge test | 24–48 h | Classes A, D | 100 | <90% | 85 | 100 | $ | + | Poor sensitivity for NDM producers, poor specificity when AmpC is present |
| Carba NP test | 5–120 min | Classes A, B, D | 72.5–100 | 100 | 100 | 69.2–92.2 | $ | + | Poor sensitivity for OXA-48 producers, poor sensitivity for mucoid isolates |
| Modified carbapenem inactivation method (mCIM) | 24–48 h | Classes A, B, D | 97 | 99 | ND | ND | $ | + | None known |
| **Molecular detection** | | | | | | | | | |
| Real-time multiplex PCR | 60–120 min | Classes A, B, D | 100.0 | 89.9–100.0 | 46.6–16.6 | 100.0 | $$ | ++ | Unable to detect novel carbapenemase |
| LAMP (e.g., eazyplex SuperBug complete A) | 25–60 min | Classes A, B, D | 100.0 | 100.0 (83.0 for OXA-48) | ND | ND | $$ | + | Unable to detect novel carbapenemase |
| MALDI-TOF MS | 75 min–12 h | Classes A, B, D | 95.2–100.0 | 90.0–100.0 | ND | ND | $$ | ++ | None known |
| Microarray (e.g., Alere Technologies) | 2–8 h | Classes A, B, D | 98.2 | 97.4 | ND | ND | $$$ | ++ | Unable to detect novel carbapenemase |
| Next-generation sequencing platforms | >8 h to days | Classes A, B, D | 100.0 | 100.0 | ND | ND | $$$ | +++ | Unable to detect novel carbapenemase |

## Rapid Nucleic Acid-Based Tests

As described above, carbapenemases are encoded by different β-lactamase genes, which allow for direct detection of the presence or absence of resistance genes using nucleic acid-based assays. These assays provide a rapid, sensitive, and specific tool for the recognition and identification of carbapenem resistance genes [10] and can provide molecular epidemiologic data which can be essential for infection control and outbreak investigations. Most of these techniques are based on PCR technology and may additionally be followed by Sanger DNA sequencing of the amplicon to identify sequence variations.

### *Conventional PCR Assays*

The increasing frequency of carbapenemase-producing Gram-negative bacteria underlies the necessity of tools to monitor the emergence and spread of different classes of carbapenemase genes. As such, several conventional PCRs have been developed to detect and differentiate specific carbapenemase genes. In 2007, Ellington et al. [11] developed a multiplex PCR assay which successfully detects and distinguishes genes encoding five different acquired MBL families (VIM, IMP, SPM, GIM, and SIM) in a single reaction. The assay displayed excellent performance, correctly distinguishing and identifying 11 known reference MBL-producing strains producing IMP-1, IMP-2, IMP-4, IMP-7, IMP-12, VIM-1, VIM-2, VIM-7, SIM-1, GIM-1, and SPM-1, respectively.

In 2011, Poirel et al. [12] developed a multiplex PCR assay for detection of 11 carbapenemase genes belonging to different classes. The assay consisted of three multiplex PCR reactions and was able to detect several common carbapenemase genes belonging to Ambler classes A (KPC), B (NDM, IMP, and VIM), and D (OXA-48), as well as several newly identified carbapenemase genes encoding DIM-1, BIC-1, AIM-1, etc. The assay was rapid and reproducible and provided a convenient molecular tool for detection of both common and "minor" carbapenemase genes, thereby allowing for better evaluation of the prevalence of these clinically relevant carbapenemase genes.

Whereas the two assays described above focused solely on carbapenemase genes, other researchers have developed assays that also target AmpC β-lactamase and ESBL genes. In 2010, Dallenne et al. [13] developed a set of six multiplex PCRs and one simplex PCR for rapid detection of the most frequently encountered β-lactamase genes, including OXA-1-like broad-spectrum β-lactamases, ESBLs, plasmid-mediated AmpC β-lactamases, and class A, B, and D carbapenemases. An evaluation of the assay was performed using a collection of 31 clinical *Enterobacteriaceae* strains displaying resistance to broad-spectrum third-generation cephalosporins or carbapenems. Direct sequencing from PCR products was subsequently carried out to identify β-lactamase gene variations. Most PCR amplicons

contained major substitutions, allowing the identification of different clusters of β-lactamase genes (e.g., differentiating broad-spectrum β-lactamase SHV genes from ESBL-type SHV genes).

In an updated version of the assay, Voets et al. [14] described an additional set of 7 multiplex PCR assays for detection of an additional 25 β-lactamase families, including plasmid-mediated AmpC β-lactamases (ACC, ACT, DHA, CMY, FOX, LAT, MIR, and MOX), metallo-carbapenemases (GIM, NDM, SIM, and SPM), serine carbapenemases (IMI, SME, and NMC-A), and OXA β-lactamases (OXA groups 1, 2, 4, 23, 24, 48, 51, and 58). The combination of the two PCR assays [13, 14] can therefore detect a wide range of β-lactamase genes using the same amplification conditions. This enables the identification of the majority of clinically important β-lactamases responsible for resistance to third-generation cephalosporins and carbapenems.

More recently, Lee et al. [15] developed a rapid and accurate PCR assay using 62 primer pairs, designed through an elaborate optimization process. To investigate the applicability of this large-scale *bla* detection method (named $_{\text{large-scale}}$*bla*Finder by the authors), the assays were performed on a number of previously reported bacterial control isolates/strains. With 100% specificity and 100% sensitivity in 189 control strains and 403 clinical isolates, the $_{\text{large-scale}}$*bla*Finder detected almost all clinically recognized *bla* genes, along with 24 previously unreported *bla* genes. This PCR-based system is therefore able to detect nearly all *bla* genes existing in a clinical isolate, providing an important aid for monitoring the emergence and dissemination of *bla* genes, and potentially minimizing the spread of resistant bacteria.

The aforementioned conventional PCR assays can be easily adapted by different laboratories around the world and require limited resources and have been widely used in CRE detection and epidemiological studies. However, conventional PCRs are performed on bacteria grown in pure culture and usually involve gel electrophoretic analysis of multiple bands, which is both time-consuming and less practical for direct detection of *bla* genes in primary clinical samples. By contrast, real-time PCR assays overcome most of these limitations and offer several advantages including greater sensitivity, minimal post-amplification analysis, and lower risk of PCR-based laboratory contamination.

## *Real-Time PCR Assays*

### In-house Real-Time PCR

At present, many clinical laboratories use "in-house" or laboratory-developed tests (LDT) involving real-time PCR-based methods, to overcome the limitations of phenotypic detection methods and conventional PCRs and to reduce detection time. Real-time PCR assays performed directly on bacterial colonies or primary specimens can generate results within hours, with excellent sensitivity and specificity. A number of multiplex real-time PCR methods have been described in the literature

[16–41]. These real-time PCR assays typically use sequence-specific probes (e.g., molecular beacon or TaqMan probe) or nonspecific double-stranded DNA (dsDNA)-binding dyes (e.g., SYBR Green), followed by melting curve analysis, for the detection of amplified DNA products.

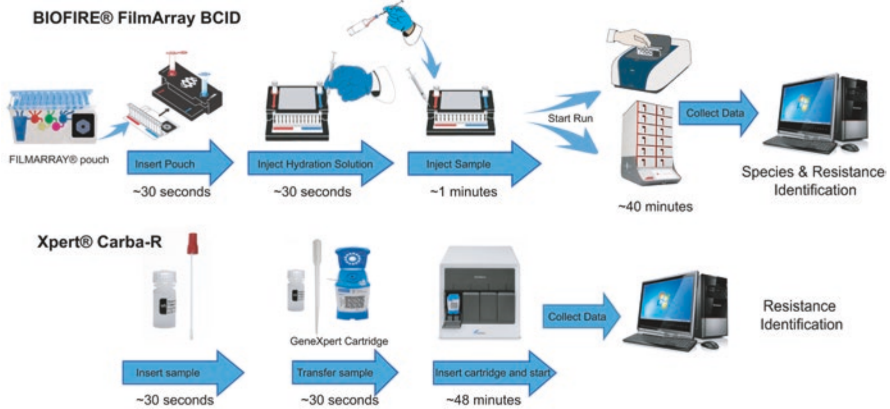
Mendes et al. [41], for example, described one of earliest multiplex real-time PCR assays for detection of metallo-β-lactamase-producing Gram-negative bacteria. The assay is a single-tube reaction, requiring a total of 2 h following colony selection. MBL identification is based on differentiation of characteristic amplicon melting curves. Shortly thereafter, Bisiklis et al. [42] reported another real-time PCR assay which is able to specifically detect $bla_{\mathrm{VIM}}$ and $bla_{\mathrm{IMP}}$ genes from Gram-negative bacteria within 1 h. The authors showed that melting curve analysis of the real-time PCR products clearly differentiates the target genes into four groups: (i) $bla_{\mathrm{VIM\text{-}1}}$-like, (ii) $bla_{\mathrm{VIM\text{-}2}}$-like, (iii) $bla_{\mathrm{IMP\text{-}1}}$-like, and (iv) $bla_{\mathrm{IMP\text{-}2}}$-like.

In 2011, Chen et al. [39] developed a multiplex real-time PCR assay using molecular beacons (MB-PCR) for rapid and accurate identification of $bla_{\mathrm{KPC}}$ variants. The assay consists of six molecular beacons and two oligonucleotide primer pairs, allowing for detection and classification of a number of $bla_{\mathrm{KPC}}$ variants ($bla_{\mathrm{KPC}}$-2 to $bla_{\mathrm{KPC}}$-11). The described real-time PCR can distinguish between different $bla_{\mathrm{KPC}}$ variants and therefore provides information of both epidemiological and evolutionary significance. Subsequently, the same group [43] described a multiplex real-time PCR assay capable of identifying both the epidemic *K. pneumoniae* ST258 clone and $bla_{\mathrm{KPC}}$ in a single reaction using molecular beacon probes. The assay displayed excellent sensitivity (100%) and specificity (100%), providing an effective tool for screening of KPC-producing *K. pneumoniae* isolates and surveillance of the epidemic ST258 clone. More recently, Chavda et al. [44] reported a multiplexed molecular beacon-based real-time PCR assay to identify prominent extended-spectrum-β-lactamases, plasmid-mediated AmpC β-lactamases (pAmpC), and carbapenemase genes directly from perianal swab specimens. The assay included two linear-after-the-exponential PCR (LATE-PCR) assays with melting curve analysis in order to improve the performance for single-mutation-based SHV- and TEM-ESBL detection. This assay is one of few real-time PCR methods able to detect SHV- or TEM-type ESBLs without further sequencing requirements. The assay was evaluated using 158 perianal swabs collected from hematopoietic stem cell transplant recipients and demonstrated that it was highly sensitive and specific for detection of CTX-M-, AmpC-, and KPC-producing *Enterobacteriaceae* compared to culture on chromogenic agar [44].

**Commercial Real-Time PCRs**

Over the past few years, commercial manufacturers have likewise recognized the need to have low-to-moderate complexity tests for carbapenemase detection available for rapid detection and institutional surveillance purposes. Several commercial real-time PCR-based platforms have been developed. A few have obtained FDA clearance for clinical testing, while the majority are available for research use only (RUO).

(A) Real-time method




(B) Microarray method

**Fig. 1** Workflow of FDA-approved rapid molecular detection platforms for CP-CRE. (**a**) Real time method. (**b**) Microarray method

## FDA-Approved Real-Time PCR Assays

(a) **BioFire FilmArray**

BioFire Diagnostics, LLC (Salt Lake, UT, USA), now part of bioMérieux, has developed an integrated diagnostic platform known as the BioFire® FilmArray, which fully automates the detection and identification of multiple organisms from a single sample in about 1 h. An unprocessed clinical sample is subjected to nucleic acid purification, reverse transcription, a high-order nested multiplex PCR reaction, and amplicon melt curve analysis (Fig. 1a). Biochemical reactions are enclosed in a disposable pouch, minimizing PCR contamination risk [45]. Their FilmArray blood culture identification (BCID) panel can identify >25 pathogens and 4 antibiotic resistance genes from positive blood cultures in 1 h [46]. At the end of the run, a report is automatically generated which documents any detectable organism(s) as well as the antimicrobial resistance genes: *mecA*, *vanA/B*, or $bla_{\mathrm{KPC}}$. FilmArray BCID was the first FDA-cleared (June 2013) diagnostic test to directly query the $bla_{\mathrm{KPC}}$ gene. A recent large multicenter study evaluated 2207 positive blood cultures (1568 clinical and 639

seeded) collected in 8 clinical microbiology laboratories in the USA [47]. The assay displayed both 100% sensitivity and specificity in detecting KPC gene from 6 clinical KPC-positive specimens and 33 seeded specimens. Recently, a research use-only antimicrobial resistance panel of FilmArray systems covering a wide range of resistance mechanisms in Gram-negative bacteria was also developed [48]. The panel consists of assays for 22 resistance determinants, including ESBLs (CTX-M, TEM, SHV), AmpCs (CMY, DHA, FOX), carbapenemases (KPC, NDM, VIM, IMP, OXA), and quinolone resistance determinants (*gyrA*, *parC*, QnrA/B/S/D, QepA). Currently, this panel is under optimization, and it is expected that implementation of the antimicrobial resistance Gram-negative panel will benefit clinical laboratories interested in rapid molecular detection of CRE.

(b) **GeneXpert Carba-R Test**

In 2013, Cepheid described a GeneXpert® (Cepheid, Sunnyvale, CA, USA) as a real-time PCR platform with ready-to-use cartridges for rapid detection of clinically relevant carbapenemase genes ($bla_{KPC}$, $bla_{VIM}$, and $bla_{NDM}$) directly from rectal swabs or perirectal swabs (Xpert MDRO assay) (Fig. 1a) [49]. An updated assay, the Xpert® Carba-R, was subsequently developed to allow for detection of $bla_{KPC}$, $bla_{NDM}$, $bla_{VIM}$, $bla_{IMP}$ (subgroup 1), and $bla_{OXA-48}$-like (**e.g.**, $bla_{OXA-48}$, $bla_{OXA-162}$, $bla_{OXA-163}$) carbapenemase genes, making it one of the earliest commercially available assays able to detect $bla_{IMP-1}$. However, the Xpert Carba-R assay is unable to detect several important $bla_{OXA-48}$ variants, e.g., $bla_{OXA-181}$ and $bla_{OXA-232}$ [50]; a later version (Xpert Carba-R version 2) was subsequently updated to allow for efficient detection of $bla_{OXA-181}$ and $bla_{OXA-232}$.

The Xpert Carba-R kit version 2 (v2) was tested on a collection of 150 well-characterized enterobacterial isolates, including several different $bla_{OXA-48}$**-like** variants (20 $bla_{OXA-48}$, 2 $bla_{OXA-162}$, 9 $bla_{OXA-181}$, 5 $bla_{OXA-204}$, 3 $bla_{OXA-232}$, and 2 $bla_{OXA-244}$) [51]. The Xpert Carba-R v2 was able to detect all $bla_{KPC}$, $bla_{NDM}$, $bla_{VIM}$, and $bla_{OXA-48}$ variants, including $bla_{OXA-181}$ and $bla_{OXA-232}$. In addition, the assay's performance was evaluated within the context of the daily workflow of a hygiene unit in a setting with low CP-CRE prevalence [52]. The Xpert® Carba-R v2 assay correctly detected 12 OXA-48-like, 1 KPC, and 1 OXA-48-like/NDM carriers with 100% sensitivity and 99.13% specificity and with 85.71% and 100% positive and negative predictive values, respectively [52]. This study demonstrated that the Xpert® Carba-R v2 kit is well adapted for rapid screening of high-risk patients in low-prevalence regions, with turnaround times of <1 h versus 24/48 h for culture [52].

In March 2016, the Xpert Carba-R assay obtained initial FDA clearance for detection and differentiation of carbapenemase genes in pure bacterial isolates, followed by expanded clearance in June 2016 for analysis of direct rectal swab specimens, thereby positioning the Xpert Carba-R kit as a valuable tool for identification of colonized patients and as an aid to infection control efforts.

Commercially Available Research Use-Only (RUO) Assays

The Check-Direct CPE kit (Check-Points, Wageningen, The Netherlands) is a new commercial multiplex real-time PCR assay designed to simultaneously detect the most prevalent and clinically important carbapenemase genes (*bla*$_{VIM}$, *bla*$_{OXA\text{-}48}$, *bla*$_{NDM}$, and *bla*$_{KPC}$) directly from rectal swabs. The Check-Direct CPE assay is able to differentiate between *bla*$_{KPC}$, *bla*$_{OXA\text{-}48}$, and *bla*$_{VIM/NDM}$, obtaining results within 3 h; however, it is not able to differentiate between NDM and VIM, as the probes corresponding to these targets share the same fluorescent tags [24]. Also, IMP carbapenemases are not targeted by this assay [53]. In a multicenter evaluation of the Check-Direct CPE assay for direct screening of carbapenemase-producing *Enterobacteriaceae* from rectal swabs in Belgium, the assay showed 100% sensitivity and 94% specificity when compared with selective culture [54]. In another study, Lau et al. [55] evaluated the clinical performance of Check-Direct CPE for carbapenemase detection directly from 301 perirectal swabs (258 patients) in a non-outbreak setting. Check-Direct CPE demonstrated a sensitivity value, specificity value, positive predictive value (PPV), and negative predictive value (NPV) of 100% (all *bla*$_{KPC}$), 88%, 21%, and 100%, respectively. False positives accounted for 79% (n = 34) of samples and were all due to targets with low incidence in the USA, such as *bla*$_{NDM/VIM}$ and *bla*$_{OXA\text{-}48}$. The authors suggested that Check-Direct CPE will likely prove most useful in high-prevalence areas or outbreak settings where rapid carbapenemase detection is critical for infection control management [55].

Other carbapenemase surveillance assays currently in development or clinical trials include BD MAX™ CRE assay (Becton-Dickinson, USA), RenDx Carbaplex assay (Renshaw, UK), and Amplidiag CarbaR+VRE (Mobidiag, Espoo, Finland). These assays detect a variety of carbapenemases, typically including KPC, NDM, OXA-48, and VIM (VIM is not available for BD MAX). These commercial assays offer a reliable method to detect bacteria with clinically significant carbapenemases. Whether clinical laboratories choose to perform molecular testing, and the subsequent choice of test, will ultimately depend on the cost, intended throughput, target gene prevalence, and ability to fit into local workflows.

## *PCR/ESI-MS*

Endimiani et al. developed a PCR-based PCR/electrospray ionization-mass spectrometry (PCR/ESI-MS) method for the detection and identification of *bla*$_{KPC}$ genes among *Enterobacteriaceae* in 2010 [56]. The PCR/ESI-MS technology measures the exact molecular mass of PCR products and interprets the data as DNA sequence information. As such, it is a promising genotyping system possessing high multiplexing capacity and can be used for detecting different genes

present in a single strain. This system can also detect single-nucleotide polymorphisms, including mutations corresponding to changes in key amino acids. In their study, Endimiani and colleagues detected 100% of the KPC producers, and all *bla*$_{KPC-2}$-possessing and *bla*$_{KPC-3}$-possessing strains were correctly reported [56]. Given its rapid performance, the PCR/ESI-MS-based platform could be used in hospitals to improve the outcome of infected patients, as well as to perform epidemiological and infection control studies where isolates need to be rapidly detected.

## *Loop-Mediated Isothermal Amplification*

Despite its unparalleled success as a molecular biology tool, there are inherent limitations associated with PCR, such as the cost involved in purchasing consumables and the inactivation of *Taq* polymerase by inhibitors (such as heparin) in crude biological samples. In order to overcome these deficiencies, the loop-mediated isothermal amplification (LAMP) assay, a relatively simple and field-adaptable platform which only requires a temperature-controlled water bath to ensure isothermal conditions, has been developed [57]. Autocycling strand displacement DNA synthesis is performed in the presence of the *Bst* DNA polymerase under isothermal conditions, using a set of four to eight primers that attach to unique sites on the target DNA sequence, ensuring highly specific amplification. Several in-house LAMP assays for carbapenemase genes, including *bla*$_{OXA-48}$, *bla*$_{VIM}$, *bla*$_{IMP-14}$, and *bla*$_{KPC}$, have been reported, all demonstrating high sensitivity and specificity and rapid turnaround time [38, 58–60].

The eazyplex® SuperBug CRE system (Amplex Biosystems GmbH, Giessen, Germany) is a commercially available LAMP assay, consisting of a freeze-dried, ready-to-use mixture which facilitates an isothermal amplification reaction that targets carbapenemase variants of the VIM, NDM, and KPC families, several members of the OXA family (OXA-48, OXA-23, OXA-40, and OXA-58 for eazyplex® SuperBug complete A and OXA-48, OXA-23, OXA-40, and OXA-181 for eazyplex® SuperBug complete B), as well as CTX-M-1 and CTX-M-9 ESBL families. The eazyplex® SuperBug CRE system can directly detect carbapenemase producers from bacterial colonies, rectal swabs, or positive blood cultures, allowing for detection within 15–30 min (depending on sample type) without DNA extraction. Amplification products are visualized by real-time fluorescence detection of a fluorescent dye bound to double-stranded DNA, using a portable Genie® II instrument. Garcia-Fernandez et al. tested a collection of 94 previously genotypically characterized and 45 prospectively collected carbapenemase-producing strains [61]. The eazyplex® SuperBug CRE system correctly detected *bla* carbapenemase genes with or without *bla*$_{CTX-M}$ genes in 100% of the molecularly characterized strains.

### *Microarray*

DNA hybridization techniques in microarray formats allow for simultaneous detection of numerous sequences. Microarray technology utilizes a number of DNA probes that hybridize to DNA targets, including carbapenemase genes. Microarrays can be paired with PCR amplification of target genes or used to directly query DNA sequences within bacterial isolates. The advantage of an array platform compared to PCR assays is in the number of targets available for interrogation; while PCR can typically accommodate a maximum of four to five targets per assay, microarrays can include hundreds of targets, depending on the platform.

Check-Points has several commercially available microarray kits for epidemiological use (http://www.check-points.eu/). The Check-MDR CT101 array targets carbapenemase genes $bla_{\mathrm{KPC}}$ and $bla_{\mathrm{NDM}}$, AmpC β-lactamase genes, and $bla_{\mathrm{CTX\text{-}M}}$, $bla_{\mathrm{SHV}}$, and $bla_{\mathrm{TEM}}$ (both wild type and ESBLs). The Check-MDR CT102 array also includes carbapenemase genes $bla_{\mathrm{VIM}}$, $bla_{\mathrm{IMP}}$, and $bla_{\mathrm{OXA\text{-}48}}$ but omits the AmpC targets. Check-MDR CT103 XL contains all of the targets in Check-MDR CT101 and CT102 and includes additional carbapenemase genes typically identified in *Acinetobacter baumannii* or *Pseudomonas aeruginosa*, such as $bla_{\mathrm{OXA\text{-}23}}$, $bla_{\mathrm{OXA\text{-}24/40}}$, and $bla_{\mathrm{OXA\text{-}58}}$, as well as gene encoding some emerging carbapenemases (**e.g.**, $bla_{\mathrm{GIM}}$, $bla_{\mathrm{GES}}$, $bla_{\mathrm{SPM}}$) and ESBLs (**e.g.**, $bla_{\mathrm{VEB}}$, $bla_{\mathrm{PER}}$, $bla_{\mathrm{BEL}}$, $bla_{\mathrm{GES}}$), thereby making the Check-MDR CT103 XL array one of the most clinically relevant β-lactamase gene detection commercial panels. The assays usually work on bacterial cultures and involve DNA extraction, ligation-mediated PCR, amplification of ligated probes, and hybridization on the microarray. These assays provide highly accurate detection of known resistance genes within several hours, thereby facilitating rapid implementation of isolation measures and appropriate antibiotic treatment [62]. Bogaerts et al. [63] evaluated the performance of Check-MDR CT103 XL in 223 well-characterized *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp. strains. Specificity and sensitivity values of 100% were recorded for most *bla* genes, with a slightly lower signal observed for $bla_{\mathrm{IMP}}$.

The Verigene Gram-negative blood culture (BC-GN) assay (Nanosphere, Inc., Northfield, IL) is an FDA-approved (Jan 2014), automated, multiplexed nucleic acid microarray-based test for rapid Gram-negative bacterial speciation and antimicrobial resistance detection from blood cultures. A workflow of Verigene BC-GN assay is shown in Fig. 1b. The assay allows for detection of the eight most commonly isolated Gram-negative organisms, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Enterobacter* spp., *Citrobacter* spp., *Proteus* sp., *Acinetobacter* spp., and *P. aeruginosa*, as well as six classes of resistance genes: $bla_{\mathrm{CTX\text{-}M}}$, $bla_{\mathrm{KPC}}$, $bla_{\mathrm{NDM}}$, $bla_{\mathrm{VIM}}$, $bla_{\mathrm{IMP}}$, and $bla_{\mathrm{OXA}}$ (https://www.luminexcorp.com). The assay exhibits 92.2–100% positive agreement for speciation and 95.3–100% for resistance gene identification, when compared with the reference method (www.nanosphere.us). In a recent study, Walker et al. [64] evaluated the clinical impact of implementing the Verigene BC-GN assay for detection of Gram-negative bacteria in positive blood cultures

obtained from hospitalized patients. The BC-GN panel yielded a positive identification in 87% of Gram-negative cultures and was accurate in 95/97 (98%) of the cases compared to results using conventional culture. Verigene BC-GN had significantly shorter turnaround times for organismal identification (mean, 10.9 h versus 37.9 h; $P < 0.001$). Moreover, length of ICU stay, 30-day mortality, and mortality associated with multidrug-resistant organisms were significantly lower in the Verigene BC-GN intervention group ($P < 0.05$). The results showed that the Verigene BC-GN assay is a valuable addition for early identification of Gram-negative organisms that cause bloodstream infections and can significantly impact patient care, particularly when resistance markers are detected. However, it is important to note that the BC-GP assay is not a target amplification assay; instead, amplification occurs within the blood culture bottle during incubation. A recognized limitation is that polymicrobial cultures are subject to false-negative results and thus lower sensitivity, due to the slower growth of some Gram-negative bacilli in mixed cultures [65, 66]. Consequently, the bacterial concentrations present in the sample may be lower than the limit of detection for species-specific and resistance gene targets.

The CarbDetect (Alere Technologies GmbH, Loebstedter, Jena, Germany) platform is a novel oligonucleotide microarray-based assay designed for bacterial cultures [67, 68]. RNA-free, unfragmented genomic DNA from pure and monoclonal culture material is amplified ~ 50-fold and labeled with biotin-11-dUTP using a linear amplification protocol, of which only one antisense primer per target is used to generate single-stranded DNA products in order to simultaneously label and amplify an essentially unlimited number of sequence-specific targets. However, the amplification sensitivity is lower than that of a standard PCR assay, and consequently the method is restricted to pure culture and cannot be performed on swabs or other primary specimens. The biotin-labeled ssDNA is transferred and hybridized to DNA oligonucleotide microarrays bearing 238 probes for 35 carbapenemase genes; 26 ESBLs, narrow-spectrum β-lactamases, and AmpC genes; as well as 48 other relevant antibiotic resistance genes (e.g., aminoglycoside resistance). Additionally, eight species markers are provided, including *E. coli* (including enteroinvasive strains), *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, *C. freundii*, *Shigella* spp., *Salmonella* spp., and *Enterobacter* spp. (CarbDetect AS-2 Kit, https://alere-technologies.com). This assay is currently the most comprehensive resistance gene detection test commercially available and includes additional resistance targets beyond β-lactamase genes. The assay was evaluated with DNA extracted from 117 clinical *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii* strains collected from urinary, blood, and stool samples, which was then used to identify bacterial species, carbapenemases, ESBLs, and narrow-spectrum β-lactamase genes in a single reaction, with 98.2% and 97.4% sensitivity and specificity, respectively [68]. The newly developed assay thus provides an accurate and convenient tool to identify and discriminate the most clinically relevant carbapenemases.

## *Next-Generation Sequencing (NGS)*

The "first generation" of DNA sequencing technology, commonly referred to as Sanger sequencing, was the primary sequencing technology from 1975 to 2005. Sanger sequencing produces relatively long (500–1000 bp) high-quality DNA sequences and has long been accepted as the gold standard for DNA sequencing. The introduction of pyrosequencing technology by 454 Life Sciences in 2005 began the "next-generation sequencing" (NGS) revolution [69]. This high-throughput technology allowed for the generation and detection of thousands to millions of short sequencing reads in a single run with no need for cloning. Since then, a number of NGS technologies have emerged, and the development of various high-throughput platforms has paved the way for the application of whole-genome sequencing to the study of bacterial pathogens and antimicrobial resistance.

Using NGS technology, a large amount of sequence data can be generated in a relatively short time, with read lengths ranging from 100 to 300 bp (e.g., Illumina HiSeq/MiSeq platforms) to >20,000 bp (e.g., PacBio and MinION platforms) [70]. The primary steps in this process include DNA isolation from bacterial culture, library preparation, DNA fragment sequencing, and data analysis. Depending on the type of information needed, NGS can include either DNA or RNA (in the form of cDNA) as sequencing material. DNA NGS captures the entire genomic content and can be used to identify the presence of antimicrobial genes or genetic mutations, while RNA NGS (or RNA sequencing) can detect global gene expression, including that of genes indirectly contributing to antimicrobial resistance (e.g., porin or efflux pump genes). Coupled with appropriate bioinformatics pipelines for identification of antimicrobial resistance genes, NGS offers the unprecedented advantage of rapidly providing genetic information at the whole-genome level, thus making it ideal for identifying all possible genetic determinants of antimicrobial resistance within a microbial genome [71]. In addition, NGS can be directly performed on primary/uncultured clinical samples, which allows for detection of all pathogens and mining of the resistance information (resistome) in a bacterial community, i.e., metagenomics analysis.

NGS facilitates molecular characterization of bacterial pathogens on many levels, without the need for a priori selection of targets as is required for PCR. The sequence data generated can be used for many purposes, including speciation and strain discrimination; resistome, plasmidome, and virulome identification; outbreak investigations and source tracking; and transmission and evolution studies. With regard to antibiotic resistance monitoring, NGS offers especially great promise, as the full repertoire of resistance genes, the sequences of plasmids that bear them, and the chromosomal background of the host strains can all be deduced from the same data. In the case of CRE, NGS also allows for differentiation of carbapenemase producers from isolates that are resistant by virtue of other mechanisms, which can be important for infection control. Furthermore, with the advancement of long-read sequencing, the sequences of complete genomes and plasmids can be readily

obtained without the need for transformation, conjugation, or PCR gap closure, thereby facilitating downstream bioinformatics analysis and accurate prediction of antimicrobial resistance.

Currently, real-time integration of NGS into clinical laboratories has been hampered by processing speed, financial costs, and automated data analysis [72]. In addition, the complexities of NGS require an evolving set of standards in order to ensure testing quality. Regulatory and accreditation requirements, professional guidelines, and best practices that help ensure the quality of NGS-based tests are needed [73]. However, with decreasing cost and turnaround time, improved sample preparation workflow, and development of user-friendly bioinformatics tools, it is only a matter of time until NGS becomes a key tool for antimicrobial surveillance and infection control, with widespread implementation in clinical microbiology settings.

## Rapid Non-nucleic Acid-Based Tests: MALDI-TOF MS

Matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS) is increasingly utilized in clinical microbiology laboratories for identification of bacteria and yeasts. Several MALDI-TOF MS assays for detection of β-lactamase activity, including carbapenemases, have been developed in recent years [74–84]. The methodology utilized typically involves the following steps: a fresh bacterial culture, usually grown overnight, is suspended in a buffer and centrifuged; the pellet is then resuspended in a reaction buffer containing the β-lactam molecule; after incubation at 35 °C for 1 to 3 h, the reaction mixture is centrifuged, and the supernatant is mixed with an appropriate matrix and measured by MALDI-TOF MS. The resulting spectra displaying peaks representing the β-lactam molecule, its salts, and/or its degradation products are then analyzed [85].

A MALDI-TOF MS assay for detection of carbapenemases was published in 2011 by Hrabak et al. [74]. This method allows for detection of resistance to carbapenems in *Enterobacteriaceae* carbapenemase-mediated hydrolysis, without false-positive results. Several modified MALDI-TOF MS assays were subsequently published, with varying carbapenem targets and methodological details [75–84]. The MALDI-TOF MS carbapenemase detection assays usually yield high sensitivity (~95%–100%) and specificity (~95%–100%) in comparison with phenotypic detection methods. In addition, this approach can be directly used for clinical specimens, such as blood culture and urine samples [84, 86–91]. Detection of carbapenemases by MALDI-TOF MS can be a powerful, quick, and cost-effective method for microbiological laboratories, without false-positive or false-negative results [76]. In addition, MALDI-TOF MS has been used to characterize porin function in relation to carbapenem resistance, with one study suggesting that compared with SDS-PAGE, MALDI-TOF MS is able to rapidly identify porin-deficient strains within half an hour with greater sensitivity and less cost [92].

In summary, MALDI-TOF MS is becoming an essential tool in clinical microbiology laboratories, not only for rapid identification of bacterial pathogens but also

for resistance detection. However, while various methods and models have been described in the literature, a notable limitation associated with detection of enzymatic carbapenem degradation by MALDI-TOF MS is the lack of well-standardized protocols. Moreover, whereas MALDI-TOF MS can detect production of carbapenemases and carbapenemase activity, it cannot differentiate specific carbapenemase types and is consequently less informative for molecular studies.

## Conclusion

Carbapenemase-producing *Enterobacteriaceae* have now spread worldwide and become a major public health issue, challenging not only treatment solutions but also detection methods. However, appropriate treatment and infection control rely largely on efficient and timely identification of carbapenem-resistant bacteria. Therefore, there is an urgent need for rapid and accurate detection of carbapenemases, and it is necessary to introduce molecular methods into clinical diagnostic workflows. Clinical susceptibility testing provides valuable phenotypic resistance information for therapeutic decision-making but usually takes more than 48 h, which may delay appropriate treatment. Culture-based methods for carbapenemase detection, such as the MHT, Carba NP test, and mCIM, can provide rapid carbapenem resistance information but are unable to differentiate specific carbapenemases, which may be important for infection control and epidemiological investigations of CRE transmission. Currently, there are a variety of molecular-based methods able to detect most of the major carbapenemase gene families in global circulation, but they are largely limited to known carbapenemase sequence targets and can potentially miss novel variants or carbapenem resistance mechanisms. NGS, which can detect the entire genomic content or expression profile of a bacterial strain, is currently the most promising platform in antimicrobial resistance detection; however, further work is required to improve the workflow, including shortening turnaround times, reducing costs further, and improving automatic data-analyses pipelines. In summary, while no single detection platform can encompass all possible genes or resistance mechanisms, one can envision that future testing might incorporate rapid methods for both molecular detection of common carbapenemases and rapid non-nucleic acid-based determination of antimicrobial susceptibility, thus enabling timely identification of CRE and facilitating effective antibiotic therapy and infection control measures to prevent further CRE dissemination.

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# Advanced Methods for Screening and Identification of Methicillin-Resistant *Staphylococcus aureus*

**Raymond Widen and Yi-Wei Tang**

## Clinical Relevance

*Staphylococcus aureus* strains, both methicillin sensitive (MSSA) and methicillin resistant (MRSA), are recognized as significant pathogens and are associated with community-acquired and nosocomially transmitted infections. Methicillin resistance in *S. aureus* was first noted in the 1960s [1], and since the 1980s strains of MRSA have evolved to become a worldwide problem [2]. Initially, MRSA infections were primarily health care associated (HA-MRSA) with evidence of nosocomial transmission. Endemic MRSA in the health care setting led to increases in bloodstream and other serious infections [3]. MRSA-related bacteremia leads to greater cost of care related to increased length of stay and greater challenges in treatment [4, 5]. It is important to be able to rapidly differentiate MSSA and MRSA as effective treatment differs greatly. In serious infections such as sepsis, MRSA is not effectively treated with most beta-lactams and requires agents such as vancomycin; however, for MSSA vancomycin is less effective than beta-lactams [6] so rapid differentiation of MSSA and MRSA is critical for optimal patient management.

Community-acquired MRSA (CA-MRSA) was first recognized in in the early 2000s. CA-MRSA is often found colonizing otherwise healthy individuals; however, serious infections may originate from carriage of CA-MRSA [7, 8]. Preventing the dissemination of MRSA in health care facilities requires vigorous infection control guidelines. Challenges include identification of reservoirs of transmission and

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strict adherence to infection prevention methods once sources are identified. As noted by Francois and Schrenzel in the previous edition of this topic and others, screening of patients upon admission for MRSA carriage has proven useful in addressing nosocomial transmission [9]. It has been recognized that MRSA carriage is a risk factor for developing MRSA infection [10, 11]. Two different approaches have been taken to attempt to address nosocomial MRSA transmission with both showing efficacy. One approach is to screen for MRSA colonization in all patients upon admission to the institution and initiate decolonization efforts (mupirocin nasal treatment and chlorhexidine baths) along with isolation protocols. The second is to bypass MRSA screening and go directly to decolonization efforts. The argument against universal MRSA screening relates to the cost of this approach [12, 13]. The proponents of the screen first for presence of MRSA by NAAT or culture and decolonize only the individuals that test positive note the increasing incidence of mupirocin resistance and even resistance to chlorhexidine. There is no consensus in the literature as to which approach is the most cost-effective and most beneficial.

## Molecular Epidemiology

The molecular basis of MRSA was reviewed previously [5, 9, 14, 15]. There are 11 recognized SCC*mec* types currently [16] (http://www.sccmec.org/Pages/SCC_TypesEN.html). Figure 1 depicts the location and organization of the resistance marker.

It has been demonstrated that *mecC* is a more recently recognized variant of the *mecA* gene that encodes a PBP2 that has significant differences in gene and amino acid sequence compared to classical *mecA* encoded PBP2 [17]. As noted later in this chapter, *mecC* has created challenges for detection of MRSA due to the fact that the differences in gene and protein sequence are sufficient to lead to failure of some detection methods to correctly classify *mecC* containing *S. aureus* as methicillin resistant leading to false-negative MRSA screening tests.

## Culture-Based MRSA Detection

The prior version of this chapter provided a comprehensive review of culture-based methods for detection of MRSA [9], and there has not been a significant change in these methods in the recent years. They all still rely on 18–48 h. incubation of the cultures and detection by specific color characteristics of MRSA on the selective/differential media as a means to provide an earlier indication of the presence of MRSA in a sample. Nonselective media necessitate additional steps to screen for MRSA including the differentiation of MRSA from MSSA using additional tests. Confirmation of MRSA can be achieved employing antimicrobial susceptibility testing or PBP2 detection [18, 19]. However, the emergence of *mecC* as a resistance

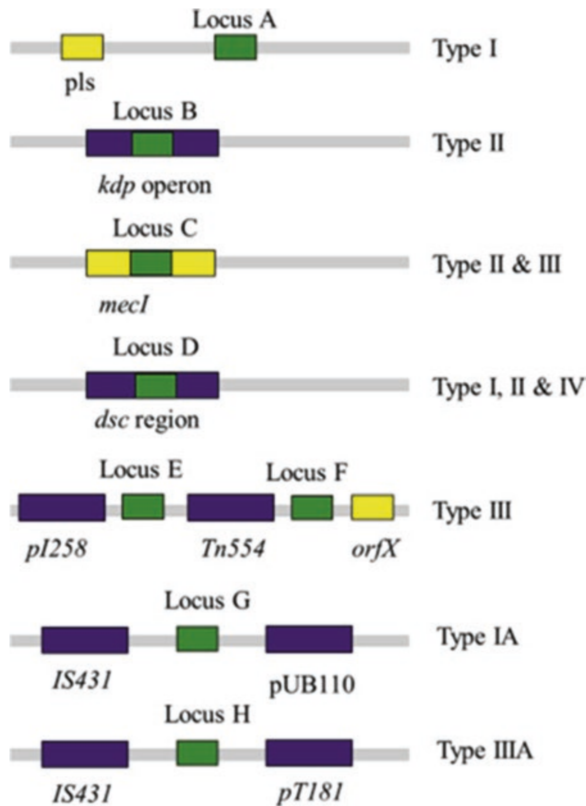


**Fig. 1** Specific loci for SCC*mec* types. Locus A was located at the downstream of the *pls* gene and was specific for SCC*mec* type I; locus B was internal to the *kdp* operon which was specific for SCC*mec* type II; locus C was internal to the *mecI* gene present in SCC*mec* types II and III; locus D was internal to the *dcs* region present in SCC*mec* types I, II, and IV; locus E (specific for SCC*mec* type III) was located in the region between integrated plasmid *pI258* and transposon *Tn554*; locus F (specific for SCC*mec* type III) was located in the region between *Tn554* and the chromosomal right junction (*orfX*). Locus G (specific for SCC*mec* type IA) was located at the left junction between *IS431* and pUB110, and locus H (specific for SCC*mec* type IIIA) was located at the left junction between *IS431* and *pT181*. Genes are marked by yellow blocks. Mobile elements are marked by purple blocks. Loci are marked by green blocks. (Adapted with permission from Liu et al. [16])

mechanism for MRSA has complicated the picture [20, 21]. PBP2 from *mecC* is genetically and antigenically distinct enough that it is not detected by current PBP2 assays [22, 23]. MRSA possessing *mecC* also is not reliably detected in several of the automated susceptibility testing methods using oxacillin as the antimicrobial and is only detected reliably when cefoxitin is utilized [24–26]. It is important for users of the various instruments to be aware of the limitations in detection of *mecC*.

Chromogenic agars are available that allow the laboratory technologist to detect MRSA based on a specific color development on the selective medium. The selec-

**Table 1** Chromogenic agar for MRSA detection

| Vendor | Test name | Website |
|---|---|---|
| BIO-RAD | MRSA select | bio-rad.com |
| BD BBL | Chromagar MRSA II | bd.com |
| bioMerieux | ChromID MRSA | biomerieux.com |
| Oxoid/Thermo fisher | Brilliance MRSA2 | thermofisher.com |
| Hardy diagnostics | MRSA screen plate | hardydiagnostics.com |
| Remel | Spectra MRSA | remel.com |
| EO labs | Colorex MRSA | eolabs.com |

tive medium contains inhibitors for Gram-negative organisms, an antimicrobial to suppress MMSA and a substrate that is specific for *S. aureus*. Table 1 summarizes the available MRSA chromogenic agar. Reviews of the performance of the various commercially available MRSA chromogenic agars generally do not indicate clear consistent superiority of one over the other [27, 28]. The chromogenic media perform well with either *mecA*- or *mecC*-positive MRSA [27]. The sensitivity of chromogenic agar for detecting MRSA may be increased by a prior enrichment broth step however that adds 18–24 h to the time for results [27].

## Molecular Methods for MRSA Screening for Surveillance

In order to provide more rapid TAT for detection of MRSA in clinical samples, both for surveillance and diagnostics, rapid molecular assays have been developed over the years. As mentioned earlier antibody-mediated PBP2 detection from MRSA has allowed for more rapid identification from culture; however it has no utility from clinical samples since methicillin-resistant coagulase-negative staphylococci (MRCoNS) also harbor *mecA* gene. With recognition of *mecC* and the demonstration that the current PBP2 assays fail to react with the PBP2 encoded by *mecC*, the utility of such assays will be limited until assays that detect PBP2 both from *mecA* and *mecC* possessing MRSA are developed and validated.

The initial MRSA detection molecular assays were designed for screening patients for colonization. Most are FDA cleared for detection in nares only; however investigators have documented utility in other sample types as well, and some believe targeting multiple sites could lead to greater identification of colonized patients [29]. The first generations of MRSA NAATs targeted the chromosome/SCC region and worked reasonably well; however it later became apparent that some tests were false positive due to “cassette dropouts” where the *mecA* gene was lost [30, 31]. In these “dropouts” the NAAT would be positive for MRSA; however the phenotype is MSSA. The next generation of tests added the *mecA* gene itself as a target to address the problem with *mecA* dropouts. As noted earlier, *mecC* as an alternative resistance gene was identified necessitating inclusion of *mecC* gene as a target in order to reliably detect MRSA. Therefore, most of the currently available

**Table 2** MRSA NAAT nares screening assays

| Vendor | Test name | Detects | Website |
|---|---|---|---|
| Becton Dickinson | BD MAX MRSA XT | MRSA | bd.com |
| Becton Dickinson | BD MAX staph SR | MRSA/MSSA | bd.com |
| Cepheid | Xpert NxG | MRSA | cepheid.com |
| Cepheid | Xpert SA nasal complete | MRSA/MSSA | cepheid.com |
| Elitech | MRSA/SA ELITe MGB® | MRSA/MSSA | elitech.com |

assays include detection of *mecA*/*mecC* in addition to the chromosome/SCC junction. FDA-cleared assays, and those in development, for MRSA detection in nares samples are summarized in Table 2. They all offer significant improvements in TAT for detecting MRSA compared to culture, and some provide results for the presence of MSSA as well. The ability to detect MRSA with very rapid TAT allows for near real-time decisions relating to isolation of patients to control potential nosocomial spread.

## Molecular Methods for MRSA Diagnosis in Active Infection

NAATs have been developed for rapid detection of MRSA in a variety of clinical samples. Table 3 lists NAATs that are currently available or are in development.

## Detection of MRSA/MSSA in Positive Blood Cultures

Several NAATs are available for detection of MRSA as part of a panel of agents performed directly from positive blood cultures [32]. These allow for identification of MSSA or MRSA within a few hours from the time a culture flags as positive. The *Staphylococcus* QuickFISH method (an updated version of PNA FISH) allows for rapid detection of *Staphylococcus* species from positive blood culture bottles providing a total test-to-result time of less than 30 min [33]. The GeneOhm StaphSR was the first PCR amplification-based device approved by the US Food and Drug Administration (FDA) for identification of MSSA and MRSA from positive blood culture [34]. This assay has been gradually replaced by user-friendly, integrated systems such as the Xpert MRSA/SA blood culture assay, which offers differentiation between MRSA and MSSA within about 1 h [35, 36]. The KeyPath MRSA/MSSA assay uses mixed lytic bacteriophage to differentiate *S. aureus* from other bacteria and predicts methicillin susceptibility [37].

MRSA/MSSA detection/differentiation also is included in multiplex NAAT panels performed on positive blood cultures. There are four FDA-cleared panels that include direct detection of MRSA/MSSA. The Luminex Verigene system relies on the Gram stain result from the positive blood culture bottle to guide the laboratorian

**Table 3** Rapid detection of MRSA in positive blood cultures

| Vendor | Test name | Detects | Website |
|---|---|---|---|
| BioFire | BCID | MRSA/MSSA and other pathogens | biofire.com |
| Cepheid | Xpert® MRSA/SA BC | MRSA/MSSA | cepheid.com |
| Great Basin | Staph ID/R blood culture panel | MRSA/MSSA and other staphylococci | gbscience.com |
| iCubate | iCubate gram-positive BCID | MRSA/MSSA and other GPC | iCubate.com |
| Luminex | Verigene® gram-positive BCGP | MRSA/MSSA and other GPC | luminex.com |
| GenMark | BCID gram-positive panel | MRSA/MSSA and other pathogens | genmark.com |

as to which panel to set up – Gram positive or Gram negative. The Gram-positive panel is performed therefore after Gram-positive cocci are observed in Gram stain. It detects and differentiates MRSA and MSSA along with several other Gram-positive cocci in a reaction that takes about 4 h [38]. The bioMerieux BioFire used a different approach in development of its multiplex blood culture identification (BCID) panel. They developed a more highly multiplexed panel on the FilmArray system that detects Gram negative, Gram positive, and some yeast targets as well as resistance markers. The same panel is performed regardless of Gram stain results [39]. The assay time on the FilmArray system is about 1 h. More recently iCubate recieved FDA clearance on their gram positive pathogen assay direct from positive blood culture that detects and differentiates MSSA/MRSA along with several other gram positive bacteria. Reaction time for the iCubate assay is approximately 5 h [40, 41]. GenMark has developed a similar system for their ePlex multiplex PCR/array instrument. Similar to the Verigene approach, their panels are driven by the blood culture gram stain result. The ePlex assays however will include a pan Gram-negative target and a pan yeast target in their Gram-positive panel and the reverse in their Gram-negative panel as a flag for the possibility of a missed organism in the Gram stain [42]. The assay is CE IVD marked for use in the European Union (EU), and the company plans to pursue FDA IVD clearance. The time to result is in the 1.5 h range. Regardless of the platform routine subculture of the positive blood culture bottle will be required for additional susceptibility testing and to detect organisms not included in the panels; however the rapid results allow for more timely introduction of appropriate antimicrobial therapy targeted to the specific pathogen detected.

With short turnaround times and broad species coverage, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) offers the possibility of accurate, rapid, inexpensive identification of bacteria, fungi, and mycobacteria isolated in clinical microbiology laboratories. Overall turnaround time for direct identification varied from minutes to hours. Accuracy and reproducibility of MALDI-TOF MS-based methods for detection and identification of staphylococci from blood-bottle media have been shown in clinical studies [43, 44]. Both commercially available systems, the MALDI Biotyper and the Vitek MS, are equally good choices in terms of analytical efficiency for routine identification procedures. The Accelerate Pheno System took a different approach to detect and identify

MRSA/MSSA, along with other pathogens, directly from positive blood culture. The system combines pathogen-specific FISH probes to identify the pathogen with direct microscopic monitoring of pathogen growth (or lack thereof) to detect antimicrobial resistance in a phenotypic approach [45]. The system has the unique ability to identify and provide phenotypic MIC and categorical AST results in a few hours directly from positive blood culture bottles and support accurate antimicrobial adjustment.

## Detection of MRSA in Other Sites

Cepheid offers an FDA-cleared assay for skin and soft tissue infection, the Xpert SSTI cartridge [46, 47]. Our laboratory has validated the use of the BD MAX StaphSR assay for rapid detection of MSSA/MRSA in wound infections [48]. There have been numerous publications documenting the utility of either the Cepheid or BD systems for detection of MRSA/MSSA in sites of infection including respiratory tract, tissues, and various body fluids [49]. Several assays are in commercial development for detection of MRSA and other pathogens in lower respiratory tract samples. Curetis has assays in development to detect MRSA in a variety of other sample types (abdominal fluid, joint infection, others) as part of a broader panel to detect a variety of pathogens within hours. BioFire is developing a lower respiratory tract FilmArray panel that includes MSSA/MRSA along with about 30 additional pathogens. Curetis also has a panel for detection of lower respiratory tract infections that includes MRSA/MSSA as targets. GenMark has a lower respiratory highly multiplexed panel in development that includes MRSA/MSSA among the targets.

## Direct Detection of MRSA in Blood

Direct culture-independent detection of pathogens in blood samples within hours represents a long-awaited development in microbiology [32, 50]. This has become a reality with the release of the FDA-cleared T2 *Candida* assay [51, 52] and the more recently developed bacterial panel (pending FDA approval at the time of this writing) from T2 Diagnostics. The assays use PCR to generate amplicons that are detected by magnetic resonance in the reaction cartridge. The bacterial assay includes *S. aureus* although it does not differentiate MSSA from MRSA. Even so this represents a major time savings (3–5 h versus 18–120 h for culture). The IRIDICA BAC BSI assay has demonstrated the capability for culture-independent detection and identification of bacteria and yeast pathogens including staphylococci in the blood within 8 h [53, 54]; unfortunately, it was discontinued recently by Abbott. Qvella is developing a FAST ID BSI assay that is designed to lyse and amplify multiple pathogen DNA including *S. aureus* within 1 h direct from a peripheral blood sample [55]. Like the T2 assay it does not differentiate MSSA vs

MRSA. The Qvella assay is still in development but is likely to enter clinical trials in 2018. LiDia (DNAe) is also developing a broad range of panel to detect pathogens, including MSSA/MRSA directly from blood samples within 2–3 h using PCR on an array utilizing detection of H ions in real time, similar to the technology used in the Ion Torrent sequencing system [56]. This assay will include resistance markers for Gram-positive and Gram-negative agents allowing for simultaneous identification of the pathogen and detection of resistance markers. Nolling et al. reported a pathogen identification system which uses duplex DNA-invading γ-modified peptide nucleic acids for rapid detection and identification of bacterial and yeast pathogens directly from crude blood [57].

All of these assays of course cannot definitively determine that an organism is susceptible to an antimicrobial agent but instead can only indicate that it is resistant since new resistance mechanisms evolve regularly, and it would be impossible to update the assays quickly enough to include all of the new resistance mechanisms in the assay. All of these systems could potentially be validated by the laboratory on alternative sample types following the CAP/CLSI guidelines for lab-developed tests.

## Next-Generation Sequencing (NGS) for Rapid Detection of MRSA

In the last few years, the utility of NGS in detecting pathogens has been documented [58, 59]. Using either metagenomic or targeted amplification, one can directly identify an essentially unlimited array of pathogens and resistance markers simultaneously. Two recently published articles demonstrate the utility of NGS for studying the epidemiology of outbreaks of MRSA [60, 61]. Since one can perform deep sequence analysis with NGS data, it is possible to detect and simultaneously acquire information to determine if organisms are identical, closely related, or unrelated without the need for an isolated colony. At the time of this publication, the factors that are limiting the adoption of NGS for pathogen detection using the most common NGS systems, Illumina and Thermo Fisher Ion Torrent, include cost of the instrumentation, cost of reagents/consumables, lack of standardized protocols, and the slow time to result (2–3 days). It is likely that over time the turnaround time will be reduced as well as the cost per result. The Oxford Nanopore NGS system has been used for pathogen detection with same day results and using less expensive instrumentation [62, 63].

## Conclusions

It is abundantly clear that rapid identification of *S. aureus* and differentiation of MSSA vs MRSA are critical for optimized patient care. Rapid identification of MRSA is of great importance in controlling nosocomial spread of infection as well.

Molecular methods offer the best approach currently by combining high sensitivity and specificity along with rapid (1–2 h) as compared with culture methods which are not as sensitive and take 48–72 h for a definitive result. Several highly automated molecular platforms exist for MRSA screening. In the last few years, rapid detection of MRSA has proven to be useful in rapid detection of MRSA in infected tissues or other patient samples. These rapid assays allow the treating provider to make informed decisions relating to escalation or de-escalation of therapy. New technology is on the horizon that will allow detection of MRSA, along with other pathogens, directly in peripheral blood samples. NGS, once more rapid and cost-effective approaches develop, will provide yet another approach to rapid detection of MRSA with the advantage of being able to detect essentially any pathogen present. That said, culture will remain an important tool. We will have to be on constant lookout for novel resistance mechanisms that NAAT could miss (e.g., *mecC*).

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# Advanced Techniques in Diagnostic Parasitology

**Bobbi S. Pritt**

## Introduction

Parasites are a significant source of morbidity and mortality worldwide [1–4]. They can be found in nearly all regions of the world and infect individuals in both resource-rich and resource-limited settings [3, 4]. Many parasites such as the filaria, *Plasmodium* spp., trypanosomes, and intestinal helminths are found primarily in tropical settings and disproportionately impact impoverished individuals who lack access to adequate sanitation and health care. Malaria, for example, caused an estimated 216 million cases in 2016, with 445,000 associated deaths [5]. Most cases occur in sub-Saharan Africa, with children under 5 years of age most vulnerable to fatal outcomes. The World Health Organization has also recognized 20 categories of neglected tropical diseases (NTDs), of which 12 have a parasitic etiology: Chagas disease, dracunculiasis, echinococcosis, food-borne trematodiasis, human African trypanosomiasis, leishmaniasis, lymphatic filariasis, onchocerciasis, scabies and other ectoparasites, schistosomiasis, soil-transmitted helminthiases, and taeniasis/cysticercosis [4]. Other parasites such as *Giardia duodenalis*, *Cryptosporidium* spp., *Enterobius vermicularis* (pinworm), and *Pediculus humanus capitis* (head lice) have a cosmopolitan distribution and affect both rich and poor. Giardiasis and cryptosporidiosis, specifically, are reportable diseases in the United States. In 2016, there were 16,310 and 13,453 reported cases of giardiasis and cryptosporidiosis, respectively, in the United States and US territories [6]. The US Centers for Disease Control and Prevention (CDC) has also identified five neglected parasitic infections (NPIs) that have been targeted for public health action based on the severity of illness, availability of methods for treatment/prevention, and number of individuals infected in the United States [3]. These infections are Chagas disease, neurocysticercosis, toxocariasis, toxoplasmosis, and trichomoniasis [3]. Increased recognition

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of parasitic infections has prompted greater national and global efforts for development of useful diagnostics and therapeutics, but much work needs to be done to achieve effective control [4].

Parasitic infections manifest in numerous ways, ranging from asymptomatic colonization to serious disease [2]. At one end of the spectrum are the nonpathogenic protozoa that inhabit the human alimentary tract. These organisms are relevant to the field of clinical microbiology since they must be differentiated from similar-appearing, genetically related pathogens when seen in stool specimens. Other parasites cause severe infections which must be diagnosed and treated promptly to prevent fatal outcomes. At a minimum, the clinical microbiology laboratory must offer rapid diagnostic services for detection of potentially life-threatening infections such as malaria and primary amebic meningoencephalitis. If the skills and resources for detecting these parasites are not available locally, then the laboratory needs to make arrangements with a local facility that can provide rapid diagnostic services [7]. Laboratory detection of parasites relies heavily on traditional morphology- and serology-based assays. However, there have been recent advancements in the field that allow for more rapid, sensitive, and/or specific detection. This chapter will review how advanced laboratory technologies are being used for parasite detection and give specific examples of organisms for which advanced diagnostics show great promise for routine practice.

## Advanced Technologies in Clinical Parasitology

### *Mobile Phone Microscopy*

Microscopic examination remains a commonly used diagnostic modality in the clinical parasitology laboratory and is arguably the gold standard for diagnosis of certain parasitic infections such as malaria [7]. It is also used for identification of uncommon or novel parasites, for which adequate nucleic acid sequence databases do not exist. A good example of the continued need for microscopy is the calculation of the percent parasitemia in patients infected with *Plasmodium* or *Babesia* species. This calculation is performed using microscopic examination of thin or thick blood films and is used to guide clinical decisions based on decades of research and experience with the percent parasitemia. Quantitative nucleic acid amplification tests (NAATs) cannot easily replace this traditional calculation since the quantitative DNA or RNA value does not directly correlate with the number of parasite-infected cells. Several reasons exist for this discrepancy; NAATs detect all parasite nucleic acid in the sample, including extracellular parasite forms, free DNA from nonviable organisms, and gametocytes, and these entities are excluded from the microscopic parasitemia calculation. Also, NAATs detect DNA from multiple parasites inhabiting a single infected erythrocyte, and by microscopy, a multiply infected cell would only be counted once. Given the traditional reliance of morphology-based methods for parasite diagnosis, it is not surprising that multiple

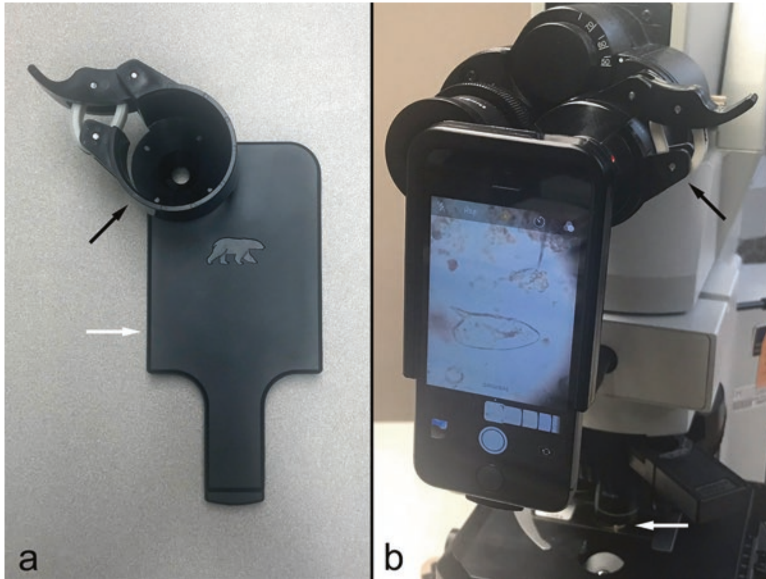


**Fig. 1** Example of a mobile phone adaptor for a standard light microscope. This particular adaptor (Magnifi™, Arcturus Labs, Lawrence, Kansas) consists of a molded plastic case to hold the mobile phone (**a**, white arrow) and an adjustable attachment that fits over the ocular. When mounted on the ocular (**b**, black arrow), the phone's camera can be used to acquire a still image or video from the slide on the stage (white arrow) as viewed through the ocular

groups have developed alternate tools for capturing and analyzing microscopic images, including small portable field microscopes, thus applying advanced technologies to a traditional art [8].

One technology that is gaining traction for parasite diagnosis is the use of mobile "smart" phones with high-resolution image and video acquisition capabilities. This imaging technology can be used in a variety of manners [8]. Perhaps the simplest is to use the mobile phone's camera to capture an image of a macroscopic object, such as a worm or arthropod, and other objects, such as a lateral flow chromatographic assay (e.g., malaria rapid antigen diagnostic test) [8]. The images can then be sent to a skilled health-care worker for evaluation or analyzed using software applications (apps) and algorithms (see section on *Digital Image Analysis* below).

The phone's microscope can also be used to capture an image through the ocular of a light microscope, either by manually holding the phone up to the ocular or by using an adaptor (Fig. 1). This form of "phone-assisted microscopy" has been used with conventional microscopes, as well as with small portable microscopes such as the Foldscope®, an "origami-based paper microscope"(Foldscope instruments, Palo Alto, CA) [9], and CellScope® (http://cellscope.berkeley.edu/technology/). Ephraim et al. [10] compared the performance of a mobile phone-mounted reversed-lens CellScope and a Foldscope for detecting *Schistosoma haematobium* eggs in centrifuged urine, using conventional microscopy as the gold standard.

Both portable microscopes achieved moderate sensitivities (67.6% and 55.9% by the Foldscope and CellScope, respectively) and specificities of >90%. Other investigators used phone-assisted microscopy for detection of *Giardia duodenalis* cysts and *Plasmodium falciparum*, also with moderate sensitivities [8]. The Newton Nm1 microscope (Newton microscopes, Bedford, UK) is another portable microscope that shows promise for field use, although studies using it in conjunction with a mobile phone have not yet been published. The Nm1–600 XY portable field microscope was compared to conventional microscopy for detecting *S. haematobium* from filtered urine and *S. mansoni* from Kato-Katz stool smears and provided sensitivities of 91.7% and 81.1%, respectively. These values were notably higher than those achieved by the CellScope in this study (35.6% and 50.0%, respectively) [11]. The Nm1 microscope also showed low to high sensitivities for detecting various intestinal protozoa [11]. Further enhancements to small portable microscopes may improve their use for field diagnostic work, either alone or in conjunction with mobile phones.

Another means for using a mobile phone for parasite diagnosis is to mount a small portable lens directly onto a mobile phone, thus turning the phone itself into a microscope. Lens-mounted mobile phone microscopy has been used for detection of *Schistosoma haematobium* eggs in urine and helminth eggs in stool [12], although observed sensitivities were low. Other investigators demonstrated improved sensitivities by using lenses with a larger field of vision and higher spatial resolution [8]. Phone-mounted microscope lenses can be purchased from numerous online vendors.

Mobile phones with video capacity offer an additional tool for parasite identification by detecting characteristic parasite motility patterns in unfixed specimens. The primary advance in this area has been in the detection and quantification of motile *Loa loa* microfilariae in whole blood specimens using a specialized device called the CellScope Loa [13]. This device houses an Apple iPhone® 5 s and reversed iPhone camera lens module within a custom plastic case, along with a thin (4-mm-wide, 50-mm-long, and 200-μm-deep) glass capillary tube. Blood is collected via fingerstick and drawn into the capillary tube, which is then inserted into the plastic case and viewed through the iPhone camera in conjunction with the additional reversed lens. A motorized carriage moves the capillary tube along a linear rail so that multiple (4 × 3.16 mm) fields of view can be analyzed using a custom iPhone app. The app detects microfilariae in each field by their motility and provides a total count of microfilariae per mL blood from the fields examined. The CellScope Loa can be seen in action at https://www.youtube.com/watch?v=Iyzzg7dTuvY. Results are delivered in just 2 min, with an additional 1 min of required hands-on time. The investigators showed an excellent correlation ($r$ = 0.99) with gold standard microscopic quantification of microfilariae in Giemsa-stained blood films [13] and subsequently applied their device successfully in a "test and not treat" onchocerciasis control strategy in Cameroon where a high prevalence of coexisting loiasis exists [14]. Current mass drug administration efforts for onchocerciasis (African river blindness) rely on administration of ivermectin to at-risk populations in endemic settings. Unfortunately, ivermectin cannot be safely administered to individuals

with concurrent loiasis when a high *L. loa* microfilaremia (i.e., >20,000 microfilariae per milliliter blood) is present due to the risk of severe adverse events such as fatal encephalopathy. Individuals can be excluded from receiving ivermectin if they are known to have a high degree of *L. loa* microfilaremia; however, traditional light-microscopic microfilariae quantification is impractical in many endemic settings, thus limiting the range of onchocerciasis control programs. The authors showed that the CellScope Loa provided a viable alternative method to traditional microscopy for quantifying *L. loa* microfilaremia in endemic settings [14]. They analyzed the blood of 16,259 individuals living in the Okola health district in Cameroon and were able to identify 340 (2.1%) individuals who had >20,000 microfilariae/ml blood. These individuals were excluded from receiving ivermectin. An additional 397 individuals were excluded for other conditions (e.g., pregnancy, serious acute illness), while a total of 15,522 (95.5%) individuals received ivermectin based on negative or low *L. loa* microfilariae counts [14]. No severe adverse reactions were observed in this study, thus supporting the safety of the CellScope Loa screening and exclusion ("test and not treat") method. It is important to note that the CellScope Loa cannot differentiate between *L. loa* and other microfilariae such as *Wuchereria bancrofti* and *Mansonella perstans* which may also present in *L. loa*-endemic settings. However, the authors argue that *M. perstans* rarely reaches high levels in the blood and is thus unlikely to negatively impact CellScope Loa microfilaria counts, while *W. bancrofti* microfilaremia occurs primarily at night between 10 pm and 2 am [14]. In contrast, *L. loa* microfilaremia occurs primarily between 10 am and 2 pm, and this is when blood should be collected for CellScope Loa testing. Further use of mobile phone video microscopy could conceivably be applied to the detection of other motile parasites such as *Strongyloides* larvae, various intestinal protozoa, and the blood stage of trypanosomes.

Finally, an exciting new use of mobile phone microscopy is to pair the camera capabilities of a mobile phone with portable "lab-on-a-chip" devices to detect parasite antigens or DNA [8]. Stemple et al. took advantage of a phone's ability to illuminate and detect scatter/absorption to measure *P. falciparum* histidine-rich protein-2 (HRP-2) in whole blood samples [15]. Another group used a mobile phone recorder to detect fluorescence produced following loop-mediated isothermal amplification (LAMP) in a microfluidic chip [16].

Incorporation of mobile phones into diagnostic parasitology offers several advantages. Mobile phones are widely available, even in some remote and resource-limited settings and are more easily accessible and portable than light microscopes. They also have limited electricity requirements and can be used in the field for several hours without needing to be charged. Portable or battery- or solar-powered chargers can greatly extend the use of the phone in the field. Finally, mobile phones allow for electronic image transmission to others such as a skilled reader at a local or reference laboratory. Thus the use of mobile phone microscopy allows healthcare workers to overcome major burdens that are present in resource-limited settings: insufficient access to expensive instruments, reagents, skilled microscopists, and electricity [8]. Continued refinement of portable microscopes, attachable phone lenses, microfluidic devices, and software applications for acquiring and analyzing

images will likely enhance our use of this widely available tool. Of note, apps and equipment will continually need to be updated to accommodate the latest mobile technology and operating systems.

## *Digital Image Analysis*

Still images and videos acquired by both conventional methods and mobile phone cameras have the potential to be analyzed by computational algorithms to automate and enhance detection of parasites from clinical specimens including blood [17–20] and stool [21–23]. Several investigators have shown utility in this approach, although it remains primarily in the research realm at this time. With further advancements and production of commercially available and FDA/CE in vitro diagnostic (IVD) products, digital image analytic systems have the potential to revolutionize parasite diagnostics.

Use of computer-aided diagnosis has perhaps been the most widely explored in the field of malaria diagnostics [18, 19]. Conventional malaria diagnosis is traditionally accomplished by microscopic examination of Giemsa-stained thick and thin blood films by a highly trained reader. Microscopy remains the gold standard for laboratory diagnosis of malaria and allows for detection of as few as 10–50 parasites per microliter of blood (0.002–0.001% parasitemia) under ideal conditions [24]. However, microscopy under standard field conditions in endemic settings is commonly less sensitive (100–500 parasites/μL blood) due to lack of microscope maintenance, reagent quality, and appropriate training [24, 25]. Competency is also difficult to maintain in non-endemic settings where there is a paucity of positive cases. Given these challenges, there has been a long-standing desire to supplement or replace manual and subjective human interpretation with computer-aided diagnostics [18–20]. Computer-aided malaria diagnosis typically involves several steps, including digital image acquisition from stained slides, image enhancement to remove noise and sharpen objects of interest, detection and segmentation of individual blood cells, identification of specific diagnostic features, and application of a computer algorithm for categorizing and classifying cells (e.g., infected vs. non-infected) [18]. A number of different methods have been used for image acquisition (e.g., light microscopy, fluorescent microscopy, image-based cytometry, scanning electron microscopy) and computer-based classification (decision trees, basic artificial neural networks, deep learning algorithms) with variable degrees of success for detecting, differentiating, and quantitating *Plasmodium* parasites [18]. There are currently no commercially available systems for this purpose in the United States, but several manufacturers are working on creating and improving systems for future commercial use. One such system is the Parasight Platform by Sight Diagnostics (https://www.sightdx.com) that uses a small desktop instrument to capture and analyze approximately 800 images from a liquid blood monolayer sample in 4 min. EDTA blood is first stained for 10 min with a proprietary fluorescent dye solution (Fig. 2); thus total processing and analytic time is <15 min. Computer vision and

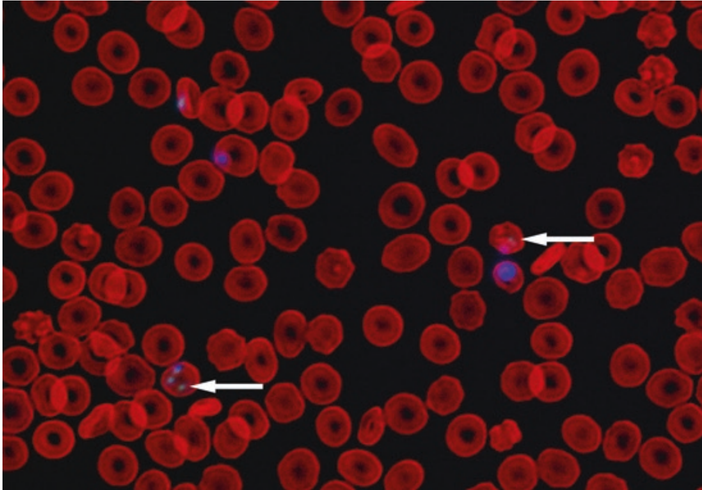

**Fig. 2** Screen capture of the Parasight blood film analysis showing DNA indicated by faint green fluorescence (corresponding with the presence of intraerythrocytic *Plasmodium* parasites; arrows) and RNA/lysosomes indicated by blue/purple fluorescence. Howell-Jolly bodies will also stain green and thus must be differentiated from true parasites by the computer algorithm. This is one example of computer-based parasite detection using deep-learning algorithms

statistical models detect infected cells, determine the infecting species, and calculate percent parasitemia. A recent two-site trial in Chennai, India, and Nairobi, Kenya, showed the Parasight to have 99% and 100% sensitivities compared to gold standard microscopy and PCR, respectively, with specificities 98–100% for detection of *Plasmodium falciparum* and *P. vivax* in endemic settings [17]. The calculated lower limit of detection of the Parasight was 20 parasites/μl, thus equivalent to traditional microscopy. Further modifications are needed to accurately differentiate other human *Plasmodium* species and obtain lower detection limits [17].

## *Molecular Diagnostics*

As with all areas of clinical microbiology, the past few decades have witnessed an explosion of molecular diagnostic methods for detection of pathogenic microorganisms. While parasite molecular diagnostics have lagged somewhat behind those for viruses, bacteria, and fungi, a vast number of laboratory-developed tests (LDTs) and, eventually, commercially available kits have become available. Most described molecular diagnostics for parasitic infections utilize PCR, but other forms of NAATs and sequencing assays have been described and applied to various clinical settings. The following sections highlight the most significant of these methodologies and advances.

### Nucleic Acid Amplification Methods

A variety of NAATs have been described for virtually every parasite capable of infecting humans [26–29]. Most are LDTs, but several commercial methods also exist. A few tests are now cleared/approved by the US Food and Drug Administration (FDA) for IVD use, and many more are CE-marked. Within the past decade, several multiplex panels including gastrointestinal and sexually transmitted parasitic pathogens have received clearance from the FDA and have been readily adopted by both small and large clinical microbiology laboratories [30]. The advantages and disadvantages of NAATs for routine clinical diagnosis are discussed in previous chapters, and the following section will focus only on those issues relevant to detection of parasite identifications.

In general, NAATs offer the highest sensitivity and specificity of the laboratory diagnostics currently available for parasitic infections and are less subjective than microscopy-based morphologic assessments. These characteristics make them ideally suited for detection of low-level infections (e.g., asymptomatic malaria in endemic settings), differentiation of morphologically identical or similar parasites (e.g., *Entamoeba histolytica* vs. *E. dispar*, *Plasmodium falciparum* vs. *Babesia microti*), identification of parasites with suboptimal microscopic morphology (e.g., *Plasmodium* species in patients taking antimalarial drugs), and detection of coinfections [26]. However, NAATs have several significant limitations as well: they are usually high-complexity methods requiring expensive reagents and instruments, highly trained personnel, and a suitable laboratory setting with a constant source of electricity [26]. Also, as mentioned above, quantitative results may not correlate with traditional microscopic measurements (e.g., malaria percent parasitemia), and therefore reflex microscopy may still be required for positive cases [7, 31]. These limitations significantly constrain the use of NAATs in resource-limited settings where many parasitic infections are endemic. Additionally, NAATs are not generally performed on a rapid basis, particularly in situations where it is most cost-effective to test specimens in batches. This is not appropriate for potentially life-threatening infections like malaria, where testing must be performed within several hours of the specimen being obtained [7].

Assay design poses another important challenge. Parasites comprise an incredibly diverse range of organisms, including protozoa, helminths, and arthropods, and their phylogeny is not adequately reflected by their traditional classifications [32]. For example, the protozoa comprise many genetically unrelated eukaryotic organisms, and it is impossible to create a single set of primers to amplify all of them without amplifying non-targeted (e.g., host) DNA. The same holds true for the helminths and the arthropods. Obtaining sufficient assay inclusivity may also be problematic, particularly when many different species in one parasite genus can infect humans (e.g., *Cryptosporidium* spp.). In these instances, it may be difficult to detect all species in a single assay with equal sensitivity. Similarly, it is challenging to capture all of the parasites that may be found in a single source; for example, there are a large number of nematodes, trematodes, and cestodes that can inhabit the intestinal tract and biliary tree and be detected in stool. On the other hand, some

parasites are genetically similar to related organisms, and thus, non-targeted organisms may be detected (i.e., cause false-positive results) when present in high amounts. This is the case with the nonpathogenic ameba, *Entamoeba dispar*, using the FilmArray® GI panel (Biomérieux, BioFire Diagnostics, Salt Lake City, UT); the presence of high amount of *E. dispar* (i.e., ≥100,000 cysts/mL) causes false-positive results for the true pathogen, *Entamoeba histolytica* [33]. The commonly used genetic targets and clinical indications for select parasitic infections are listed in Table 1. The parasites for which the greatest number of commercial assays exists are *Trichomonas vaginalis* and select gastrointestinal pathogens (Tables 2 and 3, respectively). Gastrointestinal multiplex molecular assays are discussed in greater detail below to illustrate the diversity and variability that exist among commercially available options.

Gastrointestinal parasites infect billions of individuals worldwide and cause a broad spectrum of clinical manifestations including diarrhea, dysentery, anemia, intestinal obstruction, rectal prolapse, and vitamin B12 deficiency [4, 28]. Several protozoan parasites are relatively common causes of diarrhea and are traditionally detected using microscopic examination (e.g., ova and parasite exam) and antigen detection methods (e.g., enzyme immunoassay, direct fluorescent antibody tests). Most recently, several protozoa have been included on commercial multiplex molecular panels for bacterial, viral, and parasitic pathogens [34]. At this time, the molecular multiplex panels that are FDA approved/cleared are the FilmArray Gastrointestinal Panel (GIP) (BioFire Diagnostics, A bioMérieux company, Salt Lake City, UT) [33, 35, 36], xTAG® Gastrointestinal Pathogens Panel (GPP) (Luminex®, Austin, TX) [35, 37–40], and the BD Max™ Enteric Parasite Panel (EPP) (Becton-Dickinson, Franklin Lakes, NJ). Other commercially available multiplex molecular panels are the NanoCHIP panels (Savyon Diagnostics, Ashdod, Israel) (https://savyondiagnostics.com/product/nanochip-gastroentestinal-combi-i/) [37, 41] and EasyScreen™ (Genetic Signatures, Newtown, Australia) [42]. All panels detect *Cryptosporidium parvum*, *C. hominis*, *Entamoeba histolytica*, and *Giardia duodenalis,* while *Blastocystis hominis*, *Cyclospora cayetanensis*, and *Dientamoeba fragilis* are less common targets. In general, these panels provide excellent sensitivity and specificity for their parasite targets (>90%) and allow for similar or increased detection when compared to conventional methods [28]. However, they are relatively expensive, and significant differences exist in assay methodologies, complexity, and throughput.

At one end of the spectrum is the FilmArray GIP, a moderate complexity test that performs extraction and nested PCR for 22 viral, bacterial, and parasitic within a self-contained cartridge [33]. Results are available in approximately 1 h, with minimal (~2 min) hands-on time. It is the fastest, yet lowest throughput, FDA/CE IVD system currently available, producing a result for one patient per cartridge. Cartridges can be tested one at a time on the FilmArray 2.0 instrument, while 2–12 cartridges can be tested at once on the new scalable FilmArray Torch instrument (http://www.biofiredx.com/products/the-filmarray-panels/).

One the other end of the spectrum are the NanoCHIP panels and the xTAG GPP, which are high-complexity methods for detection of multiple bacterial and parasitic

**Table 1** Commonly used genetic targets of select parasites and clinical indications for nucleic acid amplification testing[a]

| Parasite (disease[s]) | Commonly used nucleic acid target(s) | Comments |
|---|---|---|
| *Ascaris lumbricoides* (ascariasis) | ITS1, ITS2, Cyt b | Higher sensitivity than with O&P. No FDA-approved/cleared tests |
| *Babesia* species (babesiosis) | 18S rDNA, 16S-like rRNA gene, ITS, thiamine pyrophosphokinase gene | No FDA-approved/cleared tests. PCR LDTs available at select reference laboratories |
| *Brugia malayi* (lymphatic filariasis) | Hha I repeat region | No FDA-approved/cleared tests |
| *Cryptosporidium* species (cryptosporidiosis) | 18S rDNA, *Cryptosporidium parvum*-specific 452-bp fragment, *Cryptosporidium* oocyte wall protein (COWP) gene, DnaJ-like protein gene | Component of FDA and CE IVD multiplex gastrointestinal pathogen panels |
| *Cyclospora cayetanensis* | SSU rDNA, ITS | Component of FDA and CE IVD multiplex gastrointestinal pathogen panels |
| *Dientamoeba fragilis* (*D. fragilis* infection) | SSU rDNA, 5.8S rDNA, ITS1 | No FDA-approved/cleared tests |
| *Entamoeba histolytica* (amebiasis) | SSU rDNA | Component of FDA and CE IVD multiplex gastrointestinal pathogen panels |
| Free-living amebae (amebic keratitis, granulomatous amebic encephalitis, primary amebic meningoencephalitis) | 18S rDNA | Equal sensitivity to agar culture for detecting *Acanthamoeba* spp. and *Naegleria fowleri*. No FDA-approved/cleared tests |
| *Giardia duodenalis* (giardiasis) | SSU rDNA, β-giardin gene, glutamate dehydrogenase gene, elongation factor 1(EF1)-α gene, triosephosphate isomerase gene | Component of FDA and CE IVD multiplex gastrointestinal pathogen panels |
| Hookworms (hookworm infection, ancylostomiasis) | ITS2 | Higher sensitivity than with O&P. No FDA approved/cleared tests |
| *Leishmania* species (leishmaniasis) | kDNA, 16S rDNA, glucose phosphate isomerase gene, glucose-6-phosphate dehydrogenase gene, ITS1, ITS2, GPI gene | FDA-approved qualitative test (SMART Leish) for detection of *Leishmania major*; limited availability. Species-specific and sequencing-based assays used for identification of species causing mucocutaneous leishmaniasis in order to guide treatment |

(continued)

**Table 1** (continued)

| Parasite (disease[s]) | Commonly used nucleic acid target(s) | Comments |
|---|---|---|
| *Loa loa* (loiasis) | Expressed sequence tags (LLMF72 and LLMF269), 15r3 repeat region | No FDA-approved/cleared tests |
| *Onchocerca volvulus* (onchocerciasis, African river blindness) | O-150 repeat sequence | No FDA-approved/cleared tests |
| *Plasmodium* species (malaria) | 18S rDNA (most common), mitochondrial DNA, reticulocyte binding protein 2 (rbp2) gene, *P. ovale curtisi* and *P. o. wallikeri* tryptophan-rich antigen (*poctra* and *powtra*) genes, *dhps/dhft*, *Pfcrt*, *Pfmdr*, *PfATPas6/pfmdr*, *PfKelch13* | Primary diagnosis where available (if performed rapidly), detection of low levels of parasitemia, confirmation of blood smear analysis (particularly when morphology is suboptimal), differentiation of *P. ovale* subspecies, detection of drug resistance markers; multiple commercial kits; no FDA-approved/cleared tests |
| *Schistosoma* species (schistosomiasis) | Highly repeated short 0.64 kb DNA sequence, cytochrome c oxidase gene | Higher sensitivity than with O&P. No FDA-approved/cleared tests |
| *Strongyloides stercoralis* (strongyloidiasis) | SSU rDNA, 28S | Higher sensitivity than with O&P. No FDA-approved/cleared tests |
| *Toxoplasma gondii* (toxoplasmosis) | RE (REP-52) and B1 genes | Performed on CSF, amniotic fluid, ocular fluid and tissue. Clinical sensitivities of 64%–100%. Recommended for testing amniotic fluid in women with documented acute toxoplasmosis during pregnancy. Multiple commercial kits available; no FDA-approved/cleared tests |
| *Trichomonas vaginalis* (trichomoniasis) | 16S rRNA, RNA probe (no amplification) | NAATs have the highest sensitivity and specificity of all methods; many commercial options; FDA-approved/cleared singleplex assays and component of FDA-approved/cleared multiplex panels for sexually transmitted infections and causes of vaginitis/vaginosis |
| *Trichuris trichiura* (trichuriasis) | SSU rDNA | Higher sensitivity than with O&P. No FDA-approved/cleared tests |

(continued)

**Table 1** (continued)

| Parasite (disease[s]) | Commonly used nucleic acid target(s) | Comments |
|---|---|---|
| *Trypanosoma cruzi* (Chagas disease) | 18S rDNA, kDNA, nuclear DNA (minisatellite TCZ region) | No FDA-approved/cleared tests for clinical diagnostics; LDTs used for blood donor screening in some endemic settings |
| *Trypanosoma brucei* (human African trypanosomiasis) | 177-bp satellite repeat, ITS1 rDNA, 18S rDNA, SRA gene, expression-site-associated genes 6 and 7 (ESAG6/7) | No FDA-approved/cleared tests |
| *Wuchereria bancrofti* (lymphatic filariasis) | 188 bp DNA sequence Ssp-1 | No FDA-approved/cleared tests |

Abbreviations: *bp* base pair, *DNA* deoxyribonucleic acid, *CE* Conformité Européenne, *FDA* US Food and Drug Administration, *ITS* internal transcribed spacer, *IVD* in vitro diagnostic, *kDNA* kinetoplastid DNA, *LDT* laboratory-developed test, *NAATs* nucleic acid amplification test, *nDNA* nuclear DNA, *O&P* ova and parasite exam, *rDNA* ribosomal DNA, *RNA* ribonucleic acid, *SSU* small subunit

[a]Information obtained from the corresponding manufacturer product package inserts and websites and from [26–28, 55]

targets requiring separate extraction, amplification, and detection steps. While 24 (xTAG) or 96 (NanoCHIP) specimens can be tested at once, testing time takes 5 h or more.

Given the significant differences among tests, the laboratory director must give serious consideration to which system would be best suited for his or her needs and how the test would be best used in clinical practice. A thorough analysis of FDA-approved/cleared panels as well as the pros and cons of using large multiplex molecular panels as the first-line method for detecting intestinal pathogens were recently published [30, 36, 43, 44]. Regardless of whether conventional methods or multiplex molecular panels are used for testing, most professional societies agree that the decision to perform testing should be based on a careful evaluation of the patient and assessment of disease severity and risk factors. Given that most cases of gastroenteritis manifesting with vomiting and diarrhea are self-limited, testing and treatment are not required [45, 46]. Furthermore, most cases of infectious diarrhea are due to viruses or bacterial toxins, and not parasites, particularly in non-endemic settings. Testing should be reserved for patients with warning signs for severe disease (e.g., bloody diarrhea, fever) or risk factors for severe infection (e.g., immunocompromised state) [30, 36, 44]. The American College of Gastroenterology has recognized that molecular diagnostic tests can provide a greater diagnostic yield when testing is indicated, when compared to conventional tests [46].

It is important to note that a major limitation of singleplex and multiplex molecular assays is that only targeted analytes are detected. For example, helminths are an uncommon, but important, cause of diarrhea in certain populations, and none are included in the currently available commercial multiplex molecular assays. Helminths can also cause other significant clinical manifestations, and molecular amplification-based tests have consistently been shown to provide increased

**Table 2** Comparison of select commercially available molecular tests for *Trichomonas vaginalis*[a]

| Test (manufacturer) | Instrument(s) | Amplification method | Throughput | FDA-approved/ cleared specimen types | Regulatory IVD status |
|---|---|---|---|---|---|
| AmpliVue® trichomonas assay (Quidel) | No specific brand required | Helicase-dependent amplification | 1 specimen in 50 minutes | Clinician-collected vaginal swabs. Symptomatic and asymptomatic patients. | FDA cleared, CE marked |
| Aptima® *Trichomonas vaginalis* assay (Hologic) | Panther™ Tigris™ | Transcription mediated amplification | *Panther*: 275 samples in 8 h; up to 750 in 16 h *Tigris*: 450 samples in 8 h; up to 1000 in 13.5 h | Clinician-collected vaginal swabs. Clinician-collected endocervical swabs. Specimens in PreservCyt solution. Urine specimens (F). Symptomatic and asymptomatic patients. | FDA cleared, CE marked |
| BD max™ CT/GC/TV assay (BD)[b] | BD max™ system | Real-time multiplex PCR | 1–48 specimens in 8 h | Patient-collected vaginal swabs. Clinician-collected endocervical swabs. Urine specimens (F). Symptomatic and asymptomatic patients. | FDA cleared, CE marked |
| BD max™ vaginal panel (BD)[b] | BD max™ system | Real-time multiplex PCR | 1–48 specimens in 8 h | Vaginal swabs. Symptomatic patients only. | FDA cleared, CE marked |
| Probe Tec™ TV Q[x] (BD) | BD viper™ | Strand displacement amplification | >700 specimens in 8 h | Patient-collected vaginal swabs. Clinician-collected endocervical swab. Urine specimens (F). Symptomatic and asymptomatic. | FDA cleared, CE marked |

(continued)

**Table 2** (continued)

| Test (manufacturer) | Instrument(s) | Amplification method | Throughput | FDA-approved/ cleared specimen types | Regulatory IVD status |
|---|---|---|---|---|---|
| Solana® trichomonas assay (Quidel®) | Solana instruments | Helicase-dependent amplification | 1 specimen in 35 min | Clinician-collected vaginal swabs. Urine specimens (F). Symptomatic and asymptomatic. | FDA cleared, CE marked |
| Xpert® TV assay (Cepheid®) | GeneXpert® I, II, IV, and XVI systems or GeneXpert infinity (48 and 80 modules) | Real-time PCR | 1 specimen cartridge in ≥40 min | Patient-collected vaginal swabs. Clinician-collected endocervical swabs. Urine specimens (F and M). Symptomatic and asymptomatic. | FDA cleared, CE marked |

Abbreviations: BD, Becton Dickenson; CE, Conformité Européenne; F, female; FDA, US Food and Drug Administration; M, male; NOS, not otherwise specified; PCR, polymerase chain reaction; rRNA, ribosomal RNA

BD. Women's Health and STIs. 2018. http://moleculardiagnostics.bd.com/syndromic-solutions/womens-health-stis/. Accessed 7/6/2018

[a]Information obtained from the corresponding manufacturer product package inserts and websites.

[b]The BD Max CT/GC/TV detects DNA of *Chlamydia trachomatis*, *Neisseria gonorrhoeae,* and *T. vaginalis*, while the BD Max Vaginal Panel detects DNA of *T. vaginalis*, *Candida* spp., and several bacterial flora whose levels may be disrupted in bacterial vaginosis (*Lactobacillus* spp., *Gardnerella vaginalis*, *Atopobium vaginae*, *Megasphaera*-1, and bacterial vaginosis-associated bacteria-2)

sensitivity over the traditional ova and parasite examination. The commonly used targets for intestinal helminths are outlined in Table 1 and were recently reviewed [27]. Another limitation of multiplex panels is that a change or addition of any primer or probe sequence necessitates revalidation of the entire test, including detection sensitivity and specificity for each targeted analyte.

## Sequencing-Based Approaches

Targeted and unbiased sequencing-based approaches overcome some of the limitations of singleplex and multiplex NAATs mentioned above and provide expanded options for parasite detection. While Sanger sequencing is still commonly used in the clinical laboratory setting, next-generation sequencing (NGS) holds promise for more rapid, high-throughput detection of parasites and the ability to detect mixed infections [28, 29]. Testing is performed primarily in the research setting at this

**Table 3** Comparison of select commercially available molecular multiplex tests for gastrointestinal parasitic pathogens[a]

| Test (manufacturer) | Instrument(s) | Amplification/ detection method | Classes of organisms detected | Parasites detected | Workflow and throughput | Specimen types | Regulatory IVD status |
|---|---|---|---|---|---|---|---|
| BD max™ enteric parasite panel (BD) | BD max™ system | Multiplex real-time PCR/ hydrolysis probes | Parasites | *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Entamoeba histolytica*, *Giardia duodenalis* | Automated from extraction to answer; 24 specimens per 4.5 h including ~50 min hands-on time | Unpreserved stool, stool in 10% formalin | CE marked, FDA cleared |
| EasyScreen™ enteric protozoan detection (genetic signatures) | Multiple compatible extraction and amplification platforms | Multiplex real-time PCR | Parasites | *Cryptosporidium* spp., *Blastocystis hominis*, *Dientamoeba fragilis*, *Giardia duodenalis*, *Entamoeba histolytica* | Separate extraction and amplification steps; 96–384 specimens/3–5 hours (excluding sample processing) | Not specified | CE marked |
| FilmArray gastrointestinal panel (BioFire, Biomérieux) | FilmArray 2.0 or FilmArray torch | Multiplex nested PCR (first step is RT-PCR)/ intercalating fluorescent DNA dye with melting temperature analysis | Bacteria, parasites, viruses | *Cryptosporidium* spp.,[b] *Cyclospora cayetanensis*, *Giardia lamblia*, *Entamoeba histolytica* | Fully automated, extraction to answer; 1 specimen per ~1 hour, including ~2 minutes sample processing | Stool in Cary-Blair medium | CE marked, FDA cleared |

(continued)

**Table 3** (continued)

| Test (manufacturer) | Instrument(s) | Amplification/ detection method | Classes of organisms detected | Parasites detected | Workflow and throughput | Specimen types | Regulatory IVD status |
|---|---|---|---|---|---|---|---|
| NanoCHIP® gastrointestinal COMBI and parasites panels (Savyon diagnostics) | Extraction system not specified, 3 recommended amplification platforms, NanoCHIP® instrument for detection | Multiplex conventional PCR/ complementary capture oligonucleotides in an electronic microarray | Bacteria (only on COMBI I and II panels), parasites | COMBI I panel: *Cryptosporidium* spp. *Giardia duodenalis*, *Entamoeba histolytica* Parasites panel I and COMBI II panel: Same as COMBI I but also *Blastocystis hominis*, *Dientamoeba fragilis* | Separated extraction and amplification and NanoCHIP detection steps; 96 specimens/run; total time not provided | Unpreserved stool, stool in Cary-Blair medium | CE marked |
| xTAG® gastrointestinal pathogen panel (Luminex®) | Magpix® or Luminex® 100/200™ system | Multiplex conventional RT-PCR/bead-based tag-sorting | Bacteria, parasites, viruses | *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Entamoeba histolytica*, *Giardia lamblia*[c] | Separate extraction, amplification, and detection steps; 24 specimens per ~5 h | Unpreserved stool, stool in Cary-Blair medium | CE marked, FDA cleared |

Abbreviations: *BD* Becton Dickenson, *CE* Conformité Européenne, *FDA* US Food and Drug Administration, *IVD* in vitro diagnostic use, *PCR* polymerase chain reaction, *RT-PCR* reverse transcription PCR

[a]Information obtained from the corresponding manufacturer product package inserts and websites

[b]Detects *C. hominis*, *C. parvum*, and other less common *Cryptosporidium* spp. that infect humans

[c]Intended use of the FDA-cleared version states "xTAG® GPP positive results are presumptive and must be confirmed by FDA-cleared tests or other acceptable reference methods"

point, although some sequence-based applications for parasite diagnosis and discrimination are used at specialized reference facilities. For example, the CDC has largely replaced isoenzyme analysis of cultured *Leishmania* parasites with Sanger-based sequence analysis of the internal transcribed spacer 2 (ITS2) gene for species identification [47]. By using a generic primer set for amplifying parasite DNA for sequencing, CDC scientists overcame the limitations of the existing singleplex *Leishmania* species, genus, and complex-specific PCR assays which vary significantly in their sensitivity and specificity [47].

### *Proteomics*

Proteomic approaches such as matrix-assisted laser-desorption time-of-flight (MALDI-TOF) mass spectrometry have revolutionized many areas of clinical microbiology, including bacteriology, mycology, and mycobacteriology. Unfortunately, these technologies have been significantly slower in penetrating the field of clinical parasitology due to the complex nature of commonly analyzed specimens (e.g., stool) and lack of commonly used culture techniques for parasites. The primary use of proteomics in clinical parasitology has been for acquiring parasitic proteome data [48–50] and characterizing/differentiating protozoa isolated using culture, filtration, or other techniques [51, 52]. MALDI-TOF has also been used for identifying insect disease vectors [53]. Lastly, investigators have used LD-TOF for detection of hemozoin (malaria pigment) directly from whole blood after red cell lysis [54]. This method reports detection levels of 10 parasites/μL blood and is thus equivalent to the sensitivity of the thick blood film. *Plasmodium* species identification and calculation of percent parasitemia are not possible by this method, and thus reflex blood film examination must be performed [54].

## Conclusions

Several advanced diagnostic techniques show promise for revolutionizing clinical parasitology, moving it from a field that is dependent on manual morphologic examination by the unaided human eye, to a field that embraces cutting-edge technology in digital imaging, computer-aided image analysis, deep-learning algorithms, molecular amplification, next-generation sequencing, metagenomics, and proteomics. The paucity of commercially available tests with FDA approval/clearance or CE-marked status has been a major limiting factor in the widespread adoption of advanced diagnostic techniques in the United States. However, we have seen IVD tests such as molecular multiplex panels for gastrointestinal pathogens, and sexually transmitted infections become rapidly incorporated into routine laboratory testing menus and algorithms once they become available. Thus it is apparent that having expanded options for commercial IVD tests using advanced technologies will strongly advance their adoption into the clinical parasitology laboratory.

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# Advanced Methods for Detection of Foodborne Pathogens

**Heather Harbottle**

## Introduction

Methods for the detection of bacterial and viral foodborne pathogens to assure the safety and cleanliness of human food have been successfully utilized for many decades. While traditional microbiological methods have been used and trusted for years, the emergence and acceptance of molecular methods to identify and characterize foodborne pathogens have been increasing exponentially in the past decade. Most notably, since the publication of the first edition of this chapter [1], whole genome sequencing (WGS) for identification and characterization of bacterial foodborne pathogens is beginning to be commonly used to supplant more traditional methods in public health surveillance. This update will not describe in detail the traditional methods of detection and characterization of foodborne pathogens described in the previous edition of this chapter; instead, this review will focus exclusively on the comparison of traditional methods to the current state-of-the-art molecular techniques, indicating where possible, which of these methods have become accepted as standard.

In 2011, the CDC listed the top four foodborne pathogens as norovirus, non-typhoidal *Salmonella*, *Clostridium perfringens*, and *Campylobacter* spp. [2]. The leading cause of foodborne illness resulting in hospitalization and/or death was attributed to *Salmonella enterica* serotypes. According to the CDC's latest data from 2013 to 2016, *Campylobacter* spp. have been identified as the leading cause of foodborne infections followed by *Salmonella enterica*, *Shigella*, STEC, *Cryptosporidium*, *Yersinia*, *Vibrio*, *Listeria*, and *Cyclospora* spp. [3]. In 2013, the CDC published a report entitled the "Antibiotic Resistance Threats in the United States" that ranked the most urgent, serious, and concerning antimicrobial-resistant

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bacterial infections detrimental to human health [4]. The rankings descend in importance from urgent (the most threatening resistant infections involving high-consequence antimicrobials and urgent action), to serious (those infections that are significant antibiotic resistance threats but do not require urgent action at this time), and finally to concerning (those infections that cause serious illness but at this time multiple therapeutic options may be available). These rankings were based on several factors including the estimated burden of illness in the USA, as well as the number of available antibiotics which could treat these resistant infections. The leading foodborne infections from 2011 and 2016, *Salmonella enterica* serotypes and *Campylobacter* spp., are both ranked as serious resistance threats to public health by the CDC [2–4]. Any of these zoonotic strains which may also be resistant to carbapenems are classified as urgent threats, including *Salmonella enterica* serotypes, *Campylobacter* species, and pathogenic *Escherichia coli* [4]. Therefore, a major focus of this chapter will describe detection and characterization of these leading causes of foodborne infections that threaten human health.

## Traditional Methods

Traditional methods, consisting of bacterial and/or viral culture of food samples using microbiological media with biochemical identification of bacterial genera or cell culture techniques for viruses, continue to be considered the most reliable and successful methods for foodborne pathogen detection and currently remain the gold standard. The Food and Drug Administration's (FDA) Bacterial Analytical Manual (BAM) currently describes the officially accepted methodology for detection of bacteria, viruses, yeast, and molds [5]. These fundamental microbiological assays remain the cornerstones of most pathogen detection schemes, involving standard sample collection, selective agar plating, and characterization via biochemical tests for proper identification. However, these traditional culture methods are slow, labor intensive, and can require specialized skills. In a typical bacterial foodborne disease outbreak, a minimum of 5–7 days is required to culture and identify an isolated colony following BAM recommendations. The time necessary for microbiological and biochemical identification of the bacterial strain may delay the proper diagnosis and subsequent treatment regime, resulting in a longer hospital stay [2]. Therefore, a significant demand for a more rapid detection of pathogens (minutes, rather than days) has arisen. Alternate molecular methods, including culture-independent diagnostic techniques (CIDT), have been developed in an attempt to reduce or eliminate rate-limiting steps and thus reduce the time required to provide public health officials the identity of the cause of a foodborne disease outbreak. A partial list of some rapid methods and alternative molecular methods are listed in the FDA BAM in Appendix I, although these are not methods officially used or endorsed by the FDA [5].

## Serotyping

Following the identification of a bacterial foodborne pathogen utilizing selective media and biochemical testing, common and successful methods for further characterizing these strains involves the use of antibodies. For bacteria such as *Salmonella enterica* strains, serotyping per the Kauffman-White scheme is one of the oldest and most successful subtyping methods available [6]. Serotyping is based on antibody recognition of the O and the H antigens present on *S. enterica* flagella, and typing is achieved via agglutination testing using monoclonal antibodies specific for each variant. There are over 2500 serotypes of *Salmonella enterica* now recognized. Although serotyping is a widely used and specific method to characterize *S. enterica* strains, it is laborious, time consuming, requires specialized skills, and the logistics for maintaining adequate stocks of antisera can be challenging.

Molecular serotyping methods, such as multiplex polymerase chain reaction (PCR), real-time PCR systems, probe detection, gene sequencing, single-nucleotide polymorphism, and whole genome sequencing methods, have been established for some of the most common foodborne serotypes of *S. enterica* and *E. coli* found in the USA and Europe [7–19]. Leader et al. [7] tested over 700 strains of *Salmonella* serotypes using a multiplex PCR system capable of detecting the 50 most common serotypes in the USA with an accuracy of 89%, when compared to traditional serotyping. Taking multiplex PCR of the O and H antigens of *S. enterica* serotypes one step further, a technology whereby multiplex PCR products are detected via a liquid array of fluorescently labeled antigen-specific probes coupled to beads was developed to increase the throughput and specificity of the multiplex PCR molecular serotyping of *Salmonella* serotypes [10, 11]. McQuiston et al. [11] utilized this technology amplifying the *fliB* and *fliC* genes of the H antigen to characterize 500 serotypes of *S. enterica* in parallel with traditional serotyping techniques. This method correctly identified 461 (92.2%) isolates, partially serotyped 47 (9.4%) isolates, and characterized 13 (2.6%) isolates as monophasic or nonmotile strains. Only 39 (7.8%) strains were not correctly identified. The authors suggest that this methodology is sufficiently high throughput to screen 100 isolates per day and is useful for outbreak detection when used in combination with the similar O-antigen scheme developed by Fitzgerald et al. [10]

Single-nucleotide polymorphism (SNP) typing has been investigated for molecular serotyping. Highly informative sequence variations in the *gnd* gene encoding for the serotype of *E. coli* have been utilized to screen retail beef for *E. coli* O157 and the "big six" *E. coli* non-O157 serotypes that are flagged by the US Department of Agriculture (USDA) as contaminants of public health concern [12]. In order to develop SNP types correlating to these *E. coli* serotypes of concern, the *gnd* region was sequenced in a "collection of 195 STEC isolates, including isolates belonging to O157:H7 (n = 18), O26(n = 21), O45 (n = 19), O103(n = 24), O111 (n = 24), O121 (n = 23), O145 (n = 21), and ten other STEC serogroups (n = 45)." Subsequent to this analysis, additional informative SNPs were identified for molecular serotyping.

Twelve informative SNPs have been identified and multiplexed into a SNP typing assay by single base pair extension chemistry. Using this technology, SNP types were determined for the seven clinically important STEC serogroups and, although multiple SNP types per serogroup were identified, "there were no overlapping SNP types between serogroups." [12]

Microarray methods to determine serotype of *Salmonella enterica* strains have been developed but to date do not have a 100% correlation with the traditional Kauffman-White method. The *Salmonella* genoserotyping array (SGSA) detects 57 of the most commonly reported serovars through detection of the genes encoding surface O and H antigens [13]. This microarray was evaluated and validated by testing 1874 isolates from human and nonhuman sources at 4 laboratories in 3 countries, correctly identifying 96.7% of isolates from the target 57 serovars. Test specificity and sensitivity was greater than 98% for *S*. Enteritidis and 99% for *S*. Typhimurium. However, the SGSA array has its greatest utility as a rapid screen for those most common serotypes included in the 57 targets and cannot correctly detect other serotypes including those which may be unusual or on the rise [13]. Patel et al. [14] developed a custom *E. coli* pan-genome *E. coli* microarray (the FDA *E. coli* identification or FDA-ECID array) as a "molecular toolbox" for use in bacterial characterization and outbreak tracking. The FDA-ECID array was designed to represent the core genome of all *E. coli* isolates based on WGS sequence analysis of all publicly available *E. coli* sequences available in the public domain. The FDA-ECID array is capable of molecular serotyping using 25-tiled 11-mer probes per target O or H antigen gene target capable of detecting SNPs including "211 unique probe sets for identifying 152 O types and 54 probe sets for all known H types." Validation of this array was accomplished by testing 103 *E. coli* isolates from the *E. coli* reference collection and diarrheagenic *E. coli* collection for comparison of the molecular serotype determined by the array to WGS data and traditional serotyping. Ninety-nine of the 103 isolates were correctly identified by O-type, and all but 15 were correctly identified by H-type by the FDA-ECID array. The authors state that errors were due to the absence of particular O-type antigen probes, mistyping by serology, and nonmotile strains [14]. While this array is capable of multiple types of molecular characterization of *E. coli* isolates simultaneously, the limitation of the array versus whole genome sequencing or traditional serotyping lies in the fact that it can only detect the specific number of O and H antigen types that are designed into the array and cannot detect those which are not included or are unusual.

The community-wide adoption and decreasing per strain cost of whole genome sequencing of foodborne bacterial strains has resulted in a large amount of isolate-level sequencing data which can be analyzed using bioinformatics to determine the serotype of foodborne bacterial strains. One such system is called SeqSero and is a web-based tool developed to accurately identify *Salmonella enterica* serotypes based on the matching of sample sequence data to well-curated databases "of *Salmonella* serotype determinants (*rfb* gene cluster, *fliC* and *fljB* alleles)." The SeqSero tool can "determine serotype rapidly and accurately for nearly the full spectrum of *Salmonella* serotypes (more than 2,300 serotypes), from both raw

sequencing reads and genome assemblies." [15] These authors tested SeqSero's capability to accurately determine the serotype of each isolate using three types of sequencing data. The first type of data included the "raw reads from genomes of 308 *Salmonella* isolates of known serotype" from the Centers for Disease Control. The second type of data consisted of raw WGS "reads from genomes of 3,306 *Salmonella* isolates sequenced and made publicly available by GenomeTrakr, a U.S. national monitoring network operated by the Food and Drug Administration." These isolates included metadata submitted by the submitting agency, which included an indicated serotype. The third type of data consisted of 354 other publicly available draft or complete *Salmonella* genomes, with metadata describing the serotype. After comparison of the sequence data with the known serotypes or submitted metadata serotype, the SeqSero tool's serotype prediction matched the known serotypes in 98.7% of the 308 CDC isolates, 92.6% of the serotypes submitted in the metadata of the GenomeTrakr isolates, and 91.5% of the metadata submitted serotypes of the publicly available isolates. Two hundred serotypes successfully correlated to known or metadata submitted serotypes, which included 85 of the top 100 *Salmonella* serotypes associated with human infections. Errors were attributed to variability in the H antigens, and unknown serotypes were not adequately represented in the database [15]. This platform may be considered for official adoption in public health laboratories and national surveillance systems and is undergoing validation (S. Ayers, personal communication).

In 2015, Public Health England implemented routine whole genome sequencing as a part of their foodborne pathogen surveillance and serotype identification for *Salmonella* serotypes [16]. Public Health England utilized a multilocus sequence typing (MLST) approach using whole genome sequence data of housekeeping gene alleles to predict the serotypes of 6887 human gastroenteritis cases of *S. enterica* subspecies I using the MLST scheme and database reported by Achtman et al. [17] This report showed that a majority of sequence types (ST) of *S. enterica* clustered by serotype due to the evolutionary relatedness strains with the same seven housekeeping gene alleles. Metadata including serotypes for a majority of the strains are housed in the database [17]. In this study, MLST sequence for the 6887 isolates were assigned a sequence type, and the associated serotype was predicted using the database reported in Achtman et al. [17] Of the strains tested by Public Health England, 6616 (96%) showed concordance between MLST-predicted serotype and phenotypic serotyping information in the metadata. The 4% that did not match were due to process errors, incorrect data entry regarding serotype, and some instances where two serovars belonged to the same sequence type (ST). Seventy isolates belonged to STs that did not belong to a defined serotype in the database, and those serotypes were determined phenotypically. Due to the success and robustness of this method, it was recommended that Public Health England adopt this scheme for serotyping *S. enterica* isolates [16].

Building on the concept of using genetic determinants for the O and H antigens as are used in the SeqSero method and allelic diversity of conserved housekeeping genes employed in MLST, Yoshida et al. [18] developed a bioinformatics platform

to analyze WGS data of *Salmonella* isolates to determine serotype called the *Salmonella* In Silico Typing Resource (SISTR). This platform rapidly performs simultaneous in silico analyses on draft *Salmonella* genome assemblies. SISTR predicts serovars utilizing several methods for sequence-based serotyping (genoserotyping) based on the O and H antigens as in other platforms and integrating phylogenetic sequencing schemes including MLST, ribosomal MLST (rMLST), and core genomeMLST (cgMLST). Yoshida et al. [18] validated the SISTR platform by analyzing 4129 sets of *Salmonella* WGS data available in the public domain by comparing the predicted serotype from the SISTR analysis with the indicated serotype from the strains' metadata. SISTR correctly identified the serotype of 94.6% of the finished genomes and WGS draft assemblies. Errors in correct serotype prediction were identified as incorrect serotypes in the metadata submitted with strains and some quality issues associated with the sequencing data. However, coupling the cgMLST and genoserotyping of O and H antigen genes in the SISTR platform provided the most accurate serotype prediction [18].

In 2017, a group from PulseNet Canada compared three of the molecular serotyping methods described herein (SeqSero, SISTR, and MLST) with traditional serotyping to ascertain which method was the most concordant with traditional serotyping [19]. Serotype was most accurately predicted for 813 clinical and laboratory *S. enterica* strains using the SISTR method (94.8%), with the SeqSero and MLST methods resulting in 88.2% and 88.3% concordance with traditional serotyping, respectively. The authors conclude that this validation indicates that each of these methods "would be suitable for maintaining historical records, surveillance systems, and communication structures currently in place;" however, the authors maintain the importance of traditional serotyping for the foreseeable future [19].

Although molecular serotyping methods are much faster and orders of magnitude less labor intensive, they are not 100% accurate and have not been established for all 2500 serotypes of *S. enterica*, particularly veterinary strains. Moreover, very limited information can be gleaned from establishing a serotype, although this method of detection is considered a first step in the broad characterization of a *S. enterica* or *E. coli* strain.

## Enzyme-Linked Immunosorbent Assay (ELISA)

In addition to traditional serotyping, which uses specific antibodies to detect and characterize foodborne pathogens such as *Salmonella enterica* and *E. coli*, other types of antibody-mediated methods are available for the detection of foodborne pathogens with varying levels of specificity, detection versus characterization capabilities, and time for required for results. Enzyme-linked immunosorbent assays, or ELISAs, are an example of one such method. Nyman et al. [20] evaluated three ELISA platforms for the detection of *Salmonella* serotype Dublin in bovine bulk milk for potential use in surveillance in the Swedish *Salmonella* Control program.

Samples were randomly "collected within the Swedish bulk milk sampling scheme and analyzed with three ELISAs; a Danish in-house Dublin ELISA, PrioCHECK(®) *Salmonella* Ab bovine Dublin ELISA and PrioCHECK(®) *Salmonella* Ab bovine ELISA." Each ELISA resulted in high specificities for the detection of *S*. Dublin in bulk milk at 99.4%, 99.4%, and 97.9%, respectively. Therefore, the authors concluded that these ELISA tests were sufficiently specific to be included as a screening step for Swedish *Salmonella* surveillance; however, an obvious limitation to this test is the inability to detect other *Salmonella* serotypes [20].

Another example of a commercially available automated system is the VIDAS system (bioMerieux SA, Marcy l'Etoile, France), which detects *Salmonella enterica*, *E. coli*, *Campylobacter*, *Vibrio*, and *Listeria* strains from a mixed culture via an immunoassay strip-based method, whose inner surfaces are coated with specific antibodies. The VIDAS allows for automated rapid detection of *Salmonella* in 1–2 days, versus the longer process of identifying via traditional culture methods (5–7 days) and serotyping (5–7 days). This system was reviewed in the previous version of this chapter [1] and is still currently used in some US federal foodborne pathogen detection labs as a rapid method of detection and first step for screening for foodborne pathogens which are then confirmed with official BAM methods (S. Ayers and K. Blickenstaff, personal communication).

Although traditional ELISA methods can be sensitive and specific for the detection of foodborne pathogens, those conducted using a plate/well scheme are time consuming and can require large volumes of antibody or sample for accurate detection. Therefore, immunoassays that function similarly to ELISA have been developed. These immunoassays are faster, can detect more samples as well as further characterize the strains, and are more sensitive. A newly developed antibody-based microarray that detects the foodborne pathogens *E. coli* and *Salmonella* with comparative sensitivity to ELISA and returns results in 1 h was reported by Karoonuthaisiri et al. [21]. Other technologies based on immunoassays, such as microbead-based immunoassays (discussed in the serotyping section of this review), are replacing traditional ELISA methods. Microbead assays that are capable of detecting a multiplex of 40–100 or more different targets including foodborne pathogens and associated virulence genes are faster, more reproducible, and more sensitive [22]. An immunoassay utilizing gold nanoparticle aggregation linked to a polyclonal antibody specific for *Salmonella enterica* was described by Hahn et al. [23] for sensitive detection of *Salmonella enterica* on the surface of tomatoes. These researchers have detected *Salmonella* serovars Typhimurium, Javiana, and Newport to a level of detection of 10 CFU/g of tomatoes. Cho et al. [24] developed an in situ immuno-gold nanoparticle network-based ELISA biosensor platform to detect *S.* typhimurium and *E. coli* in food matrices with high sensitivity. This sensor system includes a sample concentration step based on immuno-magnetic separation of the pathogenic microorganisms to increase sensitivity to "3 cells/mL of *E. coli* O157:H7 and *Salmonella* typhimurium in buffer and 3 CFU/mL of *E. coli* O157: H7 and 15 CFU/mL of *S*. typhimurium" in food matrix conditions within 2 h of inoculation.

## Bacteriophage

Bacteriophages are viruses which infect bacteria via recognition of strain-specific antigens. Bacteriophages are ubiquitous in nature, and their selective properties make them ideal for the detection of bacteria. Anany et al. [25] utilized the natural specificity and selectivity of bacteriophages (phage) to develop a "dipstick" paper device impregnated with phage to detect foodborne bacteria such as *Escherichia coli* O157:H7, *E. coli* O45:H2, and *Salmonella* Newport in spinach, ground beef, and chicken homogenates. When coupled with quantitative real-time PCR, "a detection limit of 10–50 colony-forming units per ml was demonstrated with a total assay time of 8 h, which was the duration of a typical work shift in an industrial setting." Junillon et al. [26] developed a multiple foodborne pathogen detection system based on the use of bacteriophage tail fibers affixed to a solid phase surface and an intracellular metabolic marker to visualize the bacterial presence on the device surface. The solid phase support surface was affixed with bacteriophage tail fibers specific for *Escherichia coli* O157: H7, *Listeria* spp., and *Salmonella* spp. and added directly to a stomacher bag of food sample artificially inoculated with the pathogens of interest. Bacterial capture was visualized "*in situ* as a result of the bacterial reduction of the colorless soluble substrate triphenyltetrazolium chloride (TTC) (present in the primary culture medium) to an intracellular red insoluble formazan product." The authors state that this system is faster than traditional microbiological methods by eliminating post-stomaching incubation and is practical for use in industrial food environments [26].

While bacteriophages are natural and exquisitely specific, this form of detection simply identifies bacteriophage specific strains in food matrices and provides no further information about the strain. Due to their specificity, variants of strain types may not be detected. Further limitations of bacteriophage include the requirements for microbiological culture for propagation and a cold chain for maintenance of testing stock.

## Polymerase Chain Reaction (PCR), Real-Time PCR, and Reverse Transcriptase PCR

Polymerase chain reaction is one of the gold standard methods for detecting and characterizing foodborne pathogens. Because PCR can be conducted on impure samples as well as on mixed samples, and can be performed without the time-consuming microbiological culture and isolation methods, it is one of the fastest, most robust, and most reliable methods to date. Methods to detect and characterize the major foodborne pathogens (*Salmonella*, *Campylobacter*, *E. coli*, *Listeria*, and *Vibrio*, *spp.*, to name a few) have been developed for contamination detection in a variety of food products and were comprehensively reviewed in Mangal et al. [27], including commercially available PCR detection systems.

While PCR is used for the first step of foodborne pathogen detection, the use of PCR for inter- and intra-strain characterization is discriminatory and popular. PCR speciation of *Campylobacter jejuni* and *C. coli* is a common method for species identification from food production environments and for surveillance of retail meats in the National Antimicrobial Resistance Monitoring System [28, 29]. MLST schemes have been developed for foodborne bacterial species such as *Salmonella enterica*, which has been shown to group strains by serotype and evolutionary relatedness by identifying single or multiple nucleotide changes in well-conserved housekeeping genes. Sangal et al. [30] used MLST and a database of thousands of sequence types contributed by researchers all over the world to study the relatedness and population structure of five major serotypes of *Salmonella*, with a focus on *Salmonella* Newport and its MDR-AmpC phenotype expressing resistance to nine antimicrobials. Achtman et al. [17] proposed MLST as a replacement for traditional serotyping. However, primary identification systems such as bacteriological culture and isolation must be used prior to MLST characterization for strain detection. As useful as MLST or any variant multilocus scheme is to define strains as a standalone method, a combination of PFGE and other methods such as MLST have been shown to be the most discriminatory [31, 32]. MLST schemes have been incorporated into whole genome sequencing analyses to group related strains and reign as the most discriminatory combination to date [18].

Real-time PCR (qPCR) and reverse transcriptase PCR (RT-PCR) are common and regularly utilized methods to detect and quantify bacterial foodborne contamination events. Due to the popularity of qPCR methods, commercial kits have been developed and validated by the AOAC for diagnostic tests for food products. A comprehensive review of commercial kits available was reported by Mangal et al. [27]. For example, two kits were developed by Roche and/or BIOTECON Diagnostics to individually detect *Listeria monocytogenes* and *Salmonella enterica* in a variety of food matrices using a qPCR scheme. The foodproof kit allows for rapid isolation of the DNA from food matrices such as peanut butter, milk, vegetables, retail meats, and many other food products [33, 34]. These foodproof qPCR detection kits have been evaluated to be equivalent in performance to the FDA-BAM reference method, however, much more rapidly. The ability to test for more than one pathogen concurrently is a characteristic essential to the rapid diagnosis of a foodborne illness. qPCR is easily manipulated to test for multiple targets and was used by Fukushima et al. [35] to detect the causative agents of 21 foodborne outbreaks in 2 days. Therefore, the benefits of using real-time PCR to detect the foodborne pathogen contamination in food products or in an outbreak include the rapidity of the method over traditional microbiological identification, increased sensitivity and specificity, quantification of the pathogen, and the ability to multiplex the reaction. However, neither of these methods can differentiate between the detection of live or dead bacterial cells. One method to detect viable bacterial cells in food was reviewed in depth by Zeng et al. [36], "whereby biological dyes such as ethidium monoazide and propidium monoazide (PMA) are used to pretreat samples before DNA extraction to intercalate the DNA of dead cells in food samples, and then proceed with regular DNA preparation and qPCR." The intercalation of the

dyes into DNA interferes with subsequent PCR amplification and thereby excludes dead cell DNA from being amplified with DNA from live bacteria in food. These authors reviewed in detail the detection of viable *Salmonella* serotypes, *Campylobacter* species, *E. coli*, and other foodborne pathogens using this method; however, limitations to this method include the incomplete exclusion of dead cell DNA in complicated food matrices.

Reverse transcriptase PCR (RT-PCR) is a method capable of detecting live bacterial cells via the isolation of mRNA with subsequent conversion by reverse transcription to cDNA for further amplification and quantitation. Miller et al. [37] tested the sensitivity and rapidity of the detection of *Salmonella* Typhimurium from spiked samples of lettuce and tomatoes via RT-PCR of the *invA* gene. These authors could show that RT-PCR identified *S*. typhimurium at 6 log CFU/25 g of lettuce spiked with high inocula *Salmonella* without pre-enrichment and at 4 log CFU/25 g at low inocula levels with a 6-h enrichment. For tomatoes, *Salmonella* strains were detected at 6–7 log CFU/100 g without enrichment and at 4 log CFU/100 g with 6-h enrichment at a low inocula. Therefore, this method can detect *Salmonella enterica* contamination in produce within 24 h.

Zhang et al. [38] compared qPCR, RT-PCR, and loop-mediated isothermal amplification (LAMP) to the FDA BAM method for the efficiency of the molecular methods to identify *Salmonella* serovars in six high-risk produce commodities: cilantro (coriander leaves), lettuce, parsley, spinach, tomato, and jalapeno pepper. *Salmonella* serovars were spiked into 25 g samples of each commodity at two different levels, $10^5$ and $< 10^1$ CFU/25 g. All four methods detected as little as two CFU of *Salmonella* cells/25 g of produce. Compared to the BAM method, each of the molecular methods, qPCR, RT-PCR, and LAMP resulted in equally sensitive detection levels but more rapidly. RT-PCR additionally has the advantage of detecting live *Salmonella* serovars, an important feature in food safety screening in six high-risk produce commodities.

## Microarray

Microarrays have been used with success to identify and characterize foodborne pathogens such as *E. coli*, *Salmonella*, and *Campylobacter* spp. in purified or mixed samples since their first description in 1995 and were reviewed in the previous edition of this chapter [1, 39–42]. Microarrays are a high throughput and information-dense tool that are particularly useful when screening multiple pathogen types with multidrug-resistant phenotypes and virulence types in foodborne pathogen surveillance [39, 43–46]. While an exhaustive review of microarrays will not be explored in this chapter, it is of note that custom, high-density microarrays have been developed which provide almost sequencing type data on a microarray slide. Photolithographic microarrays, such as Affymetrix arrays, were designed for foodborne pathogens, which can accommodate millions of probes due to the photolithographic technology (Affymetrix Inc., Santa Clara, CA). These information-dense,

high-throughput microarrays contain probes for the entire genomes of foodborne pathogens and can define a single strain. Jackson et al. [47] used this technology to define and describe the genomic content of *E. coli* isolates from a reference collection and human illnesses. Patel et al. [14] utilized the FDA *E. coli* identification (FDA-ECID) custom *E. coli* microarray as discussed previously to identify the molecular serotype of 103 diverse *E. coli* strains. Additionally, the FDA-ECID array is designed to include probes representing the core *E. coli* genome, detect virulence genes, and identify SNPs which correlate to phylogeny, thereby providing strain-level characterization of tested isolates. Data generated from screening via the FDA-ECID array were validated against WGS of 103 diverse *E. coli* strains including those associated with past foodborne illnesses. "A 99.7% phylogenetic concordance was established between microarray analysis and WGS using SNP-level data for advanced genome typing" [14]. Therefore, the array provides a plethora of genomic information and would best be used for in-depth screening when WGS is not available.

Although microarrays remain useful as screening tools for foodborne pathogen detection, characterization of strains, and source tracking, whole genome sequencing has become affordable for almost all public health laboratories and may become secondary to the more powerful and informative WGS for food safety surveillance.

## PFGE

For the last 20 years, pulsed-field gel electrophoresis (PFGE) has maintained its status as the gold standard for outbreak tracking and molecular subtyping of zoonotic foodborne bacteria such as *Salmonella enterica*, *Campylobacter* species, *Escherichia coli*, *Shigella*, *Vibrio cholerae*, and *Listeria monocytogenes* [48–50]. The PulseNet program, a molecular subtyping program consisting of state and public health laboratories and the CDC, operates via sharing macrorestriction digest gel fingerprints of each strain of foodborne bacteria within a common database and can identify indistinguishable patterns which may be linked in a foodborne outbreak. Surveillance networks that utilize PFGE include the National Antimicrobial Resistance Monitoring System (NARMS), CIPARS in Canada, and many other international surveillance systems in PulseNet International including the USA, Europe, Canada, Asia Pacific, Latin America and the Caribbean, Middle East, and Africa [51–53]. The benefits of the PFGE method include national and international validation and standardized methodology, a full genome "fingerprint" or banding pattern that is stored electronically, and a shared database between the state, local, and federal food safety agencies. Although single- or two-enzyme PFGE analysis provides a whole genomic snapshot of the bacterial strain, and does provide a high level of discrimination between very similar strains or serotypes, the actual sequence of these genomic differences is not identified. Additionally, plasmids, due to their small size, often are not visible on the PFGE fingerprint and often are not identified when only using the PFGE method. Finally, microbiological culture and isolation/

identification of the bacterial pathogen must be conducted before PFGE can be conducted, resulting in a wait time of about 10 days before the results are realized.

Due to the limitations of the method and the community-wide adoption of whole genome sequencing, the PulseNet program has officially committed to transition to whole genome sequencing as the primary molecular subtyping method for food-borne outbreak characterization in the USA [48]. Whole genome sequencing delivers the entire genome of the foodborne pathogen, whereby characterization via single-nucleotide polymorphisms results in multiple methods for analyzing the data in one assay. Molecular serotyping, as discussed in the serotyping section above, as well as relatedness typing via whole genome MLST (wgMLST) or core genome MLST (cgMLST), single-nucleotide polymorphism strain typing, virulence typing, plasmid detection, and the identification of antimicrobial resistance genes can be accomplished with multiple analyses from the data from a single whole genome sequence of the foodborne pathogen [48, 51]. Therefore, while PFGE remains in wide use, whole genome sequencing will soon completely replace PFGE as the primary method for molecular subtyping of foodborne pathogens in the USA [48, 51].

## Whole Genome Sequencing (WGS)

The advancements in sequencing technologies in the last two decades, in addition to the plummeting per reaction cost of performing these methods, have rendered the use of whole genome sequencing feasible for foodborne pathogen surveillance. The ability to identify and subtype strains involved in a disease outbreak is now a reality [54]. As mentioned in the previous section describing PFGE, the reigning gold standard primary method for molecular subtyping of foodborne pathogens in the USA, whole genome sequencing is replacing PFGE and is being adopted by the CDC-led PulseNet program for foodborne outbreak surveillance [48, 51]. The benefits of using whole genome sequencing versus PFGE are many. While PFGE provides strain discrimination that can reliably identify clusters of outbreak strains, whole genome sequencing provides data that can be analyzed to identify the serotype, phylogenetic relatedness of strains, antimicrobial resistance and virulence genes, and plasmids or other mobile elements [51]. However, the time it takes to achieve results, purely from isolation of the foodborne bacterial strain to the generation of a PFGE profile or WGS dataset, is not markedly different. Generating whole genome sequence from foodborne bacterial strains still relies on the time-consuming microbiological isolation of a pure culture from food or an ill consumer, which can take up to 5 days. However, once the data are obtained, the ability to analyze WGS data and obviate the need to perform further traditional characterization testing, including serotyping or further PCR/sequencing to characterize the strain, is a major benefit. A recognized limitation to the use of whole genome sequencing includes the need for complex bioinformatics tools and personnel expertise to analyze the data, set standards to define requirements for calling strains related, the need to set a

national and international agreement on the appropriate method for analyzing the WGS data [using SNP differences, whole genome MLST (wgMLST), or core genome MLST (cgMLST)], the need for databases with comprehensive and defined nomenclature to identify genetic elements by the same names, and a common repository to store the immense amount of data generated per strain. Despite these challenges, US public health laboratories surveillance systems such as NARMS and CDC's PulseNet Program are beginning to use WGS as a primary method of identification of foodborne pathogens [51].

A number of studies have provided proof of principle for this emerging technology in the study of food-related disease outbreaks, including the 2013 pilot outbreak detection program for *Listeria monocytogenes* by CDC, FDA, USDA-FSIS, NCBI and local, state, and international partners [51, 55]. This pilot project prospectively performed WGS on all available *L. monocytogenes* isolates collected from food, food processing environments, and patients in the USA to evaluate the usefulness of WGS in real-time foodborne disease surveillance. CDC's PulseNet program, including state and local health departments, performed WGS on all human cases of *L. monocytogenes* in 2013, USDA-FSIS performed WGS on isolates from food processing environments, and FDA's GenomeTrakr network contributed WGS data from food sources of *L. monocytogenes* in 2013. All *L. monocytogenes* WGS data from all partners were submitted to NCBI under a single BioProject, which functioned as a single repository for deposition of the WGS data. PFGE was performed in parallel by many of the partners on the *L. monocytogenes* strains. While two different methods of analysis were employed, core genome MLST (cgMLST) by CDC and high-quality SNP analysis (hqSNP) by the other partners, the authors report that the two "methods equally distinguish isolates belonging to an outbreak from sporadic cases with high epidemiological concordance." When comparing WGS to PFGE, the authors found that more clusters were distinguished and in a more rapid time frame than using PFGE alone. In September 2012 to August 2013, the year before WGS was piloted, 14 outbreak clusters were identified. After WGS implementation, 19 outbreak clusters were detected in the first year, and 21 clusters were detected in the second year. While two outbreaks were solved using molecular subtyping pre-WGS, five were solved in the first year of utilizing WGS, and nine were solved in the second year, linking to more conclusive food sources. The authors conclude that WGS is a preferable method for use in *L. monocytogenes* outbreak detection versus PFGE because WGS analysis could delineate clusters with diverse PFGE patterns, determine the source of cold cases, refine outbreak case definitions, link sporadic illnesses to food sources, and confirm outbreaks following product testing [55]. Subsequently, CDC's PulseNet and state and public health laboratories began to transition to using WGS for foodborne outbreak detection, recognizing that the standard for defining the number of SNPs which may diverge in strains that cluster together has not been set, and epidemiological information is necessary to meaningfully group outbreak strains. *L. monocytogenes* outbreaks involving ice cream from a single manufacturer in three facilities from 2014 to 2015 and Hispanic-style cheese in 2013 were successfully detected and characterized using PFGE and WGS, with WGS emerging as the more

discriminatory and meaningful method for outbreak tracking [56, 57]. The *L. monocytogenes* outbreak traced back to cheese is recognized as the first use of WGS in US regulatory investigation of an outbreak [58].

As mentioned previously, in response to the community-wide interest in using WGS for outbreak tracking, the FDA has organized a network of participating state and federal public health and FDA field labs generating WGS data on outbreak and foodborne disease-related isolates in 2012 called GenomeTrakr. This network, currently comprised of 28 state health and independent labs and 15 FDA labs in the USA,[1] was initiated to centralize the deposition of WGS data generated in the public health and field labs into 1 publicly available repository at NCBI, which syncs data nightly with global DNA databases in Europe and Japan [European Molecular Biology Laboratory (EMBL) and DNA Database of Japan (DDBJ)]. As of 2017, GenomeTrakr has added 20 international locations to the network and continue to add participants [1]. Thereby, GenomeTrakr and NCBI provide a platform for global comparison of the rapidly uploaded draft genomes, including critical metadata such as food source and geographical location, for foodborne disease outbreak identification to support timely investigations [58].

Whole genome sequencing has been successfully used to improve discrimination of foodborne outbreak clusters of *Salmonella enterica* serotypes including *S.* Enteritidis, a serotype which is historically difficult to differentiate via PFGE due to the phenomenon that most strains fall into only 3–5 PFGE profile types. Whole genome sequencing of these isolates in retrospective and prospective studies using outbreak isolates was capable of subclustering strains into discrete outbreak clusters which was not previously possible using PFGE [59, 60]. Another example of *Salmonella s*erotype foodborne outbreaks being solved by WGS includes *S.* Heidelberg, one of the top serotypes in human infection. An outbreak of 146 *Salmonella* Heidelberg infections in 2014 in 24 states was retrospectively analyzed by conducting WGS, successfully tracking the food source of the outbreak to chicken at a catered party. While whole genome sequencing is rapidly being validated as the most useful method for outbreak tracking and surveillance, multiple food sources can be confounding making epidemiologic information inclusion necessary for the most successful foodborne outbreak resolution [61]. Foodborne strains of *Campylobacter* species have also been successfully analyzed with greater discriminatory power using WGS than PFGE or MLST and were comprehensively reviewed by Llarena et al. [62].

Due to the successes in foodborne outbreak resolution for *Salmonella enterica* and *Campylobacter* species, the NARMS program has begun to use WGS as the primary method of foodborne bacterial characterization and discrimination for these two pathogens. As discussed previously, WGS has begun to officially replace gold standard methods such as traditional serotyping and PFGE for foodborne pathogen detection and characterization in US surveillance systems and outbreak tracking programs such as CDC's PulseNet. Antimicrobial susceptibility testing is

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[1] https://www.fda.gov/Food/FoodScienceResearch/WholeGenomeSequencingProgramWGS/ucm403550.htm

important to perform for foodborne pathogens to provide a baseline of resistance and characterize trends in antimicrobial resistance development to inform human medical treatment of gastroenteritis from food. Because WGS data analysis can reveal antimicrobial resistance gene presence in foodborne bacterial strains, several proof of concept studies have been conducted to assess the predictive value of the detection of antimicrobial resistance genes to phenotypic antimicrobial resistance for *Salmonella enterica* serotypes and *Campylobacter* species [63, 64]. McDermott et al. [63] performed WGS on 640 retail meat and human infection *Salmonella* serotypes from the NARMS program from 2011 to 2012 and assessed the correlation between the detection of antimicrobial resistance genes in those isolates to phenotypic Clinical and Laboratory Standards Institute (CLSI) resistance breakpoints and epidemiological cutoff values. Overall concordance between the methods was shown to be 99% for all the isolates, whereby a resistance gene was identified that could predict the resistant phenotype assessed by microbroth dilution per CLSI standards. A match was not identified in 20 instances, resulting in an overall sensitivity of 98.8%, and these cases involved aminoglycosides, beta-lactams, sulfasoxazole or trimethoprim-sulfasoxazole, and quinolones. A total of 65 unique resistance genes have been identified for which antimicrobial resistance was not tested phenotypically, highlighting the ability of WGS to identify antimicrobial resistance phenotypes which may be missed by the constraints of phenotypic testing. However, the authors also recognize that unknown resistance genes that confer resistance will not be detected if WGS is the sole manner of characterizing decreased antimicrobial resistance and maintain that phenotypic antimicrobial susceptibility testing will be conducted in the NARMS program in some fashion for the foreseeable future. Looking at the ability of WGS to predict reduced susceptibility in *Campylobacter* species, Zhao et al. [64] compared in vitro antimicrobial susceptibility testing results to WGS of 114 *C. jejuni* and *C. coli* from retail meats, cecal samples, and human infections from 2000 to 2013 from the NARMS program. The authors found that "phenotypic and genotypic correlation was 100% for tetracycline, ciprofloxacin/nalidixic acid, and erythromycin, and correlations ranged from 95.4% to 98.7% for gentamicin, azithromycin, clindamycin, and telithromycin" [64]. An overall correlation of 99.2% between the methods was identified, suggesting that WGS is a reliable indicator of resistance for foodborne *Campylobacter* species in the USA. Limitations identified by the authors of both studies include the fact that short reads from the benchtop sequencers used preclude closing the genomes, whereby some antimicrobial genes can be missed or locations not accurately identified. Further, plasmids are difficult to close using short-read sequencers, and comprehensive databases for plasmid gene identification are not yet publicly available, rendering the ability to use WGS for plasmid identification incomplete. Although the NARMS surveillance program supports the use of WGS to predict phenotypic resistance in foodborne pathogens and forecasts the replacement of antimicrobial susceptibility testing by WGS, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disagrees [65]. In 2017, the EUCAST published a paper exploring the ability for WGS to completely replace phenotypic antimicrobial susceptibility testing for clinical therapy guidance, and this group feels that there is currently

insufficient evidence to support a complete transfer of methodology to WGS. Some of the limitations listed by this group, in addition to those cited by the NARMS group [63, 64], include the importance of setting international standards and quality control metrics to predict resistance from all WGS participants that epidemiological cutoff values should be used to predict non-susceptibility versus clinical resistance breakpoints and the importance of a single, comprehensive database for identifying mutations and resistance-conferring genes. Therefore, while the sole use of WGS to predict decreased antimicrobial susceptibility is gaining support in the US foodborne pathogen surveillance systems such as NARMS, the international community has not yet committed the same level of confidence in the replacement [65].

Overall, whole genome sequencing is becoming accepted as the primary method of epidemiological outbreak and source tracking foodborne pathogen studies and is being used in real time to identify contaminant point sources in the USA. Real-time outbreak detection with the capability to simultaneously identify important characteristics of the foodborne pathogen-like serotype, resistance phenotype, and virulence gene presence are important during high-priority public health events and will become more efficient as standards in quality metrics and bioinformatics pipelines are adopted.

## Metagenomics

According to a study from 2011, the number of foodborne illnesses that cannot be attributed to a specific cause is estimated at 38.4 million cases [2]. In order to decrease the number of unattributed cases, researchers have been employing culture-dependent methods such as WGS and newer technologies made possible by the affordability of WGS to identify and characterize more foodborne pathogens than ever. Culture-independent diagnostic techniques (or CIDT), such as PCR conducted without microbiological identification and isolation of the pathogen, have been increasingly utilized by medical professionals to decrease the time to treatment and achieve better clinical outcomes. However, PCR and other CIDT are limited by the number of antigens that can be detected in a multiplex simultaneously, by the known variants of foodborne pathogen strains, and by known pathogens. Metagenomics, or the identification of genetic material using sequencing technology directly from samples, is a growing field for CIDT. Metagenomics can be conducted without microbiological isolation of strains, and because sequencing is performed on all DNA in the sample, none of the potential pathogens are missed. This method is faster than traditional culture-dependent techniques, and multiple pathogens in the millieu can be identified simultaneously including the presence of antimicrobial resistance and virulence genes. However, although virulence and antimicrobial resistance genes can be identified, it is difficult to assign these genes to a host pathogen or determine if the pathogen was viable in the sample [66].

To test the ability to use metagenomics in foodborne pathogen outbreaks, Huang et al. [66] performed a proof of concept study using two outbreaks in 2013

determined using culture-based methods by the CDC and state health labs to be *S*. Heidelberg. These two outbreaks, occurring in Alabama and Colorado, were indistinguishable via PFGE, occurred in the same month, and were originally suspected to be identical but were resolved using WGS to be two distinct outbreak strains. Using shogun metagenomics on the original patient stool samples, Huang et al. [66] compared the metagenomics results to the culture-dependent methods to solve the outbreak. In this comparison, metagenomic investigations were consistent with the culture-based findings. Additionally, the intrapopulation diversity of *S*. Heidelberg in the samples was identified, as well as the "possibility of coinfections with *Staphylococcus aureus*, overgrowth of commensal *Escherichia coli*, and significant shifts in the gut microbiome during infection relative to reference healthy samples." A bioinformatics pipeline was designed to address challenges associated with the analysis of clinical samples, including the high frequency of contaminating human DNA sequences. This study described the successful use of metagenomics to detect and characterize foodborne outbreaks while addressing some of the gaps in the validation of these methods [66].

While there are many advantages to the use of metagenomics to reduce the time to treatment in human infections, the loss of the microbiological isolation of the causative bacterial strains for secondary testing has caused problems for foodborne pathogen characterization and surveillance. In November 2015, the CDC sent a letter to US state and territorial epidemiologists and public health labs stating that the use of CIDT as a sole method of detection of enteric pathogens "are a serious and current threat to public health surveillance, particularly for Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella*." [67] Without a cultured isolate, secondary testing such as antimicrobial susceptibility testing cannot be performed, and the presence of a live causative agent may not be confirmed because metagenomics methods detect DNA of both living and dead cells. While a multitude of characteristics of patient or food samples can be determined, antimicrobial resistance genes cannot be attributed to a specific strain in the mixture when solely using CIDTs, making the attribution of antimicrobial resistance to an outbreak strain difficult. The authors feel that the sole use of CIDT may compromise the ability to link ill patients to each other, definitively link ill patients to a causative common food source, and link dispersed cases. A lack of isolates may cause outbreaks to go undetected, causing contaminated products remaining on the market, and reopen gaps in the food safety system. Adding reflexive culturing of CIDT positive strains may alleviate some of these pitfalls; however, the added cost to perform reflexive culture of patient samples has rendered some diagnostic labs resistant to conduct this isolation. Obtaining causative isolate cultures with future storage also provides the ability to retest these strains with the next "gold standard" methodology developed in the future and thus maintaining historical information on outbreaks. Considering the ever-advancing technology, this point may be the most critical advantage of reflex culturing positive CIDT samples. Future research and validation of methods to conclusively distinguish between viable and nonviable cells as well as link antimicrobial resistance genes to the host organisms will be important for metagenomics utility in foodborne pathogen detection and characterization schemes.

## Conclusions

With food safety and antimicrobial-resistant foodborne infections drawing national attention due to recent outbreaks involving retail meats, peanut butter, cheese, and fresh vegetables, it is imperative that the programs which protect the US food supply from accidental or intentional contamination remain strong, reliable, and incorporate state-of-the-art molecular methods. Traditional methods, while validated and internationally accepted, are often laborious, time consuming, and lack detailed genetic information necessary to adequately detect and characterize a foodborne pathogen outbreak and indicate treatment strategies. New and advanced technologies, such as whole genome sequencing and CIDTs including metagenomics, are becoming regularly used for surveillance of the food supply, recognizing the limitations associated with these methods. Extensive multi-laboratory validations are being conducted for whole genome sequencing as this method is officially becoming the gold standard for foodborne pathogen outbreak detection. New bioinformatics tools are being designed to accurately delineate related strains and predict antimicrobial resistance, serotype, and evolutionary relatedness. However, epidemiological information remains essential for use with molecular technologies to meaningfully characterize outbreaks and must be preserved in parallel with these exciting and emerging technologies to preserve human health and the safety of our food.

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# Technical and Clinical Niches for Point-of-Care Molecular Devices

**Glen Hansen and Ellen Jo Baron**

In 1900, speaking about the clinical and ward laboratory, William Osler, physician-in-chief at Johns Hopkins from 1889 to 1905, said, "They [lab tests] are to the physician just as the knife and scalpel are to the surgeon" [1]. Since Osler made his observation, the availability of laboratory testing has increased and expanded to all fields of medicine. As diagnostic testing has advanced, so too has the goal of applying diagnostic testing within the context of the patient-physician experience, which in turn has led to the availability of point-of-care testing (POCT).

## Defining POCT: The Debate

Over time, many descriptive names have been applied to POCT, including bedside testing, near-patient testing, physician office-based testing, decentralized testing, off-site testing, ancillary/alternative site testing, and testing performed by non-laboratory-trained personnel. The College of American Pathology defines POCT testing as "tests designed to be used at or near the site where the patient is located, that do not require permanent dedicated space, and that are performed outside the physical facilities of the clinical laboratories" [2]. Further insight into POCT provides a working definition as "diagnostic testing that will result in a clear and

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actionable management decision, such as when to start treatment or to require a confirmatory test, within the same clinical encounter" [3].

While debates over various definitions of POCT exist, there can be little debate regarding the intent of POCT. In order for POCT to provide tangible benefits, results should be actionable and lead to improved health outcomes and increased patient satisfaction. Therefore, the key objective of POCT is to produce results quicker than could be routinely offered by contemporary or centralized testing and ideally within the context of a discrete patient visit.

Pai and colleagues recently expanded upon this working construct encompassing POCT by describing POCT under unique "test target profiles" (TTPs) [3]. TTPs define the user, the location, and the intent of a given test, providing a greater understanding of how POCT should be applied to nontraditional laboratory environments such as clinics, pharmacies, or homes. For example, a rapid 3-minute test performed in a clinic setting is of little use as a POC test if test results cannot be delivered, performed, or used to direct patient management within the context of the patient encounter. Conversely, rapid testing performed in centralized laboratories often shortens the turnaround time of many POC tests because dedicated laboratory staff are available to perform testing which can then be resulted during patient visits.

Many POC tests carry Clinical Laboratory Improvement Act (CLIA) waivers allowing testing to be performed in nontraditional laboratory settings by non-laboratory personnel. Over time a test which receives CLIA waiver is often taken as a benchmark that defines the success of a given POC test. The example provided above helps demonstrate that simplicity of a given test does not define POC testing. Rather, POC testing encompasses a spectrum deeper than just the test device itself. Additionally, technology itself does not define POCT; rather use and application of rapid testing defines POCT.

POCT is one of the largest growing segments of laboratory testing; however for POCT to be successfully implemented, POCT programs often require additional staff, resources, and infrastructure for success. Such factors frequently increase costs associated with POCT programs. In many instances the need for coordinated infrastructures such as staffing, testing space, medical reporting, availability of associated therapies, or medical interventions is as important as the test result itself. Suggestions for POCT environments include areas that can safely accommodate at least one person working in comfortable working conditions, including air-conditioning and sufficient light. POC laboratories should take into account storage of instruments, equipment, and reagents which may require refrigeration. Storage of waste, including biohazardous waste and potentially contaminating amplicons, needs to be well planned. Additionally, clean testing environments should take into account pretest scenarios which can compromise accuracy of test results and generate false-positive reports. Numerous studies have reported false-positive results due to administration of vaccine delivery in areas where both specimen testing and specimen collection occur [4–7].

The importance of infrastructure needed to support POCT is highlighted by recent experience demonstrating that applying POC molecular testing for tuberculosis in

high endemic areas at the primary care level is feasible but must be accompanied by financial, operational, and logistical support in order for POCT to be practically applied [8, 9].

## Why Point-of-Care for Clinical Microbiology?

There are several reasons to develop a POC test for an infectious disease. These include:

- The need to quickly provide highly targeted therapy. Current algorithms for seriously ill patients depend on empiric treatment based on the most likely pathogens for a given clinical presentation; however, this approach involves use of broad-spectrum therapy to cover the likely possibilities. Knowing the exact identification of the pathogen will allow more focused therapeutic decisions. If a molecular method also detects important resistance factors in the pathogen, then a therapeutic decision can be made specifically to both treat the pathogen and limit development of resistant organisms.
- POCT infectious disease molecular assays may be developed to detect specific infections for which a rapid response is desirable. Examples include common outpatient infections such as group A streptococcal pharyngitis where immediate diagnosis saves follow-up efforts or *Chlamydia* and gonorrhea, where rapid results may allow immediate treatment of patients who might otherwise be lost to follow-up. There is the potential for both clinical and public health benefits from this class of test.
- Another potential objective of a POC test is to recognize quickly which patients require infection control precautions as they are admitted to the healthcare institution to prevent the spread of the agent to other patients or to caregivers. Some POC assays are meant for surveillance only, and in such cases interventions are taken to break transmission routes and prevent the development of infections in other patients. Increasingly, healthcare institutions are being asked to become more cost-effective, and rapid applications of infection control activities have been shown to be most effective. The potential for POCT to impact infection control is particularly significant for long-term care facilities and other healthcare settings without on-site laboratories.

## Technologies for POCT: The Right Technology for the Right Job

Early interest and application of POC testing focused on resource-poor settings and the need for user-friendly, cheap, instrument-free testing which defined the “assured” criteria surrounding POCT (Table 1) [10].

**Table 1** Components of ASSURED criteria in POCTs

| A | Affordable |
|---|---|
| S | Sensitive |
| S | Specific |
| U | User-friendly (simple to perform with minimal operating steps) |
| R | Robust and rapid (storage at room temperatures and quickly delivered results <30 mins |
| E | Equipment-free; free from electrical requirements |
| D | Deliverable to individuals who need testing and results |

With experience, the value of POCT is now viewed in the context of the larger patient care experience, and the emphasis on low costs and simple design now incorporates ease of use and clinical outcomes associated with POCTs. In the industrialized world, POCT is most commonly used to provide results within medical decision-making or infection control intervention actionable timeframes in order to accelerate and streamline care. The timeframes needed to deliver POC results differ depending on the nature and acuteness of the infectious process, but for purposes of this discussion, a range of 15 min to 4 h reasonably defines the lower and upper limits for expected turnaround times needed for POCT from sample collection to results delivery. In some clinical situations, this will be too long, and clinical decisions must be made without test results for guidance; thus POCT should account for the timeframe needed to routinely impact care.

Generally, two broad types of technology support POCT: small benchtop analyzers and handheld, single-use devices. Benchtop systems are smaller versions of laboratory analyzers that have been automated to reduce the need for specialized laboratory practices needed to perform the testing. Handheld devices have been developed using microfabrication techniques. They are outwardly simple but internally complex devices that do several tasks—for example, separate cells from plasma, add reagents, and read colorimetric or other endpoints.

POCT applications for clinical microbiology initially focused on lateral flow enzyme immunoassays (EIAs) for bacterial, viral, fungal, or parasitic antigens. Lateral flow tests or strip tests rely on the binding of a microbial antigen present in the clinical sample to a primary antibody conjugated to a signal, typically a gold-impregnated molecule or a fluorescent marker. Bound antibody-antigen complex(es) then migrates either under the effect of a lysis buffer or by capillarity in a solid substrate to generate detectable signals.

Sandwich EIAs are variations of standard enzyme-linked immunoassays (ELISAs) which quantify target antigens containing at least two antigenic epitopes capable of binding two layers of antibodies described as capture and signal antibodies in a sandwich format. Sandwich EIAs are commonly used in a number of group A streptococcus tests as well as applications for detection of HIV and hepatitis viruses. EIA-based tests are available in several formats, and testing can be performed in 5–30 min. Results are interpreted either visually or by using compact readers that detect the chromogenic detection or fluorescent signal associated with

the results [11, 12]. Rapid EIA tests are widely used in clinical practice because they can be performed quickly by non-laboratory-trained personnel at relatively low cost. However, POC EIA tests often provide lower sensitivity and specificity than nucleic acid-based tests or more complex laboratory tests, and interpretation in some settings is operator-dependent leading to false-positive and false-negative test results [13, 14]. To overcome poor performance of some rapid EIA testing for microbiology applications, nucleic acid amplification tests (NAATs) have recently been used for a number of POC applications [15]. NAATs offer increased confidence in test performance because they target pathogen-specific RNA or DNA sequences. NAATs include many variations of molecular chemistries including PCR and isothermal amplification methods such as transcription-mediated amplification (TMA), strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), and helicase-dependent amplification (HDA).

POC PCR technologies adapt and refine traditional molecular testing performed in centralized laboratories onto automated platforms capable of one-step processes. As a result, POC PCR platforms [15] provide higher levels of sensitivity than traditional EIA testing because they provide $\log^2$ amplification across 30–40 cycles of a typical PCR assay, lowering the limit of detection and improving sensitivity.

While PCR assays have emerged as a gold standard diagnostic, their reliance on instrumentation, limitations of thermocycling chemistry, and high costs associated with performing such testing outside of the traditional setting have limited their adoption. Recent developments have now focused on NAAT techniques that can be performed with minimal instrumentation requirements at a single temperature (isothermal amplification).

Loop-mediated isothermal amplification (LAMP) is a form of isothermal technology which can be used to amplify DNA using a polymerase enzyme operating at a constant temperature of 60–65 °C. LAMP assays eliminate the need for specific thermocyclers, making NAATs cheap, energy-saving, and easy to perform in the POC laboratory [14]. Current developments include the rapid diagnosis of malaria [15], tuberculosis, and Buruli ulcer [16].

While NAATs offer the provision of more accurate and specific testing near to patient encounters, a number of limitations regarding NAAT testing at POC exist. Recent experience suggests that different molecular chemistries may not perform equally in real-world experiences and that differences in test performance exist between different NAAT-based POC assays [15, 16]. Genetic diversity which often translates into geographical and regional differences between pathogens may impact molecular-based assays; and while these factors also impact centralized laboratory testing, the expertise and oversight needed to troubleshoot and validate POCT are often lacking at the patient intake location. NAATs have risk of contamination during nucleic acid extraction or from carryover of previously amplified material, such as vaccines [2, 7, 17], or from leakage of test cartridges, which may affect test results.

Novel test systems (e.g., viewing of malaria smears via mobile phones) continue to inform POCT, and their use may further define the operational definitions of POCT [18].

## Clinical Situations for Which POCT for Microbiology Are Used

Clinical microbiology and infectious diseases lend themselves to POCT, in part because test results are often linked to treatment decisions. A number of infectious diseases are well established as avenues for POCT.

*Streptococcus pyogenes* Pharyngitis (sore throat), which results in an estimated 15 million medical visits annually in the United States, can be caused by viral or bacterial pathogens [19]. *Streptococcus pyogenes* is one of the key pathogens, accounting for up to 30% of cases in children. Group A streptococcus (GAS) can be detected directly using a number of different POCT technologies including the most commonly used EIA lateral flow assays with sensitivities ranging from 76% to 83% [20, 21] (ref). While other causative agents of acute pharyngitis include Lancefield carbohydrate antigen groups C and G streptococci, *Mycoplasma pneumoniae*, viral agents, and other targeted pathogens such as *Fusobacterium necrophorum*, antibiotic therapy is recommended for GAS but not for most other causes of pharyngitis, so diagnosis of streptococcal pharyngitis is important to inform treatment to prevent nonsuppurative sequelae [22]. Numerous studies have demonstrated that introduction of rapid group A streptococcus tests in clinical practice can reduce the number of unnecessary prescription of antibiotics in pharyngitis and LRTIs [23–25].

**Cryptococcal Antigen Screening** Latex agglutination assays for the detection of cryptococcal polysaccharide (cryptococcal antigen [CrAg]) are widely used as both POCT and on-demand laboratory testing. Currently, CrAg testing is considered a laboratory-based assay; however, a report from the World Health Organization recently noted that the low cost, rapid results, lack of required infrastructure, and the ability to be performed by personnel with little training satisfy most of the WHO criteria [10] for POC tests making it an attractive application for near-patient testing [26, 27]. Recent experience with CrAg testing at the bedside in resource-poor settings demonstrates that CrAg testing can provide further risk stratification from fingerstick blood samples [28]. Current recommendations by the WHO suggest that serum or plasma CrAg screening be considered prior to antiviral use in HIV patients with a CD4 count <100 cells/mm$^3$ in regions with a high prevalence of cryptococcal antigenemia [27].

**HIV-1/2 Testing** Rapid HIV testing represents one of the most successful and longest tenured applications for a POCT. Several rapid antibody lateral flow assays have received CLIA waiver for fingerstick whole blood, venipuncture whole blood, plasma, and oral specimens. Rapid HIV antibody testing was subsequently approved in the United States in 2012 as an over-the-counter test for use with oral fluid specimens. Oral fluids may be more acceptable to patients due to the noninvasive nature of the specimen collection. The advantages of POCT for HIV allow testing to occur in value-added settings such as physician offices or even testing by non-healthcare professionals in home use (Fig. 1).

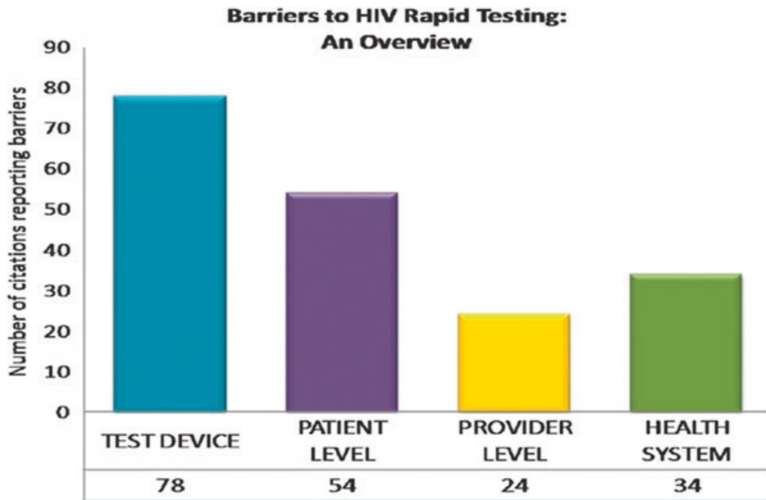


**Fig. 1** Barriers to rapid HIV testing

Longitudinal experience using high prevalence settings concludes that rapid HIV testing is both feasible in resource-poor settings and can provide both highly sensitive and specific testing [29]. Currently, fourth-generation HIV testing which combines both antibody and antigen detection is cleared for POCT. Diagnostic performance of currently approved fourth-generation testing, able to co-detect both antibody and antigen in suspected primary HIV infection, has proven to be highly sensitive (91–100%) compared to centralized laboratory testing [30, 31]. Recently, rapid POCT utilizing nucleic acid detection able to differentiate HIV-1 and HIV-2 in 25-μL whole blood or plasma samples has been highly sensitive and specific in clinical settings [32].

Rapid POC HIV testing represents one of the best examples of how POCT can impact disease prevalence by providing diagnosis which in turn links patients to care cascades [33]. The opportunity to detect primary HIV infection at the time of a patient visit allows healthcare professionals to link newly diagnosed patients to care, and providing early antiviral therapies to newly infected patients facilitates suppression. This "cascade," which begins with rapid diagnosis, supports large public health initiatives beyond individual patient care as community HIV suppression, which defines groups of people in larger communities, has been shown to significantly reduce the number of HIV transmission events [33, 34].

**Sexually Transmitted Diseases (STDs)** *Chlamydia trachomatis*, human papillomavirus, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Treponema pallidum*, and herpes simplex virus are among some of the most common causes of STDs. STDs disproportionally affect women and target socioeconomically vulnerable populations. Many STDs initially present asymptomatically prior to long-term consequences, highlighting the need for reliable screening tests. Additionally, utilization of POC for STD screening applications is increasingly viewed as a method that

can drive patient satisfaction metrics for hospitals, clinics, and healthcare systems. In resource-poor countries, lack of access to medical facilities and cost-effective testing is often cited as a reason why public health services which target STDs have failed to provide measurable outcomes in reducing STD rates.

Among current assays for STDs, the Cepheid CTNG GeneXpert assay, a multiplex rapid PCR, has recently been evaluated as a POCT as part of a "sample first" approach to public health screening during STD visits. While this approach provides reliable testing in a clinic setting, it still failed to provide results in time to direct management in 78.6% of patients (unwilling to wait for 70 minutes), demonstrating the difficulties in utilizing POC testing for *N. gonorrhoeae* and *C. trachomatis* in a clinic setting [35]. A number of lateral flow EIA tests, including the aQcare Chlamydia TRF test (Medisensor Inc., Korea), the QuickVue CT and the QuickVue TV tests (Quidel Corp., San Diego, CA), the Alere Determine Syphilis TP (Alere Inc., Waltham, MA), and the SD BIOLINE (Standard Diagnostics), are also POCT used for STD testing. While the benefits of POCT for STDs are widely supported, concerns exist over the performance needed for POC testing to be safely used as stand-alone tests. In a recent literature review of *N. gonorrhoeae* and *Trichomonas vaginalis* tests, sensitivity and specificity of testing ranged from 12.5% to 100% and 38% to 98%, respectively [36].

In-home STD testing kits are now available to patients and offer additional options where privacy issues may otherwise prevent testing. Patients could collect their own sample at home and either bring the specimen into a laboratory or run the test at home. Currently, in-home antibody tests for HIV in saliva specimens can provide results in 20 minutes. (http://www.oraquick.com/). FDA-cleared in-home sample collection kits for HIV and hepatitis C from fingerstick blood samples allow the patient to mail their specimen to a laboratory for testing. Additional kits for GAS, gonorrhea, and chlamydia will likely be available soon [36].

Novel approaches to POC STD testing include portable bioactive paper-based sensors that have the potential to detect herpes simplex virus DNA at the POC with limits of detection as low as 97 copies/ml [37]. Microfluidics, including applications for cell phone-based DNA testing, have been shown to provide highly sensitive, rapid, cost-effective diagnoses of *C. trachomatis* in an emergency department (ED) setting [38].

**Community-Acquired Respiratory Tract Infections** Community-acquired respiratory tract infections have long been targeted for POC microbiology testing because such tests can be used quickly to help triage bacterial versus suspected viral etiologies and influence antibiotic or antiviral prescribing. In the United States alone, >56,000 deaths per year are attributed to pneumonia, making it the eighth leading cause of death [39]. The age-adjusted death rate for pneumonia and influenza has demonstrated an increase of nearly 10% per year since 2012, illustrating the serious impact of pneumonia on the general population [40]. Additionally, overlapping symptoms associated with infectious and noninfectious respiratory illness necessitate the use of laboratory-based diagnostic support to help narrow down the broad differential diagnosis.

Severe community-acquired pneumonia generally justifies an extensive attempt at an etiological diagnosis [41]. The availability of POC tests for *Streptococcus pneumoniae* and *Legionella pneumophila* allows clinicians to safely assess their initial empirical therapy, restricting unnecessary antibiotic use, and, in the case of *Legionella pneumophila*, further investigate the suspected etiology of disease in an unresponsive patient. *S. pneumoniae* is among the most frequently documented agents of community-acquired pneumonia, and patients frequently present with undetermined etiology. Urine-based immunochromatographic assays detecting the C-polysaccharide common to the cell walls of all pneumococcal serotypes have been widely used since their approval in 1999, including as POCTs used outside the laboratory [42]. The availability of the result in 15 min offers a diagnostic alternative, especially when good-quality sputum cannot be obtained.

Diagnostic tests for legionellosis, based on specific detection of a lipopolysaccharide portion of the *Legionella* cell wall antigen shed in urine, became available shortly after the recognition of *L. pneumophila* respiratory illness in 1976 [43]. The availability of reliable testing for *L. pneumophila* serotype 1 significantly aided the diagnosis of legionellosis in atypical and community-acquired pneumonia cases and contributed to the observed global increase of cases of legionellosis [44]. Recent guidelines published by both the Infectious Diseases Society of America and the American Thoracic Society recommend the use of both *S. pneumoniae* and *Legionella* urinary antigen testing [41]. Rapid etiological diagnosis of community-acquired pneumonia provided by POCT in this setting has been shown to decrease mortality through facilitation of prompt empiric antimicrobials [42, 45, 46].

The sensitivity of the urine pneumococcal assay was investigated against standard microbiological cultures of lower respiratory tract samples and blood cultures [42] and ranged from 66% to 70% [25, 26], increasing in the setting of severe/bacteremic pneumonia to 80–94% [25, 27]. Although a high specificity of 90–100% has been demonstrated [25–27], as many as 21% of healthy children younger than 12 months of age also present with a positive urinary antigen, correlating with nasopharyngeal pneumococcal carriage [28].

**Influenza Infection** Rapid influenza tests are among the most widely accepted POC microbiology tests. Clinical guidelines from the US Centers for Disease Control and Prevention (CDC) and other expert groups recommend initiating antiviral treatment within 48 h of onset of symptoms to prevent deaths, shorten duration of symptoms, and limit spread of secondary infections [47]. As such, reliance on rapid testing is needed to allow clinicians to make informed treatment decisions for symptomatic patients. EIA testing is widely used at the point-of-care for suspected influenza patients. A recent survey of US hospitals indicated that over 60% of healthcare systems rely on rapid EIA testing as their sole diagnostic test for influenza [48]. However, concerns over poor performance of rapid influenza assays exist [49]. Reports describing the sensitivity of rapid EIAs vary widely from <40% sensitive to upward of 80–90% [11, 50, 51]. Circulating seasonal variations among influenza strains resulting in antigenic drift and antigenic shift, differences in vaccination effectiveness, and the target populations tested all impact performance of rapid EIA

influenza test performance [52]. New molecular technologies are starting to bridge the gap between accuracy and speed. The Roche Cobas Liat Influenza A + B and the Alere i Influenza A/B were among the two first molecular assays to receive CLIA waiver. The Liat assay also recently received CLIA waiver for combined influenza/RSV testing, opening the door for additional molecular-based testing as true options for POCT that can be reliably performed by non-laboratory personnel in a number of nontraditional laboratory settings such as EDs and physician offices [53]. As molecular POCT expands, a number of clinical applications demonstrating the value of rapid reporting at or near the patient encounter have already been demonstrated.

*Mycobacterium tuberculosis* **(M.tb)** With the increasing incidence of tuberculosis (TB) including its drug-resistant variants, POCT for M.tb offers the ability to detect and treat cases rapidly. Efficient light-emitting diode (LED) fluorescence microscopy technology that is affordable for resource-limited settings and has a long lifespan of up to 50,000 h has led to the WHO recommendation that LED microscopy be phased in as an alternative to Ziehl-Neelsen smears prepared and read in centralized reference laboratories [54, 55]. In an African-based study examining 221 sputum specimens submitted for culture and laboratory-based microscopy, the sensitivity and specificity of light diode microscopy were 73.6% and 99.8%. The mean time to read a negative smear was 1.4 min with fluorescence microscopy and 3.6 min with light microscopy, reflecting a time savings of 61% with fluorescence microscopy [56]. Concerns over missing clinically important cases of TB with rapid microscopy [57] has fostered interest in applying rapid PCR testing for M.tb with select rifampin resistance markers.

Various commercial methods for amplification of nucleic acids of M.tb exist. The first FDA-cleared NAAT assay was the transcription-mediated amplification test (Amplified Mycobacterium Tuberculosis Direct [MTD] test) by Hologic (San Diego, Calif.) which detects ribosome targets directly from acid-fast smear-positive respiratory [58]. Commercial PCR assays include the Roche Amplicor PCR assay (Roche Molecular Systems, Pleasanton, CA) [58, 59] and the Seegene Anyplex assays Anyplex™ II MTB/MDR/Anyplex™ II MTB/XDR assays. Neither the Roche or Seegene assays cleared for use within the United States, but both assays support M.tb detection in areas outside the United States. The Roche Amplicor PCR assay carries regulatory approval via Canada-IVD, CE-IVD, Japan-IVD, and CE-IVD, respectively. The Seegene Anyplex assays Anyplex™ II MTB/MDR/Anyplex™ II MTB/XDR assays are both CE-IVD cleared [60].

Advances in molecular testing for M.tb have decreased time to diagnosis in M.tb-positive patients while allowing M.tb negative to be effectively removed from isolation [61, 62]. However, many molecular assays for M.tb require multiple laboratory steps, trained operators, and dedicated laboratory facilities; thus while many research opportunities for handheld POC M.tb tests exist [63–65], the Cepheid GeneXpert MTB/RIF assay is currently the only FDA-cleared TB-PCR assay amenable for use as a POCT. Contemporary studies examining the Cepheid GeneXpert

MTB/RIF assay, in the POC setting, conclude that using the assay in POC environments facilitates rapid initiation of appropriate TB treatment over traditional TB-testing approaches [66]. In addition to detection of M.tb, the Xpert MTB/RIF assay allows rapid assessment of rifampin resistance using a hemi-nested PCR design that allows five molecular beacons to bind to different regions of the *rpoB* gene. Under wild-type susceptible genetics, all five regions bind to distinct fluorescent beacons. If one or more of the regions fails to bind its specific beacon, but at least two regions are present, the M.tb strain is reported as resistant [67].

## The Transition of POC to "On–Demand" Hospital Testing

Traditional approaches to POCTs provide testing outside the walls of traditional hospital or centralized laboratories and place testing in the hands of non-laboratory personnel. However, as technologies advance, they offer the provision of rapid, laboratory testing that can deliver results from the hospital laboratory quickly during patient visits. Recent experience with the BioMerieux BioFire® respiratory panel demonstrates that on-demand laboratory-based testing can be used to provide results in the ED, which further impact metrics such as antibiotic stewardship practices and reductions in length of hospital stays [68]. These forms of testing offer new insight into how testing can be applied to improve patient care visits.

**Methicillin-Resistant Staphylococcus aureus (MRSA)** Real-time PCR (RT-PCR) assays for detection of nasal colonization of MRSA (MRSA) at or near patient encounters have been widely studied [69]. Colonized patients can be placed into contact isolation, decolonization protocols can be initiated, and appropriate surgical prophylaxis can be used [70]. Use of rapid MRSA detection with the United States Veterans' Administration hospitals is one factor credited with lowering healthcare-associated MRSA infections 59% since universal screening and additional infection control interventions were implemented. Contemporary experience using PCR, as a rapid MRSA reporting tool, combined with hand hygiene and infection prevention practices continues to support sustained reductions in MRSA transmissions and healthcare-associated infections [71]. Selected nosocomial infections due to *Clostridium difficile* and vancomycin-resistant enterococci (VRE) also decreased [72]. Two further reports on the use of surveillance for MRSA illustrate the effectiveness of this intervention. With rapid results available within hours of patient admission, the NorthShore Hospital System showed 69.6% decrease in hospital-associated MRSA disease over the study period [73]. In contrast, another healthcare institution used a slower method for MRSA nasal surveillance with results available more than a day later, and results were disappointing [74].

Recently, some studies have challenged the impact that universal screening, accomplished with POC MRSA testing, has on hospital MRSA acquisition rates [75, 76]. Such studies demonstrate that differences in hospital MRSA rates and operational support needed to act on rapid POCT results directly impact the value

and outcomes associated with POCT for MRSA. Despite these findings, molecular POC tools are virtually the only method possible to achieve the most effective infection control. Additional assays that detect both MRSA and methicillin susceptible staphylococci in patients' skin and soft tissue wound sites and in nares are also available [77, 78].

*Clostridium difficile* Testing feces for the presence of toxigenic *C. difficile* is another use of rapid molecular technology today [79]. Many assays including both molecular and EIA platforms are available for the direct detection of toxigenic genes or antigen present in fecal samples (Table 3). RT-PCR platforms and a loop-mediated isothermal amplification (LAMP) platform are FDA-cleared. They each employ different targets. The LAMP assay seeks a genetic locus in the TcdA gene of *C. difficile*, whereas the PCR assays either identify a portion of the toxin B gene (TcdB) [80] or a second FDA-cleared assay presumptively identifies the epidemic, hypervirulent 027 strain by detecting both a binary toxin sequence and a deletion in the toxin regulatory gene, in addition to the TcdB gene [81, 82].

To date, no molecular-based *C. difficile* assay has received CLIA waiver as a POCT, but many applications of current testing have been examined in the POC setting. A recent UK-based study examined the feasibility and clinical utility of providing a *C. difficile* result at the POCT [83]. Nurses and laboratory technicians in the ICU wards were trained to perform the Cepheid GeneXpert assay as a POCT over a 22-month period; all samples were tested in parallel with the centralized laboratory for comparison. Examination of 335 stool samples revealed that offering *C. difficile* diagnosis as POCT reduced the time to diagnosis by over 16 h and contributed to a reduction in follow-up testing [83]. These types of studies provide evidence that *C. difficile* can be managed as POCT. The availability of easy-to-use, highly sensitive molecular assays capable of reporting results within 30–60 minutes provides additional opportunities for *C. difficile* testing as a POCT. Additional studies examining *C. difficile* in the hospital setting demonstrate that access to POC *C. difficile* testing can be performed by a wide variety of operators and significantly reduces time to diagnosis for hospitalized patients eliminating one of the major bottlenecks in *C. difficile* testing among hospitalized patients [83]. In one study, adoption of PCR testing reduced the average number of CDI cases per day per 350 beds from 9.4 to 8.5 while improving isolation by using single bedrooms which was a cost-effective process [84]. Rapid molecular-based testing further provides a cost-effective strategy for *C. difficile* testing via increased identification of suspected cases and reduced transmission and adverse events among misdiagnosed patients [85].

**Antibiotic Resistance Detection** The threat and growth of antibiotic resistance represents a major challenge to healthcare. The availability of point-of-care testing has reduced the burden of patients requesting unnecessary antibiotics and holds tremendous potential for guiding appropriate antimicrobial prescribing practices. The current shift in healthcare away from focusing on treatment as an end goal to prevention of disease necessitates the use of rapid diagnostics. The earlier detection happens, the easier and more cost-effective treatment decisions can be applied.

Thus, POCT for rapid antibiotic resistance testing is an area of current research representing a national “challenge” in US healthcare. The Antimicrobial Resistance Diagnostic Challenge is a $20 million federal prize competition, jointly supported by the National Institute of Allergy and Infectious Diseases (NIAID) and the office of the Assistant Secretary for Preparedness and Response (ASPR) Biomedical Advanced Research and Development Authority (BARDA), seeking innovative, rapid POC laboratory diagnostic tests to combat the development and spread of drug-resistant bacteria [86].

An FDA-cleared RT-PCR assay can be used to detect gastrointestinal colonization with VRE using rectal swabs [87]. The US version of the test was developed to detect the *vanA* gene only because *vanB* VREs are uncommon in the United States today and because there are more *vanB*-containing non-enterococci than enterococci in feces. A commonly used FDA-cleared PCR platform hosts another enterococcal assay that detects *vanA* and *vanB*, but specificity of the *vanB* marker is poor, and the format is not optimized for POC [88]. Recently, additional systems, such as the Becton Dickinson BD Max assay, have been approved for VRE screening, offering additional options for rapid, automated non-culture approaches to VRE detection [89]. Rapid detection of VRE direct from patient specimens can now be performed with sensitivity levels between 1 and 10 CFU/mL rivaling that of culture-based approaches [90]. Further studies examining the utility of rapid VRE detection indicate that rapid PCR testing provides strong negative predictive value (>99%), eliminating the need for sampling using multiple swabs. [91].

Detection of organisms carrying extended spectrum beta-lactamase genes, including carbapenemase genes, has become important given the global dissemination of resistant organisms carrying these antibiotic resistance elements. Molecular testing offers the advantage of culture-independent reporting, which decreases the time needed to provide laboratory results compared to culture-based phenotypic testing. Currently, only one assay has an FDA-cleared application direct from patient samples.

The T2 Biosystems Candida assay directly detects four different species of *Candida* directly from the bloodstream within 3–5 h using a combination of molecular probes and magnetic resonance detection. The sensitivity and specificity of the assay, established in clinical trials, for *C. albicans/C. glabrata/C. topicalis/C. parapsilosis*, were 91–99% and 98.9%, respectively; and testing detected a number of true candidemic events that were not detected with conventional blood cultures [92, 93]. An FDA-cleared direct blood culture test for bacteria is expected within 2018 and provides tangible evidence that testing directly from patient specimens is an achievable diagnostic goal.

Poor patient outcomes associate with ineffective treatment including treatment that begins too late to provide optimal outcomes. In a study of neutropenic patients bacteremic with carbapenemase-producing bacteria, nearly 90% of patients received ineffective therapy, and median times of 55 hours were required for cultures to provide the results needed to safely guide patient management [94]. Clinical recognition of septic patients and time to appropriate therapy are now measured in hours,

emphasizing the importance of timing as a contributing factor in optimal patient outcomes. The need for rapid POCT that can quickly detect not only pathogenic bacteria but also genetic forms of resistance tied to infection will be important future targets of POCT in microbiology.

No assay is currently approved, as a CLIA-waived test, for rapid resistance testing direct from patient samples in a POCT. However, a number of assays, such as the GeneXpert CARBA-R and MRSA assays, could be applied in POC settings such as nursing homes or EDs [95]. Direct testing from positive microbiology cultures in the conventional microbiology laboratory decreases times needed to detect bacteria and associated genetic resistance elements. The BioFire FilmArray® BCID is a multiplex PCR assay that detects 24 microorganisms commonly encountered in blood inclusive of gram-positive bacteria, gram-negative bacteria, and *Candida* spp. Testing also provides relevant resistance profiling targeting the $bla_{KPC}$ carbapenemase gene and *mecA*, *vanA,* and *vanB* genes in *Staphylococcus* and *Enterococcus* spp. During the US clinical trials, at least one organism was detected by the assay in 88.1% of 1382 positive clinical specimens [96], and testing has proven to be >90% sensitive for the detection of both organism and resistance identification in clinical practice [97].

The Luminex Verigene® BC-GN and BC-GP assays are microarray-based methods that detect the common gram-negative and gram-positive bacteria from positive blood cultures. This assay expands the detection limits to additional carbapenemases (IMP, KPC, NDM, OXA, and VIM) and CTX-M extended beta-lactamase. In a US multicenter study, the positive predictive agreements for identification of resistance determinants were blaCTX-M, 98.9%; blaKPC, 100%; blaNDM, 96.2%; blaOXA, 94.3%; blaVIM, 100%; and blaIMP, 100%. All resistance determinant targets demonstrated >99.9% negative predictive agreement [98]. Additional PCR-based assay which provide rapid resistance profiling [99, 100] including a potential POCT direct from respiratory specimens [101] are also available in research-use-only applications.

## Infectious Syndromes and Syndromic POC Testing in Clinical Microbiology

POC applications for microbiology have initially focused on detection of a single key pathogen by either simplified molecular or enzyme-immunoassay methods. As the spectrum of infectious agents (viruses, bacteria, parasites) capable of causing illness continues to expand, so too does the realization that few infectious processes are limited to a single potential pathogen. Diagnosis in the POC setting requires rapid assessment of presenting clinical symptoms by a knowledgeable healthcare provider and rapid diagnostic test response. However, clinical assessments based on presenting symptoms often lack the accuracy needed to establish a diagnosis, and an accurate diagnosis is needed to target therapeutic interventions.

The assortment of clinical signs, symptoms, and findings which collectively address a distinct clinical entity, recognizable and differentiated by its associated features, defines a "syndrome." The patient presentation with overlapping symptoms attributable to a common illness but caused by a wide array of potential pathogens is now driving a comprehensive "syndromic" approach to diagnostic testing utilizing multiplex- or panel-based assays (e.g., EIA, PCR) capable of simultaneous detection of 2–20 pathogens for a given disease. The movement toward syndromic testing is based on the premise that access to a definitive diagnosis can direct care and facilitate treatment approaches or eliminate antibiotic treatment for illness caused by viruses. Etiological agents associated with endocarditis, pericarditis, pneumonia, pharyngitis, osteitis, and uveitis are not being assessed through multiplexed assays.

A syndromic approach for respiratory illness caused by respiratory viruses was the first FDA-approved multiplex PCR panel, introduced in 2008. Since this introduction, PCR panels for the co-detection of respiratory, gastrointestinal, and meningitis pathogens have been approved by the FDA and are commercially available, and more such panels are likely to become available. These assays can detect multiple pathogens capable of causing disease in a specific anatomical site/organ or associated syndrome. Although these initial tests were developed for centralized laboratories, over time advances in technology, ease of use, and smaller footprints allow syndromic multiplex PCR panels to deliver test results in less than 2 h, allowing diverse options for placement, facilitating timely decisions about hospital admission, treatment, infection control, and patient return to work and family. Such assays offer the provision of near-patient care testing with minimal training and labor cost.

The value and role of syndromic multiplex PCR testing in the POC environment have yet to be fully evaluated; however, a number of studies suggest that the use of POC multiplex testing reduces healthcare costs, and the rapidity of results is extremely satisfying to both patients and medical providers [102]. Contemporary studies demonstrated the value of rapid, POC syndromic testing. Gilbert and colleagues recently demonstrated that combining rapid procalcitonin results with the BioFire FilmArray® RP multiplex PCR panel used as part of extended bundles for community-acquired pneumonia (CAP) quickly identified a diagnosis in 70% or more of patients, a >30% increase in diagnostic yield over traditional approaches to the diagnosis of CAP [103]. A recent randomized controlled trial examining standardized molecular POC testing for respiratory viruses in adults presenting to the hospital with acute respiratory illness demonstrated that POCT for respiratory infections failed to impact empiric antibiotic use. However, patients who received POC testing were given overall fewer antibiotic doses, had an overall shorter length of hospital stay, and improved antiviral response due to rapid influenza detection suggesting that POC testing can impact some important hospital and patient metrics [104].

Discussions surrounding syndromic POC testing often focus on large panel-based testing of 10–20 potential causative organisms. However, syndromic-based testing can also be applied to a small number of potential pathogens which co-circulate together or which in combination represent ≥80% of the most frequent

pathogens. Combination of influenza and respiratory syncytial virus testing is likely to detect nearly 80% of all viruses seen in the ED during respiratory season [105, 106]. Since 2003, public health recommendations indicate the role for combination screening recommendations for both *Chlamydia* and gonorrhea among at risk patient populations such as those infected with HIV and sexually active persons [107, 108]. Among current POC tests for *Chlamydia*, gonorrhea, and *Trichomonas vaginalis*, Cepheid's CTNG GeneXpert assay uses multiplex, rapid real-time polymerase chain reaction and fluorescence detection.

Dual POC rapid tests detect antibodies to both HIV and *T. palladium* and have the potential to simplify training, streamline procurement, ensure testing for both HIV and syphilis, and improve patient experiences. The similarities in screening recommendations for combined HIV/syphilis testing offer opportunities at the point of patient care to engage the care of patients who might be otherwise lost to healthcare systems, particularly among pregnant women [109, 110]. Dual rapid HIV/syphilis tests make integrated screening more feasible, and there are currently at least five manufacturers of these tests (Chembio Diagnostics Systems Inc., Medford, NY; MedMira Inc., Halifax, Canada; Standard Diagnostics Inc., Gyeonggi-do, Republic of Korea; Premier Medical Corp Ltd., Watchung, NJ; bioLytical Laboratories Inc., British Columbia, Canada) and one dual assay prequalified by WHO (Alere SD Bioline HIV/Syphilis Duo test) [36].

Consistent with the 2030 Agenda for Sustainable Development, the World Health Organization (WHO) has developed draft global health sector guidance on sexually transmitted infections (STIs) defining (i) access to health coverage, (ii) access to health services, and (iii) the importance of the public health sector in control and prevention of STDs. POC testing can facilitate achievement of the vision set forth for STIs, and the recognition that STD may involve multiple co-pathogens lends itself to multiplexed approaches at the POC. Previous experience with STD testing clearly demonstrates that providing combination or multiplex testing for multiple STD pathogens increases diagnostic yield. Similarly, increasing frequency and access through POC testing increases the numbers of accurate diagnoses and cost-effectiveness [111, 112]. Dual POC rapid tests able to detect antibodies to both HIV and *T. palladium* have the potential to simplify training, streamline procurement, ensure testing for both HIV and syphilis, and improve patient experiences [109].

These examples demonstrate the value of multiplex and syndromic testing, which are likely to expand into POC testing settings alongside the expansion of syndromic panels and menus offered in centralized laboratories. Importantly, the menu of any given POC syndromic test should evaluate the actionable event associated with increasing diagnostic evaluation such as the abatement of antibiotics given for virial illness and the need to establish key pathogens that are likely to be present in the vast majority of syndromes. Consideration of syndromic testing approaches should evaluate what is needed and reasonable as part of the test profile which may require evaluation of different targets for specific geographic regions. This also means that the menu of any POC syndromic kit may vary slightly from one geographic area to another, to take into account the most prevalent pathogens in that region. [113].

## Limitations of Current POC Technologies

Limiting the spread of infectious diseases and ensuring timely and appropriate management require accurate, easy to use diagnostic testing methods (Table 2). The potential impact of POCT in microbiology has been recognized for well over a decade now. In 2006 a report from the Global Health Diagnostics forum analyzed the contributions that good diagnostics can have for a number of infectious diseases ranging from HIV to respiratory infection to STDs. For example, modeling projects showed that POCT for malaria in children with sensitivity and specificity near 95% could prevent more than 100,000 deaths and 400 million unnecessary treatments annually [114]. While POC testing focuses largely on technological advances that allow testing to occur closer to the patient, advances in technology are only one side of the coin, [115] and even when tests with acceptable performance are available, there are considerable challenges and difficulties in introducing new tests (Table 2). In order for POCTs to provide desired outcomes, they must be surrounded by programs and personnel that support POC testing. In many cases, the infrastructure needed to support POC testing adds considerable costs to POCT. Therefore, technological advances must also be balanced with the resources needed in health systems and the goals of and intent of providing POC testing.

In an analysis of 132 publications examining rapid POC tests for HIV infection, Pai and colleagues described 190 barriers to adoption of POCT for HIV (Table 3). Concerns over rapid POC HIV testing were observed for test devices and at the patient, provider, and health system levels. Interestingly, although 190 barriers to adoption were noted in the series, factors such as test performance, costs, workflow, and who should perform testing were noted as common variables across each level [116]. Concerns voiced from laboratory communities over POCTs suggest that when clinical microbiology expertise is available in a healthcare setting, the

**Table 2** Limitations of some current POC technologies

| |
|---|
| The impracticality of performing some methods in a random access, non-batched mode |
| Efficiency of testing needed to provide throughput in non-laboratory testing settings |
| The need for space for instruments and other supplies and physical infrastructure that do not exist at most POC locations |
| The delay incurred when an additional sample is received fortesting once a testing process has commenced that cannot be stopped in the middle<br>(*Note*: I merged two cells and deleted second bullet here) |
| The need to test all necessary controls with individual samples rather than groups of patient samples |
| Quality control and oversight needed to provide confidence in POCT results |
| The need for additional instruments for sample preparation or pre-amplification |
| The possibility of contamination |
| The need for electronic reporting and connectivity which allows POC results to be captured, documented, charged, and reported to medical records |
| Storage needed for reagents needed to perform testing |
| Are POCT sufficiently sensitive to guide safe medical management of patients in POC settings? |

**Table 3** Current FDA-approved assays for detection of *C. difficile* toxin/antigen from fecal specimens

| Assay | Methodology | Target | Extraction | Time to result (min) |
|---|---|---|---|---|
| **Available FDA-cleared molecular assays for detection of *Clostridium difficile* toxin direct from fecal samples** | | | | |
| BD GeneOhm | qPCR/molecular beacons | *tcdB* | Manual | 75–90 |
| BD Max | Achromopeptidase lysis; qPCR/molecular beacons | *tcdB* | Automated | 120–150 |
| ProDesse ProGastro Cd | qPCR | *tcdB* | Automated | 180–200 |
| Cepheid GeneXpert | Multiplex qPCR | *tcdB/ cdtA tcdC*[a] | Automated | 29–45 |
| Meridian Illumigene | Loop-mediated isothermal amplification | *tcdA* | Manual | 70 |
| Focus Technologies Simplexa | qPCR, bifunctional fluorescent probe-primers | *tcdB* | None | 60 |
| Great Basin Portrait Analyzer | Helicase-dependent amplification; microarray detection | *tcdB* | Manual | 80 |
| Quidel AmpliVue *C. difficile* | Helicase-dependent amplification; visual evaluation of results using handheld cassette | *tcdA* | Manual | 80 |
| Luminex Verigene | PCR combined with gold particle probe capture and silver signal amplification on an array | *tcdB/ cdtA tcdC*[a] | Automated | 150 |
| GenePOC | Microfluidic PCR | *tcdB* | Automated | 70 |
| Roche Liat | Taqman PCR | *tcdB* | Automated | 21 |
| BioMerieux BioFire | Multiplex PCR | *tcdB* | Automated | 75 |
| **Available FDA-cleared enzyme immunoassays for detection of *Clostridium difficile* in fecal samples** | | | | |
| TechLab C. Diff Quik Chek complete | Lateral flow EIA | *Ab/Ag toxA/ toxB* | None | <30 |
| Meridian premier toxins A & B | Microwell EIA | *Ab toxA/ toxB* | None | 60–70 |
| Remel Xpect *C. difficile* A/B | Lateral flow EIA | *Ab toxA/ toxB* | None | <30 |
| Remel ProSpecT | Microwell EIA | *Ab toxA/ toxB* | None | 60 |
| Wampole Tox A/B Quik Chek | Lateral flow EIA | *Ab toxA/ toxB* | None | <30 |
| Vidas *C. difficile* toxin A/B | Solid-phase, enzyme-linked fluorescent assay | *Ab toxA/ toxB* | None | 30–60 |
| ImmunoCard toxin A & B | Lateral flow EIA | *Ab toxA/ toxB* | None | <30 |

*tcdA* toxin A gene, *tcdB* toxin B gene, *cdt* binary toxin gene, *PCR* polymerase chain reaction, *EIA* enzyme immunoassay
[a]both assays detect a deletion in the *tcdC at nucleotide 117*

laboratory should retain oversight of the quality assurance of infectious disease diagnostic tests, even those that are performed outside the laboratory [16].

A future POCT requires reliable sensitivity, the ability to test different types of clinical specimens in different patient populations, cost-effectiveness, and the ability to fit into the infrastructure and staffing where testing will be performed and used. Training, competency assessment, and proficiency testing will be cornerstones for the performance of such assays. After these factors have been considered, the remaining current technologies include polymerase chain reaction, isothermal loop-mediated amplification, and direct DNA hybridization. Other methods are in earlier stages of development but may show potential in the future. Molecular methods, when brought to routine POC use, have the potential to provide performance equivalent to that of laboratory-based methods, which will drive further development of POCTs and eliminate concerns over patient risk and POCTs.

Methods and application of POCTs must be chosen carefully. The tests should be able to detect small numbers of organisms in limited sample volumes. This is the situation which currently challenges laboratory-based diagnostics in a number of infectious diseases, for example, tuberculous meningitis, where the paucibacillary nature of the cerebrospinal fluid has challenged the development of effective molecular assays [117]. Use of molecular methods at POC will bring new challenges to those who administer and perform POCT. In addition to the usual quality assurance (QA) and quality control (QC) associated with any POCT, molecular POCT will require procedures for controlling contamination with both amplified material and patient-derived materials. QC of each stage of the analytical procedure, extraction, amplification, and detection, may make troubleshooting more challenging. The phenomenon of inhibited specimens may require operators to report results more complex than "positive" or "negative." POC molecular instruments are likely to be more complex than current systems such as glucose testing systems and may, initially, lack some of the sophisticated POC management tools associated with traditional POC platforms [118].

Diagnosis at the POC can be lifesaving in select patients; however, the variables which make POC testing successful may not translate between healthcare systems. Current research is needed to further define which variables and under which conditions POCT provides positive patient outcomes. The benefit of scale and efficiency developed by centralized laboratory testing process almost always provides more cost-effective testing options than those obtained through POCTs. As healthcare systems struggle with increasing costs and declining reimbursements, the need to demonstrate medical and financial value associated with POCTs will be required but is currently underrepresented in the form of well-done studies. Cost-effectiveness research is needed to determine whether the cost of a POC test is justified based on important contemporary healthcare metrics such as patient morbidity and mortality, reduction in disease transmission, decrease of overall hospital costs, and impact of testing on hospital admission and readmission rates. Current and emerging clinical needs; increased acuity of inpatient care, expanded outpatient care, and an increasingly mobile population; and the need to control healthcare-acquired infections and novel antibiotic resistance mechanisms will all drive new technologies, including molecular microbiology, to the POC.

If the importance of rapid, reliable testing could be acknowledged by Sir William Osler more than a century ago, there is optimism that we can improve patient access to important testing, change the way POCT is perceived, and realize the benefits of POCTs as a pathway to better patient care [119].

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# The Human Coronaviruses

**Oliver Schildgen**

## Introduction

Although human coronaviruses (CoV) are known as human pathogens since the 1960s, their virus family has gained notoriety in 2002 and 2003 with the first outbreak of the SARS coronavirus epidemic and with the recent emergence in 2012 of the MERS coronavirus.

Coronaviruses belong to the family Coronaviridae and are enveloped single-stranded RNA viruses with positive RNA-genomes [1]. Their genome is about 26–32 kilobases long and thus represents the longest know viral RNA genome. The name coronaviruses is based on electron microscopy photographs which stimulated the imagination of early electron microscopy analysts who thought that the viruses have a crown-like surface. Consequently, these researchers named the viruses according to the Latin word for crown, i.e., *corona* [2]. Until today, all known coronaviruses share a similar genome organization and expression profile of their genomes: 16 nonstructural proteins (named nsp1–16) are encoded by an open reading frame (ORF) named 1a/1b which is located at the 5′ terminus of the genome, followed by the structural proteins (spike/S, envelope/E, membrane/M, nucleocapsid/N) that in total are encoded by ORFs located 3′ of the viral genome.

Within the family of coronaviruses, four genera exist which are named alpha-CoV (or group 1), beta-CoV (group 2), gamma-CoV (group 3), and delta-CoV (group 4), whereby group 2 coronaviruses comprises four lineages named A, B, C, and D, respectively [2]. In this context it is worth mentioning that the lineage A viruses of the group 2 CoVs encode a smaller protein called hemagglutinin esterase (HE), which appears to be functionally similar to the S protein [3].

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## HCoV Genome Organization

As mentioned previously, the human coronaviruses have a non-segmented positive-stranded RNA genome. Approximately 60–70% of this genome consist of two large and overlapping open reading frames (ORF1a and ORF1b) that encode for the polyproteins pp1a and pp1ab that in turn are processed into the 16 nonstructural proteins 1–16. The structural proteins E, M, N, and S share the rest of the ORFs of the viral genome while being accompanied by a variable number of the so-called accessory proteins [2]. The long genomes are believed to originate from a unique replication fidelity that in turn is originated by a set of viral enzymes harboring RNA-processing functions [4].

## Clinical Symptoms

In humans, HCoV infections in general result in self-limiting disease courses that involve the upper respiratory and the gastrointestinal tract. Symptoms may vary from mild to serious and (sometimes) life-threatening infections in permissive patients and range from a common cold to bronchitis and pneumonia; occasionally renal involvement is seen [5–15].

In this context it is important to note that the clinical manifestations of the two most serious (but also least frequent) HCoVs, namely, SARS coronavirus and MERS coronaviruses, are more serious and frequently are life-threatening. However, despite the ongoing endemic MERS outbreak in the Arabian region and single outbreaks in South Korea, these two pathogens remain limited to single outbreaks (in case of SARS-CoV) and endemic zoonotic transmissions in the Middle East area.

In any case, none of the remaining human coronaviruses can be identified on clinical symptoms alone, and coinfections with other respiratory viruses are as common as with other respiratory pathogens, making it difficult to identify which is the “leading” pathogen in multiple infections [16–22].

## Epidemiology

To date, six human coronaviruses have been discovered, i.e., the human coronaviruses OC43 and 229E, NL63 and HKU1, and the SARS and MERS coronaviruses. Except for the latter two, all human coronaviruses have been noted to occur worldwide and are mostly associated with a seasonality that follows the typical flu-like symptom season [23–31]. As the nomenclature of coronaviruses is far from being logical, these viruses are described in the next section in more detail according to their systematic order.

## Human Coronavirus 229E (Group 1/Alpha-Coronavirus)

Occurring globally, the human coronavirus type 229E was initially discovered in 1966 during a trial to identify several newly recognized pathogens associated with the common cold [32, 33]. The clinical symptoms associated with 229E include malaise, headache, sneezing, sore throat, sometimes fever, and cough. The time span between infection and clinical symptoms is reported between 2 and 5 days with clinical symptoms lasting between 2 and 18 days [34–37]. Anyway, as mentioned earlier, there is no clinical difference between 229E infections and other respiratory infections caused by viral pathogens such as rhinovirus or influenza A [34–37].

Recently it has been postulated that 229E originated from a recombination event between the alpaca alpha-coronavirus. This recombination event occurred within the S gene and was followed by a deletion in the same gene [38].

## Human Coronavirus NL63 (Group 1/Alpha-Coronavirus)

Discovered in 2004, the human coronavirus NL63 has been found worldwide since then and is mainly associated with respiratory infections in children, the elderly, and immunocompromised patients. The virus was consecutively discovered in two separate laboratories in the Netherlands, one in Amsterdam and one in Rotterdam [39, 40]. NL63 infections in general present with mild respiratory symptoms such as cough, rhinorrhea, tachypnea, fever, and hypoxia [11, 13, 41–44] and are self-limited. A frequently observed “complication” is croup which is present in approx. 5% of NL63 infections [45].

## Human Coronavirus HKU1 (Group 2/Betacoronavirus, Lineage A)

Starting with the description of the human metapneumovirus in 2001, a new era in virology began; this era focused on viral discovery methods that combined classical techniques of virology with modern molecular methods. The resulting wave of virus discoveries led to another trend in molecular diagnostics in which singleplex step by step methods were replaced with multiplexing technologies able to screen for several pathogens simultaneously. During this time, HKU1 was detected in 2005 at the **H**ong **K**ong **U**niversity (which is also the institution from which the name HKU1 was derived). The isolation of HKU1 was from an elderly patient who suffered from bronchiolitis and pneumonia [46–48]. Fatal infections occur rarely, and the infections are indistinguishable from other viral respiratory infections. As the other “common cold” coronaviruses, HKU is circulating globally [49–54].

## Human Coronavirus OC43 (Group 2/Betacoronavirus of Lineage A)

The strain OC43 belongs to the longest known human coronaviruses and was identified in 1967 [55, 56]. The discrimination between OC43 and 229 can be performed exclusively by molecular methods or serologically, and both viruses have the same morphology and clinical spectrum [55, 56].

## SARS Coronavirus (Group 2 Coronavirus/Betacoronavirus of Lineage B)

Much has been speculated; even more has been confirmed about the SARS coronavirus since it was first detected in 2002/2003 during an outbreak in China. The subsequent pandemic that was beginning was halter due to strict hygienic procedures and intervention measures before a worldwide disaster could occur. As a matter of fact, the discovery of this virus was possible solely by the first alarming observations reported by Dr. Carlo Urbani [57], a physician who was confronted with patients suffering from fever, myalgia, headache, malaise, and chills followed by a dry cough, dyspnea, and respiratory distress; in some cases infections of the liver, kidney, gastrointestinal tract, and brain occurred [58–62]. The overall mortality rate is 9% but is higher with increasing age. To date, the SARS coronavirus has caused only a single outbreak followed by spread to other locations as a result of travel. This initial SARS coronavirus outbreak is now known to be an archetypic zoonosis outbreak of this virus or other SARS-like coronaviruses. Such coronaviruses circulating in their natural reservoirs should not be excluded during and outbreak and require a narrow mesh of surveillance.

## MERS Coronavirus (Group 2/Betacoronavirus, Lineage C)

The MERS coronavirus first came to the attention of the scientific community in 2012 when the virus was isolated for the first time in Saudi Arabia. It causes severe pneumonia with acute respiratory distress (ARDS) and is frequently associated with gastrointestinal symptoms. Importantly, renal impairment is frequently observed. Especially patients with an underlying comorbidity are permissive for MERS-CoV infections and have a high mortality rate [63–75]. It is important to note that, although the virus appears to be endemic, spontaneous outbreaks due to imported cases are possible, as most recently reported from South Korea, where the roommate of an index patient left the hospital on his own account and thereby caused a

local outbreak [76–79]. It is worth noting that in terms of the MERS-CoV, it is assumed that the viral spike protein enables the virus to evade the immune system by preventing the binding of neutralizing antibodies.

## Virus Ecology of Human Coronaviruses

To date it appears that the coronaviruses NL63, HKU1, 229E, and OC43 are well-adapted human viruses that remain in the human reservoir; these coronaviruses originated from zoonotic transmission long ago [38, 80–83]. In contrast, MERS-CoV and SARS-CoV are less adapted to the human host and most likely represent zoonoses, originating from their natural reservoirs camels and bats, respectively [82–90].

## Diagnostics

The diagnostic confirmation of a human coronavirus infection does not necessarily lead to a specific therapeutic decision. While coronaviruses NL63, HKU1, OC43, and 229E do not require "special" attention, isolation of patients is strictly required in case of SARS-CoV and should be considered in case of MERS-CoV.

As diagnostic methods, neither cell culture-based nor electron microscopy methods are the first choice. Instead, molecular methods such as RT-qPCR, LAMP, or multiplexing methods should be used. RT-qPCR protocols have been described by several groups and are the method of choice for the new coronaviruses. For MERS coronavirus it is recommended by Corman and coworkers to use the upE region and the Orf1a as targets for the PCR, while Orf1b has a reduced sensitivity [91]. In addition, it is recommended to sequence parts of the RdRp- and/or the N-gene to confirm the results. Internal and external controls should be included in every PCR run and are available, e.g., from Public Health England.

For the other coronaviruses, several validated and approved multiplex assays are available, such as the RespiFinder assay (Pathofinder, Maastricht, Netherlands), the film array (former IDAHO film assay, meanwhile produced and distributed by bioMerieux, Lyon, France), or the Luminex RVP (Luminex, Austin, Texas, USA). All of these assays have the advantage of a high sensitivity combined with the simultaneous detection of several other pathogens. Moreover, the novel Light Mix Modular Assays from Roche/TIBMOLBIOL could serve as an alternative for coronavirus diagnostics.

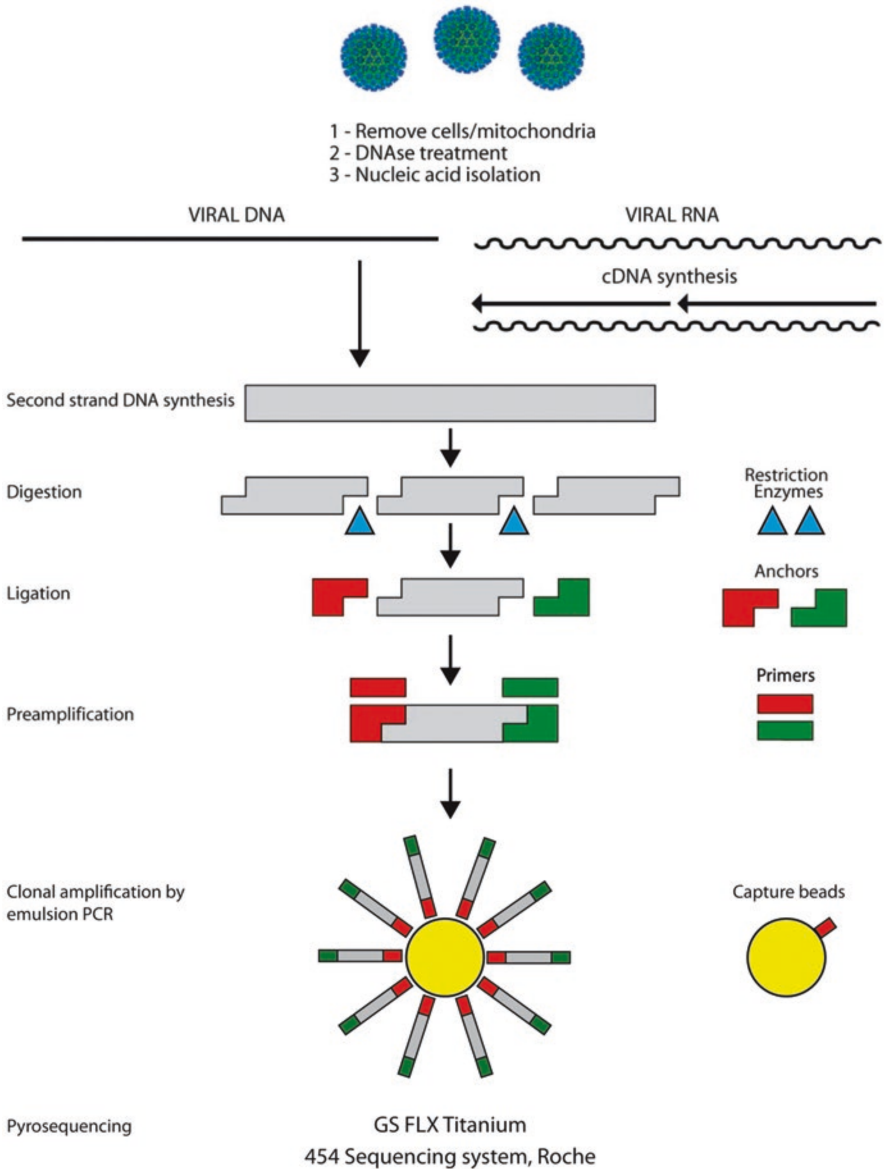


**Fig. 1** Overview of the novel high-throughput VIDISCA method. (From de Vries et al. 2011, PLoS One [92]. Original picture published under the Creative Commons Attribution (CC BY) license in PLoS One [92])

## Advanced Molecular Techniques Relevant to Human Coronaviruses

The detection of novel coronaviruses within the last 15 years are excellent examples for the necessity of advanced molecular techniques that have to be combined with classical virological methods. As an example, the discovery of the SARS coronavirus has become possible solely due to the sophisticated combination of detailed and timely clinical observation followed by attempts to isolate the virus in cell culture (classical method) and subsequent characterization by modern molecular techniques. The latter method used for the identification of the novel genome of the SARS coronavirus was called random reverse transcriptase PCR and led to the amplification and subsequent sequencing of the first known SARS genomes [62].

A further example is the discovery of the human coronavirus NL63 by van der Hoek and coworkers [39]. These researchers established a novel method called VIDISCA (virus discovery cDNA-AFLP). For this method, the viral DNA or cDNA is digested with enzymes targeting short recognition sequences that are virtually present in all viruses. These fragments are then ligated to adaptors and amplified by an adaptor-specific PCR. The VIDISCA method meanwhile was refined (Fig. 1) and is applicable as a sensitive assay for virus discovery also from clinical samples [92].

## Concluding Remarks

Coronaviruses have been recognized as a major player in serious airway infections. The recent experiences with the MERS coronavirus and the outbreak experience with the SARS coronavirus have shown that these zoonotic viruses are able to cross the species barrier and along with influenza viruses are the most likely candidates for future outbreaks. In concert with newer studies on virus ecology, it has become obvious that coronaviruses are ubiquitous pathogens infecting a broad range of mammals that often are in contact with humans, thus providing the basics for future zoonotic outbreaks.

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# The Role of the Human Bocavirus (HBoV) in Respiratory Infections

**Oliver Schildgen and Verena Schildgen**

## Introduction

The current classification of human bocaviruses is based on the latest recommendations of the International Committee for the Taxonomy of Viruses (ICTV) (https://talk.ictvonline.org/taxonomy/). The variant 1 of the human bocavirus (HBoV-1) that causes respiratory infections in primates and humans belongs to the family of Parvoviridae, subfamily Parvovirinae and genus *Bocaparvovirus* and was discovered originally in 2005 by Tobias Allander [1] and co-workers and represents together with the strains HBoV-3 and the gorilla bocavirus the species *Primate bocaparvovirus 1* [2].

The discovery of HBoV-1 was one among a series of virus discoveries that occurred during the first 15 years of this century. These discoveries were based on novel virus discovery systems using molecular approaches developed in order to reduce the considerable number of cases in which a clinical diagnosis of a respiratory infection could not be confirmed by the laboratory detection of a pathogen. Following the initial description of the virus, a huge number of clinical studies and case reports have been published which were supplemented by some basic research reports. In parallel, several related viruses have been newly identified, such as a swine bocavirus, a feline bocavirus and a novel canine parvovirus, of which of them share some biological features with HBoV [3, 4]. In 2016, an additional novel bocavirus variant occurring in chimpanzees was identified, which along with the gorilla virus gives rise to the assumption that a long co-evolution between primates and bocaviruses exists [5–7].

Unfortunately, HBoV research still relies on clinical studies and case reports with accompanying cell culture studies as the major source of information on HBoV

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pathophysiology, because to date no animal model has been identified. Preliminary data on the use of ferrets as a model for gene therapy with HBoV capsid-based vectors suggests that ferrets might be a possible model for future research on HBoV-host interactions and vaccinations [8].

## HBoV Biology

The human bocavirus (HBoV) was initially discovered in clinical samples from the respiratory tract of children suffering from respiratory infections of unknown aetiologies [1]. To date, HBoV is the fourth most detected respiratory virus, but as there is still no animal model or a broadly convertible cell culture available, Koch's modified postulates have not been experimentally fulfilled yet [9], but a case study from the group of Maria Söderlund-Venermo, Klaus Hedman and Olli Ruuskanen has shown that human-to-human transmission is most likely [10]. This report describes an intra-family infection chain that was characterized by both symptomatic and asymptomatic infections/transmissions, subsequent reactivation of the virus and hints for latency of HBoV.

Nevertheless, HBoV is the second parvovirus known so far that is capable of infecting humans with the potential to cause clinical disease. Until HBoV was discovered, the parvovirus B19 was the sole human parvovirus, which is difficult to culture in in vitro cell cultures, likely because infection strongly depends on the optimal cell cycle phase [11–20]. This latter fact hampered the development of potent and specific antivirals; tenacity studies and the development of disinfectants active against human parvoviruses as surrogate pathogens with animal pathogenicity were used. The narrow parvoviral host tropism also hampered the development of cell culture systems that support the replication of human bocavirus.

The discovery of HBoV has resulted in several molecular findings that are of major interest regarding the pathophysiology of human parvovirus. Within a primary cell culture in which the human bocavirus was replicating, it was possible to identify the HBoV transcriptome including splicing variant of viral RNA [21]. This cell culture demonstrated for the first time a potential tool for the investigation of human parvovirus in its natural infectious setting, enabling investigations of the molecular biology of human parvoviruses in general and HBoV in particular. Unfortunately, the primary cell culture that enables HBoV growth in vitro is very expensive and requires a highly specialized laboratory. Moreover, this is an error-prone cell culture, which means the availability of this technology is limited to several laboratories worldwide, which in turn will delay further research. In search for a broadly convertible replication system, the group headed by Dr. Jianming Qiu from the University of Kansas Medical Center made a significant step forward: this group has established a plasmid-based replicon-like system that has identified additional RNA species that are transcribed during the HBoV replication cycle [22]. The system is based on plasmids that contain the complete published HBoV sequence but are flanked by ITR regions of the adeno-associated virus (AAV); the ITR regions

are terminal repeats containing palindromic sequences that form hairpin-like structures which in turn are required for the replication of parvoviruses according to the so-called rolling hairpin mechanisms of replication [23]. With this first replicon system, Chen et al. have shown that HBoV types 1 and 2 express a similar RNA pattern like other parvoviruses. In particular, they identified a spliced NS-1 transcript that was not recognized before and have shown that the NP-1 transcripts are expressed abundantly [22]. In this context it is worth to note that the viral NP1 protein, which is a small NS protein encoded by the middle open reading frame, is required for the expression of viral capsid proteins (VP1, VP2 and VP3), whereas the other NS proteins (NS1, NS2, NS3 and NS4) are not essential for the expression of VP proteins [24].

Although the hairpin-like structures of HBoV were not described when the first genomic analyses were performed, it has been postulated that the HBoV genome also is flanked by such structures and that HBoV replicates its genome by the rolling hairpin mechanism, although this assumption is exclusively based on phylogenetic analogous conclusion rather than on experimental evidence. In theory, the rolling hairpin replication results in progeny genomes that occur in equal amounts of both polarities, whilst packaging of viral genomes is dependent on additional factors [25–31]. For almost four decades, it is postulated that all parvoviruses replicate according to this mechanism, although this replication model is solely based on experimental data obtained by the research on rodent parvoviruses. The model is characterized by a terminal hairpin-dependent self-priming initiation of the viral genome replication and concatemeric replication intermediates of head-to-head or tail-to-tail replication intermediates. Based on an early publication of the postulated model in 1976 in Nature, this replication model became a dogma in the field of parvovirology and was deemed to be true for all parvoviruses. Interestingly it was impossible to identify both genome polarities in clinical samples containing HBoV-infected cells [32]. Thereby, NASBA analyses revealed that all HBoV strains package negative-strand genome, whilst only a minority also packages the plus strand; this observation is compatible with another replication mechanism known as rolling circle replication. In order to test the hypothesis if rolling circle replication may occur in HBoV infection and in order to decipher the unknown terminal hairpins, a couple of systematic PCR-based analyses were performed [33].

This approach has identified DNA sequences that contain head-to-tail genome fragments linked by a newly identified linker stretch that has a partial by high homology to the minute virus of canine (MVC) ITR and to the ITR of bovine parvovirus. Most recently it was shown that these sequences most likely represent the missing terminal hairpin-like structures [33, 34]. Despite identifying the terminal sequences in both clinical samples and cell cultures, a lack of self-priming activity of HBoV genomes as well as the lack of intermediates typical for rolling hairpin replication has been noted. Instead the samples contained head-to-tail structures. Additional groups have published similar observations, all questioning the dogma of parvovirus replication [35–38]. It is thereby important to know that the head-to-tail episomal form of HBoV differs from formerly described circular parvoviral

episomes that have been shown to consist circular-closed genome dimers of head-to-head and tail-to-tail orientation [39].

Although the role of the linker sequence and the head-to-tail junction remains unclear, these findings were surprising as they support the hypothesis that HBoV replicates differently from non-human parvoviruses by possibly initiating a rolling circle mechanisms, at least as an alternative route of replication.

Based on the newly identified sequences, the structure of the putative terminal repeats of the HBoV genome was predicted in silico [34]. In addition, the Kansas group has developed a true full-length vector clone of HBoV which can be transfected to HEK-293 cells and produced a “recombinant wild-type” human bocavirus that in turn is infectious for differentiated CuFi-8 cells [40]. CuFi-8 cells are derived from a patient with cystic fibrosis and can be grown as monolayer cultures that can be differentiated into a polarized respiratory epithelial structure by changing the culturing media. This polarized respiratory epithelial structure in turn supports HBoV replication [40]. It is worth noting that CuFi-8 cells experience a serious cytopathic effect that is able to destroy the cellular glycocalyx structures (Fig. 1) and is accompanied by a loss of cilia [41]. This novel cell culture moreover supports the hypothesis that HBoV is a serious pathogen as it induced a remarkable cytopathic effect in the polarized CuFi-8 cell line which in turn is compatible with the assumption that the clinical symptoms of an HBoV infection are caused by tissue damages related to viral replication. Thereby, this infection model harbours a surprising feature that is a further hint for an alternative replication of the human bocavirus: if the full-length HBoV plasmid containing the hairpin sequences is transfected into HEK293 cells, infectious progeny virions are produced although based on the rolling hairpin model this process should be impossible, as the free (!) hairpin sequences are believed to be essential for the replication. In contrast, replication is possible in the plasmid although they are flanked by the vector’s backbone sequence and no helper plasmids are required as known for the dependoviruses. This simple observation strongly contradicts the model of rolling hairpin replication but in turn favours other replication models known for circular DNA, as, for exam-

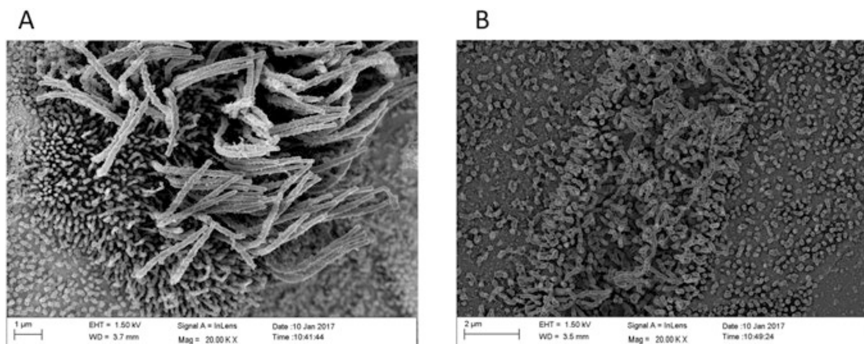


**Fig. 1** Loss of cilia from glycocalyx of HBoV-infected cells in comparison to mock-infected cells

ple, the rolling circle replication, which in the natural infection would produce head-to-tail concatemers. However, it has to be mentioned that despite these conflicting data, the minimal essential origin of replication was identified in the right-end hairpin sequence [42]. Thereby, unlike other parvoviruses, the HBoV-NS1 protein did not specifically bind to the oriR in vitro, indicating that other viral and/or cellular components or oligomerization of NS1 is required for NS1 binding to the oriR. Of note, NP-1 and other viral nonstructural proteins (NS1–4) co-localized with the viral replication centres [42]. During the viral replication cycle, it appears that the expression of viral capsid proteins is regulated by polyadenylation mechanisms of the viral RNA transcripts [43]. It was shown that in addition to a distal polyadenylation signal named (pA)d, a further distal polyadenylation site named (pA)d2 is present in the right-end hairpin sequence, which does not contain the typical hexanucleotide polyadenylation motif. Moreover, the viral replication is strongly dependent on a newly identified small non-coding RNA named BocaSR within the 3′ non-coding region (nt 5199–5338) [44]. This RNA is transcribed by the RNA polymerase III from an intragenic promotor at amounts similar to the RNAs of the nonstructural genes. BocaSR accumulates in the replication centres within the nucleus and is suspected to directly influence the viral DNA replication.

Furthermore, clinical observations give rise to the hypothesis that the HBoV replication can be triggered or influenced by human herpesviruses such as HHV-6, CMV and herpes simplex virus. In this context it is noteworthy that herpesviruses, especially HSV, are capable of initiating a rolling circle replication mechanism of replication in trans as shown for SV40, which has a circular double-stranded genome [45].

Thereby herpesviruses may either act as a trigger that arrests the host cell at transition from G1- to S-phase of the cell cycle, or they could directly interact with the HBoV DNA supporting the replication by the herpesviral replication enzymes. The latter appears likely, as head-to-tail intermediates are a feature of the rolling circle replication that may be initiated by a couple of viruses including the human herpesviruses type 1 and type 6 [45–52]. These viruses (e.g. the adeno-associated virus, AAV) in turn are able to act as helper viruses for the parvoviral subclass dependoviruses that require those helper viruses for their replication [48–52]. Recently, a clinical case was observed in which the HBoV infection appeared to depend on a co-infection and co-replication of human herpesvirus type 6. In this case the HBoV infection persisted because of an immune disease but was terminated by antiviral therapy with cidofovir which is directed against HHV6 [53]. This was the key observation leading to the assumption that HBoV is either sensitive to cidofovir or that a possible rolling circle HBoV replication is triggered by HHV6, which in turn would explain the high frequency of co-infections observed in case of HBoV [52, 54, 55].

In 2011, two severe cases of respiratory failure in adults associated with HBoV infection and herpesvirus co-infection, with a history of lung fibrosis likely related to the presence of chronic HBoV infection [56], strongly suggest that the head-to-tail structures could have been episomal reservoirs enabling the virus' persistence as postulated by Kapoor and co-workers [35]. It may be speculated as to whether the persistence of HBoV episomes in the lung of the patients is analogous to a HBV

infection, in which episomal cccDNA persists in the infected cell until the cell is targeted by the immune response or subjected to apoptosis and in which this chronic state frequently produces a mild inflammation that is subclinical but could induce fibrosis over time. The persistence of HBoV episomes in the lung could have led to mild chronic inflammation eventually resulting in fibrosis of the lung, which would not be easily compensated as in the liver. In the context of a putative chronic HBoV infection or a persistence of HBoV at a subclinical level, it thus appears possible that HBoV could directly or indirectly, by interactions with the immune system, contribute to chronic lung disease such as idiopathic lung fibrosis.

Another, recently detected novel feature of HBoV is the expression of more nonstructural proteins that concluded from our previous knowledge on parvovirus replication studies. Shen et al. have shown that besides NS1 three novel proteins named NS2, NS3 and NS4 are expressed during the viral replication, of which NS2 is believed to have a crucial role during the viral life cycle [57].

Moreover it is important to mention that the HBoV replication cycle is independent of the cell cycle phase. As early as in 2010, it was shown in A549 cells that the expression of HBoV-1 proteins, unlike the parvovirus B19 infection, does not induce cell cycle arrest and apoptosis [22]. In contrast, two recent studies have shown that the DNA damage repair system is involved in HBoV-1 replication [58, 59]. Thereby the hallmarks of the DDR response, the phosphorylation of H2AX and RPA32, are activated accompanied by the activation of all three PI3KKs. In addition, the polymerases Pol-η and Pol-κ, both being part of the DNA repair system, are recruited to the viral replication sites, thus providing additional evidence that parvovirus DNA replication has to occur in cell cycle-arrested cells.

## Epidemiology

Like all respiratory pathogens (except SARS and MERS coronavirus) causing respiratory infections, HBoV-1 is distributed worldwide and has been detected in patients from several regions of each continent [60–112]. However, unlike most other viruses that are known to peak seasonally in autumn and winter, HBoV infection peaks do not seem to be restricted to these seasons.

Although the route of transmission was not yet systematically investigated, it is widely accepted that the transmission of HBoV most likely occurs by smear or droplet infections or aerosols and nasal or oral uptake as described for the majority of “common cold viruses.” The transmission route passes through airway excretions but could also be via the gastrointestinal route, as HBoV is shedded also by stool (Figs. 2 and 3).

The HBoV seroprevalence is high and reached 95% and more in children up to the age of 5 years [113, 114]. This seroprevalence remains high in most adults [76, 82] but decreases from 96% to 59% in European adults if antibodies against HBoV strains two to four were depleted. Thus in 41% of patients, no long-term immunity could be generated, supporting the assumption that the virus is able to persist and

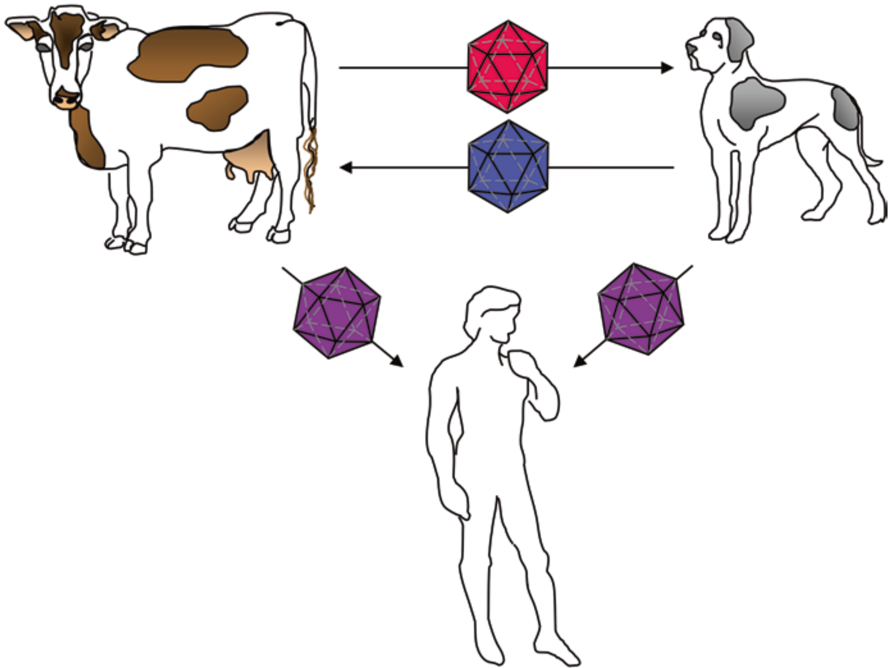

**Fig. 2** Overview of the putative zoonotic transmission of animal bocaviruses to the human population. Based on sequence analyses, especially of the terminal sequences, a zoonotic event is likely, as HBoV-1 contains genome structures highly conserved from the *Canine minute virus* and the *Bovine parvovirus*

could also reinfect elderly patients [115]. Surprisingly, HBoV-1 DNA can also be detected in blood and blood products from healthy Chinese blood donors with a lower seropositivity compared to the above-mentioned cohorts [116].

In recent months a few studies have been published that demonstrated that human bocaviruses are also stable in the environment. As an example, Iaconelli et al. have shown the frequent detection of HBoV in urban sewages, an observation confirmed by a study from Egypt [117].

## Clinical Features

HBoV-1 respiratory infection is clinically indistinguishable from other respiratory infections and can only be diagnosed using molecular assays. The spectrum of HBoV infections ranges from asymptomatic [67, 118, 119] to mild upper respiratory infections [67, 120–122] up to serious and life-threatening lower respiratory tract infections [70, 109, 123–133] in all age groups [70, 71, 109, 119, 123–136]. The immune response against HBoV starts with an IgM response and is followed by

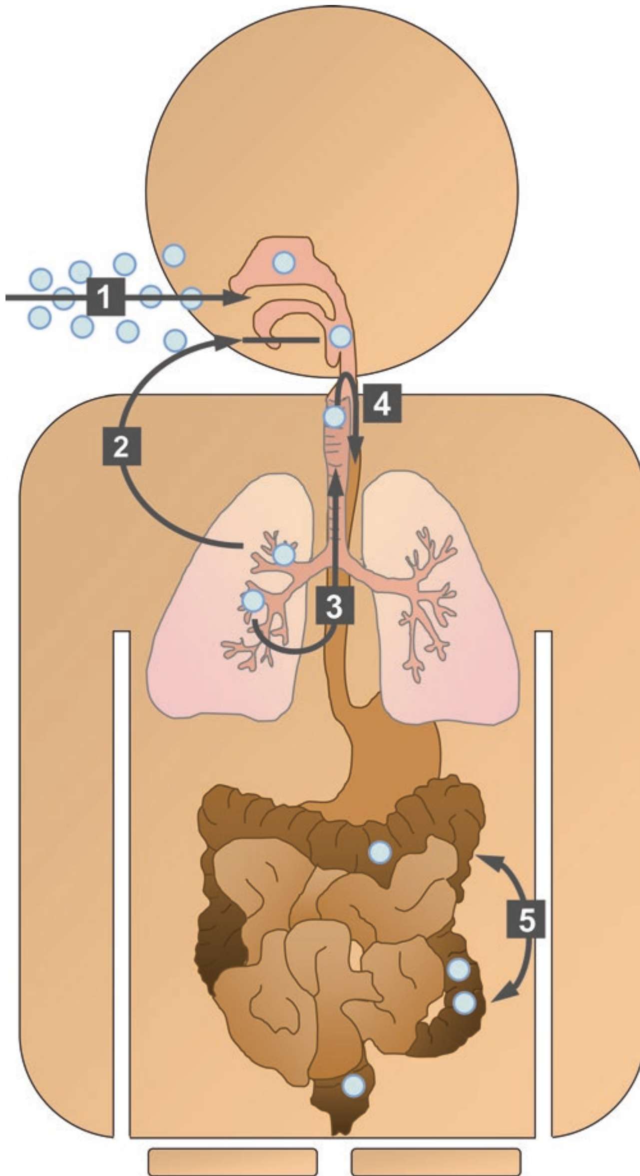


**Fig. 3** Schematic overview of the HBoV life cycle. (1) Entry through the nasopharyngeal space, (2) infection of the lung, (3) and (4) swallowing of the expectorated infectious secretion, and (5) infection of the gastrointestinal tract. Additionally, the virus spreads via the bloodstream and causes classical viremia (not indicated)

the formation of IgG [113, 114], but no lifelong immunity is generated in at least 40% of patients due to the original antigenic sin [i.e. Hoskins effect] [76, 82, 137].

The general HBoV-1 infections appear to start in the upper airways; in 2014 Proenca-Modena and co-workers demonstrated that hypertrophic adenoid is a major infection site with 25.3% of tested tissues positive for viral RNA and DNA, followed by nasopharyngeal secretions (10.5%), tonsils (7.2%) and peripheral blood (1.5%) [138]. Thereby it is worth to note that tonsils are suspected to be a major site of persistence as hypothesized by Clement and colleagues [139]. Subsequently the virus most likely initiates a downstream infection caused by swallowing of virus-containing secretions, which then enter the gastrointestinal tract where active viral replication occurs and is accompanied by a true viremia. Persistence of HBoV in the respiratory tract has been confirmed by a novel pyrosequencing approach by Wagner and co-workers, who observed primary infections and recurrence in a large cohort of paediatric patients [140].

HBoV-1 is able to infect the central nervous system and induces clinical symptoms of encephalitis or necrotizing encephalopathies [96, 98, 141]. HBoV-1 has been identified as a putative cause of idiopathic lung fibrosis [56] supported by the fact that a set of profibrotic cytokines were upregulated during HBoV infection in adults and their HBoV-dependent upregulation was confirmed in cell culture [142], whereas HBoV does not induce a clear Th1 or Th2 response [143]. The HBoV-dependent regulated cytokines furthermore include a subset of cytokines which are known to be involved in several cancer-associated pathways, supporting the hypothesis that HBoV may be associated with chronic diseases or even cancerogenesis [144–146]. Although this hypothesis requires further prospective studies, HBoV DNA was detected in lung and colorectal tumours. Detection of HBoV DNA, eventually associated with persistence, has been described in addition to detection in normal lung tissue [119] and in lung and colorectal tumours [146, 147]. HBoV-1 has been detected in other tissues such as tonsils [35, 139, 148, 149] and myocardium and may affect additional tissues that have not yet been tested for HBoV positivity.

Lung fibrosis, especially idiopathic lung fibrosis (IPF), is characterized by a Th2-type dominated immune response in the affected tissue (reviewed by [150–152]). The Th2 response in the lung is accompanied by increased expression levels of IL-4, IL-5, IL-10 and IL-13 and is followed by increased levels of CCL17 (TARC), CCL5 (RANTES) and others. Moreover, fibrosis is related to expression of TNF and IL-8; it is worth noting that the neutralization of TARC leads to a reduction of fibrosis in the animal model [151, 153]. In addition, an elevation of the TARC/IP-10 ratio is also characteristic for fibrosis and was previously discussed as a marker for IPF [154].

Moreover, a unique case has been described in which the infection/reactivation of HBoV occurred between two episodes of BAL sampling; the fibrosis-associated cytokines were expressed in association with the HBoV infection but not before, supporting the previously mentioned data. This data leads to the conclusion that HBoV colonization/chronic infection may be at least one trigger that could stimulate airway remodelling. However, it could be argued that not only the resident airway epithelial cells are involved in the in vivo immune response, but also additional patient-specific factors will contribute to altered profibrotic cytokine profiles. In

order to address this problem, experiments in an air-liquid interface culture of human airway epithelial cells were performed. These experiments confirmed that profibrotic cytokines were expressed by the infected cell cultures but were minimally or not at all expressed in mock-infected cells; the identified cytokines belong to the initial immune response following HBoV infection [123].

According to the literature, the two HBoV proteins VP2 and NP1 seem to influence the regulation of the interferon-beta pathway, but the data appear to be controversial as VP2 upregulates the pathway [155], whilst NP1 inhibits the IFN-beta production when overexpressed [156]. In addition, in an experimental setting with overexpression conditions, it has been shown that HBoV NS1 and Ns1–70 proteins inhibit the TNF-α-mediated activation of NF-κB by targeting p65 [157].

Moreover, based on clinical observations of a longitudinal study, Martin and colleagues concluded that HBoV infections could possibly be divided into two distinct clinical subgroups, one with a short viremic phase and short viral shedding, most likely being the primary infection, and a second group with long-term shedding; the second group is likely to be co-infections with other pathogens or a reactivation of a persistent HBoV infection [158].

## Coinfections and Persistence

Simultaneously with the discovery of HBoV in 2005, multiplexing PCR methods started to become an accepted diagnostic tool, and consequently detection of multiple infections, especially in respiratory tract diseases, has become a common phenomenon [67, 159–163]. Nowadays, multiple infections with up to six pathogens being simultaneously present in a single respiratory sample are frequent [67, 159–164] and may mislead some researcher to claim that the human bocavirus, also occurring in asymptomatic patients, is a harmless bystander rather than a pathogen [165, 166]. This hypothesis seems to be supported by the fact that a formal fulfilment of Koch's modified postulates was not yet possible for HBoV [167], as no animal model exists to date and volunteer transmission trials cannot be recommend based on our current knowledge of this virus [145].

In contrast, although there is a cohort of asymptomatic carriers [67, 119, 159, 166, 168, 169], several studies have shown that HBoV induces clinical respiratory symptoms [64, 91, 126, 127, 159, 170–176]. The asymptomatic viral shedding is meanwhile believed to originate from long-term shedding after an acute infection or from persistent viruses [34, 35, 95, 137, 177–180]. This has most recently been confirmed by a long-term prospective cohort study [67, 181]. Thereby it was shown that the rate of asymptomatic HBoV infections is similar to the rate of rhinovirus infections and no one would doubt that rhinoviruses are true pathogens [67]. Finally, HBoV is known to induce serious cytopathic effects in infected cell cultures, which is a typical feature of a pathogen [21, 40, 57, 177].

## Diagnostics

In addition to several published home-brew PCRs and real-time PCRs (reviewed by [9]), numerous commercial assays, such as the Luminex RVP assay [119, 182], the Idaho FilmArray [164, 182] or the RespiFinder assay [119], have been developed and released to the market enabling the detection of HBoV from clinical samples. However, multiplexing solely allows detection of the viral DNA in a respiratory sample without providing the essential information as to whether an active replicative infection underlies the currently clinical episode requiring laboratory testing [67]. As HBoV can be shedded for longer than 3 months after the acute symptomatic phase [67], a proper diagnostics of human bocavirus requires the proof of active replication, which can be done either by detection of a viremia in the peripheral blood [91, 107, 115, 137, 183–187] or by detection of spliced viral RNA transcripts that were shown to be present exclusively during the active phase of the replication [188]. Recently, a novel rapid antigen test was developed which could be a major advance in HBoV diagnostics [189]. Further progress in this direction can be expected from novel approaches to test for human antibodies and bocviral antigens from all four subtypes based on yeast-derived virus-like particles [190].

## Advanced Molecular Techniques in HBoV Research and Diagnostics

The discovery of HBoV has become possible due to the usage of a novel virus discovery strategy used by Allander and colleagues in 2005 [1]. These authors used a strategy of a virus screening library combined with a 96-well format high-throughput sequencing approach based on rolling circle amplification and sequencing. This technique was used subsequently also by other labs and has become a simple but work-intensive strategy to identify novel viruses and virus variants.

In addition, with the isolation and propagation of HBoV in three-dimensional air-liquid interface cell cultures, another novel method has set standards for the research on respiratory viruses in general and human bocavirus in particular [21]. This technique has meanwhile been refined, and several models are available [8, 40, 59, 177].

## Summary and Perspective

There is an increasing body of evidence showing that the human bocavirus is a serious pathogen that is associated with acute respiratory infections, sometimes with life-threatening complications. In addition, there is evidence that the human bocavirus could contribute to long-term disease of the airways resulting in lung carcinoma

or lung fibrosis. It is therefore crucial to analyse the long-term effects of HBoV infections in order to identify the mechanisms of HBoV persistence as well as for determining host factors for asymptomatic infections and to test the hypothesis that HBoV could trigger the development of lung cancer and fibrosis. Novel studies have identified the antigenic epitopes on the viral surface and may enable the development of potent vaccines or antibody-based therapies [191].

In any cases, the proper diagnostics of HBoV require additional attention as does the need for HBoV to be evaluated in terms of its interaction with other respiratory viruses that may simultaneously be detected during clinical episodes.

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# Technical Advances in Veterinary Diagnostic Microbiology

**Dongyou Liu**

## Introduction

Microorganisms (including subcellular viruses, unicellular bacteria and yeasts, multinucleate/multicellular filamentous fungi, protozoa, and helminths) make up a significant part of the biomass on earth. While many microorganisms are free-living and involved in the degradation of plant debris and other organic materials, others lead a symbiotic, mutually beneficial life within their hosts. Moreover, some microorganisms have the capacity to take advantage of transient weaknesses in their hosts, and induce notable pathogenic changes that lead to significant morbidity and mortality.

Given the shared phylogeny between humans and animals, some pathogens (i.e., zoonotic pathogens) that normally infect animals may transcend the host boundaries and establish in human hosts, causing zoonotic diseases (Fig. 1). Examples of zoonotic pathogens include *Bacillus anthracis*, *Brucella* spp., *Clostridium* spp., *Escherichia coli* O157, *Coccidioides*, and *Cryptosporidium* [1]. In addition, some plant pathogens (especially fungi) may be involved in cutaneous and systemic mycoses as well as poisonings through their toxins in humans and animals (Fig. 1).

Therefore, accurate identification and tracking of microbial pathogens affecting animal hosts not only facilitate their control but also help prevent their further spread to human hosts. As recent advances in veterinary diagnostic virology are covered in chapter "Recent advances in veterinary diagnostic virology" and reviewed elsewhere [2], this chapter summarizes the latest developments in the identification, typing, virulence determination, and antimicrobial susceptibility testing of pathogenic bacteria, fungi, and parasites affecting animals.

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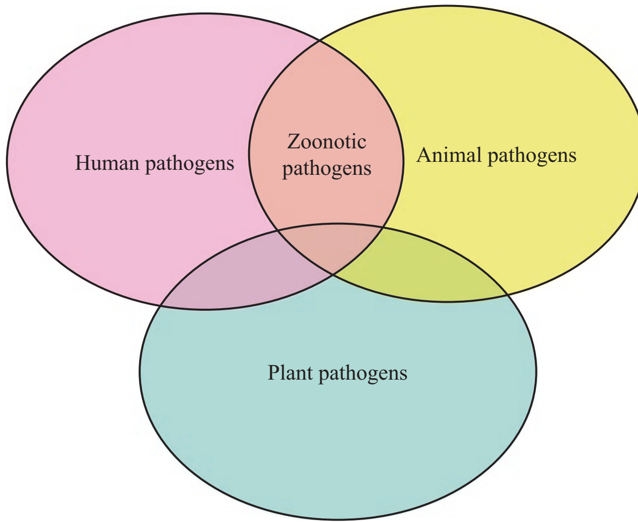


**Fig. 1** Relationships among pathogenic microorganisms affecting humans, animals, and plants

## Identification

A primary objective of veterinary diagnostic microbiology is the identification of pathogenic microorganisms involved in animal disease outbreaks. Similar to its medical counterpart, veterinary diagnostic microbiology has traditionally relied on phenotypic techniques for microbial characterization. These procedures typically assess the morphological, biological, biochemical, serological, in vitro, and in vivo properties of microorganisms and have played an essential role in the diagnosis of microbial diseases affecting animals. However, due to their limited sensitivity and/or specificity, inadequate reproducibility, and slow turnaround, phenotypic procedures are increasingly supplemented and/or superseded by molecular techniques that target the nucleic acids for the identification as well as typing of microbial pathogens affecting animals (Table 1) [3].

Morphological characterization builds on the premise that various classes of microorganisms often demonstrate distinct morphological features (e.g., size, shape, internal and external components, colony morphology) which allow their initial identification upon macroscopic and microscopic examination. Application of light microscopy or transmission/scanning electron microscopy (EM) together with relevant stains/dyes helps reveal additional morphological details. For example, hematoxylin and eosin (H&E), Gram, Giemsa, and crystal violet stains are useful for enhancing the contrast of microbes to their background; Gimenez and Pinkerton's stains for detecting rickettsial organisms in tissue sections; Ziehl-Nielsen, Kinyoun, or auramine O stains for initial identification of mycobacteria; KOH, lactophenol cotton blue, India ink, and Southgate's mucicarmine stains for detection of fungi; periodic acid-Schiff (PAS), Grocott's methenamine silver (GMS), Fontana-Masson,

**Table 1** Common laboratory techniques for identification of microbial pathogens

| Technique | Key features |
|---|---|
| Morphological | Examination of macroscopic and microscopic features (e.g., size, shape, internal and external components, colony morphology) of microorganisms allows their rapid, inexpensive identification. The use of general or specialized stains/dyes further enhances the contrast of microbes to their background. Nonetheless, morphological characterization may lack desired sensitivity and specificity, and its result interpretation is sometimes subjective |
| Biochemical | Analysis of microbial metabolic or enzymatic products (e.g., carbohydrate, protein, amino acid, fat, and enzyme) enables their discrimination at genus- and species-levels. However, the performance of biochemical tests is impacted by factors that influence microbial growth and metabolism |
| Serological | Detection of specific interactions between host antibodies and microbial antigens (e.g., protein, carbohydrate) by serological techniques provides indirect evidence for causal relationships between diseases and microbial pathogens. Serological tests have a relatively high sensitivity, specificity, and quick turnaround time but may show cross-reactivity with closely related microbial species |
| Biological, in vitro and in vivo | Assessment of biological features (e.g., host range, transmission pattern, pathological effects, geographical origin) of microorganisms helps diagnose microbial infections in cases where other relevant data are scarce. In vitro culture techniques using laboratory media and cell lines facilitate isolation and propagation of target microorganisms for subsequent morphological, biochemical, serological, and molecular characterization. In vivo assays using laboratory animals and chicken embryos allow for recovery of microorganisms that fail to grow on culture media or cell lines and help determine host susceptibility and immune response to and pathogenic effects of microorganisms |
| Molecular | Detection of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) using molecular techniques offers direct evidence on the presence of microorganisms. Application of nucleic acid amplification technologies and subsequent automation further improve the speed, sensitivity, and specificity of microbial identification |

Gridley's, and H&E stains for detection of mycotic elements in tissue biopsies; and clearing agents (e.g., beechwood creosote, lactophenol, glycine) and carmine-based stains for improved visualization of nematodes. The application of fluorescently labeled antibodies, fluorescent sensor molecules in electron microscopy, fluorescence microscopy, or time-lapse microscopy further enhances morphological characterization of microorganisms. Moreover, the atomic force microscopy (AFM) technique provides a powerful platform for analyzing the structure, properties, and functions of microbial pathogens as well as the localization, mechanics, and interactions of the individual cell wall constituents, contributing to the elucidation of the molecular bases of cell adhesion (nanoadhesome) and mechanosensing (nanosensosome) [4]. Besides unraveling paradigms of pathogen entry and pinpointing the exact intracellular location, these new techniques permit direct monitoring of the intracellular lifestyle of microbial pathogens and yield insights into the underlying mechanisms of their pathogenicity [5].

Biochemical characterization examines the metabolic or enzymatic products of microorganisms, including distinct patterns of carbohydrate, protein, amino acid, fat metabolisms, and production of particular enzymes. Biochemical tests help distinguish between aerobic and anaerobic breakdown of carbohydrates, identify carbohydrates and their specific breakdown products (e.g., formation of acids, alcohols, and gases when grown in selective liquid or solid media), determine the ability of microorganisms to utilize substrates (e.g., citrate and malonate), assess the metabolism of protein and amino acids (e.g., gelatin liquefaction, indole production, amino acid decarboxylase test, and phenylamine deaminase test) as well as of fats (e.g., hydrolysis of tributyrin), and detect production of enzymes (e.g., catalase test, oxidase test, urease test, ONPG test, and nitrate reduction). Analysis of fungal primary metabolites such as ubiquinones (coenzyme Q) is valuable for the taxonomy of black yeasts and filamentous fungi, whereas examination of fungal secondary metabolites (e.g., steroids, terpenes, alkaloids, cyclopeptides, and coumarins) by chromatographic techniques offers another means for fungal identification. A recent approach for biochemical characterization of microorganisms centers on the characteristic outer surface charges of microbes that contribute to their distinct migration under a direct-current electric field such as capillary electrophoresis (CE), leading to rapid and efficient separation, identification, quantitation, and characterization of intact microorganisms (i.e., bacteria, viruses, and fungi) [6]. Another useful technique for biochemical characterization of microbes is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), which has shown promise for specific identification of *Enterobacteriaceae* and other microbial pathogens [7].

Serological characterization measures the increased levels of specific IgA, IgM, and IgG antibody titers or seroconversion in blood, urine, and fecal materials, providing indirect evidence for causal relationships between diseases and microbial pathogens [8]. An interesting development in the serological characterization of disease-causing microorganisms is the use of chemically synthesized peptides. Generated by chemical approaches, these peptides are composed of two or more amino acids linked together by peptide bonds. By mimicking naturally occurring peptides or segments of proteins, these peptides serve as synthetic antigens in peptide microarrays as potential diagnostic tools in high-throughput immunoassays [9, 10]. Other new developments in serological characterization of microorganisms include biosensors and nanotechnology (nanoarrays and nanochips). Biosensors involve the use of a microbe-specific antibody and a transducer (e.g., electrochemistry, reflectometry, interferometry, resonance, and fluorimetry) to convert a biological interaction into a measurable signal [11]. In the particle concentration fluorescence immunoassay (PCFIA) for brucellosis, submicron polystyrene particles are coated with antigen and placed in a 96-well vacuum plate. After addition of fluorescent conjugate followed by vacuum filtration to remove unbound conjugate, the total particle-bound fluorescence is measured by front-surface fluorimetry. Nanotechnology (e.g., nanoarrays and nanochips) offers small-scale platforms to identify an array of infectious agents or serotypes on a single chip.

Biological characterization targets the issues related to the host susceptibility, transmission patterns, pathological effect(s), and geographical origin of microbial pathogens, which help achieve correct diagnosis of microbial infections in cases where other relevant data are scarce. In vitro isolation and propagation on laboratory media and cell lines offer a valuable tool for identification and diagnosis of microbial infections. The size, color, shape, and form of colonies formed by microorganisms on nutritional agar and other selective media are diagnostically informative. Microsporidia and parasitic protozoa may also be cultivated as a means of identification [12]. However, because not all microorganisms will grow in laboratory media and cell lines, embryonated eggs, insect vector, and laboratory animals (e.g., rodents) may be utilized. For example, *Trypanosoma cruzi*, the causal agent for Chagas disease, is grown in the guts of its vector triatomine bug for confirmation and diagnosis. The availability of cultured isolate/strain permits further antigenic studies, antibiotic susceptibility testing, and genetic investigations. Despite their relatively high expense and length of time required, in vitro and in vivo culture techniques have aided in the studies of microbial taxonomy, biology, epidemiology, pathogenesis, and treatment response. A recent development in the use of in vivo techniques for microbial characterization relates to the in vivo bioluminescence imaging or biophotonic imaging (BPI). Based on genetically engineered bioluminescent/fluorescent microorganisms, this technique assists the study of microbial infections and host immune responses [13]. Application of genetically engineered mice with luciferase reporters for specific microbial or host genes helps overcome the limitations of in vivo bioluminescence imaging for assessment of microbial replication, activation of key genes in host immunity, and response to tissue damage in vivo [14].

Molecular characterization exploits the superior sensitivity, exquisite specificity, and rapid turnaround of nucleic acid detection procedures for identification and typing of microbial pathogens. Progresses in the areas of genetic target selection, template preparation, transition from nonamplified to amplified approaches, and automation in product detection over the past two decades have made molecular techniques an indispensable tool in the diagnosis of microbial pathogens of both medical and veterinary importance [3].

Genetic targets for microbial identification and typing are generally of three types: nonspecific, shared, and specific. Nonspecific genetic targets include the guanine and cytosine composition (or G + C content), short random primer sites, randomly dispersed repetitive extragenic palindromes (REP), enterobacterial repetitive intergenic consensus sequences (ERIC), variable number tandem repeats (VNTR) (also known as simple sequence repeats or microsatellites), restriction enzyme sites, etc. Shared genetic targets comprise ribosomal RNA (rRNA) genes (e.g., 16/18S rRNA, 23/26/28S rRNA), internal transcribed spacer (ITS) regions, mitochondrial DNA (mtDNA), housekeeping genes, etc. Specific genes are uniquely present and enable precise identification and determination of pathogenic bacteria, fungi, and parasites.

As an essential organelle involved in the synthesis of proteins in all cellular organisms, ribosome harbors ribosomal RNA (rRNA) genes that are composed of a

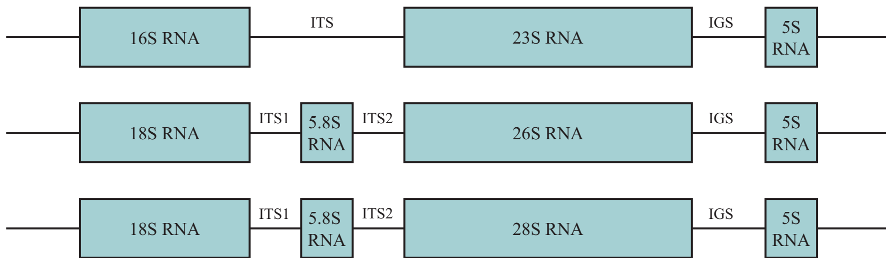


**Fig. 2** Schematic presentation of ribosomal RNA (rRNA) genes in bacteria/microsporidia (top panel), yeasts /protozoa (middle panel), and filamentous fungi/helminths (bottom panel). [IGS, intergenic spacer (or non-transcribed spacer); ITS, internal transcribed spacer]

small subunit (30 Svedberg units or 30S in prokaryotes and 40S in eukaryotes; sitting on top of large subunit) and a large subunit (50S in prokaryotes and 60S in eukaryotes; forming a dome-shaped structure). Whereas the small subunit (SSU) comprises a 16S rRNA molecule in prokaryotes or a 18S rRNA molecule in eukaryotes, the large subunit (LSU) includes 23S and 5S molecules in prokaryotes and microsporidia; 26S, 5.8S, and 5S molecules in yeast and protozoa; but 28S, 5.8S, and 5S molecules in filamentous fungi and helminths (Fig. 2).

Ribosomal RNA molecules provide a mechanism for decoding/transcribing messenger RNA (mRNA) into amino acids (at the center of small subunit) and function as petidyltransferase (large subunit) through its interaction with transfer RNA (tRNA) during the synthesis of proteins. Given their vital roles in living organisms, rRNA genes are highly conserved and present in multiple copies. Therefore, rRNA genes offer extremely valuable targets for molecular identification and typing of bacterial, fungal, and parasitic pathogens.

Preparation of nucleic acid templates from cultured isolates and clinical specimens represents an important initial step for molecular identification and detection of microorganisms. This often involves (1) disruption of cell walls, (2) denaturation, and (3) removal of contaminating proteins, polysaccharides, polyphenolic pigments, and other compounds [15]. While enzymatic digestion (e.g., using lyticase, zymolase, chitinase, gluculase, and/or proteinase K) and occasionally acid and alkali treatments may be effective for breaking up bacterial and yeast cells, mechanical grinding, sonication, or bead-beating is often necessary to disrupt the mycelial and helminth cell walls. Following the extraction with organic solvents (e.g., phenol/chloroform) and detergents (e.g., sodium dodecyl sulfate, SDS; hexadecyltrimethylammonium bromide, CTAB; and *N*-Lauroylsarcosine), which denature cytosolic proteins and lipid membranes and inactivate endogenous DNase/RNAse, nucleic acids of high purity are obtained after precipitation with ethanol or isopropanol. The recent development of various easy-to-use commercial kits has negated the need to use hazardous organic solvents in the isolation of microbial DNA/RNA. Furthermore, automated nucleic acid extraction systems have become increasingly sophisticated and affordable, contributing to the streamlining of template preparation and reduction of potential cross-contamination during manual handling.

The early-generation molecular procedures rely on nonamplified, hybridization approaches, such as DNA–DNA hybridization (for estimation of guanine–cytosine ratio or G–C content), and use of gene probes in dot blot, Southern blot, fluorescence in situ hybridization (FISH), etc. [16]. A more recent development in the DNA hybridization-based approach is DNA microarray, in which high-density oligonucleotide probes (or segments of DNA) are immobilized on a solid surface and used to hybridize (catch) any complementary sequences (labeled with fluorescent nucleotides) in a test sample.

Subsequent detection and quantification of fluorescence signal permit identification and determination of the relative abundance of nucleic acid sequences in a sample [17]. Although these nonamplified procedures have adequate specificity, they are relatively insensitive, and often require large quantity of starting materials for reliable detection. Nonetheless, some of these nonamplified techniques remain valuable for comparison of microbial genomes and for identification of species- and virulence-specific gene regions. For example, dot blot hybridization was employed for screening genomic DNA libraries of *Dichelobacter nodosus* strains causing virulent and benign foot rot and several virulent- and benign-specific gene regions with potential for differentiation of virulent and avirulent *D. nodosus* strains were identified as a result [18, 19]. This approach was also applied for identification of novel virulence-specific gene regions in zoonotic bacterial pathogen *Listeria monocytogenes* and novel species-specific gene in animal bacterial pathogen *Listeria ivanovii* [20, 21].

The mid-1980s witnessed the advent of a novel, highly efficient in vitro nucleic acid amplification technique known as polymerase chain reaction (PCR). This technique has the capacity to synthesize billions of copies from a single nucleic acid template within 3–4 h and demonstrate superior sensitivity, exquisite specificity, rapid turnover time, and amenableness to automation for high-throughput testing. Since then, PCR and its variants [e.g., nested PCR, multiplex PCR, real-time PCR, quantitative PCR, reverse transcription PCR (RT-PCR), arbitrarily primed PCR (AP-PCR), random amplified polymorphic DNA (RAPD), degenerate oligonucleotide primed PCR (DOP-PCR), sequence-independent single-primer amplification] [22, 23]. Apart from PCR, other nucleic acid amplification procedures include nucleic acid sequence-based amplification (NASBA), ligase chain reaction (LCR), strand displacement amplification, Q-b replicase-mediated amplification, linear linked amplification, loop-mediated isothermal amplification (LAMP), etc.

Conventional methods for detection of nucleic acid products are based on electrophoretic separation followed by staining with ethidium bromide, GelStar, or SYBR Green. While agarose gel electrophoresis provides a convenient, inexpensive way for separation and semiquantitation of DNA and RNA, polyacrylamide gel electrophoresis (PAGE) is useful for separating small nucleic acid fragments (<500 bp). Among the various PAGE-based procedures, single-strand conformational polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE), and temperature gradient gel electrophoresis (TGGE) are widely applied. SSCP is capable of detecting single nucleotide variations, and in combination with capillary electrophoresis (CE), SSCP-CE provides an automated system for rapid

separation of nucleic acid products. Recent advances in instrument automation and fluorescent dye chemistry permit real-time monitoring of PCR amplicons (so-called real-time PCR). Besides the use of double-stranded DNA intercalating dye (e.g., SYBR Green), specifically designed probes such as hydrolysis dual-labeled probes (TaqMan®), hybridization probes (LightCycler), molecular beacons, peptide nucleic acid (PNA) probes, TaqMan minor groove-binding (MGB™) probes, locked nucleic acid (LNA®) primers and probes, and scorpions™ may be utilized [24].

Other nucleic acid detection approaches include DNA microarray (also known as DNA chip, gene or genome chip, or gene array), biochips (biosensors), line probe assay (LiPA), enzymatic signal amplification (e.g., ELISA and flow cytometry), and DNA sequencing. Biochips (biosensors) are small analytical devices designed for nucleic acid-based electrical/optic detection (fluorescence or chemiluminescence) [25]. DNA sequencing analysis provides a most accurate way to determine the identity of microbial organisms. Whereas the classic Sanger method (also known as "chain termination method" or "dideoxy sequencing") can read up to 900 bp and produce 100 kb of sequence data per run, the "next-generation sequencing" technologies (e.g., 454 pyrosequencing-based instrument, Genome Analyser, and SOLiD System) allow longer reading (up to 400 bp) and generation of 400 Mb–20 Gb sequence data per run, respectively.

## Typing

Microbial pathogens are noted for the diversity and their ability to adapt and survive in challenging environments. The ability to type and track microbial strains and varieties involved in disease outbreaks is crucial for their control and prevention. For this reason, a number of phenotypic and molecular procedures have been developed and applied for subtyping and phylogenetic analysis of microbial strains such as restriction fragment length polymorphism (RFLP), pulse-field gel electrophoresis (PFGE), ribotyping and amplified techniques (e.g., amplified fragment length polymorphism (AFLP), PCR-RFLP, multilocus sequence typing (MLST), and mobile genetic element-PCR (MGE-PCR) (Table 2) [26].

## Virulence Determination

Many microorganisms demonstrate varied pathogenic potential within the species or genus [27]. In view of their similar epidemiology but distinct pathogenicity, the ability to accurately assess microorganisms of differing virulence is crucial for their control and prevention. For example, Gram-negative bacterium *D. nodosus* encompasses strains that cause virulent, intermediate, or benign foot rot in sheep. Since virulent and some intermediate foot rot induces lameness and severe pain in affected sheep, leading to ill-thrift and reduced weight gain, it is important to apply control

**Table 2** Common laboratory techniques for typing and phylogenetic analysis of microbial pathogens

| Technique | Key features |
|---|---|
| Biotyping | Biotyping separates microbial strains into "biotypes" on the basis of their metabolic and enzymatic activities (e.g., sugar fermentation, amino acid decarboxylation/deamination, urease activity, hydrolysis of compounds, hemagglutination, hemolysis), colonial morphology, and environmental tolerances (e.g., tolerance to pH, chemicals, dyes, heavy metals). Biotyping is generally reproducible and easy to perform and interpret. However, it has poor discriminatory power due possibly to variation in gene expression and point mutation |
| Phage typing | Phage typing distinguishes microbial strains into "phage types" by their patterns of resistance or susceptibility to a standard set of bacteriophages, depending on the presence or absence of particular receptors on the bacterial surface for phage binding. Phage typing shows good reproducibility, discriminatory power, and ease of interpretation but requires maintenance of biologically active phages and demands technical skills. Further, some strains may be nontypeable |
| Serotyping | Serotyping differentiates microbial strains into serotypes (serovars) according to the antigenic variations present on the surface structures (e.g., lipopolysaccharides, membrane proteins, capsular polysaccharides, flagella, and fimbriae). Agglutination, latex agglutination, coagglutination, or fluorescent and enzyme-labeled assays may be used for serotyping. Serotyping has good reproducibility and ease of interpretation and performance. However, serotyping depends on the availability of good quality reagents, and some autoagglutinable (rough) strains are nontypeable. Additionally, serological techniques may have limited discriminatory power due to cross-reactive antigens |
| Bacteriocin typing | Bacteriocin typing assesses microbial strains for their susceptibility to a set of bacterial peptides (bacteriocin) and has been employed to type stains of *Pseudomonas aeruginosa*, *Escherichia coli*, *Yersinia pestis*, etc. It shows good reproducibility, discriminatory power, and ease of interpretation but is technically demanding and does not work with nontypeable strains |
| Multilocus enzyme electrophoresis (MLEE) typing | MLEE typing separates strains into "electromorphs" (typically reflecting amino acid substitution that alters the charge of the protein) in accordance with their distinct electrophoretic mobilities of a set of metabolic enzymes. The technique has excellent reproducibility and ease of interpretation but shows moderate discriminatory power and requires expensive equipments |
| Antibiogram typing | Antibiogram typing compares different microbial isolates in their susceptibility to a set of antibiotics. The technique has ease of performance and interpretation and reasonable reproducibility. However, it has poor discriminating power |
| Restriction endonuclease analysis (REA) or restriction fragment length polymorphism (RFLP) | Digestion of chromosomal DNA with certain restriction endonuclease produces various fragments whose number and sizes (from 0.5 to 50 kb) are distinct among microbial strains and varieties. This technique has good reproducibility but generates complex profile of hundreds of bands that may be difficult to interpret electrophoresis |

**Table 2** (continued)

| Technique | Key features |
|---|---|
| Pulse-field gel electrophoresis (PFGE) | Based on restriction fragment length polymorphism (RFLP), PFGE uses selected restriction enzymes to yield between 8 and 25 large DNA bands of 40–600 kb in size, alternating currents to cause DNA fragments to move back and forth and resulting in a higher level of resolution of large fragments. This technique has good reproducibility and ease of interpretation. However, it requires costly reagents and equipment |
| Ribotyping | Ribotyping uses a ribosomal RNA (rRNA) probe derived from the *Escherichia coli* to detect the restriction fragment patterns of 16S rRNA, 23S rRNA, and tRNA after digestion of chromosomal DNA with appropriate restriction enzymes. Microorganisms are classified as separate species if their sequences show <98% homology and are classified as different genera if their sequences show <93% identity. As a derivative of RFLP, this technique is reproducible and is easy to interpret. However, it requires costly reagents and equipment |
| Amplified fragment length polymorphism (AFLP) | AFLP is a modification of RFLP through the addition of adaptors to restriction enzyme-digested DNA followed by PCR amplification and electrophoretic separation of PCR products, generating highly informative, polymorphic patterns of 40–200 bands for individual microbial strains. An obvious shortcoming of AFLP is its requirement for the ligation of linkers and indexers to enzyme-digested DNA from individual strains |
| PCR-restriction fragment length polymorphism (PCR-RFLP) | PCR-RFLP involves PCR amplification of one or more microbial housekeeping or virulence-associated genes followed by digestion with selected restriction enzymes and separation by agarose gel electrophoresis. The resultant band patterns allow differentiation of microbial subtypes. The technique obviates the need to ligate linkers and indexers before PCR amplification (as in AFLP) and represents a sensitive, discriminatory, and reproducible method for tracking and epidemiological investigation of microbial strains and varieties |
| Multilocus sequence typing (MLST) | In MLST, multiple DNA segments are amplified by PCR and examined by DNA sequencing analysis, leading to phylogenetic comparison of multiple isolates and definitive identification of microbial strains and subtypes. MLST is reliable and easy to interpret |
| Mobile genetic element-PCR (MGE-PCR) | MGE-PCR uses a single primer in PCR to amplify particular MGEs followed by electrophoresis to discriminate amplicon profiles. This technique has been utilized to characterize different isolates of *Trypanosoma brucei* by targeting RIME which has a relatively high copy number in the genome |

measures to stem the economic losses. On the other hand, benign foot rot causes minimal harm to affected sheep and has the tendency to self-cure; it is unnecessary and indeed wasteful to treat benign foot rot. Traditionally, the virulence of *D. nodosus* strains is determined by elastase test and gelatin gel test, which may take up to 4 weeks to complete and often demonstrate notable variability. After comparative analysis of recombinant DNA libraries from *D. nodosus* virulent and benign strains, a panel of virulent- and benign-specific genes was identified. The use of gene probes and primers derived from these genes facilitates rapid and sensitive determination of *D. nodosus* virulence [18, 19].

The Gram-positive bacterium *L. monocytogenes* is a zoonotic pathogen that encompasses a spectrum of strains with various pathogenic inclinations. While some *L. monocytogenes* strains are highly pathogenic and sometimes deadly, others are relatively avirulent and cause little harm in the host. The current laboratory techniques for assessing the virulence of *L. monocytogenes* strains include the mouse virulence assay and in vitro cell assays. While the mouse virulence assay is capable of providing an in vivo measurement of all virulent determinants, its high expense limits its application. Representing a low-cost alternative to the mouse virulence assay for assessing *L. monocytogenes* virulence, in vitro cell culture techniques measure the ability of *L. monocytogenes* to cause cytopathogenic effects in the enterocyte-like cell line Caco-2, to form plaques in the human adenocarcinoma cell line HT-29 or to cause death in chicken embryos. Several other cell lines (e.g., hepatocyte Hep-G2, macrophage-like J774, epithelial Henle 407 and L2) are also useful for studies on *L. monocytogenes* ability to adhere, invade, escape from vacuoles, grow intracellularly, and spread to neighboring cells (Fig. 3) [28]. However, these techniques are time-consuming, and occasionally variable. Following recent identification of novel virulence-specific genes (e.g., *inlJ*), the virulence of *L. monocytogenes* strains can be rapidly and specifically determined by PCR [20, 29].

## Antimicrobial Susceptibility Testing

Microorganisms have the ability to acquire resistance to drugs administered for their treatment. As drugs are often used in animals (e.g., cows, pigs, chickens, fish, etc.) that provide an important source of human food, microorganisms exposed to these drugs can develop antibiotic resistance through horizontal gene transfer events (e.g., conjugation, transduction, or transformation) and point mutations [30]. The resistant bacteria in animals due to antibiotic exposure can be transmitted to humans through the consumption of meat, from close or direct contact with animals, or through the environment [31]. For example, the use of fluoroquinolone in poultry production has been linked to the emergence of fluoroquinolone-resistant campylobacter infections in humans. Some bacteria (e.g., *Staphylococcus aureus*, *Enterococci*, *Gonococci*, *Streptococci*, *Salmonella*, and *Mycobacterium tuberculosis*) have acquired multidrug resistance. While the application of in vitro culture technique facilitates determination of MIC (medium inhibition concentration) of the strains, detection of specific gene mutations provides an alternative approach for assessment of antimicrobial drug resistance in microbial pathogens of economic importance [32, 33].

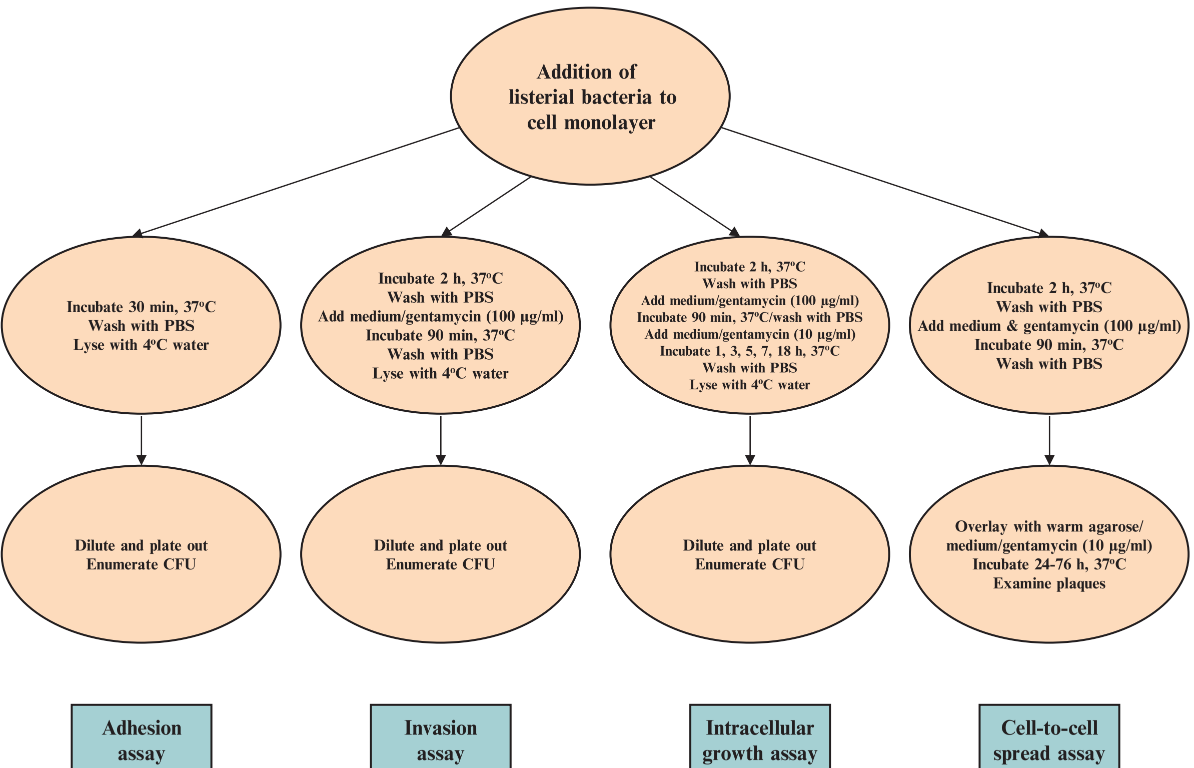


**Fig. 3** Common in vitro cell assay protocols for *Listeria monocytogenes* virulence. Variations in incubation time and gentamycin concentrations facilitate assessment of listerial pathogenicity. (CFU colony-forming units, PBS phosphate-buffered saline)

## Conclusion

Given the diversity of animal hosts that are susceptible to a wide range of microbial infections, veterinary diagnostic microbiology faces a greater challenge than its medical counterpart in achieving a correct and timely identification of culprit microorganisms causing significant economic losses in agricultural production. The threat of zoonotic pathogens (e.g., *Bacillus anthracis*) being used in bioterrorism attacks and the emergence of rapidly evolving antibiotic-resistant microorganisms and animal pathogens causing severe diseases in humans have made the development and application of improved diagnostic methods for animal pathogens increasingly important. Although phenotypic procedures are useful for microbial identification, their time-consuming nature and occasional variability have provided the impetus for the development and adoption of nucleic acid detection methodologies. Further improvement through miniature, multiplexing, and automation will extend the utility and reduce the cost of these genotypic testing procedures.

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# Recent Advances in Veterinary Diagnostic Virology

**Anbu K. Karuppannan, Alessandra Marnie M. G. de Castro, and Tanja Opriessnig**

## Introduction

Infectious diseases in animals have a high impact not only on the farming sector, but also on human welfare, food security, international trade of food products, and many other areas. The concept of One Health has been universally accepted by the contemporary scientific community and policy makers. The interconnected nature of animal, human, plant, and environmental well-being is increasingly appreciated, and has brought together expertise from many disciplines in understanding and solving the current issues of One Health. Despite advancements in various areas such as environmental engineering to improve sanitation and hygiene in animal husbandry, improved vaccine and diagnostic technologies, and increasing numbers of epidemiological studies to understand the spread of infectious disease, outbreaks of infectious diseases in farmed and wild animals are still quite common. Global trade of animal-related products ranging from meat and milk produced for human consumption, animal feed ingredients, veterinary biologicals, animal-derived biological and biochemical products, frozen semen, embryos and breeding livestock,

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animal hides, and others have increased the potential for the spread of infectious agents. In addition, the threat of bioterrorism is a serious risk in the modern world.

Only a handful of major livestock diseases such as foot-and-mouth disease (FMD), classical swine fever (CSF), and African swine fever (ASF) are being controlled in developed nations, and only one major disease, rinderpest, has been declared eradicated in 2011. The vast majority of the world nations are still suffering under the economic burden associated with farm animal pathogens. Viral infections in particular take a heavy toll due to their rapid spread and the high mutation rates of some viruses. The emergence and re-emergence of new or variants of already known viruses, some with zoonotic potential, are serious issues in veterinary and human medicine. A summary of the necessary steps of an animal pathogen to evolve into a human pathogen (Fig. 1) and methods by which this could be detected were illustrated by Temmam et al. [1]. Transboundary animal diseases (TADs), defined as highly contagious with a potential of rapid spread across national borders, have occurred in the recent past and include FMD, CSF, ASF, and influenza A. The introduction of ASF into the Caucasus region in 2007 and the subsequent uncontrolled spread to large territories of Russia and other Baltic states, the recent emergence of bluetongue virus (BTV) and Schmallenberg virus in Europe, both spread by *Culicoides* biting midges, and the emergence of pathogenic Seneca valley virus and atypical porcine pestivirus in pig populations around the world clearly illustrate that animal health authorities require a strong preparedness with continuous

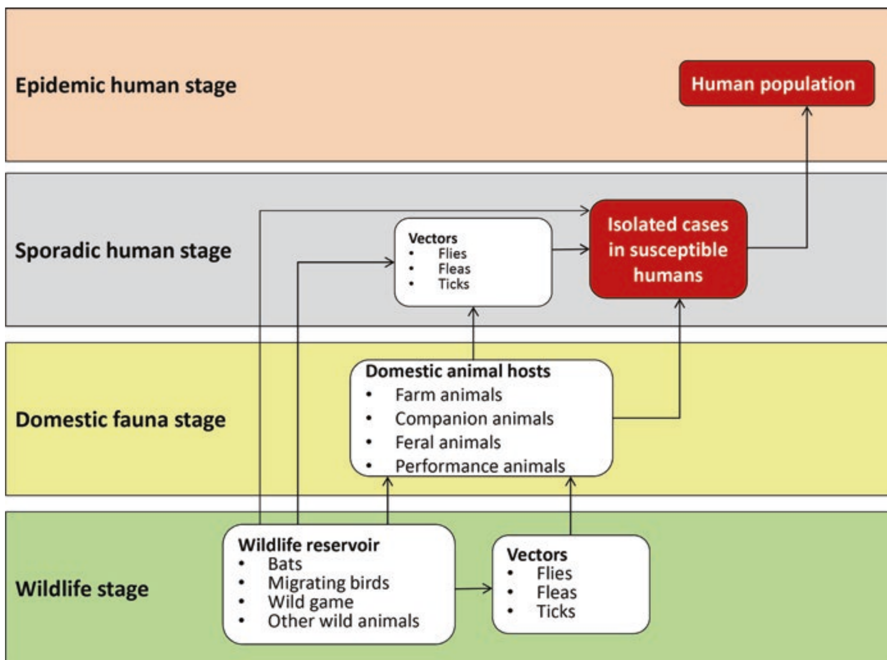


**Fig. 1** The origins of zoonotic human infections [1]

access to rapid and sensitive diagnostic tools for the successful fight against the emerging and reemerging pathogens.

Laboratory diagnosis of viral infections is based on the detection of virus particles, their genomes, or the immune response against the viruses. Isolation of viruses in cell culture or in susceptible animals is the classical gold standard to demonstrate presence of infective virus. However, virus isolation is not practical in every instance considering the long turnaround time, skilled labor, and resources required. Molecular detection of viral genomes, mainly by polymerase chain reaction (PCR) or detection of an antibody response against the virus of interest by serological assays, is rapid and reliable. While classical diagnostic methods such as virus isolation largely remain technically unaltered or show rather small changes and improvement, the molecular diagnostic methods have advanced dramatically over the last two decades with a range of novel diagnostic assays available now, including novel PCR assays, isothermal amplification methods, padlock PCR probes, and novel enzyme-linked immunosorbent assay (ELISA) systems and platforms. In order to improve on-site diagnosis in the field, basic methods and equipment were adapted, and portable PCR machines or simple thermo-platforms for isothermal amplification for viral genome detection and dipsticks as well as lateral flow devices for antigen or antibody detection are now available. Considering the wide reception of the above listed developments, *the prompt detection and rapid and exact identification of pathogens* is an important and essential task in veterinary virology. New technologies provide powerful tools for the rapid detection and identification of a wide range of causative agents, as well as supporting disease control and surveillance. For example, real-time or quantitative PCR (qPCR) assays are highly sensitive and specific methods which have gained wide acceptance for detection of various pathogens in diagnostic units, including the reference laboratories and the collaborating centers of the World Health Organization (WHO; http://www.who.int) and of the World Organization for Animal Health (OIE; www.oie.int).

## Standardization of Diagnostic Assays

The advent of novel assays, many of which are automated and high throughput, has increased the complexity of result interpretation, and comparison of results obtained by different assays can be challenging. Unlike the traditional diagnostic assays, it is often difficult to obtain a standard reference guide to interpret novel assays. It is essential for the diagnostician to understand the principles of any given assay and judge its advantages and limitations in the context of an analysis. A repository of negative and positive standard samples needs to be maintained to perform reliable and repeatable diagnostic assays. The operating range of an assay, the interval of analyte concentrations over which the method provides accuracy, defines its lower and upper limits of detection. To establish this range, a high positive reference sample, from an infected animal or spiked, is serially diluted to extinction in a negative matrix representative of the sample matrix. Negative, low positive and high positive

standards should all be within the normal operating range of the assay. A large volume of each of the controls should be prepared, aliquoted, and stored for routine use in every diagnostic run of the assay. The controls should mimic field samples, be handled and tested like routine samples, and are used to validate upper and lower control limits of the assay performance and to monitor random and/or systematic variability using various control charting methods [2]. A proficiency test or “ring test” of different clinical samples involving multiple laboratories can often be of great help to validate *in-house* assays [3].

In a broader perspective, associating with national or regional accreditation bodies such as the American Association of Veterinary Laboratory Diagnosticians (http://www.aavld.org), the European Association of Veterinary Laboratory Diagnosticians (http://www.eavld.org), the Collaborating Veterinary Laboratories (http://www.covetlab.org), or others will help maintaining the validity of diagnostic results. These organizations have prescribed practices at administrative, management, and technical levels to maintain a high level of accuracy and uniformity for various diagnostic tests. The principles behind the prescribed practices are applicable to most diagnostic virology tests.

## Sample Collection, Transportation, Storage, Enrichment, and Nucleic Acid Preparation

### *Sample Collection*

Proper sample collection is crucial for the reliable diagnosis of infectious diseases which also includes biosafety measures to avoid environmental contamination or exposure of other animals and humans to potentially infectious materials [2]. Clinical samples range from relatively clean fluids like urine, thick fluids like blood, to solid materials like stool (Table 1). Some samples may contain materials capable of sequestering nucleic acid-containing cells or different inhibitors that decrease extraction efficiency of pathogen genomes, and may also interfere with downstream molecular analysis such as PCR [4]. To increase the detection rate of a pathogen, knowledge on its epidemiology and pathogenesis should be considered during sample collection (Table 1). Tissue predilection, target organs, and duration and routes of shedding will all determine which samples need to be collected. Information on the chemicals used for anesthesia or euthanasia must also be considered, as some laboratory assays are not compatible with specific blood anticoagulants and tissue preservatives [2]. To preserve the sample integrity, it needs to be protected from desiccation (e.g., as can happen in certain freezers), frequent or extreme temperature fluctuations, UV degradation, humidity, and contamination [2, 4]. The process of carcass autolysis can destroy diagnostically relevant tissues and infectious agents, and can result in overgrowth of bacterial and fungal contaminants. Care must be taken to avoid contamination of the samples with detergents and antiseptic treatments, as these agents may interfere with the laboratory test procedures [2].

**Table 1** Sample collection for molecular diagnosis based on the knowledge on epidemiology and pathogenesis of selected diseases

| Disease | Sample collection | |
|---|---|---|
| | *Live animals* | *Dead animals* |
| Aujeszky's disease | Secretions | Nervous tissue<br>Lungs<br>Lymph nodes |
| Bluetongue | Blood | Liver<br>Spleen<br>Thoracic fluids |
| Foot-and-mouth disease | Vesicular fluid<br>Epithelial tissues<br>Esophageal pharyngeal tissues<br>Milk<br>Blood | Heart<br>Esophageal pharyngeal tissues |
| Rift Valley fever | Plasma or blood | Liver<br>Spleen<br>Brain |
| Vesicular stomatitis | Vesicle fluid<br>Epithelium covering unruptured vesicles<br>Epithelial flaps of freshly ruptured, vesicles<br>Swabs of the ruptured vesicles | Tissue with vesicles, ulcers, and erosions |
| Avian infectious bronchitis | Swabs from the upper respiratory tract | Trachea<br>Lungs |
| Avian infectious laryngotracheitis | Tracheal and oropharyngeal conjunctival swabs | Trachea<br>Lungs |
| Avian influenza A | Oropharyngeal and cloacal swab | Intestinal contents<br>Cloacal swabs<br>Oropharyngeal swabs<br>Trachea<br>Lungs<br>Air sacs<br>Intestines<br>Spleen<br>Kidney<br>Liver<br>Brain<br>Heart |
| Pig influenza A | Nasal swabs | Lungs |
| Infectious bursal disease (Gumboro disease) | Blood | Bursal homogenates |

(continued)

**Table 1** (continued)

| Disease | Sample collection | |
|---|---|---|
| | *Live animals* | *Dead animals* |
| Equine rhinopneumonitis | Nasal/nasopharyngeal swabs and blood | Fetal tissue from abortions: Lung, liver, spleen, and thymus<br>Nasopharyngeal swabs or deep nasal swabs<br>Tracheal wash<br>Bronchoalveolar lavage |
| Porcine reproductive and respiratory syndrome | Bronchoalveolar lung lavage<br>Serum<br>Oral fluids (pen-based diagnosis) | Lungs<br>Tonsils<br>Tracheobronchial lymph nodes |

## *Transportation*

During transport there is a potential for loss of sample identification and associated documentation, which needs to be avoided. To prevent potential degradation of the targeted nucleic acids, the samples are normally transported at low temperature using ice packs and are stored in −20°C (frequent) or −80°C (rare) freezers before and after shipment [2]. In addition, different filter papers or cards are commercially available and include FTA™ Cards (Whatman, GE Healthcare) which have been designed for sample transportation and storage at room temperature due to inclusion of chemicals that lyse cells, denature proteins, and protect nucleic acids from nucleases, as well as from the damage caused by heat, oxidative, and UV effects.

## *Sample Storage*

Appropriate sample storage is important to avoid sample degradation during long-term archiving of tested samples to maintain traceability of diagnostic results. Occasionally, it is necessary to check for the presence of a pathogen prior to its clinical emergence, and archived samples can be useful for this purpose. In addition, re-examination of archived samples with newer technologies will refine the understanding of the epidemiology and pathogenesis of infectious agents. Freezers that maintain a temperature of −80°C are commonly used to maintain samples for periods that may range from months to 5–10 years without problems [2]. However, ultralow temperature freezers (e.g., liquid nitrogen, cryopreservation in freezers at −140°C or lower) are considered ideal for long-term storage of biological materials, but maintenance of such equipment can be difficult and is often not feasible.

## *Sample Enrichment*

Diagnosis in veterinary virology is frequently complicated by the low amount of the targeted virus in certain types of clinical samples, including food and feed products and water samples, which may lead to false-negative results. To avoid or reduce this very important bottleneck effect, diagnostic laboratories apply a range of sample enrichment methods in order to "fish out" the targeted pathogens or their components, such as nucleic acids or proteins, from the analyzed specimens. Traditionally, ultracentrifugation has been used as a reliable method for virus concentration. Reports of methods including chromatography, immuno-affinity, or virus-binding ligands such as heparin molecules can be found in the literature [5]. More commonly, DNA enrichment is achieved by functionalized magnetic nanoparticles that are coupled with probes to which the target DNA could be hybridized [3].

## *Nucleic Acid Preparation Processes*

The proper preparation of viral nucleic acids to be used in PCR and other molecular diagnostic assays is a very important task in diagnostic laboratories. The basic viral nucleic acid preparation steps utilized in most procedures are summarized in Fig. 2. A real challenge for nucleic acid preparation in molecular diagnostics is deciding which methods or products to use. Chemicals or enzymes used during the extraction process, such as chaotropic agents or other salts, alcohols, and proteases, should be removed or inactivated before downstream analyses. Molecules like nucleases/

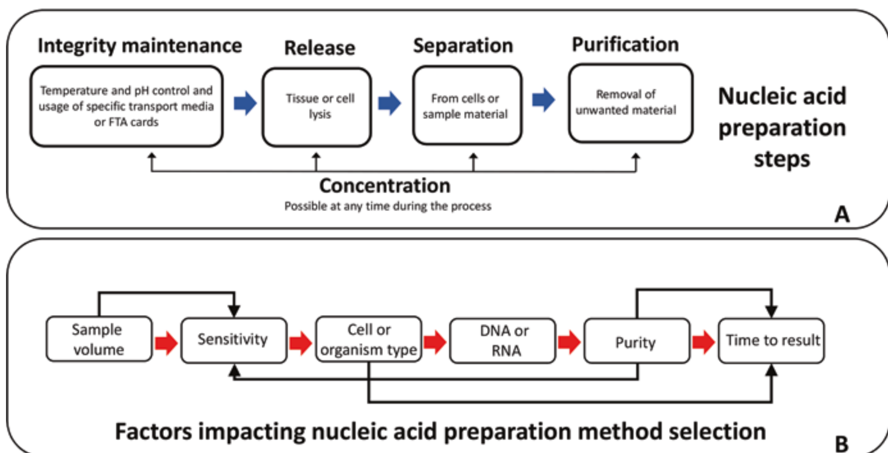


**Fig. 2** General preparation steps and decisions for nucleic acid preparation. (**a**) Basic nucleic acid preparation steps utilized in most procedures. (**b**) Considerations when selecting a nucleic acid preparation method. The connecting lines refer to relationships between decisions, with the tip of the arrow indicating what will be affected by the decision at the base of the arrow

proteins, polysaccharides, salts, and solvents also need to be removed from the nucleic acid preparations, because they can inhibit enzymatic and/or chemical reactions like PCR or interfere with visual real-time detection by blocking light or changing background fluorescence [3].

Manual genomic nucleic acid extraction procedures are standardized and work well for small numbers of samples. Simplicity and high-throughput capacity are major concerns in case of large disease outbreaks where a high number of samples have to be processed within a short period of time. It is almost impossible to complete such a task by manual extraction methods, which are labor and time intensive. In addition, personnel costs and uniformity of the extraction process need to be considered. Various kinds of automated equipment have been developed and commercialized for nucleic acid preparation and/or handling of samples and are suitable for high-throughput preparations. Available automated nucleic acid extraction platforms usually combine lysis and isolation techniques for DNA and RNA that are relatively free from impurities and can handle many sample types. Although the robotic extraction techniques increase the capacity, differences in analyte extraction efficiency compared to manual methods have been described [6]. This reiterates the need for using appropriate controls.

### *Nucleic Acid Amplification-Based Assays*

Conventional PCR reactions to detect viral genetic material are analyzed on an agarose gel at the end of the PCR cycling process, which consists often of 30–40 repeated cycles of denaturation, annealing, and extension steps, at which point the amplification reaction has reached a maximal plateau. This makes comparing the initial template quantity in samples using conventional PCR a semiquantitative estimate at the best.

Since the development of the real-time or quantitative PCR (qPCR) technique in 1996 [7], it has become a very reliable, high-throughput, and robust molecular tool for early, rapid, and sensitive detection of pathogens in both human and veterinary medicine. Compared to the conventional gel-based PCR (cPCR), the qPCR has several advantages, including high-throughput capacity, less hands-on time, lower risk of contamination, and the potential to be fully automated [8]. Quantitative PCR assays detect the amplicon in real time, at each cycling step; hence, they give a better quantitative estimate of the initial template concentration in a sample. If qPCR assays are run along with known standards, the number of copies of the template in a given sample can be estimated. Detection of the amplicon is done by using either non-specific dyes that fluoresce when they intercalate with double-stranded DNA or specific oligonucleotide-based probes which bind to a matching sequence that lies between the forward and reverse primer-binding sequences. Different types of probe chemistries have been developed by commercial vendors such as TaqMan probes, fluorescence resonance energy transfer probes, minor groove binding probes, padlock probes, etc. By using a sequence-specific probe in addition to the primers, the

specificity and analytic sensitivity of probe-based qPCR are increased. Fluorescent dye-based qPCR assays do not require probes and are therefore more cost efficient than probe-based assays. However, primer-dimer formations and non-specific amplicons may produce fluorescence similar to that in true positive samples leading to false-positive results. In order to avoid a wrong diagnosis, melting curve analysis is needed in these assays. In addition, with any PCR assay, one should consider viral genome evolution and mismatches in the regions where the primers and probes bind. If mismatches are present, the primers and probes may not bind, leading to false-negative results. Periodic monitoring of field viruses by amplification with conserved primers and Sanger sequencing will help to avoid this pitfall.

The OIE has listed selected nucleic acid amplification-based assays for detection of pandemic viruses and for identifying emerging and/or re-emerging viruses. Some examples are shown in Table 2. In the following, several examples are presented regarding specific problems, further illustrating the diagnostic application of various qPCR assays.

## Quantitative PCR Assays

### *Multiplex PCR: Detection of Multiple Viruses, Variants of a Virus, Including Vaccine Strains*

Detection and differentiation of multiple viruses or strains of a virus in a sample, at times vaccine strains versus field strains, is often desired. Multiplex assays, especially PCR-based, are suitable for simultaneous detection of different viral targets. However, multiplex PCR assays also come with the cost of reduced sensitivity.

*Detection of Virus Variants* Classical swine fever virus (CSFV) is a pestivirus within the family of *Flaviviridae*. It is the causative agent of CSF, a highly contagious disease affecting both wild boars and domestic pigs around the world. In Europe, the virus is largely maintained in the wild boar populations that serve as a reservoir for reintroduction to domestic pigs. Recently, a chimeric vaccine candidate, CP7_E2alf, has been developed and has the potential to be used as a safe and efficient marker vaccine in wild boars, which enables differentiation of infected from vaccinated animals [9, 10]. A vaccine-specific, probe-based quantitative reverse transcription PCR (probe-based qRT-PCR) assay was developed and evaluated, and a second, wild-type-specific assay was modified from an established one in such a way that both can be performed in two wells side by side in a microplate in a single run [11]. Both assays could be applied in CSFV vaccination and control programs in the wild boar population.

*Detection of Multiple Virus Variants* Rabies is a preventable disease but is still responsible for approximately 70,000 human deaths worldwide each year. Most of the human deaths occur in Asia and Africa where there is a lack of diagnostic

**Table 2** Molecular diagnostic assays for selected diseases as recommended by OIE (2016) (Manual of Diagnostic Tests and Vaccines, Office International des Epizooties, Paris)

| Disease | Method | Goal of the investigation | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Confirm that a population is free from infection* | *Confirm that an individual animal is free from infection prior to movement* | *Contribute to eradication policies* | *Confirm clinical cases* | *Investigate the infection prevalence (surveillance)* | *Determine the immune status in individual animals or populations postvaccination* |
| Bluetongue | cRT-PCR | – | +++ | – | +++ | ++ | – |
| | qRT-PCR | – | +++ | – | +++ | ++ | – |
| Japanese encephalitis | qRT-PCR | ++ | ++ | ++ | ++ | ++ | – |
| Rift Valley fever | cRT-PCR | – | – | – | +++ | + | – |
| Vesicular stomatitis | cRT PCR | – | + | – | ++ | – | – |
| Avian infectious bronchitis | cRT-PCR | +[a] | ++ | ++ | ++ | + | +[b] |
| Avian influenza (influenza A) | qRT-PCR | ++ | +++ | ++ | +++ | ++ | – |
| Equine influenza (influenza A) | qRT-PCR | – | +++ | +++ | +++ | +++ | – |
| Equine rhinopneumonitis | cPCR | – | +++ | – | +++ | – | – |
| Porcine reproductive and respiratory syndrome | cRT-PCR | +++ | +++ | +++ | +++ | +++ | – |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Influenza A viruses of swine | qRT-PCR | +++ | +++ | +++ | +++ | +++ | – |
| | cRT-PCR | – | – | – | ++ | – | – |
| Classical swine fever | cPCR | + | + | ++ | +++ | ++ | – |

*cPCR* conventional gel-based polymerase chain reaction, *qPCR* quantitative polymerase chain reaction/real-time PCR, *cRT-PCR* conventional reverse transcriptase polymerase chain reaction, *qRT-PCR* quantitative reverse transcriptase polymerase chain reaction/reverse transcriptase real-time PCR.

Key: +++ recommended method, ++ suitable method, + may be used in some situations, but cost, reliability, or other factors severely limits its application, – not appropriate for this purpose and n/a not applicable.

[a]Suitable for ensuring lack of infection during the past 10 days.

[b]Sometimes used in the evaluation of vaccines to assess protection against viral excretion but can be positive even when good clinical protection is achieved.

resources and expertise, making it difficult to develop effective prevention and control strategies. Rabies results from an infection by rabies virus, the type species of the genus *Lyssavirus*, family *Rhabdoviridae*. Even though rabies virus is responsible for most deaths, variants of the type species are also associated with similar disease [12]. Wadhwa et al. [13] developed a pan-*Lyssavirus* probe-based qRT-PCR assay called LN34 for the detection of all known rabies virus variants and other *Lyssavirus* species. LN34 assay uses a combination of multiplex primers and modified probes in addition to multiple standard controls. The assay has a high sensitivity and is important for the diagnosis of samples that have been stored or transported under sub-optimal conditions and cannot to be diagnosed by other methods [13].

*Differentiation of Infected and Vaccinated Animals (DIVA) Approaches* CSF remains endemic in some countries, including China, where vaccination with a traditional lapinized live virus is still practiced widely. Access to a DIVA diagnostic test could potentially be a powerful tool to discriminate whether pigs are naturally infected with wild-type strains or vaccinated with the lapinized live virus. The Chinese hog cholera lapinized virus (HCLV) has been through extensive serial passages in rabbits in China in the 1950s and is completely attenuated but retains its efficacy as a vaccine. This vaccine strain was introduced into European countries and named “Chinese” strain (C-strain). The Riems C-strain is one of the commercial vaccines derived from the C-strain. Differentiation of pigs vaccinated with the Riems C-strain from animals infected by CSF virus (CSFV) field strains using probe-based qRT-PCR has been investigated [14]. In a follow-up investigation, it was found that a point mutation in the primer-binding site could lead to the failure of detection of C-strain vaccine virus by the C-strain-specific quantitative RT-PCR [14].

Another qRT-PCR, based on primer-probe energy transfer technology (PriProET) for the improved detection of CSFV, has been developed [15]. The PriProET technology was developed initially as a novel qRT-PCR assay for the simultaneous detection of all serotypes of FMD virus (FMDV) [16] and subsequently was used for detection of other pathogens including swine vesicular disease virus (SVDV) [17], BTV [18], and for detection of influenza A virus of swine in outbreaks and to monitor the prevalence of disease. Following PCR amplification, the melting curve analysis allows confirmation of specific amplicons and differentiation between wild-type CSFV and the HCLV vaccine strain. Further evaluation of the assay demonstrated that in an RNA mixture of both wild-type CSFV and C-strain vaccine, the melting curves displayed only one curve, either a wild type-like or a vaccine-like, depending on the dominating RNA [19]. Therefore, the PriProET melting curve analysis could identify the presence of CSFV field strain in equivocal samples or in animals vaccinated with C-strain, but would not reliably detect infections with wild-type virus in a population vaccinated with the Riems strain.

In a recent study, a generic probe-based qRT-PCR was developed for the specific detection of three lapinized vaccine strains, namely, the Taiwanese Lapinized Philippines Coronel (LPC), the Chinese HCLV, and the European Riems C-strain [20].

The new assay could detect the Riems C-strain vaccine viral RNA in experimental samples, indicating that the assay could be a useful tool to facilitate outbreak control if a strategy using these lapinized vaccines is deployed.

## Molecular Tests for the Improved Detection of Food and Waterborne Zoonotic Pathogens

Food- and waterborne zoonotic pathogens frequently cause large outbreaks in regions where sanitation is poor. Hepatitis E virus (HEV) is an important food- and waterborne zoonotic pathogen, which is an emerging virus of global importance [21]. This single-stranded, positive-sense RNA virus belongs to the genus *Hepevirus* in the family of *Hepeviridae* that includes at least eight recognized genotypes [22]. Two qRT-PCR assays for universal detection of HEV genotypes 1–4 have been reported [23]. Both the probe-based and the PriProET assays could detect 20 viral genome equivalents per reaction. The results obtained from both qRT-PCR assays were comparable to those from a nested cPCR. However, the probe-based assay had higher reaction efficiency and performed slightly better than the PriProET assay [23]. Such tools are important not only for veterinary diagnostics but also for molecular epidemiology in humans [24].

## Proximity Ligation Assays

The proximity ligation assay (PLA) builds on the principle that recognition of target proteins by two, three, or more antibodies can bring DNA strands attached to the antibodies in proximity [25]. The DNA strands can then participate in ligation reactions, giving rise to molecules that are amplified by methods such as qPCR or rolling circle amplification (RCA) for solution-phase and sub-cellular localization detection reactions, respectively. The PLA technology uses configuration of assays for highly specific recognition of proteins and protein complexes, and the detection of the bound antibodies is brought about by amplifiable DNA strands for sensitive multiplex detection. The solution-phase proximity ligation assay detected by qPCR is presented in Fig. 3 [25]. The in situ proximity ligation assay can help to detect protein complexes and interacting proteins in cells and tissues. This assay is based on rolling circle amplification of the oligos attached to the antibodies and fluorescent probe-based detection. When two antibodies bind the same protein molecule or a pair of interacting proteins in a cell or tissue, the oligonucleotides attached to these antibodies can guide the joining of two subsequently added linear oligonucleotides to form a covalently joined circular structure by enzymatic DNA ligation. The circular DNA strand is then copied in an RCA process initiated using one of the antibody-bound oligonucleotides serving as a primer. The RCA product, including

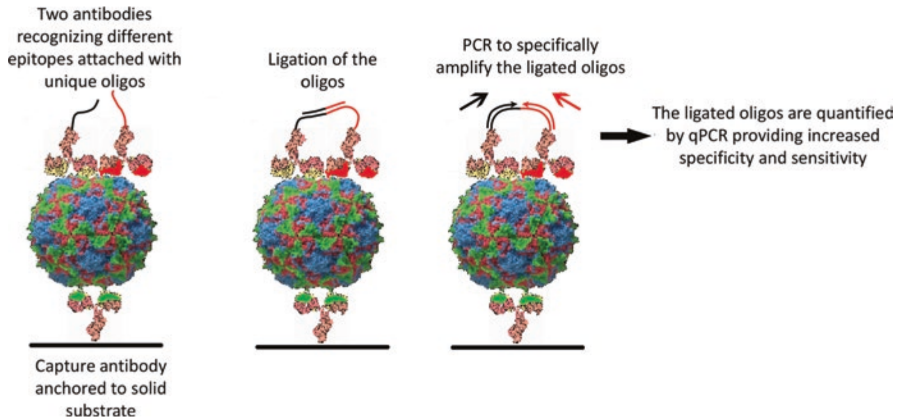


**Fig. 3** Solution-phase and in situ proximity ligation. Detection of proteins and complexes in blood and other solution-phase samples with readout via quantitative PCR (qPCR). Antibodies, with attached oligonucleotides having either a free 5′ or 3′ end, can bind to the protein complex. Upon proximal binding, the oligonucleotide pairs can hybridize to a connector oligonucleotide, guiding their ligation process. The ligation products of the proximity probes are amplified and detected by qPCR providing a measure of the amount of detected target proteins

hundreds of complements of the DNA circle, bundles up in a submicron spot, easily detected after hybridization of fluorescence-labeled oligonucleotides that are complementary to a tag sequence in the RCA product. The RCA products can be analyzed by microscopy or by using flow cytometry [25].

The solution phase detection of porcine parovirus (PPV) displays good sensitivity by qPCR and could be of value for early diagnosis of infectious disease [26]. The PLA was also demonstrated to detect antigens of avian influenza virus (AIV) and H1 2009 pandemic influenza A virus (IAV) [27, 28]. The method was four times more sensitive than a sandwich ELISA, which utilized the same antibody to detect the AIV. Compared to the widely used rapid influenza diagnostic tests (RIDTs), the PLA showed higher sensitivity (over 95%) for the detection of IAVs in clinical samples [29]. The in situ PLA technique was used to show the role of focal adhesion kinase (FAK) in endosomal trafficking of IAV. Using mini-genomes derived from H1N1, H5N1, and H7N9 viruses, the RNA replication by IAVs independent of viral entry or release was detected. The results show that FAK activity promotes efficient IAV polymerase activity and inhibiting FAK activity with a chemical inhibitor, or a kinase-dead mutant significantly reduces IAV polymerase activity [30]. A PLA using a pan-serotype reactive monoclonal antibody was developed and evaluated for the detection of foot-and-mouth disease virus (FMDV) in clinical samples. The FMDV-specific PLA was found to be 100 times more sensitive for virus detection than the commonly used antigen capture ELISA (AgELISA). Although this assay could detect diverse isolates from all seven FMDV serotypes, the diagnostic sensitivity of the PLA assay was lower than that of the qRT-PCR mainly due to a failure to detect some SAT 1, SAT 2, and SAT 3 FMDV strains [31].

## Isothermal Nucleic Acid Amplification

Isothermal nucleic acid amplification technologies can be implemented in a single step process at a constant temperature, which enables diagnostics to be conducted in small, simple, and low-power instruments. These advantages have led to considerable interest in using these techniques to conduct molecular diagnostics at the site of infection [32]. Therefore, a variety of isothermal nucleic acid amplification technologies have been developed.

Loop-mediated isothermal amplification (LAMP) is a widely used method for detection of viruses. The target, DNA or RNA, is amplified using four specific primers under isothermal conditions. The detection of the RNA template is achieved using reverse transcription LAMP (RT-LAMP) by simply adding a reverse transcriptase enzyme under identical conditions as for the LAMP reaction [33]. The assay has been used for detection of SVDV [34], Japanese encephalitis [35], porcine epidemic diarrhea virus [36], sheep pox and goat pox virus [37], BTV [38], porcine reproductive and respiratory syndrome virus (PRRSV), species 1 and 2 isolates [39] and AIV subtype H5N [40, 41], and the subtype H10 [42]. A RT-LAMP targeting the NS5B gene region detected the wild-type CSFV [43] and the C-strain vaccine [44]. The assay provides a rapid tool for the control of vaccine quality by differentially detecting the wild-type CSFV and the vaccine strain under field conditions. In the same way, a LAMP assay for detection and differentiation of glycoprotein E-deleted bovine herpesvirus 1 from wild-type virus showed a ten times higher analytical sensitivity than a cPCR assay [45].

As in other molecular techniques, variations of the LAMP assays have been developed including multiplex LAMP methods reported to detect two or more target sequences [46]. Vesicular stomatitis is endemic in Central America and northern regions of South America, where sporadic outbreaks in cattle and pigs can cause clinical signs that are similar to FMD [47]. A rapid, sensitive, and specific differential diagnostic assay is suitable for decision making in the field. A multiplex RT-LAMP assay has been developed for rapid discrimination between FMD and vesicular stomatitis virus. To permit multiplex detection, the forward inner primer and backward inner primer were modified by labeling with digoxigenin and fluorescein using previously published assays [34, 48]. The assay maintains a similar analytical sensitivity to the equivalent qRT-PCR assay [49].

Influenza virus infections represent a worldwide public health and economic problem due to the significant morbidity and mortality caused by seasonal epidemics and pandemics. A multiplex RT-LAMP using a cascade invasive reaction involving nanoparticles (mRT-LAMP-CIRN) was developed for simultaneous amplification of three subtypes of influenza viruses, such as IAV/H1N1pdm09, A/H3, and influenza B. The utilization of oligonucleotide probe-modified gold nanoparticles causes notable changes in the optical property. The analytic sensitivity of the mRT-LAMP-CIRN assay was 10 copies of RNA for both A/H1N1pdm09 and A/H3 and 100 copies of RNA for influenza B. In clinical specimen, mRT-LAMP-CIRN assay showed an overall sensitivity and specificity of 98.3% and

100%, respectively [40]. Using the same design, i.e., mRT-LAMP-CIRN, another assay was developed for simultaneous detection of A/H5, A/H7, and 2009A/H1. The analytic sensitivities of the assay were ten copies of RNA for all the three HA subtypes, and the specificity reached 100%. Clinical specimen analysis showed this assay had a combined sensitivity and specificity of 98.1% and 100%, respectively [50].

Recombinase polymerase amplification assays using real-time fluorescent detection (real-time RPA assay) and a lateral flow dipstick (RPA LFD assay) were developed targeting the gD gene of pseudorabies virus (PRV). Both assays were performed at 39° C within 20 min. The sensitivity of the real-time RPA assay was 100 copies per reaction, and it was 160 copies per reaction for the RPA LFD assay. Both assays did not cross-react with other viral DNA. Therefore, the developed RPA assays provide a rapid, simple, sensitive, and specific alternative tool for detection of PRV [51].

A uracil-DNA glycosylase (UNG)-treated reverse transcription loop-mediated isothermal amplification (uRT-LAMP) was developed for the visual detection of all subtypes of AIV. The detection limit of the uRT-LAMP assay was tenfold lower than that of the RT-LAMP without a UNG treatment. The assay can be applied for the rapid and reliable diagnosis of AIVs and can prevent unwanted amplification by carryover contamination of the previously amplified DNA [52].

## Digital PCR

The technique of digital PCR is based on end point dilution of the sample-containing target template into numerous smaller, independent PCR reactions in which the amplification is detected based on the standard qPCR chemistry such as the TaqMan system. The distribution of the target template follows a Poisson distribution in the individual reactions, and this enables the estimation of the absolute number of templates in a given sample. The digital PCR enables the quantification of the target RNA or DNA in a given sample, without the need for a known standard of the target. A recent study to quantify HEV RNA by digital PCR illustrates the platform's higher accuracy and reproducibility than the standard qPCR [53].

## Genomic Sequencing and Viral Metagenomics

Recent general changes including globalization, climate change, increasing wildlife-livestock interface, changes in agricultural practices (e.g., intensive farming), and growth in the live animal markets are some of the drivers responsible for the emergence of novel pathogens and zoonoses. The emergence of new serotypes and genotypic variants of known and/or unknown viruses are associated with the intrinsic viral characteristics (e.g., viral rate mutations) and/or the natural (e.g., migratory

birds) or artificial (e.g., animal market) movements of the host. Arthropod vectors may also play an important and sometimes necessary role in disease outbreak and spread [54, 55].

The discovery and knowledge of viruses circulating in domestic animals and wildlife enables disease control and preparation of interventions for new diseases in humans and animals. Understanding the virus diversity may be used to forecast future transmission risks or eventual outbreaks of viral diseases. Hence, identification or monitoring of the circulation of known and unrecognized viruses is one of most important requirements for the response to disease outbreaks [54, 55].

Traditional Sanger sequencing of viral genomes based on conserved or degenerate primers is widely employed to monitor circulating viruses for variants and also to identify novel viruses. Prior knowledge of viral genome sequences and conserved genomic segments is essential for Sanger sequencing of viral genomes. Evolutionary analysis of viral genes involved in host specificity, virus entry, viral pathogenicity, and immune recognition by host are important targets for molecular epidemiological studies.

Viral metagenomics is an approach using large-scale sequencing to identify/study viral genomes. Next-generation sequencing (NGS) is used as a metagenomic tool for the investigation of complex diseases, diseases of unknown etiology, and identification of emerging novel viruses in samples. In brief, NGS technologies are used to non-specifically detect pathogen genomes in a given sample, compared to detecting a predetermined gene segment of a virus by conventional sequencing methods. In addition, the NGS technologies do not require prior sequence knowledge of pathogen genomes and thus enable detection of novel pathogens. They generate a huge number of short sequence reads ranging from 100 to 800 bases which when processed yield thousands to millions of bases of sequence data in one reaction. The NGS workflow includes the following steps: sample preparation, sequence-independent amplification, high-throughput sequencing, and bioinformatics analysis as outlined in Fig. 4 [54, 55]. The commonly used NGS platforms include Roche 454, Illumina, Ion Torrent, SOLiD, PACBIO, and NANOPORE, among others. Each platform uses a different approach to generate the data with specific advantages and disadvantages (Table 3) [56].

In the following sections, several examples are given on the use of viral metagenomics to detect new and/or emerging and reemerging viruses in animals.

*Detection of Novel Viruses with NGS* The GS-FLX 454 technology was used to investigate the cause of a neurological disease of minks, termed shaking mink syndrome. The disease was first observed in farmed mink kits in Denmark in 2000 and subsequently in Sweden, Denmark, and Finland in 2001 and in Denmark again in 2002 [57]. Excluding other infectious agents, brain samples obtained after experimental infection were prepared for nucleic acid extraction and random amplification and large-scale sequencing using the GS-FLX 454 technology [58]. Analysis of the 454 sequencing data revealed eight sequence fragments similar to mink astrovirus. Based on these results, new primers were designed to determine the nucleotide sequences of the complete viral genome. The comparative analysis of complete

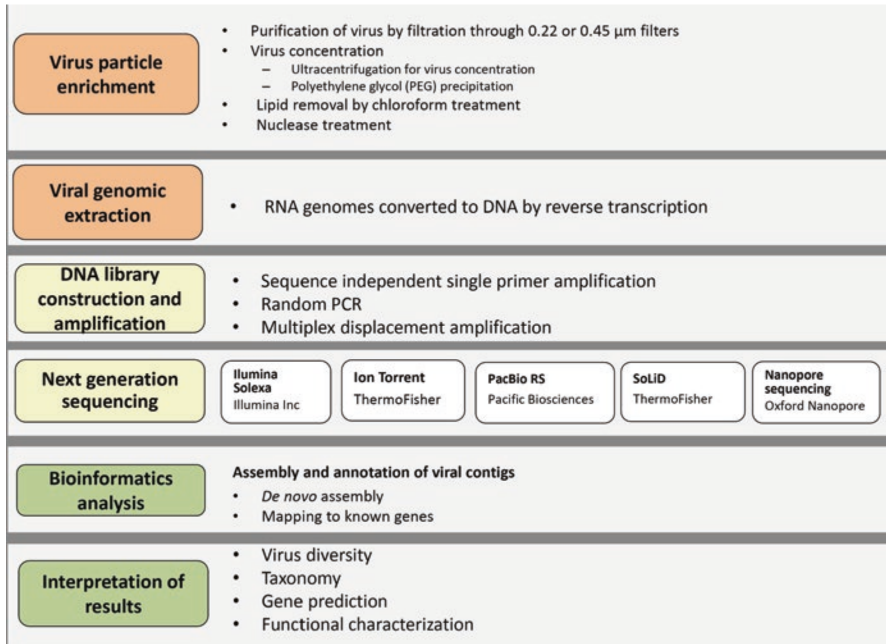


**Fig. 4** Workflow for virus detection using next-generation sequencing

genome sequences showed a similarity of 80.4% to that of a mink astrovirus causing pre-weaning diarrhea in mink. As the virus was not detected in healthy mink kits, an association between the astrovirus and the neurological disease of mink was assumed [58].

The 7382-nucleotide-long genome sequence of a new chicken astrovirus was obtained using an Illumina MiSeq System. This new virus is attributed as the cause of the "white chicks" condition recently identified in Poland [59].

A novel atypical porcine pestivirus (APPV), highly divergent from known porcine pestiviruses, was recently identified in the United States (USA) by metagenomic sequencing [60]. Five positive cases of APPV were found in 182 serum samples obtained from five US states analyzed by metagenomic sequencing. The study highlighted the widespread prevalence of the novel APPV in the United States [60].

Recently another novel virus, porcine circovirus 3 (PCV3), was identified in three unrelated cases of pigs with cardiac and multi-organ inflammation [61]. In addition to PCV3, other porcine viruses including porcine astrovirus 4 (PAstV4) were identified in all three cases [61]. This study lead to the detection of PCV3 in other parts of the world [62, 63].

*Study of Pathogenicity with NGS* PRRSV is a member of the *Arteriviridae* family in the order *Nidovirales* and causes highly significant economic losses to the swine

**Table 3** Description, advantages, and disadvantages of conventional Sanger sequencing and currently available next-generation sequencing (NGS) platforms

| Platform<br>*Company* | Sequencing principal | Read length (kb) | Accuracy | Reads/run<br>Time/run<br>Output data/run | Applications | Advantages | Disadvantages |
|---|---|---|---|---|---|---|---|
| Sanger<br>*Applied biosystems* | Dideoxy chain termination | 0.4–0.9 | 99.9% | Not applicable<br>20′–3 h<br>1.9–84 kb | *De novo*<br>Array<br>Mutation detection | High quality<br>Read length | High-cost throughput<br>Low throughput<br>Previous steps needed |
| 454 GS FLX (Roche) | Pyrosequencing | 0.4–0.7 | 99.9% | 1 Mb<br>10–20 h<br>0.7 Gb | *De novo*<br>Array<br>Mutation detection<br>Large genome | Read length<br>Fast | Expensive runs<br>Homopolymer errors<br>Low throughput |
| Hiseq<br>*Illumina* | Sequencing by synthesis | 0.04–0.3 | 99.9% | 3 Gb<br>10 days<br>600 Gb | *De novo*<br>Array<br>Mutation detection<br>Large genome<br>Re-sequencing | High throughput<br>Homopolymer sequencing | Equipment cost<br>High noise and errors in later cycles |
| Ion semiconductor<br>*Life Technologies* | Semiconductor sequencing | 0.2–0.4 | 98% | 82 G<br>2–7.5 h<br>4 Gb | *De novo*<br>Array<br>Mutation detection<br>Large genome<br>Re-sequencing | Less expensive equipment<br>Fast | Homopolymer errors |
| SOLiD<br>*Life technologies* | Sequencing by oligonucleotide ligation and twobase<br>Coding | 0.09–1.10 | 99.9% | 1.4 Gb<br>7–14 days<br>120 Gb | Array<br>Mutation detection<br>Re-sequencing | Low-cost per base<br>Accuracy | Slower than other methods<br>Short read length |

(continued)

**Table 3** (continued)

| Platform<br>*Company* | Sequencing principal | Read length (kb) | Accuracy | Reads/run<br>Time/run<br>Output data/run | Applications | Advantages | Disadvantages |
|---|---|---|---|---|---|---|---|
| PacBio<br>*Pacific biosciences* | Single-molecule real-time sequencing | 10–15 | 87% | 1 Mb<br>0.5–4 h<br>375 Mbp/cell | *De novo*<br>Array<br>Mutation detection<br>Large genome<br>Re-sequencing | Longest read length<br>Fast<br>Equipment cost | Moderate throughput. |
| NANOPORE<br>*Oxford technologies* | Single-molecule detection through a nanoscale pore | Up to 300 | 99% | 1.25 Gb<br>1′–2 days<br>Up to 12 Tb | *De novo*<br>Array<br>Mutation detection<br>Large genome<br>Re-sequencing<br>RNA directly<br>Small molecules | No PCR amplification or labeling needed<br>Ultra-long length read<br>Fast<br>Cost | Inherent disadvantages being solved |

industry worldwide. PRRSV causes changes in expression of specific genes that act to protect the host and clear the infection. Analysis of the gene expression profiles indicated a higher magnitude of differentially expressed gene in pigs infected with high pathogenic (HP) PRRSV rJXwn06 as compared to VR-2332-infected pigs [64]. Studies using NGS have been conducted to identify microevolution within a coexisting *quasispecies* population in PRRSV. The dynamics of such a mixed viral population is of increasing clinical importance due to concern on increase of virulence and pathogenesis [65]. A viral population within a host undergoes complex processes, including the onset of infection, cellular replication, selection, and migration to different tissues. In particular, it is not clear how the virus diversity generated within a cell propagates through a host to give rise to the observed degree of diversity in the *quasispecies*.

NGS was used to dissect FMDV within a host population structure. The study identified 2622, 1434, and 1703 polymorphisms in the inoculum and in the two foot lesions, respectively. Most of the substitutions occurred in only a small fraction of the population and represented the progeny from recent cellular replication prior to onset of any selective pressures. Data like this can be used to build models aimed at understanding the link between the microevolution of FMDV at the cellular scale and the population heterogeneity at the host scale [66].

Very little is known about the impact of CSFV genetics and genetic adaptations during the infection process on the manifestation of a chronic disease. In this respect, NGS was used to study the influence of the viral genome and its *quasispecies* composition in the course of CSF infection [67].

*Study of Viral Epidemiology with NGS* Characterizing and understanding the molecular epidemiology of the currently circulating viral strains in the field is essential for controlling and preventing outbreaks of diseases.

Newcastle disease virus (NDV) is a type species of an avian paramyxovirus serotype1 (APMV-1), which belongs to the genus *Avulavirus* in the family *Paramyxoviridae*. The full genomes of virulent Malaysian NDV strains, collected during 2004–2013, were characterized using NGS. All isolates were clustered within highly prevalent lineage 5 (specifically in lineage 5a); however, a significantly greater genetic divergence was observed in isolates collected from 2004 to 2011 [68].

NGS was used to associate the avian leukosis virus, classified under the *Alpharetrovirus* genus in the family *Retroviridae*, as the viral cause of mortality in a broiler flock in Malaysia. The outbreak reached a mortality rate of 10% in the 27-day-old flock ($n = 6000$) and more than 20% in the 30-day-old flock ($n = 4000$) [69].

Species 2 PRRSV strains are predominant in North America and Asia with a high diversity, while in Europe fully sequenced species 2 strains were closely related to the Ingelvac® PRRS MLV vaccine. A phylogenetic analysis performed with the whole genome of PRRSV-2/Hungary/102/2012 and 215 GenBank full genome accessions revealed that it is a member of ancient lineages 1 or 2 detected in Eastern Canada in the early 1990s. PRRSV-2/Hungary/102/2012 is the first type 2 PRRSV isolated in Europe that is not related to the Ingelvac® PRRS MLV strain. This indi-

cates that the strain was imported directly from North America during the early stages of PRRSV diversification, and the divergent evolution of the viruses in the two continents resulted in marked genetic differences among PRRSV-2/Hungary/102/2012 and other type 2 viruses [70].

The BTV-1 is the prototype species of the genus *Orbivirus*, within the family *Reoviridae*. In Europe, BTV-1 emerged in the Mediterranean Basin in 2006, and it has since been isolated in southern and northern European countries. Italian isolates obtained from 2006 to 2013 and a BTV-1 strain from an infected Tunisian sheep in 2011 were fully sequenced by NGS technology [71]. Combined results suggest that BTV-1 strains isolated in Sardinia, Sicily, and mainland Italy in 2012 and 2013 have a direct North African origin.

## xMAP Technology (Suspension Array Technology)

Detection of multiple pathogens during an exploratory diagnostic workup is often required. Popular platforms for multiple pathogen screening include multiplex qPCR which is limited by spectral overlap of the fluorescent probes utilized and in reality is limited to five probes in one assay. Even this comes with a decrease in sensitivity if more than one target is present in the sample. The Luminex® xMAP (x Multiple Analyte Profiling) technology (Luminex Corporation, Austin, TX), developed over the past two decades, is a versatile platform for high-throughput simultaneous analysis of multiple, up to over 100, analytes from a single sample [72]. The technology uses a liquid suspension array system comprising of hundreds of microsphere sets, each with a unique emission spectral signature. Chemical coupling of specific reagents to the surface of distinct microsphere sets forms the basis of the bioassays that could be adapted to the xMAP platform, such as nucleic acid amplification assays, immune assays, receptor-ligand assays, and others. An additional fluorescent signal is generated by a bioassay, and the multiple analytes are identified by the spectral character of the microspheres in combination with the fluorescent signal from the assay. The analysis of spectral emissions of the analyte bound microsphere sets is performed aligned to the principles of flow cytometry, thus enabling the simultaneous quantification of multiple analytes. However, due to the inherent nature of the assay, it is deemed semiquantitative and is best to obtain a positive/negative result rather than a quantitative perspective [72]. Various adaptations of microsphere-based multiplex nucleic acid assays (MBMNA) targeting viral nucleic acids and microsphere-based multiplex immune assays (MBMIA) targeting antibody/antigen interaction have been developed for many pathogens in human and veterinary medicine [72]. Accurate detection of multiple swine viruses using xMAP platform, PRRSV, PCV2, PRV, CSFV, and PPV based on direct hybridization with viral genomes has been reported [73]. Development of veterinary-specific respiratory, enteric, or reproductive disease panels, similar to those in human microbial diagnostics [72], would be very helpful in generating quick and comprehensive diagnostic information.

## Aptamers in Viral Diagnostics

Aptamers are single-stranded folded nucleic acids (RNA or ssDNA) that recognize a target molecule, such as proteins and other molecules, with high specificity and affinity. An in vitro process, called SELEX (systematic evolution of ligands by exponential enrichment), developed by Gold and Tuerk [74], forms the basis for development of RNA or DNA aptamers. The specificity and size of aptamers make them attractive for a variety of applications in molecular diagnostics of viruses, and aptamers are an attractive alternate for monoclonal antibodies [75]. Aptamers are used instead of antibodies in conventional ELISA platforms known as enzyme-linked apta-sorbent assay (ELASA). A hybrid assay in sandwich format with aptamers to capture PRRSV VR-2332 strain and antibodies to detect the captured virus, also known as enzyme-linked antibody aptamer sandwich (ELAAS) method, showed a sensitivity comparable to that of PCR-based detection methods [76]. Due to their specificity in binding to a wide range of molecules such as DNA, RNA, proteins, and virus particles, aptamers have immense potential as tools for affinity-based enrichment of viral components in biological and environmental samples. A recent report utilizing aptamers to capture and detect H9N2 virus based on an aptamer sandwich-qPCR detection system showed a 3-log increase in sensitivity over a similar conventional ELISA [77].

## Summary

During the last decades, substantial progress has been made in diagnostic virology, and a wide range of novel molecular diagnostic methods for the improved detection of viruses in veterinary and human medical virology have been developed. For molecular methods, upstream nucleic acid extraction is crucial for the success of the downstream diagnostic tests. The real-time PCR platform, using different chemistries, such as TaqMan and PriProET, is a very reliable, highly sensitive, and specific novel diagnostic tool. It offers a quantitative perspective and allows for the direct detection of a wide range of pathogens. Simultaneously, the real-time PCR technology allows the development of novel DIVA tests, which are required for the improved control of infectious diseases using marker vaccines and accompanied diagnostic packages. In parallel, technologies such as Luminex panels enable the simultaneous detection and identification of multiple pathogens in a single high-throughput test platform. The proximity ligation assay has emerged as a novel method with increased sensitivity and specificity in detecting viral proteins by building on the conventional antigen-antibody interaction-based assays. Viral metagenomics and large-scale genome sequencing have become establish powerful tools for the detection of “unknown” viruses, as well as for the identification of genetic variants and evolutionary trends in emerging and reemerging viruses. Simultaneous analysis for multiple viral pathogens and/or multiple genetic variants

of a virus by novel multiplex assays offers a comprehensive view to the diagnostician. These novel approaches strongly support the investigation of disease complexes and/or emerging novel disease scenarios in veterinary diagnostic virology, with regard to diseases in domestic animals and in wildlife including zoonotic infections.

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# Detection of Viral RNA Splicing in Diagnostic Virology

**Vladimir Majerciak and Zhi-Ming Zheng**

## Introduction

Diagnostic virology is the process in which the viral etiologic cause of infection is identified from a patient's clinical sample. In the past, diagnostic virology relied on three classical techniques to make this diagnosis of viral infection: (a) virus isolation by direct virus cultivation, (b) viral antigen detection, and (c) indirect detection of virus-specific antibodies. While the remaining important tools are utilized by diagnostic virology laboratories today, these techniques are time-consuming and require specific reagents/methods such as cultivation media, cell or tissue cultures, antibodies, or purified antigens. In the past several decades, the number of new molecular-based methods increased rapidly and became widely used in diagnostic virology laboratories. The core of these techniques constitutes of techniques based on nucleic acid detection by specific amplification, hybridization, and/or sequencing (reviewed in [1]). The majority of these nucleic acid-based diagnostic methods are simple, speedy, sensitive, and specific and thus meet the gold "four-S standard" for their application in any diagnostic laboratory. The methods are simple and speedy because only a specific primer pair and a PCR machine are required by the laboratory, and identification of a viral pathogen takes only a few hours. They are sensitive and specific and require only a small amount of patient's clinical specimen to detect a specific nucleotide sequence region. In general, these techniques can be used to detect almost all types of viral pathogens and can even identify multiple viral pathogens or their variants at the same time. In this chapter, we will focus on detection of viral RNA splicing as a new tool for diagnostic virology.

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# Principle of RNA Splicing

## *Definition of RNA Splicing*

RNA splicing was discovered 40 years ago by Susan Berget [2] and Louise Chow [3]; these investigators mapped adenovirus transcription and identified intervening sequences (introns) in type 2 adenovirus primary transcripts. Subsequently, RNA splicing was recognized as an essential nuclear event for mammalian gene expression and for virus replication of almost all DNA viruses and some RNA viruses. Most mammalian genes consist of multiple segments called exons which are separated by noncoding or intervening sequences named introns. Genes which are composed of exons and introns are "split" genes. After transcription, a nascent or primary transcript (pre-mRNA) contains both exons and introns. The introns are removed from the pre-mRNA by a molecular process called "RNA splicing" resulting in production of spliced mature mRNA. RNA splicing takes place both in coding as well as in noncoding primary transcripts. RNA splicing has been considered a posttranscriptional event; however, recent studies have demonstrated that RNA splicing often occurs co-transcriptionally [4, 5]. Only those transcripts which are fully processed are eventually exportable from the nucleus to the cytoplasm for protein synthesis.

## *Molecular Mechanism of RNA Splicing*

All introns are defined by three *cis*-elements: a 5' splice site (donor site), a branch point, and a 3' splice site (acceptor site) with a polypyrimidine track immediately upstream (Fig. 1a). These *cis*-elements allow cellular splicing machinery to recognize and remove the intron from pre-mRNA. Most mammalian introns start with GU dinucleotide on its 5' end and an AG dinucleotide on its 3' end ("GU-AG" introns). The GU-AG pairs are conserved sequences and define the exon-intron boundaries. Introns with an AU on its 5' end and an AC on its 3' end are rare, and this set of the introns are known as "AU-AC" introns [6, 7]. The presence of splice

**Fig. 1** (continued) These initial recognitions of the intron elements by the components of RNA splicing machinery are essential for spliceosome formation on a pre-mRNA, leading to intron removal. (**b**) RNA splicing is catalyzed by two transesterification reactions. During RNA splicing, the intron (gray) between two exons (black) is removed by two transesterification reactions. First, the intron is recognized by cellular splicing machinery via splicing factors binding to intron-specific sequences as described in (**a**). Splicing factors carry out the first transesterification reaction between the branch point and the 5' donor site, resulting of an RNA cleavage at the 5' donor site and releasing exon 1 and formation of a lariat intermediate. Subsequently, the free 3'end OH group of exon 1 attacks the 5' end phosphate of exon 2 and joins with the 5' end of exon 2 via the second transesterification reaction to form a mature mRNA. Intron is removed in the form of lariat structure and quickly degraded

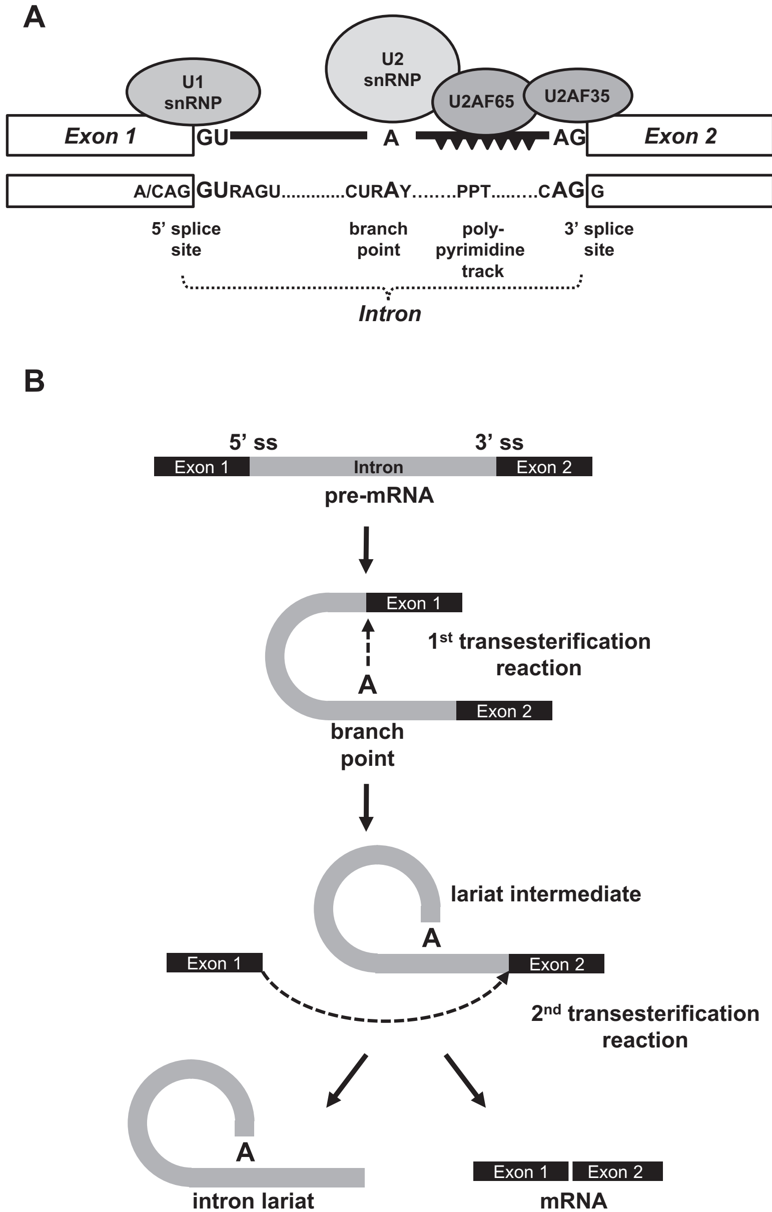



**Fig. 1** Pre-mRNA structure and splicing reactions. (**a**) Structure of a pre-mRNA containing an intron (solid line) and two exons (empty boxes). An intron is defined by several specific sequence motifs which allow intron recognition by cellular splicing machinery. A 5' splice site or donor site GU at the intron 5' end is recognized by U1 small nuclear ribonucleoprotein (snRNP), a major component of the cellular splicing machinery. A 3' splice site or acceptor site at the intron 3' end consists of an AG dinucleotide, an upstream polypyrimidine track (PPT) and a further upstream branch point, which are recognized correspondently by U2AF35, U2AF65, and U2 snRNP.

sites is not sufficient for intron definition. All introns must contain an additional element called a "branch point," which is located 20–50 nts upstream from the 3'splice site, and have a consensus sequence CU(A/C)A(C/U) where A is a most conserved base. The sequence between the branch point and the acceptor site is a run of 15–40 pyrimidines (mostly U) and is referred to as a "polypyrimidine track."

RNA splicing is catalyzed by cellular splicing machinery, which consists of the following components: (1) small nuclear ribonucleoproteins (snRNPs, U1, U2, U4, U5, and U6) and (2) splicing factors. During the initial step, snRNP, U1, and U2 recognize the intron sequences at the 5' splice site and the branch point via complementary base-pairing involving the U2 accessory proteins U2AF65 and U2AF35, which associate with the polypyrimidine track and 3'splice site, respectively (Fig. 1a). Intron recognition is a signal for the formation of a large protein complex called the "spliceosome" where intron removal takes place [8, 9] by two transesterification reactions (Fig. 1b). First, the primary transcript is cleaved at the intron 5' end (5' splice site) to leave the upstream exon free; this step is followed by branching of the cleaved intron 5' end to the branch point (usually A) to create a looped structure named "lariat intermediate". In the second step, the hydroxyl group of the free exon attacks the intron 3' splice site leading to the 3' splice site cleavage and lariat formation. Simultaneously, a covalent bond is created between two exons to create a mature mRNA. In general, the lariats are quickly released from the spliceosome and degraded in the nucleus.

The efficiency of RNA splicing is regulated at multiple levels, and both RNA *cis*-elements and cellular splicing factors play major roles in the regulation of RNA splicing. As described above, the level of conservation of the sequences at the splice sites and the branch point will affect the strength of the binding of core splicing factors and thus determine the splicing efficiency. RNA splicing is also modulated by a large family of cellular splicing factors containing serine-arginine-rich proteins (SR proteins) and heterogeneous nuclear ribonucleoproteins (hnRNPs). Most of the splicing factors that are differentially expressed in a specified tissues and/or in a development stage are the RNA-binding proteins, which bind to specific RNA *cis*-element (splicing enhancers or silencers) located within introns and exons [10]. It is now well documented that splicing factors binding to the *cis*-elements either increase or decrease RNA splicing efficiency depending on the type of splicing factors, the positions of the binding sites, and the overall spliceosome composition [11, 12]. Current studies demonstrate that in addition to splicing factors, other processes such as RNA polymerase rate and chromatin structure also affect RNA splicing [13, 14].

## *Alternative RNA Splicing*

Although all introns in a pre-mRNA could be constitutively spliced out, and all exons are supposedly included in a mature mRNA, there are many examples where an RNA splice site may be not selected constitutively, but instead skipped, during

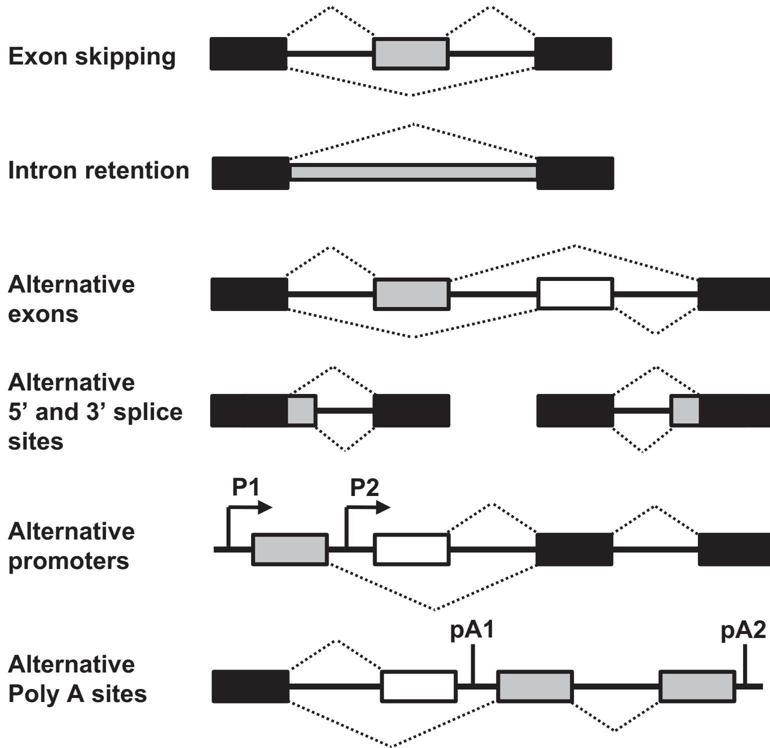



**Fig. 2** Alternative RNA splicing. Alternative RNA splicing allows production of multiple splicing isoforms from a single pre-mRNA species. Constitutive exons (black boxes) are included in all splicing products, while alternative exons (gray and white boxes) are either included or excluded in various isoforms of mature mRNAs. Major forms of alternative RNA splicing include exon skipping, intron retention, usage of alterative exons, and usage of alternative splice sites. In addition, usage of alternative promoters (P1 or P2) or polyadenylation sites (pA1 or pA2) may affect exon composition in a final mature transcript.

RNA splicing. Consequently, this alternative RNA splicing leads to the production of RNA isoforms with different exon compositions and production of different protein isoforms. In general, there are four major classes of alternative RNA splicing (Fig. 2), including exon skipping, intron retention, usage of alternative exons, and usage of alternative 5' and alternative 3' splice site [15]. In addition, alternative promoter or polyadenylation usage can further complicate the alternative RNA splicing. The reason why some of the exons or introns in a pre-mRNA are alternatively spliced is either because of the presence of weak or suboptimal splice signals in the pre-mRNA or due to the lack of a particular splicing factor. It has been noticed that usage of weak splice sites is highly dependent on auxiliary splicing factors binding to the regulatory *cis*-elements. Since the expression of these factors is variable from cell to cell and from tissue to tissue, alternative RNA splicing is often

associated with a specific cell type, tissue, or stage of cell differentiation [16]; this results in the production of various isoforms of transcripts from one gene [17]. Current analysis also revealed that the number of genes with alternative RNA splicing increases with complexity of the organism and ranges from 0.05% in yeast up to 66.8% in human. Thus, alternative RNA splicing is the driving force behind the complexity of the proteome in higher organisms in addition to the total number of the mapped genes.

## Molecular Methods for Detection of RNA Splicing

The mRNA generated by RNA splicing is different from its pre-mRNA. First, mRNA is smaller in size than its pre-mRNA due to RNA splicing which removes the introns found in the pre-mRNA. In contrast, the pre-mRNA is not only larger than the spliced mRNA but also has the same size as its DNA template. Second, mRNA contains exon-exon junctions with the sequence not present in DNA or its primary transcript, allowing designing primers or probes to specifically identify a particular mRNA isoform due to alternative RNA splicing. Although an alternatively spliced mRNA may translate a truncated protein, which could be detectable with a specific antibody, the molecular techniques based on detection of nucleic acids are more commonly used to detect RNA splicing.

### *Northern Blot*

Northern blot is one of the oldest techniques used to detect RNA splicing. First, RNA molecules isolated from samples are separated based on their size by electrophoresis in agarose or polyacrylamide gel. After transfer to a nitrocellulose or nylon membrane, the individual RNA transcripts are detected by an antisense probe specific for the detecting RNA. The probes used for the Northern blot are usually labeled with $^{32}$P isotope, enzyme (e.g., alkaline phosphatase), digoxigenin (DIG), or biotin and can be derived from a constitutive exon or an exon-exon junction (Fig. 3a). Constitutive exon-based probes would detect all spliced RNA isoforms and the remaining, unspliced pre-mRNA; these exon-based probes are recommended when the size difference between spliced RNA isoforms and unspliced pre-mRNA is sufficient enough to be separated. If the size difference is too small for two RNA isoforms to be distinguished, an exon junction probe can be used to specifically detect a spliced product. In addition, a specific probe from an alternative exon or intron can be also designed for detection of individual splicing isoforms derived from exon/intron inclusion.

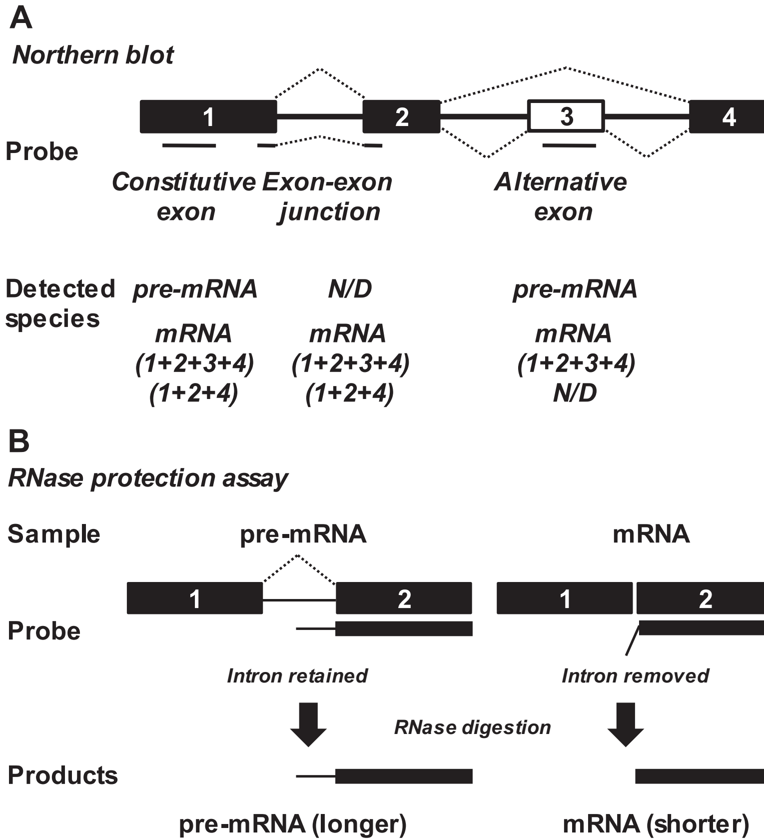



**Fig. 3** Detection of RNA splicing products by Northern blot and RNase protection assays (RPA). (**a**) Diagram of a pre-mRNA with constitutive exons (black boxes), introns (solid lines), and an alternative exon (empty box). Solid lines below indicate positions of antisense probes commonly designed for Northern blot to detect products by each probe. N/D – not detectable. (**b**) Detection of spliced RNA products by RPA. Diagram shows an antisense riboprobe spanning over an intron region (a solid thin line) between two exons (black boxes or solid thick lines) and possible detection products. As an RNase used in the assay digests a single-stranded RNA region only, an RNA region base-paired with an antisense probe uniformly labeled with isotope $^{32}$P will be protected from RNase digestion. In this diagram, the probe remains intact when binding to the pre-mRNA, while the probe binding to the spliced mRNA (lack of the intron) will cause the digestion of the probe intron region (single stranded) resulting in production of smaller protected products corresponding to each exon

## *RNase Protection Assay*

The RNase protection assay (RPA) requires $^{32}$P-labeled single-stranded antisense RNA probes complementary to the transcripts of interest. The prepared probe(s) is consequently hybridized with sample RNA to form an RNA-RNA hybrid.

Unhybridized single-stranded RNA is then removed by RNases A and T1, which digest single-stranded RNA only. The protected RNA fragments are separated in the gel by electrophoresis, and their sizes are determined by molecular markers. To distinguish a spliced RNA product, the probe should contain at least one partial intron region that will be digested from the probe due to the lack of the intron sequence in the spliced mRNA. As a result, the probe protected by the corresponding exon regions of the detecting mRNA is shorter and will run faster in the gel (Fig. 3b). In general, RPA is more sensitive than Northern blot in detection of RNA splicing.

Both Northern blot and RPA are commonly used in research laboratories. Their main advantage is high specificity. However, both methods are very laborious and low-throughput requiring isolation of large amount (usually a few micrograms) of the total RNA from samples and preparation of specific probes often labeled with radioisotopes, which limits their use in clinical diagnostics.

## *RT-PCR*

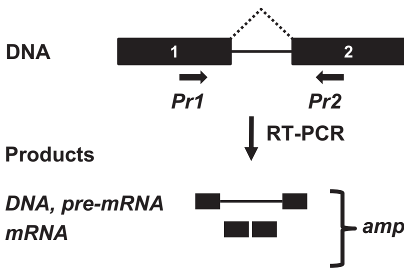
The RT-PCR (reverse transcription-polymerase chain reaction) is one of the most commonly used methods for detection and quantification of RNA molecules. During RT-PCR, RNA transcripts are converted into complementary DNA (cDNA) by reverse transcription using random hexamers or oligo-dT- (a short sequence of deoxy-thymidine nucleotides) or transcript-specific primers. The resulted cDNA is then used as a template in subsequent PCR with a pair of transcript-specific primers.

In principle, the detection of spliced RNA transcripts by RT-PCR depends on amplicon selection and primer design. The most common approach is the amplification over the intron regions by a set of primers in flanking exons. The resulted RT-PCR products vary in sizes depending on how the detecting transcript is spliced. A larger product than the predicted size may represent an unspliced pre-mRNA, or contaminating genomic DNA. The latter can be determined by a minus RT amplification (PCR). A spliced mRNA always gives a smaller RT-PCR product than its pre-mRNA due to removal of intron sequences by RNA splicing (Fig. 4a). Another approach is to specifically amplify a spliced product by using an exon junction primer because the sequence at exon-exon junction is not present in pre-mRNA, nor in genomic DNA. Similarly, a primer based on an alternative exon would amplify only the transcript with the inclusion of that exon (Fig. 4b). After amplification, the size and amount of RT-PCR products are analyzed by gel electrophoresis. Because of nonlinear nature of PCR amplification, classical PCR only provides semiquantitative data on the abundance of various spliced RNA isoforms.

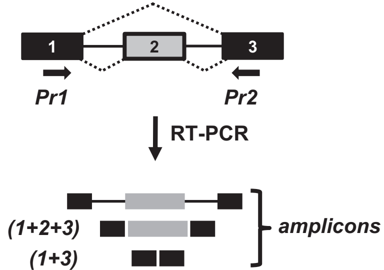
Introduction of real-time quantitative RT-PCR (RT-qPCR) with a broad (10 [7]) dynamic range has significantly improved the sensitivity of RT-PCR. Because of its high sensitivity, real-time RT-qPCR is able to detect and amplify RNA directly from

**A**

*I. Constitutive RNA splicing*

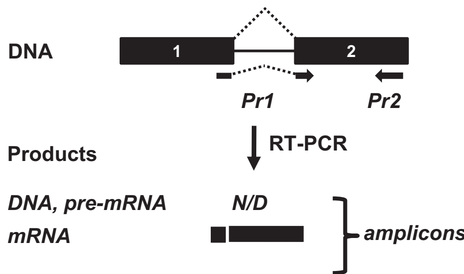



*II. Alternative RNA splicing*




**B**

*I. Constitutive RNA splicing*




*II. Alternative RNA splicing*

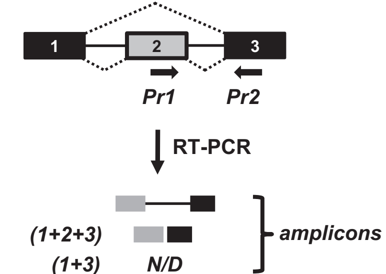
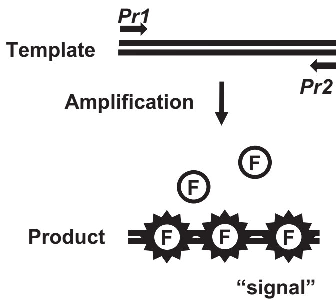
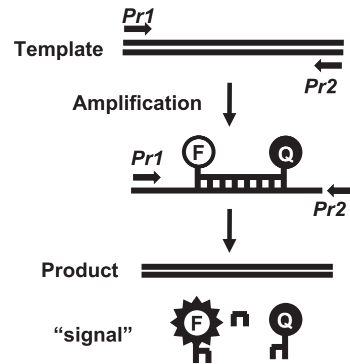


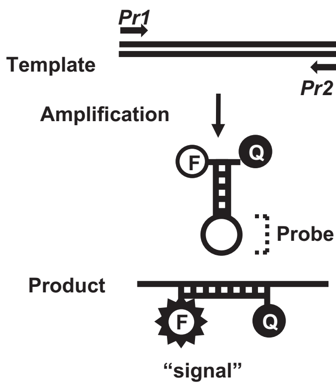

**Fig. 4** Detection of spliced RNA products by RT-PCR. Intron removal from a pre-mRNA could be detected by RT-PCR based on the size of a product (**a**) or in a selective amplification (**a** and **b**). (**a**) A pair of primers (*Pr1* and *Pr2*) used for amplification are derived from two constitutive exons (black boxes) over an intron (solid line), and the amplified mRNA product (spliced) will be smaller in size than the products amplified from template DNA or unspliced pre-mRNA. In an alternative RNA splicing assay, a pair of primers is derived from exon 1 and exon 3 spanning over alternative exon 2 (gray box). Alternative RNA splicing will result in multiple-spliced RNA products of various sizes amplified by RT-PCR. (**b**) Specific amplification of spliced RNA products during constitutive or alternative RNA splicing. The specific splicing products could be obtained by using a set of primers in which one represents a splicing junction (*Pr1*). Because of the lack of this sequence in unspliced pre-mRNA or DNA, only the spliced product will be selectively amplified. A selective amplification could be also used to amplify by RT-PCR a specific RNA isoform derived by alternative RNA splicing by using one primer in an alternative exon (exon 2) in combination with a primer in exon 3. In this case, only the spliced product with exon 2 inclusion will be selectively amplified by RT-PCR

a single cell without RNA extraction. In addition, it automates the quantification and does not require electrophoretic separation of RT-PCR products.

Currently there are four major detection chemistries widely used in real-time RT-PCR: SYBR green (Molecular Probes), *Taq*Man™ probes [18], Molecular Beacons [19], and Scorpions™ probes [20]. The principles of these different



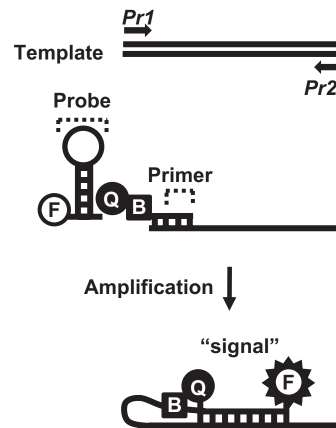




**Fig. 5** Major chemistries of real-time PCR. PCR product detection by four commercially available chemistries in real-time PCR: SYBR green (*I*), *Taq*Man™ (*II*), Molecular Beacon (*III*), and Scorpions™ probes (*IV*). The mechanism of each chemistry is described in detail in the chapter section III-in the corresponding chapter. The sections are not numbered anymore. Double lines represent a double-stranded DNA template generated by RT-PCR, while a single line is correspondent to single-stranded DNA. Horizontal arrows mark primer (*Pr1* and *Pr2*) positions. *F* stands for quenched "fluorophore moiety" and could be present in two stages: quenched nonfluorescent stage (empty circles) or in activated stage (empty circles with spikes) with generation of detectable signal. *Q* in black circles represents "quencher." Scorpion probes contain amplification stop sequences named "blocker" (*B* in black box)

detection chemistries are described in Fig. 5. As of today, SYBR green and *Taq*Man™ probes represent the most common detection chemistries. SYBR green is a fluorescent dye which has low fluorescence when in solution; however, it becomes highly fluorescent upon binding to double-stranded DNA. On the other hand, *Taq*Man™ probes, Molecular Beacons, and Scorpions probes employ fluores-

cence resonance energy transfer or Förster resonance energy transfer (FRET, also known as resonance energy transfer [RET] or electronic energy transfer [EET]) to generate a fluorescent signal. The FRET combines the donor fluorescent dye (fluorophore) with a nonfluorescent quenching moiety (quencher). When the fluorescent dye is in close proximity of the quencher, the quencher molecule absorbs the energy and thus blocks fluorescence emission from the fluorophore when excited by light. TaqMan probes are 18–22 bp oligonucleotide probes that are labeled with a reporter fluorophore at the 5' end and a quencher at the 3' end; these are thus in close proximity. Each probe is complementary to a region in the middle of the detecting target between the two primers used in the PCR reaction. When *Taq* polymerase extends the primer to synthesize the nascent strand, the 5' to 3' exonuclease activity of the *Taq* polymerase degrades the *Taq*Man™ probe annealed to the targeted region and releases the fluorophore from *Taq*Man™ probe and thereby breaks the close proximity to the quencher. As a result, the fluorophore when excited by cycler's light emits fluorescence, which marks the presence of PCR product. The method determines the amount of product by generation of fluorescent signal, which is measured in "real time" during the entire cycle time of the RT-PCR reaction, which allows the calculation of the amount of PCR product after each amplification cycle. Similar to *Taq*Man™, the Molecular Beacon and the Scorpions™ both use probes to detect specific PCR product. However, instead of probe degradation, the signal is generated by physical separation of the fluorophore and quenching moieties after hybridization of the specific probe to the PCR product during amplification (Fig. 5).

Each detection chemistry has its own advantages and disadvantages, which need to be considered during experimental design. SYBR green represents a simple, easy-to-use method and is the most economical real-time RT-PCR method. The disadvantage of SYBR green is its binding non-specifically to any DNA including primer dimers and non-specific PCR products; therefore, it is not useful for multiplex amplification of several products in the same reaction. In contrast, *Taq*Man™, Molecular Beacons, and Scorpions™ probes specifically detect only a PCR product complementary to probe sequence enabling to distinguish specific from non-specific products. The disadvantage of these detection chemistries is that each PCR product requires synthesis of its own specific probe, which increases the cost per reaction. On the other hand, labeling individual probes with fluorophores of different emission spectrums allows multiplexing with simultaneous detection of several products and thus reduces the cost and labor.

The usage of real-time RT-PCR for splicing detection requires special considerations. Since real-time RT-PCR techniques omit electrophoretic separation, the spliced product cannot be distinguished based on size. Therefore, it is important that only the desired product is amplified. In this case, the usage of SYBR green chemistry is the most challenging due to the lack of specificity. Probe-based methods provide higher specificity due to probe hybridization to selected sequences that are not present in non-specific products. To detect only the desired spliced product, the probe and primer should be complementary to a specific exon-exon junction or to an alternatively spliced region. Many commercial manufacturers of synthetic oligos

provide free online tools for the design of the most optimal and specific primer pairs based on the provided template sequence. Several other factors must be considered when using RT-PCR-based techniques in diagnostics; these include (1) RNA sample quality and preparation, (2) *Taq* polymerase inactivating contaminants in clinical samples, and (3) amplification bias. Other considerations are false positivity and PCR cross-contamination.

## *Splicing Microarrays*

DNA microarrays (also known as DNA chips) are composed of large number of probes (often several thousand probes) spotted on very small area in 2D format on a solid surface (glass or plastic). The probes represent DNA oligos of various length and chemistry. Each probe has specific DNA sequence allowing detection of corresponding DNA with a complementary sequence. Currently, there are two major technologies involved with DNA arrays and the manufacturing of a microarray: (a) direct synthesis of probes on the array and (b) printing arrays from a library of presynthesized probes. Each DNA microarray allows rapid profiling of large number of DNA molecules at the same time. Today DNA microarrays are widely used to study gene expression profiling and RNA posttranscriptional modifications including RNA splicing [21].

The analysis of RNA transcripts by DNA microarrays requires a conversion of RNA samples to DNA by reverse transcription, following amplification and labeling with fluorescent dye. After labeling, the samples are hybridized with the probes on the array. Unbound samples are washed away, and the fluorescent signal is captured and analyzed by the microarray reader (Fig. 6a). The intensity of the fluorescent signal corresponds to the number of bound molecules and thus allows the determination of the level of RNA in the original sample by use of a mathematical algorithm.

There are two different approaches to probe design for the study of RNA splicing using DNA microarrays: (1) tiling and (2) exon arrays [22] (Fig. 6b). In tiling arrays, the set of overlapping probes cover the full length of the nascent primary transcript including exons and introns. The analysis of fluorescence for each probe allows the identification of exons and introns based on the difference in signal intensity (Fig. 6b*i*). The advantage of tiling arrays is their ability to identify known splice

**Fig. 6** (continued) (*ii*) Detection of RNA splicing by co-hybridization of two probes labeled with acceptor (*A* in circle) or donor (*D* in circle) fluorophores binding to exonic regions (black boxes) flanking the intervening intron (solid line). When bound to unspliced transcript, the binding sites of two probes are separated by intron preventing energy transfer by FRET, and thus, no signal is generated. After intron removal by RNA splicing, two probes are bought to proximity for FRET to occur. The energy transfer from the donor to the acceptor leads to excitation of the acceptor fluorophore (A in circle with spikes) and generation of detectable signal. The diagram is modified from Blanco and Artero [31]

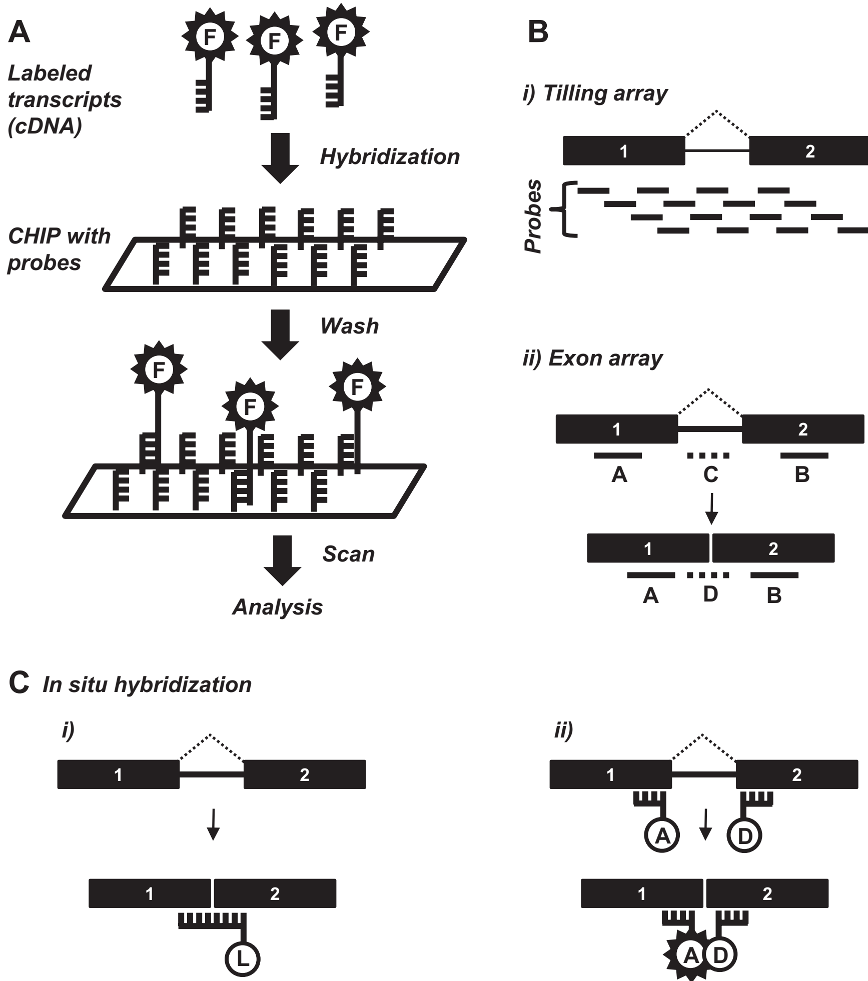



**Fig. 6** Splicing microarrays and in situ hybridization. (**a**) A workflow for microarray assay. First, sample RNAs are converted into cDNAs simultaneously labeled with specific fluorophore *(F)*. The labeled cDNAs are hybridized with oligonucleotide probes attached to a solid surface. The unbound cDNAs are washed away, and the remaining fluorescence signals resulted from specific hybridization are collected by an array-scanning device and analyzed. (**b**) Microarrays in RNA splicing detection. (*i*) Tiling arrays represent a large set of overlapping probes (short solid lines) to cover the selected genes. Because an mRNA has no intron after RNA splicing, introns (lines) can be detected with drop on signal intensity when compared to neighboring exons (solid boxes). (*ii*) Exon arrays consist of combination of two types of probes: probes binding to exon regions (short solid lines) and probes binding to intron or exon-exon junction (short dashed lines). Splicing events are calculated by analysis of signal intensity between exon, intron, and junction probes. (**c**) Detection of spliced transcript by in situ hybridization. (*i*) Probe spanning over the exons (solid boxes) junction specifically binds to spliced product but not to unspliced pre-mRNA containing intron (solid line) or genomic DNA. The probe detection depends on type of labeling (L in empty circle) including isotope, biotin, digoxigenin, fluorophore, or others.

events as well as new splice events. Therefore, the tiling arrays are often used as discovery tools. The disadvantages are the requirement of a large amount of probes, which results in time-consuming data analysis. The exon arrays are more commonly used but require the knowledge of splicing events. Several types of probes hybridizing to flanking exons, intron, and exon-exon junctions are designed to detect each splicing event (Fig. 6b*ii*). The fluorescence intensity is detected for each probe, and a mathematical model is applied to determine the occurrence of splicing event. The advantage of exon arrays is the smaller number of probes required, which means simpler data analysis. However, the exon arrays detect only known or predicted splicing variants. Due to their large capacities, the exon arrays can be designed to detect splicing in multiple viral pathogens simultaneously.

## *In Situ Hybridization*

Tissue sections historically represent an important tool for the diagnosis of pathological changes during viral infection as well as the detection of viral pathogens at the cellular level. There are two major types of tissue sections: frozen and formalin-fixed, paraffin-embedded (FFPE). Both are routinely used for the detection of viral antigens by various types of staining, but their use in the detection of nucleic acids including spliced transcripts is still relatively rare. The improved sensitivity of current nucleic acid isolation and amplification techniques allows the recovery of nucleic acid from tissue sections for further analysis by PCR and RT-PCR with selective isolation of only the cells of interest by the use of laser capture microdissection to add an additional level of specificity [23, 24]. However, the detection of nucleic acids by in situ hybridization (ISH) directly on tissue sections can provide additional information about gene expression linked with spatial distribution of specific RNA transcripts in a morphological context often at the cellular or even subcellular level. In the past, the nucleic acid molecules including RNA transcripts by ISH were detected by DNA probes labeled with radioisotope ($^{35}$S, $^{33}$P, $^{3}$H) [25], which were later replaced by nonradioactive DNA probes labeled with biotin or digoxigenin and detected by chromogenic methods using enzyme-labeled antibodies (CISH) [26]. Labeling probes specific for different transcripts with different fluorophores (FISH) allows detection of multiple targets at the same tissue section. However, the sensitivity was always a limiting factor of ISH techniques. This was caused mainly by the use of DNA probes, which suffer from low affinity to complementary RNA targets and are sensitivity to degradation of RNA-DNA hybrids by RNase H. Development of tyramide signal amplification (TSA) has dramatically improved the sensitivity of DNA probes [27]. Further improvement was seen with the introduction of locked nucleic acid (LNA) and peptide nucleic acid (PNA) probes with high affinity to RNA molecules and resistance to RNase H degradation [28–30]. Detection of RNA splicing by ISH requires a probe to specifically bind

only to spliced mRNA without binding to unspliced pre-mRNA or to the genomic DNA in the sample. Historically, this was achieved by designing a probe over exon-exon junction containing sequences present only in spliced transcripts (Fig. 6c*i*). Another approach in the detection of spliced transcripts by IHS is using co-hybridization of two probes labeled with donor and acceptor fluorophore and the generation of signals by FRET. In principle, each splicing event is monitored by a set of two probes complementary to exonic sequences flanking an intron region. One probe carries a fluorophore acceptor, while the second probe is labeled by a fluorophore donor. When probes bind to genomic DNA or unspliced nascent transcript, their binding sites are separated by intron regions resulting in the distance between the donor and acceptor being too big for the two fluorophores to engage in FRET. However, intron removal by splicing brings the probe binding sites to a proximity close enough for FRET to occur resulting in generation of measurable fluorescence [31] (Fig. 6c*ii*).This results in high specificity and low background. Using a set of probes with different fluorophores allows detection of multiple spliced transcripts or various spliced isoforms of the transcript. In summary, ISH hybridization methods provide a useful tool for the investigation of the distribution not only of protein-encoding transcripts but also of the rapidly growing number of virus-encoded noncoding RNAs, which their role in viral pathogenesis often remains elusive [32]. In situ hybridization methods could be especially suitable in retrospective analysis of archived samples in collections.

## ***RNA*-seq**

Next-generation sequencing (NGS) represents a new generation of analytical tools for genome and transcriptome analysis [33]. This method is based on generation of a large amount of short sequences in parallel sequencing reactions. Advantages of NGS are the requirement of less amount of the initial sample, deep coverage, and nucleotide resolution. NGS also does not require any previous knowledge of the detecting sequence. Currently the main platforms are the Illumina HiSeq 2500 or HiSeq 3000/HiSeq4000 for high-throughput sequencing and generating the sequencing reads of various lengths.

Sequencing of RNA samples converted to cDNA is called *RNA-seq*. *RNA-seq* provides a comprehensive picture of whole genome transcriptome and has been successfully used for analysis on gene expression and posttranscriptional processing including RNA splicing. However, NGS may be costly and time-consuming as well as requiring sophisticated data analysis; this currently makes NGS less suitable for clinical diagnostics. However, *RNA-seq* does not require any prior knowledge of detecting sequence composition and therefore allows detecting unknown or unpredicted RNA sequences. This may be especially beneficial in discovery of new pathogens including viruses [34]. In addition, *RNA-seq* instantly analyzes a transcriptome including spliced transcripts in any type of cell or tissue.

## RNA Splicing in Clinical Virology

RNA splicing does not occur in prokaryotes and is a hallmark of the eukaryotic gene expression. In eukaryotes the number of genes which undergo splicing varies highly from organism to organism, with only about 5% of all genes being spliced in yeasts to 95% in human [35, 36]. Viruses as intracellular parasites replicate inside of host cells and hitchhike many cellular processes for their replication including RNA splicing. By using constitutive and/or alternative RNA splicing, most of DNA viruses and some of RNA viruses increase the complexity of their proteome without the requirement of additional genetic materials.

Detection of spliced viral mRNAs in clinical samples would provide several benefits. While detection of viral genomes in clinical samples indicates virus infection, the result does not provide information about the stage and dynamic of the virus infection. In many cases the progress of viral replication can be assumed from changes in viral load, but this approach requires multiple sampling during infection and varies between individuals. One major advantage for detection of spliced viral transcripts is that viral RNA splicing reflects viral gene expression and thus indicates active viral infection, providing important information about the status of infection without requiring multiple sampling. The production of viral transcripts and their RNA splicing products are often the first sign of virus replication detectable before the increase of viral load or occurrence of viral-specific antigens or antibodies. Therefore, the detection of active viral infection by RNA splicing may be particularly important for early diagnosis of viral infection and may be critical for successful treatment. Because of direct association of spliced viral transcripts with the level of active viral replication and by monitoring viral RNA, one might be able to provide essential information early enough for initiation of antiviral therapy. A rapid shutoff of viral transcription and RNA splicing could be also the first sign of the blockage of viral replication visible even before the change in viral load by genome copy numbers. In the case of ubiquitous and common viruses, such as members of herpesvirus or parvovirus family, which establish latent infection in the host, detection of RNA splicing of a viral early gene would assist to distinguish viral latent infection from active lytic infection. Such a diagnosis is critical for recipients of the transplant organs where reactivation of latent viruses often leads to transplant rejection.

In addition, interpretation of the detection of RNA splicing results is straightforward without concern for carry-over DNA contamination, because spliced RNA is smaller than its corresponding DNA template. As described above, there are many techniques currently available for RNA splicing assay. These techniques are not only easy to set up with a low cost compared to virus isolations and immunological methods, but can be quickly applied to detect new emerging viruses for which the cultivation of the virus is difficult or impossible and/or no immunological method is available. This is particularly true for the combination with RNA-*seq*, which can rapidly provide sequence information about a viral transcriptome and RNA splicing of the viral messages.

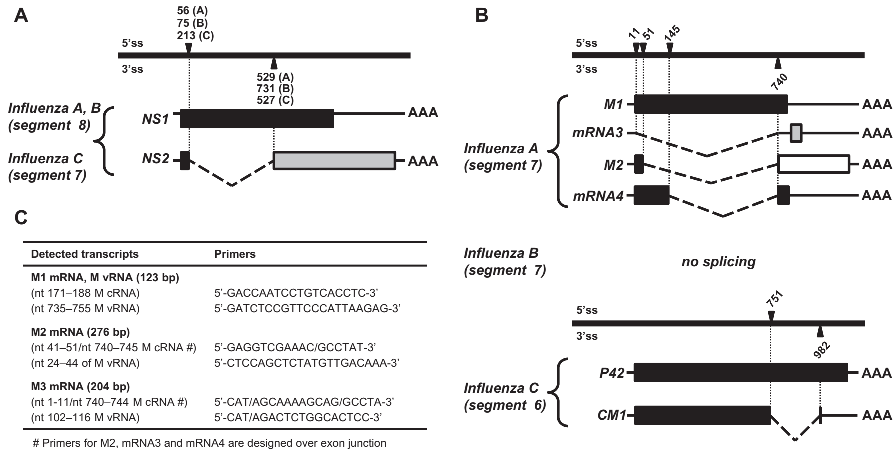
## RNA Splicing in RNA Viruses

### *Influenza Viruses*

Influenza virus infection affects millions of people every year. Influenza viruses, including influenza virus A, B, and C, are the members of the *Orthomyxoviridae* family. Influenza viruses are enveloped RNA viruses with a segmented, single-stranded RNA (ssRNA) genome of negative polarity. The number of segments may vary between virus species, with influenza viruses A and B genome having eight segments and influenza C seven segments. In contrast to the majority of RNA viruses, the influenza viruses replicate in the nucleus of host cells because of their dependence on cellular expression machinery [37]. During replication, the viral RNAs are produced by viral RNA-dependent RNA polymerase. However, viral RNA genomes use short sequences with a cap structure generated by host RNA polymerase II for priming to initiate viral transcription. During infection viral polymerase produces two types of RNAs: one for protein synthesis and the other serving as a template for viral genome replication (see review [38]).

RNA splicing in influenza viruses was first detected in an RNA transcript from the smallest segment 8 in influenza A and B as well as in their corresponding segment 7 in influenza C. This transcript encodes two nonstructural viral proteins: larger NS1 space and smaller NS2 [39]. In influenza A, NS1 protein is encoded by an unspliced primary RNA transcript (~890 nts), whereas NS2 protein is expressed from a spliced RNA (~350 nts) generated by removal of a 473-nt intron from its primary RNA transcript. This results in influenza A NS1 and NS2 proteins sharing the same AUG start codon as well as the first nine amino acid residues. Translation of NS2 protein continues in +1 frame after RNA splicing, that results in the C-terminal NS2 partially overlapping the NS1 by 70 amino acid residues [40] (Fig. 7a). A similar splicing event for production of NS1 and NS2 proteins has been detected from influenza B infections [41] as well as from influenza C [42] infections.

The influenza A segment 7, which encodes M1 and M2 proteins, produces 3 RNA species by alternative RNA splicing events. The unspliced RNA, which is collinear with the genome, encodes M1 nucleoprotein composed of 252 amino acid residues. The two alternatively spliced RNAs, M2 and mRNA3 [43], share the same 3' splice site at nt 740 position, but use different 5'splice sites for alternative RNA splicing (Fig. 7b). M2 RNA uses a 5' splice site at nt 51 position, whereas mRNA3 employs another 5' splice site at nt 11 position from the beginning of viral-specific sequences. The M2 protein has ion channel activity and shares 8 amino acid residues with the M1 N-terminus; it also overlaps with 14 amino acid of M1 C-terminus. The mRNA3 contains a short open reading frame in its exon 2 with the potential to encode a short peptide of 9 amino acid residues. However, the expression of this peptide has never been experimentally confirmed. The role of this transcript during virus replication remains unknown.




| Detected transcripts | Primers |
|---|---|
| **M1 mRNA, M vRNA (123 bp)** | |
| (nt 171–188 M cRNA) | 5'-GACCAATCCTGTCACCTC-3' |
| (nt 735–755 M vRNA) | 5'-GATCTCCGTTCCCATTAAGAG-3' |
| **M2 mRNA (276 bp)** | |
| (nt 41–51/nt 740–745 M cRNA #) | 5'-GAGGTCGAAAC/GCCTAT-3' |
| (nt 24–44 of M vRNA) | 5'-CTCCAGCTCTATGTTGACAAA-3' |
| **M3 mRNA (204 bp)** | |
| (nt 1-11/nt 740–744 M cRNA #) | 5'-CAT/AGCAAAAGCAG/GCCTA-3' |
| (nt 102–116 M vRNA) | 5'-CAT/AGACTCTGGCACTCC-3' |

# Primers for M2, mRNA3 and mRNA4 are designed over exon junction

**Fig. 7** Diagrams of RNA splicing of influenza virus NS and M transcripts. Thin lines represent noncoding sequences. Dashed lines indicate splicing directions. Transcripts are polyadenylated (AAA) at the 3' end. Black boxes mark ORF in primary transcripts. Small black boxes and white boxes are ORF in other frames created by RNA splicing. RNA splicing of NS1 (**a**) and M1 (**b**) transcripts in influenza A, B, and C viruses is diagramed according to Lamb and Horvath [38]. (**c**) Oligo primers used for RT-PCR to detect spliced RNA products from segment 7 of influenza A/WSN/33 (H1N1) as described [207]

While M2 and mRNA3 transcripts are detectable in cells infected with all influenza A viruses, some strains, such as A/WSN/33, produce an additional spliced transcript named mRNA4 [44]. The spliced mRNA4 transcript is generated by usage of additional 5' splice site at position nt 146 and shares the same 3'splice site with M2 and mRNA3 at position nt 740 (Fig. 7b). The mRNA4 has the potential to encode a peptide with 54 amino acid residues, and its first 37 amino acid residues are identical with M1 protein. Sequence analysis of more than 6000 influenza strains revealed that about 20 influenza A strains have this conserved mRNA4 splice site [45]. Sequence information of all influenza viruses can be found at https://www.fludb.org/.

The primary RNA transcript of segment 7 in influenza virus B does not undergo alternative splicing to produce M2 protein as happens in the case of influenza A. RNA splicing in the M transcript takes place with segment 6 of influenza C [46]. The two transcripts generated from segment 6 in the infected cells are the full-length primary and a single-spliced transcript created by removal of an intron located at the 3'end of the primary transcript. The primary transcript contains a 374 aa-long ORF (P42), but the spliced message contains a shorter ORF encoding 242-aa residues (CM1) due to generation of a stop codon after RNA splicing from a 5' splice site at nt 751 to a 3' splice site at nt 982 (Fig. 7b). The P42 protein is consequently processed by internal cleavage, resulting in production of a predominant CM2 protein containing the C-terminal 115-aa residues of P42 protein [47, 48].

In summary, there are two instances of viral RNA splicing in influenza infection: one conserved between all three species (NS1) and the second being highly variable

in each species (M). Thus, the combination of NS1 and M RNA splicing assays would reveal not only active influenza virus infection but also be able to specify the infection with a specific influenza virus species.

## Human Retroviruses

### *HIV-1* and *HIV-2*

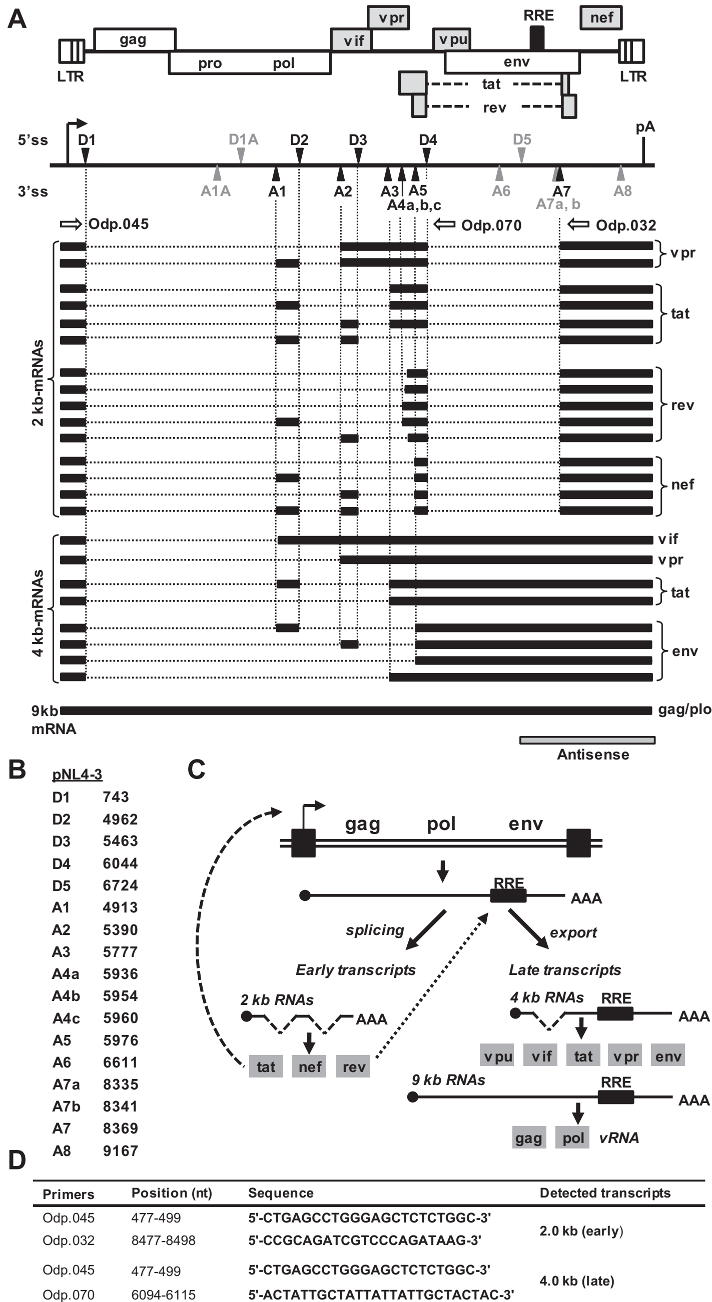
Human immunodeficiency virus (HIV) is a member of *Lentivirus* genus in retrovirus family and causes the acquired immunodeficiency syndrome (AIDS). HIV infects cells of the immune system and consequently causes the failure of immunity that is associated with occurrence of opportunistic infections that may result in death. HIV infection is considered to be a pandemic with about 0.6% of the world population being infected. Two types of HIV viruses have been characterized. Although closely related, HIV-1 differs from HIV-2 in infectivity and geographical distribution, with HIV-2 being much less pathogenic and predominantly occurring in several West African countries.

HIV is an enveloped virus and carries two copies of a single-stranded RNA genome of 9 kb having positive polarity (ssRNA+). After initial infection, the viral genomic RNA is converted by virus-encoded reverse transcriptase into DNA, which then can integrate into the host genome where this integrated viral genome subsequently resides as a provirus. Later, the integrated provirus serves as a template for continued transcription of viral transcripts.

In contrast to simple retroviruses, the HIV genome has a high coding capacity. In addition to encoding viral structural and replication proteins (*gag, pol, env*), HIV encodes a large number of accessory proteins by production of over 40 RNA isoforms, which are derived from a single RNA transcript due to extensive alternative RNA splicing [49] (Fig. 8a).

Three groups of HIV transcripts can be observed by size in Northern blot analysis. The first group represents an unspliced 9-kb transcript, which serves a template for expression of *gag* and *gag/pol* as well as serving as genomic RNA for newly formed virions. The second group represents single-spliced RNA transcripts of ~4 kb, which encode *env*, *vif*, *vpr*, and *vpu* proteins. The third group of transcripts of ~2 kb consists of multiple-spliced RNA transcripts that encode accessory proteins *tat*, *rev*, *nef*, and *vpr*. During virus infection, HIV generates a wide variety of RNA transcripts by using at least five alternative 5' splice sites as well as eight to nine alternative 3’ splice sites [50, 51] (Fig. 8b). In addition, several antisense transcripts from several 3’ long terminal repeats (3’ LTR) have been detected in HIV-1-infected cells [52].

Recent studies have demonstrated that HIV alternatively RNA splicing is largely regulated by viral RNA *cis*-elements as well as cellular splicing factors and is orchestrated for completion of the HIV life cycle during virus infection. Multiple-spliced transcripts of the 2-kb family are expressed in the early stage of virus infec-




| Primers | Position (nt) | Sequence | Detected transcripts |
|---|---|---|---|
| Odp.045 | 477-499 | **5'-CTGAGCCTGGGAGCTCTCTGGC-3'** | 2.0 kb (early) |
| Odp.032 | 8477-8498 | **5'-CCGCAGATCGTCCCAGATAAG-3'** | |
| Odp.045 | 477-499 | **5'-CTGAGCCTGGGAGCTCTCTGGC-3'** | 4.0 kb (late) |
| Odp.070 | 6094-6115 | **5'-ACTATTGCTATTATTATTGCTACTAC-3'** | |

**Fig. 8** RNA splicing of HIV-1 transcripts. (**a**) Schematic diagram of HIV-1 genome with structural and replication protein ORFs marked with empty boxes and accessory protein ORFs with gray boxes. *LTR*, *long terminal repeats*. Shown below the diagram are positions of four 5' splice sites (D1-4) and seven 3' splice sites (A1-7) identified in a 9-kb full-length primary transcript which encodes *gag* and *pol* proteins. Alternatively spliced HIV-1 transcripts in the size of

tion and express *tat*, *rev*, and *nef*. This group of spliced RNAs is produced by using the 3' splice site A3–A5 located in the central part of the viral genome with an A3 site for expression of *tat*, A4a-c for *rev*, and A5 splice site for *nef* proteins. During late stage of HIV infection, nuclear import and accumulation of *tat* together with *rev* protein allow the *rev* protein to bind to a *rev-responsive element* (RRE) in partially spliced 4-kb and unspliced 9-kb RNA transcripts located in the *tat/rev* intron between the D4 and A7 splice sites; this *rev* protein mediates the export of later transcripts into the cytoplasm for translation [53] (Fig. 8c). Sites A1A and D1A are involved in pre-mRNA stability [54]. Strains from the IIIB family of HIV viruses use additional A6 and D5 splice sites to generate a small exon in the *env* region; transcripts containing this exon express the tripartite *tat-env-rev* fusion protein, *tev* [55, 56].

Regulation of HIV RNA splicing depends on the selection of 3' splice sites, which are, in general, weak in contrast to stronger and highly active 5' splice sites. In addition, numerous positive and negative splicing regulatory *cis*-elements identified in the HIV RNA genome bind various cellular splicing factors and thus affect the selection of individual 3' splice site (see review [49]). Comparison of nucleotide sequences between the various clades of HIV-1 has demonstrated a high level of conservation of splice sites among the different clades of HIV-1 strains (except D4a, b, c).

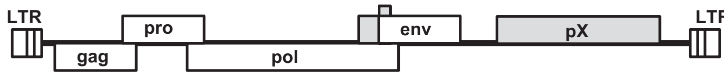
## *HTVL-1* and *HTVL-2*

Human T-cell leukemia virus type 1 (HTLV-1) and type 2 (HTLV-2) were the first two retroviruses discovered in humans [57]. HTLV-1 is etiologically linked to adult T-cell leukemia/lymphoma (ATLL), an aggressive malignancy of CD4+ T lymphocytes, as well as to a neurological disorder named *HTLV-1-associated myelopathy/tropical spastic paraparesis* (HAM/TSP) [58–60]. HTLV-1 is endemic in Japan, Africa, Caribbean basin, and South America. HTLV-2 is linked to HAM/TSP but not to ATLL. HTLV-2 infection occurs predominantly in parts of Africa and Americas [61].

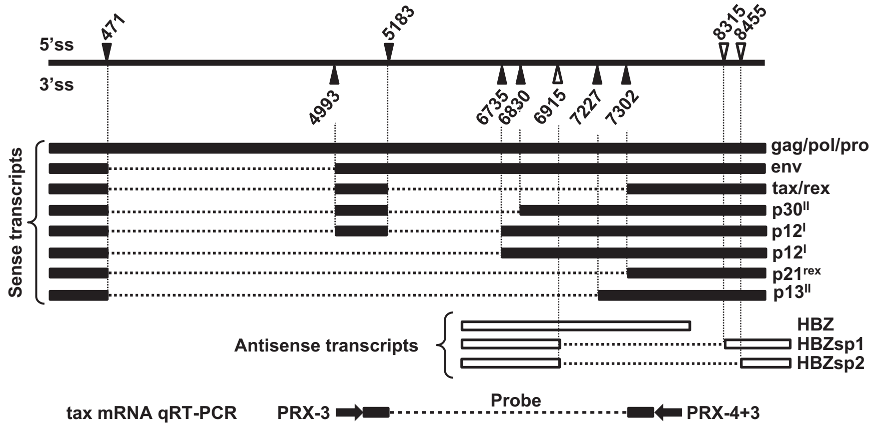
HTLV-1 and HTLV-2 are two closely related complex retroviruses that share about 70% of their nucleotide sequences. Their genome organization and replication are similar to HIV. Besides essential genes (*gag/pol/env*) expressed from the

**Fig. 8** (continued) ~2 kb and ~4 kb are grouped along with their protein-coding potentials. Solid boxes represent exons and dotted lines are introns. A gray box below the 9-kb mRNA illustrates a recently discovered antisense transcript [52]. (**b**) Nucleotide positions of all mapped 5' and 3' splice sites in a prototype HIV genome, pNL 4-3 (GenBank Acc. No. AF324493), starting from the 5' LTR [208]. (**c**) Alternative HIV RNA splicing is coupled with stages of HIV-1 infection. Multiple-spliced transcripts (2-kb group) are expressed in the early stage of the infection resulting in the expression of accessory proteins: *tat*, *rev*, and *nef*. During the late stage of HIV-1 infection, *rev* protein translocation to the nucleus promotes nuclear export of single-spliced (4-kb group) or unspliced HIV RNA via binding to an *rev*-responsive element (RRE) to express structural and replication proteins [51]. (**d**) Oligo primers used for RT-PCR, shown in (A), to detect spliced RNA products of HIV-1 pNL 4-3 [208]

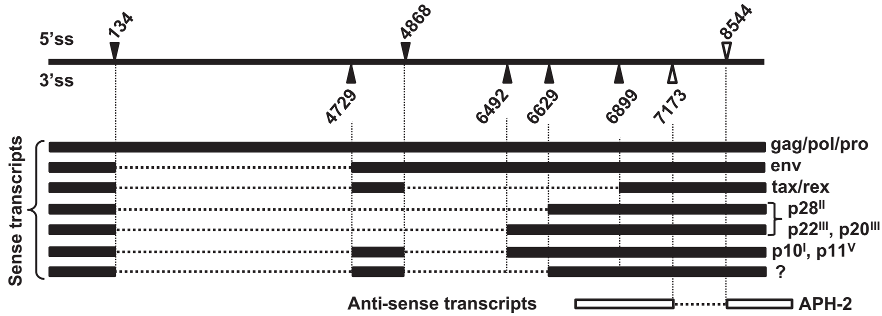
**A** **HTLV-1&2**




**B** **HTLV-1**




**HTLV-2**




**C**

| Primer | Position (nt) | Sequence |
|---|---|---|
| RPX-3 | 5096-5115 | 5’-ATCCCGTGGAGACTCCTCAA-3’ |
| RPX-4 + 3 | 7360-7338 | 5’-CCAAACACGTAGACTGGGTATCC-3’ |
| Probe | 5172-5183/7302-7312 | 5’-TCCAACACCATG/GCCCACTTCCC-3’ |

**Fig. 9** RNA splicing of HTLV-1 and HTLV-2 transcripts. (**a**) Genomic organization of HTLV-1 and HTLV-2 with structural and replication genes (white boxes) on the 5’ end and regulatory gene *pX* (gray boxes) on the 3’ end of the genome flanked with long terminal repeats (LTR). (**b**) Viral transcripts generated by alternative RNA splicing in HTLV-1- and HTLV-2-infected cells. Black boxes indicate exons and dotted line are introns. Exons in antisense transcripts are represented by empty boxes. The 5’ and 3’ splice sites are indicated by their nucleotide positions in the virus genomes. The coding potentials of each transcript are shown on the right. (**c**) Positions and sequences of the primers and probes used for detection of HTLV-1 spliced tax mRNA as described [209]. Nucleotide positions are based on a full-length HTLV-1 cDNA (GenBank Acc. No. L03562.2)

full-length or single-spliced RNA, HTLVs also encode a number of accessory proteins in a pX region located in the 3'end of the virus genome (Fig. 9a). Transcripts for encoding accessory proteins are generated by alternative RNA splicing of a full-length primary transcript using several 5' splice sites and 3' splice sites (Fig. 9b) (reviewed in [62]). In addition, transcripts antisense to pX region have been discovered in infected cells either with HTLV-1 or HTLV-2, and these antisense transcripts encode HBZ and APH-2 proteins, respectively [63, 64]. Interestingly, these transcripts also undergo RNA splicing.

In contrast to HIV-1, the regulation of HTLV RNA splicing and the roles of cellular splicing factors in HTLV RNA splicing are poorly understood.

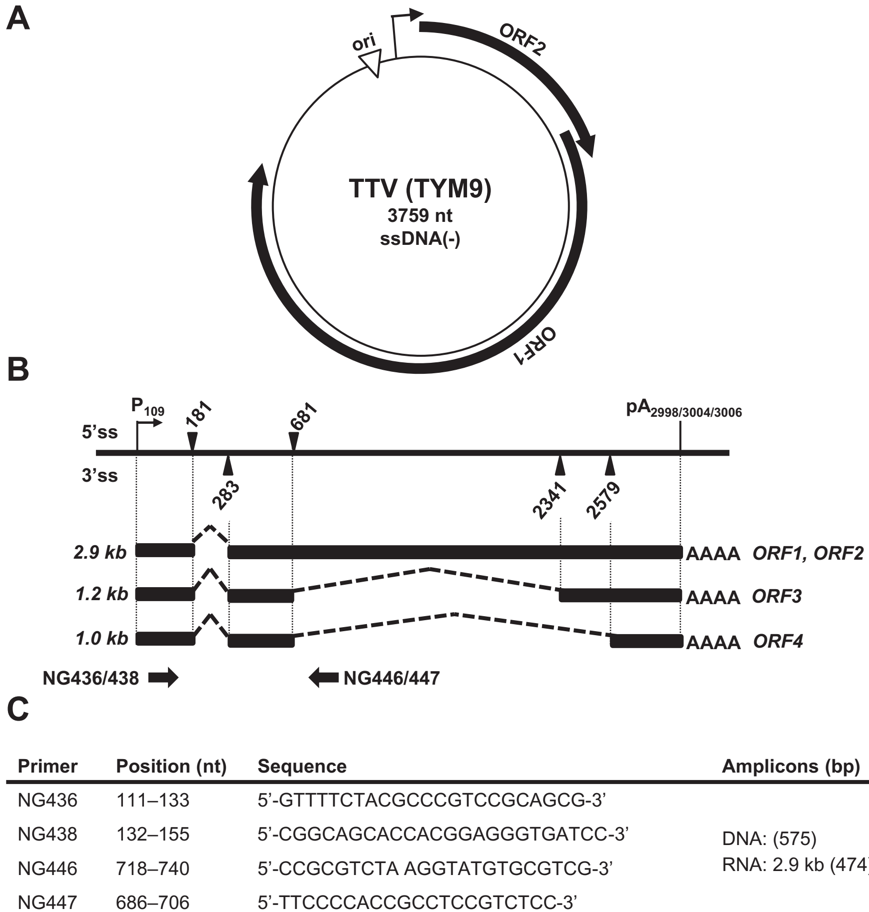
## DNA Viruses

### *Circoviruses*

Human circovirus Torque teno virus (TTV) was originally discovered in the serum of a patient with posttransfusion non-A to G hepatitis [65]. Later studies showed that TTV is present in body fluids of healthy individuals and is not associated with any pathological disorder. However, the prevalence of TTV in the general population appears highly variable and ranges from about 2% to 90% of the incidence of TTV infection. This large variation among reported studies is most likely attributable to primer selection and PCR performance [66]. TTV isolates have considerable diversity (about 30%) that can be clustered in several genotypic groups without any particular geographical distribution, indicating that TTV likely represents a ubiquitous virus. Virus replication, route of TTV infection, and association with pathological manifestations remain unclear.

TTV is small non-enveloped virus of icosahedral architecture and contains a circular single-stranded DNA genome of 3.6–3.9 kb of negative polarity (ssDNA-) [67, 68]. TTV replication is not yet fully understood, partially due to the lack of an appropriate tissue culture system. The TTV genome consists of a GC-rich noncoding region and a protein-coding region with two overlapping ORFs (Fig. 10a). Three species of RNAs of 2.9, 1.2, and 1.0 kb have been detected in infected bone marrow cells as well as in an in vitro-infected cell line [69, 70]. All transcripts originate from the same promoter and undergo alternative RNA splicing (Fig. 10b). Each RNA transcript can be translated into two different proteins by using two alternative start codons [71]. The presence of other ORFs (ORF3 and 4) in the TTV genome has been predicted, but has not yet been confirmed.

The role of TTV RNA splicing and its regulation in infection by this virus as well as TTV viral replication remained largely unknown. Another human circovirus TTV-like mini virus (TLMV) also has been identified in human sera [72]. TLMV shares the same genetic organization with TTV and other circoviruses, but its genome is only about 2.9 kb in size.




| Primer | Position (nt) | Sequence | Amplicons (bp) |
|---|---|---|---|
| NG436 | 111–133 | 5'-GTTTTCTACGCCCGTCCGCAGCG-3' | |
| NG438 | 132–155 | 5'-CGGCAGCACCACGGAGGGTGATCC-3' | DNA: (575) |
| NG446 | 718–740 | 5'-CCGCGTCTA AGGTATGTGCGTCG-3' | RNA: 2.9 kb (474) |
| NG447 | 686–706 | 5'-TTCCCCACCGCCTCCGTCTCC-3' | |

**Fig. 10** Human circovirus TTV. (**a**) Single-stranded genome of Torque teno virus (TTV) with the origin of replication (ori), promotor (arrow), and two identified overlapping ORFs. (**b**) Transcripts identified in cells infected with TTV virus with nucleotide positions of two 5' splice sites (above the line) and three 3' splice sites (below the line), together with positions of promoter (arrow) and polyadenylation site (pA). The black boxes represent exons and dashes lines are introns. The size of the spliced transcripts (in kb) is indicated on the left, and the coding potentials are on the right. Arrows below the transcripts are a primer pair used to detect the 2.9-kb transcript [69] by RT-PCR as detailed in (**c**). Primer positions in (C) are based on Torque teno virus TYM9 strain (GenBank Acc. No. AB050448.1)

## *Hepadnaviruses*

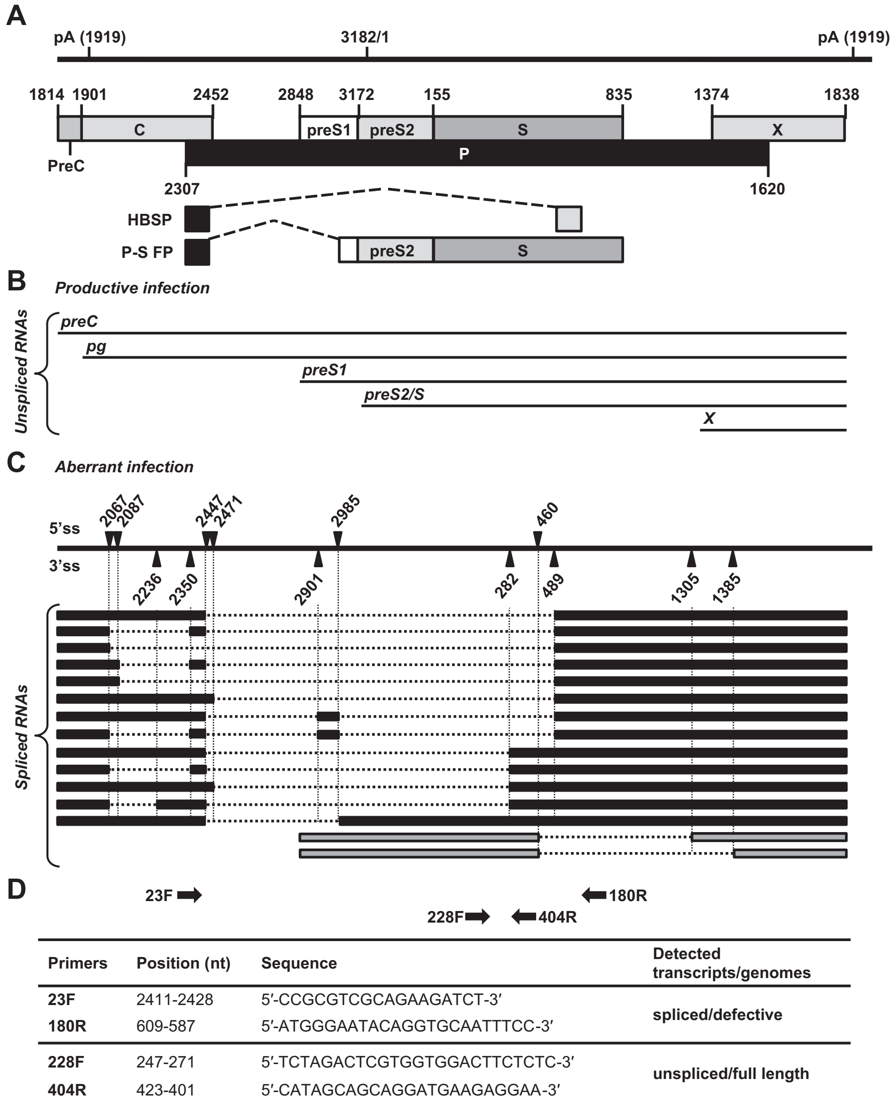
Hepatitis B virus (HBV) is a hepatotropic virus, by which chronic hepatic infection can result in the development of liver cirrhosis and hepatocellular carcinoma [73]. Despite an effective HBV vaccine that is available in many countries, HBV infection remains epidemic in many parts of the world, particularly in Asia and

sub-Saharan Africa. WHO estimates that HBV infects about 2 billion of people worldwide, with chronic infection affecting 350 million and causing death in approximately 1 million persons each year. While the vaccine can prevent HBV infection, there is no cure for already infected individuals due to the persistence of the active transcriptional template of HBV covalently closed circular DNA (cccDNA).

HBV is a non-cytopathic, hepatotropic virus. Its genome consists of a 3.2-kb-long circular and partially double-stranded DNA. HBV encodes four viral proteins: core (C), reverse transcriptase-polymerase (P), surface (S), and X proteins (Fig. 11a). In infected hepatocytes, the DNA is converted to cccDNA (covalently closed circular DNA) and serves as a template for expression of viral pre-genomic (pgRNA) and three subgenomic RNAs from several promoters. Despite the presence of several promoters, all transcripts used the same poly(A) signal for transcription termination (Fig. 11b). The core protein C and polymerase-transcriptase protein P are encoded by a bicistronic pgRNA. Subgenomic preS and S RNAs translate three surface antigens: (1) large [preS1], (2) middle [preS2], and (3) small [S] surface antigens. A short RNA of 0.7 kb encodes X protein, a nonstructural viral protein that presumably has oncogenic potential [74]. A pre-core RNA initiated upstream of pgRNA encodes HBeAg [75, 76]. Beside protein translation, pgRNA is a template for reverse transcription and results in a genomic minus DNA strand during HBV genome replication.

All HBV transcripts from cccDNA are produced by cellular RNA polymerase II. A spliced 2.2-kb RNA transcript was first identified in transfected hepatoma cells [77] and contains a single 1223-nt-long intron starting from the end of the core antigen ORF to the middle of the S antigen ORF. Subsequently, other single- and multiple-spliced forms of pgRNA have been discovered, with sizes of 2.1–2.6 kb, in both cell cultures and liver tissues of HBV patients [78–80]. So far, 13 spliced variants of pgRNA and 2 spliced isoforms of pre-S2/S RNA have been identified from HBV gene expression during infection; these spliced viral RNAs have been produced by the use of six 5’ splice sites and seven 3’ splice sites (Fig. 11c). A viral *cis*-element PRE (posttranscriptional regulatory element) as well as cellular splicing factors such as PTB (polypyrimidine track-binding protein) and SR proteins also may play roles in the regulation of HBV RNA splicing (see review [81]).

Approximately 30–50% of HBV RNA during HBV infection of human hepatoma cell lines Huh7 and HepG2 are spliced RNAs; Huh7 and HepG2 are two popular cell lines for in vitro HBV replication studies [82]. Huh7 cells seem to produce more spliced RNA than do Hep2G cells. The major spliced product is derived from 30% of pgRNA using nt 2447 5’ss and nt 489 3’ss in genotypes A, C, D, and E. Serum of infected patients or hepatocarcinoma tumor samples frequently contain HBV DNA originating from spliced variants [83, 84]. The level of spliced HBV RNAs in patients varies widely from no splicing to extensive splicing and is related to viral genotype [85]. The role of HBV RNA splicing in the HBV life cycle or in HBV pathogenesis remains to be elucidated. HBV spliced RNAs express two new proteins [86, 87]. A spliced mRNA derived from pgRNA with removal of a




| Primers | Position (nt) | Sequence | Detected transcripts/genomes |
|---|---|---|---|
| 23F | 2411-2428 | 5′-CCGCGTCGCAGAAGATCT-3′ | spliced/defective |
| 180R | 609-587 | 5′-ATGGGAATACAGGTGCAATTTCC-3′ | |
| 228F | 247-271 | 5′-TCTAGACTCGTGGTGGACTTCTCTC-3′ | unspliced/full length |
| 404R | 423-401 | 5′-CATAGCAGCAGGATGAAGAGGAA-3′ | |

**Fig. 11** Expression and RNA splicing of HBV transcripts. (**a**) Diagram of linear HBV genome structure. Numbers indicate the beginning and the end of each ORF. (**b**) Full-length viral transcripts generated from viral genome during productive HBV infection. (**c**) Alternatively, spliced viral transcripts of *preC* (black) or *preS1* (gray) primary transcripts, with exons in black or gray boxes and introns in dotted lines. Numbers above or below the linear genome are nucleotide positions of mapped 5' and 3' splice sites. It has been assumed that alternative RNA splicing of HBV *preC* and *preS1* leads to production of defective HBV viral particles. Diagrams are modified from Sommer and Heise [81]. Arrows below the transcripts are two sets of primer pairs used to detect spliced *pgRNA* products of HBV by RT-PCR [210] as detailed in (**d**) with primer positions and sequences derived from HBV TK113 genome (GenBank Acc. No. JF754635)

454-nt intron from nt 2447 to 2901 encodes a structural polymerase-surface fusion protein p43 with potential function in the entry [86]. Another single-spliced pgRNA with removal of an intron from nt 2447 to 489 translates a 93-aa fusion protein in size of 10.4 kDa, in which the first 46-aa residues are identical to the N-terminus of the viral polymerase protein followed by the 47-aa residues generated by the frameshift from the second exon. This protein has been referred as hepatitis B splice-generated protein or HBSP [87] and seems to be associated with chronical HBV infection, HBV viral cytopathogenic effect, and HBV immune evasion (see review [88]).

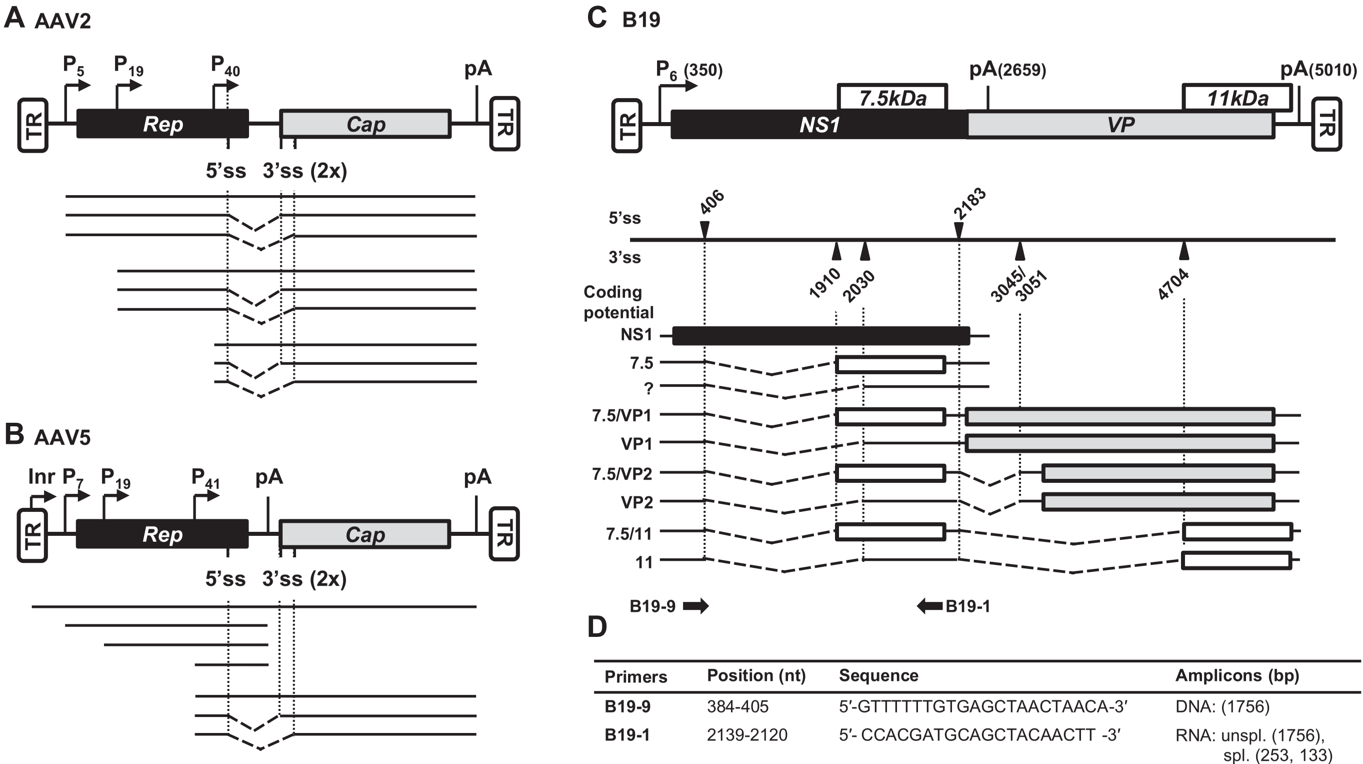
## *Parvoviruses*

Parvoviruses are a group of small non-enveloped viruses containing a single-stranded DNA genome (ssDNA) of ~6 kb. The palindromic inverted terminal repeats at the ends of the virus genome function as an origin for replication. Parvoviruses replicate via a double-stranded DNA intermediate that serves as a template for viral transcription [89]. Replication of some parvoviruses relies on "helper" viruses such as adenovirus, herpesviruses, vaccinia virus, and human papillomaviruses [90–92].

Parvoviruses are ubiquitous viruses and infect a wide range of animals. As of today, there are at least four members of the *Parvoviridae* family that are infectious to humans: adeno-associated viruses (AAV), parvovirus B19 (B19V), human bocaviruses (HuBoV), and human Parv4 [93]. Despite structural and genetic similarity, different parvoviruses use different replication and transcription strategies during viral infection and have different host tropisms for initiating a productive infection in the presence of a helper virus.

Adeno-associated viruses (AAV), currently classified as *Dependoviruses*, were the first human parvoviruses identified in the group. AAVs infect a wide range of species with AAVs-1, AAVs-2, AAVs-3, AAVs-8, and AAVs-9 being found in human [94]. Currently no disease or pathological condition is associated with AAV infection in humans. The correlation between AAV infection and fetal loss and male infertility has been proposed due to a high prevalence of AAV DNA in placental tissues and in genital tissues of men with abnormal semen [95, 96]. This has not yet been shown to be causal. Because AAV lacks pathogenicity, induces a low immune response, and infects both dividing and nondividing cells with the capability of viral DNA integration into the host genome, AAV has gained attention as a vector for gene therapy (see review [97]).

All AAV genomes consist of two open reading frames, *Rep* and *Cap*, with *Rep* for virus replication and *Cap* for structural capsid protein. AAVs use several different strategies to produce viral products. The first group represents AAV1, AAV2, AAV3, AAV4, and AAV6, and their viral transcripts originate from one of three viral promoters on the left-hand side of the viral genome and are terminated on a single polyadenylation site on the right-hand side of the genome. The middle part of the transcripts contains a ~300-nt-long intron with a non-consensus 5' splice site and




| Primers | Position (nt) | Sequence | Amplicons (bp) |
|---|---|---|---|
| B19-9 | 384-405 | 5′-GTTTTTTGTGAGCTAACTAACA-3′ | DNA: (1756) |
| B19-1 | 2139-2120 | 5′- CCACGATGCAGCTACAACTT -3′ | RNA: unspl. (1756), spl. (253, 133) |

**Fig. 12** RNA splicing of human parvoviruses. Genome organizations of AAV2 (**a**), AAV5 (**b**) and human *Erythrovirus* B19 (**c**) with terminal repeats (TR) on the ends of each genome, along with viral promoters (P), polyadenylation sites (pA), splice sites (ss), and open reading frames (boxes). As shown below each genome are viral transcripts generated by alternative transcription initiation, RNA splicing, and polyadenylation. In each panel, black boxes represent coding regions, solid lines for noncoding regions, and dashed lines for splice directions to remove the corresponding introns. (**d**) Oligo primers used for RT-PCR to detect spliced RNA products of B19 [211]. The primer positions and sequences are based on a partial genome sequence of B19-Au strain (GenBank Acc. No. M13178.1)

two 3’ splice sites (Fig. 12a).The efficient splicing requires the presence of both a helper virus and a large *Rep* protein [98]. While *Rep* protein seems to be essential for AAV2 splicing, several adenovirus proteins (E1A, E1B, E2a, E4orf6, and VA RNA) as well as some products of herpes simplex virus (UL5, UL8, UL52, and UL29) also have a stimulatory effect on AAV2 splicing [99]. AAV5 and some animal AAVs are in the second group that utilizes three upstream promoters for their transcription, but their genome contains additional polyadenylation sites in the intron region. Transcripts from two upstream promoters are polyadenylated on the internal poly(A) site, whereas spliced transcripts from P41 promoter use a poly(A) site at the right side of the genome (Fig. 12b). The only spliced transcript in AAV5 infections is the *Cap* transcript which contains a smaller (~240 nt) intron. Interestingly, the splicing of AAV5 *Cap* transcript is constitutive and highly efficient even in the absence of helper virus infection [100].

Human B19 virus, a member of *Erythrovirus* genus, was first identified in the serum of blood donor [101]. Three genotypes of B19 viruses have been identified from different geographic regions [102]. After acute infection, the virus persists in host for the rest of the life. The infection by B19 virus is generally asymptomatic, but several pathological conditions have been associated with B19 infection; these include *erythema infectiosum* (the “fifth disease) [103], *polyarthropathy syndrome* [104], transient aplastic crisis (TAC) [105], and persistent anemia/pure red cell aplasia (PRCA). B19 infection during pregnancy may associate with spontaneous miscarriage and development of nonimmune *hydrops fetalis* [106].

Like other parvoviruses, B19 virus genome encodes two large open reading frames. NS1 ORF on the left-side genome translates a 77-kDa nonstructural protein, while a VP ORF on the right-side genome produces two capsid proteins (84-kDa VP1 and 58-kDa VP2). At least nine virus-specific transcripts have been detected following B19 infection [107]; all of which are transcribed solely from a single promoter P6 located upstream of NS1 gene, but are alternatively spliced and terminated at two alternative polyadenylation sites (Fig. 12c) located either in the middle or on the far right side of the genome. By using the poly(A) site in the middle of virus genome, the P6 transcript has an intron in the NS1 ORF, and splicing of this intron from NS1 transcript may create a novel ORF encoding a small accessory 7.5-kDa protein. However, if the poly(A) site on the right-side genome is used for RNA polyadenylation, the P6 transcript becomes a bicistronic (NS1 and VP) transcript with two introns. By splicing to remove the intron 1 from the bicistronic RNA, the single-spliced P6 transcript is capable of encoding both 7.5-kDa and VP1 proteins. Double RNA splicing to remove both intron 1 and intron 2 from the P6 bicistronic transcript disrupts both ORFs for NS1 and VP1, but creates either a VP2 or a novel ORF for another accessory 11-kDa protein, depending on which alternative 3’ splice site is selected (Fig. 12c). Thus, all detected B19 transcripts are derived from a P6 pre-mRNA containing one or two introns with two possible alternative 3’ splice sites depending on the selection of one of two possible alternative poly(A) sites. All detected B19 transcripts are alternatively spliced RNA transcripts, except the unspliced full-length NS1RNA. The *cis*-elements in the central exon and intron

2 are regulatory elements and control the alternative P6 RNA splicing, with the double-spliced P6 RNAs being the predominant species in the infected cells [107].

## *Adenoviruses*

The two most common infection sites by adenoviruses in humans occur in the upper and lower respiratory tract and result in bronchitis and/or pneumonia. Adenovirus infection can also involve a wide range of other sites resulting in *conjunctivitis*, ear infection, *gastroenteritis*, *myocarditis*, *hemorrhagic cystitis*, *meningitis*, and *encephalitis*. There are 56 adenovirus types belonging to seven species (human adenovirus A–G). Types belonging to B and C are responsible for most respiratory infections, B and D for *conjunctivitis*, and F and G for *gastroenteritis* [108]. Adenoviruses were also found in other vertebrates.

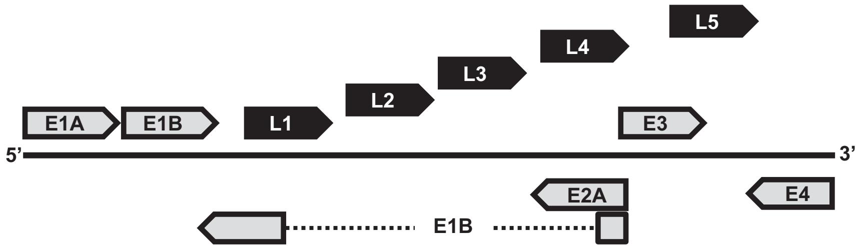
Even though the human adenoviruses are not etiologically linked to any human cancer, some adenoviruses (types 2, 5, 12, 18, and 31) can, under special circumstances, transform rodent cells in vitro and induce tumors in small animals. Transformation activities are linked to two oncogenes: (1) E1A, which bind tumor suppressor pRB, and (2) E1B, which binds tumor suppressor p53 [109].

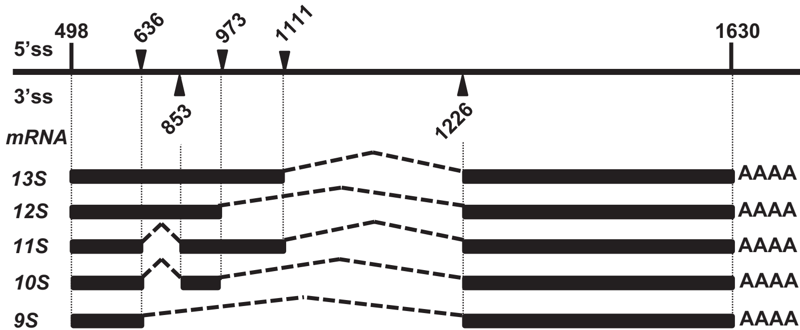
Adenoviruses are non-enveloped viruses with an icosahedral architecture that contains a linear, non-segmented, double-stranded DNA genome of ~26–45 kb that is capable of encoding 22–40 different gene products [109]. Adenoviruses replicate in the nucleus of infected cells (Fig. 13a). The early stage of virus infection is characterized by the expression of a nonstructural early protein, while viral structural proteins are expressed in the late stage of viral DNA replication marking a switch between the two infection phases.

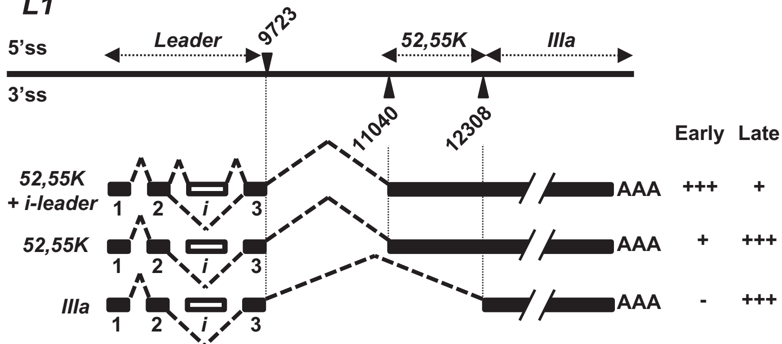
Almost all adenoviral early and late transcripts undergo RNA splicing in order to produce their corresponding viral products [110]. Here, viral E1A and L1 transcripts are used as examples for the alternative RNA splicing seen in adenoviral infections.

The adenovirus E1A primary transcript contains three 5’ splice donor sites as well as two 3’ acceptor sites and is composed of three exons and two introns. The first intron from nt 636 to nt 853 is a suboptimal, minor intron. The second intron is a major intron that uses two alternative donor sites, respectively at nt 973 and nt 1111 as well as one acceptor site at nt 1226 for RNA splicing. Alternative splicing of E1A RNA through the utilization of various combinations of splice donor and acceptor sites leads to the formation of five different species (13S, 12S, 11S, 10S,

**Fig. 13** (continued) (**b and c**) Alternatively spliced RNA transcripts of adenovirus early *E1A* gene (**b**) and adenovirus late *L1* gene (**c**). Black boxes, exons; white boxes, alternative exons; dashed lines, introns or splice directions. Nucleotide positions of each splice site are based on a complete genome sequence of human adenovirus type 2 (GenBank Acc. No. AC_000007.1). (**d**) Schematic exon compositions of *52*, *55K*, and *IIIa* transcripts and exon junction probes for specific detection of spliced *L1* isoforms from adenovirus type 2 by in situ hybridization as described [212]



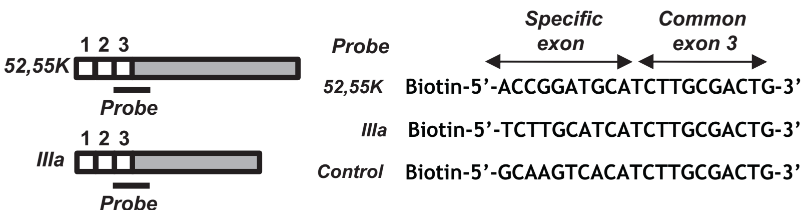




**Fig. 13** Alternative RNA splicing of adenovirus early and late transcripts. (**a**) Simplified adenovirus genome with positions and orientations of viral early (gray arrows) and late (black arrows) genes.

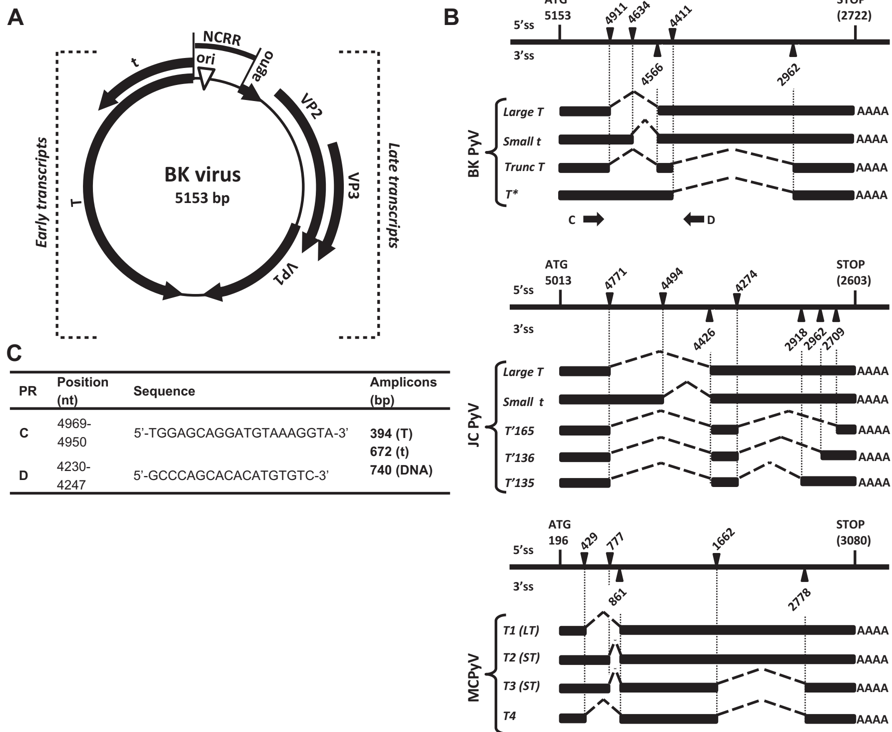
and 9S) of E1A mRNAs based on their sedimentation coefficient (Fig. 13b) and expression of individual unique proteins [111].

The transcription of late genes starts predominantly from a major late promoter. The primary late transcript is then polyadenylated at one of five polyadenylation sites, forming five groups of late transcripts (L1–L5). Each late mRNA contains a 201-nt "leader" sequence derived from three noncoding exons that function as a translational enhancer [112]. There are two variants of leader sequence with or without an *i*-leader exon. Beside the leader sequence region, L1 transcripts also are alternatively spliced by the utilization of a common 5' splice site in combination with two alternative 3' splice sites. Selection of a proximal 3' splice site results in the formation of 52, 55K RNA, while selection of a distal 3' splice site produces IIIa mRNA (Fig. 13c).

The characteristic features of adenovirus splicing depend on the stage of virus infection. For example, E1A 13S and 12S mRNA are two major spliced products that occur during early virus infection. In contrast, 9S RNA is largely accumulated in the late stage of infection [113]. Similar phenomenon has been observed in the expression of late mRNAs. Inclusion of the *i*-leader exon is generally a signature of early transcripts, but most of the late transcripts contain a classical tripartite leader. While 52,55K L1 RNA is produced during both early and late infection, the IIIa splice site is used only in the late stage of viral infection [114]. Both cellular splicing machinery and viral products have been found to regulate alternative splicing of adenoviral transcripts during the course of viral infection (see reviews [ 110, 115]).

## *Polyomaviruses*

Polyomaviruses are small non-enveloped viruses that contain a circular double-stranded DNA (dsDNA) genome of ~5000-bps. Polyomaviruses infect a wide range of mammalian and avian species, but each virus exhibits a limited host range with narrow tissue tropism. The *Polyomaviridae* family contains only one genus *Polyomavirus (PyV)*, which has nine members of the human polyomaviruses: (1) BKPyV [116], (2) JCPyV [117], (3) KI PyV [118], (4) WU PyV [119], (5) Merkel cell PyV (MCPyV) [120], (6) HPyV6, (7) HPyV7 [121], (8) *trichodysplasia spinulosa*-associated PyV (TSV) [122], and (9) HPyV9 [123]. Simian vacuolating virus 40 (SV40), a prototype virus of the family, was introduced into the human population as a contaminant in early trials of poliovirus vaccine [124]. Serological data indicate that polyomavirus infection is widespread in the general human population with initial infection occurring in childhood [125]. After the initial infection, polyomaviruses persist in the host for the rest of the life. While initial infection is usually asymptomatic, several human polyomaviruses are associated with various pathological conditions in immunocompromised patients including *nephropathy* and *cystitis* associated with BK PyV and progressive *multifocal leukoencephalopathy* associated with JCPyV [126], as well as *trichodysplasia spinulosa* presumably associated with TSV infection. Polyomaviruses expresses an oncoprotein T antigen,




| PR | Position (nt) | Sequence | Amplicons (bp) |
|---|---|---|---|
| C | 4969-4950 | 5'-TGGAGCAGGATGTAAAGGTA-3' | 394 (T) 672 (t) 740 (DNA) |
| D | 4230-4247 | 5'-GCCCAGCACACATGTGTC-3' | |

**Fig. 14** RNA splicing of polyomavirus T antigen transcripts. (**a**) Genome structure of BKPyV virus, a representative of human polyomaviruses. Black arrows represent open reading frames for early viral regulatory proteins (large T (LT) and small t (ST) antigens) and late viral capsid proteins (*VP1-3*) as well as agnoprotein (agno). *NCRR*, noncoding regulatory region; *ori*, origin of replication. (**b**) Alternative RNA splicing of large *T* and small *t* antigens among BKPyV, JCPyV, and MCPyV viruses. Black boxes, exons; dashed lines, introns or splice directions; numbers, nucleotide positions of splice donor and acceptor sites. The diagrams are modified from White et al. [213] and Shuda et al. [127]. Nucleotide positions for BKPyV are strain *Dunlop* (GenBank Acc. No. V01108), for JCPyV are strain *Mad-1* (GenBank Acc. No. J02226), and for MCPyV are isolate *MKL-1* (GenBank Acc. No. FJ173815). Arrows below shows the BKPyV transcripts are oligo primers (PR) used to detect spliced large *T* and small *t* antigen transcripts of strain *Dunlop* by RT-PCR as detailed in (**c**) [131]

and this T antigen may lead to the development of human cancer by an abortive infection as recently confirmed in a rare but aggressive Merkel cell carcinoma [127–129].

The polyomavirus genome consists of three functional regions: two protein-coding regions (early and late) divided by a noncoding regulatory region (NCRR) (Fig. 14a). Early and late transcripts are expressed in two opposite directions from promoters located in the NCRR, which also contains the origin of replication. Early transcripts encode nonstructural viral regulatory proteins (T [tumor] antigens) that are important for virus replication and modulation of cell cycle. Viral DNA replica-

tion initiates the transcription of viral late genes that encode several viral capsid proteins.

In polyomavirus-infected cells, multiple isoforms of the *T* antigen are detectable because of alternative RNA splicing. The primary transcript of the *T* antigen contains two introns, but the first intron 1 has two alternative 5' splice sites. During RNA splicing, the intron 2 retention is important for production of both large *T* and small *t* antigens. However, selection of proximal 5' splice site in the intron 1 for RNA splicing leads to production of large *T* antigen, whereas selection of a distal 5' splice site in the intron 1 results in small *t* production. Because the sequence region between the proximal 5' splice site and the distal 5' splice site has a stop codon, retention of this region in small *t* RNA splicing makes the small *t* RNA larger than the large *T* RNA, but introduction of a premature stop codon in the small *t* RNA results in production of a smaller protein (Fig. 14b). In addition, a rare tiny *t* antigen of ~17 kDa has been attributed to double RNA splicing in SV40-infected cells [130]. In this case, the transcript encoding the 17-kDa antigen shows splicing of both introns, but splicing of the intron 1 by selection of the proximal 5' ss. Similar to SV40, the multiple-spliced RNA species of early transcripts also were detected in other polyomaviruses such as the truncated *T* antigen (trunc-*T* Ag) in BKPyV [131], *T'135*, *T'136*, and *T'165* in JCPyV [132] and *T3* and *T4* early transcripts in MCPyV [127] (Fig. 14b). Alternative splicing of polymavirus early transcripts allows the expression of multiple T antigens with distinguished function during the viral life cycle. In addition to the cells with actively replicating virus, the early viral transcripts also are expressed in cells with nonproductive infection or in polyomavirus-transformed cells. These cells often do not express late gene product due to integration of the viral DNA into host genome, resulting in dysregulated viral gene expression as well as cell transformation.

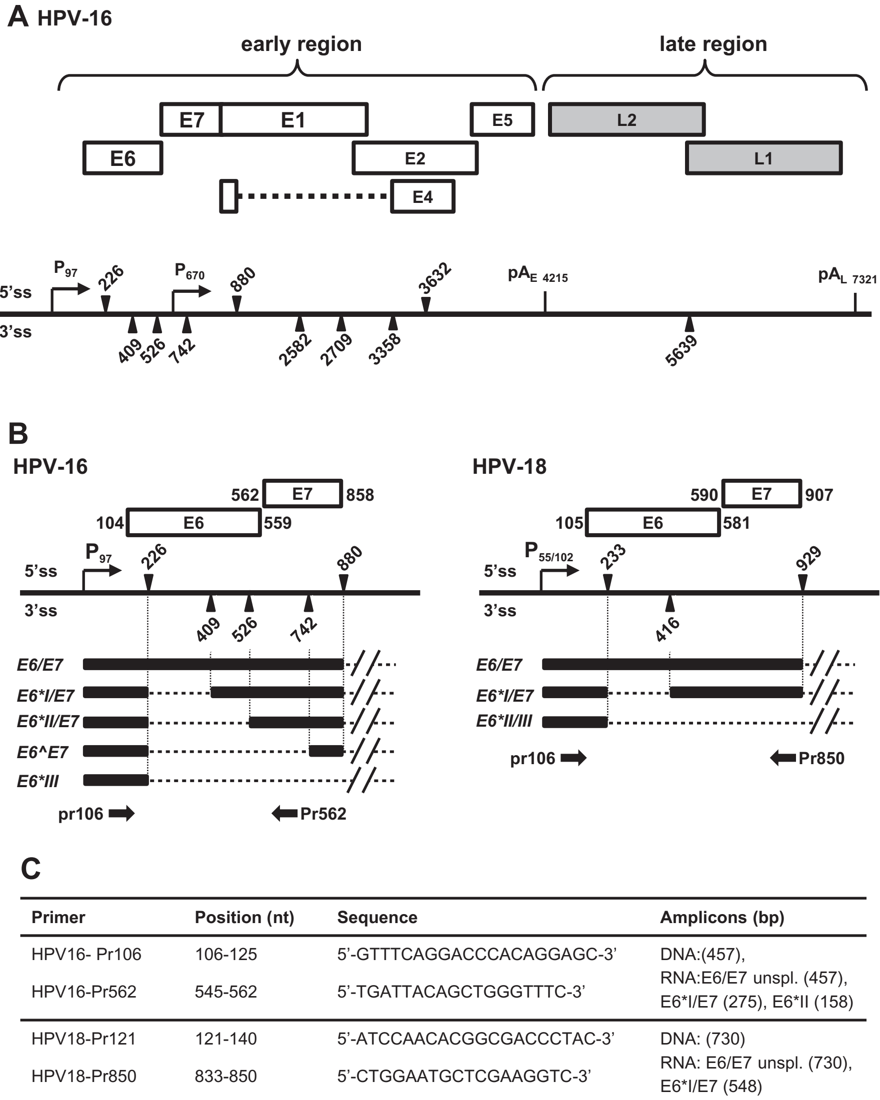
## *Papillomaviruses*

Human papillomaviruses (HPVs) are a group of small DNA tumor viruses with a genome of ~8 kb surrounded by a viral capsid. The HPV genome consists of three regions: viral early, late, and noncoding regions and generally encodes eight viral genes (E1, E2, E4, E5, E6, E7, L1, and L2). Viral early gene products are regulatory proteins responsible for virus replication and pathogenesis during a productive infection, whereas the late L1 and L2 genes encode two viral capsid proteins for virus particle formation. Interestingly, almost all viral early genes are expressed from an early promoter upstream of the viral E6 gene and are polyadenylated at an early poly(A) signal downstream of E5 gene. Thus, viral early gene transcripts are polycistronic, with several ORFs in a single RNA molecule, and undergo extensive alternative RNA splicing during viral RNA maturation. In contrast, viral L1 and L2 are commonly transcribed from E7 ORF and polyadenylated at a late poly(A) site downstream of L1 ORF (Fig. 15a). As a result, the 5' sequences of viral L1 and L2

are part of the viral early transcript sequences. RNA splicing to remove most of these early gene sequences from the RNA is important for viral L1 and L2 expression [133].

HPVs are the etiological agent of cervical cancer and presumably of other anogenital cancers. HPV is present in >95% of all cervical cancer and is required for initiation of cervical carcinogenesis and maintenance of the cervical cancer cells. Cervical cancer is a leading cause of death for women in the developing world, with about 493,000 new cases and nearly 273,000 deaths each year. More than 200 genotypes of HPVs have been identified to date and are grouped into two major groups based on their pathogenesis and association with cervical cancer [134]. The reference genome sequences are available at https://pave.niaid.nih.gov/#home. The high-risk or oncogenic HPV types are present in cervical cancers, some anogenital cancers, as well as head-and-neck cancers. The low-risk or non-oncogenic HPVs are not associated with cancers [135]. In general, women acquire HPV infection by sexual contact. A number of epidemiology studies have demonstrated that women with repeat exposure to oncogenic HPVs as well as women with persistent cervical infection by oncogenic HPVs are at high risk for developing cervical cancer [136, 137]. Infection with oncogenic HPV-16 and HPV-18, the two most common oncogenic HPV types, leads to the development of almost 70% of all cervical and other types of anogenital cancers. Viral E6 and E7 of the oncogenic HPVs are two viral oncoproteins that inactivate, respectively, cellular p53 and pRB, which are two tumor suppressor proteins essential for cell cycle control [138, 139]. In cervical cancer tissues and cervical cancer-derived cell lines, E6 and E7 oncogenes are highly expressed; the majority of the E6/E7 bicistronic RNA are alternatively spliced as diagramed for HPV-16 and HPV-18. A major spliced RNA isoform of viral E6/E7 bicistronic RNA is E6*I derived from splicing of nt 226 5' splice site to nt 409 3' splice site for HPV-16 and of nt 233 5' splice site to nt 416 3' splice site for HPV-18 (Fig. 15b). It has been demonstrated that this RNA splicing is necessary for viral E7 translation [140] and can be easily detected by RNase protection assay (RPA) or by RT-PCR methods [140, 141].

The presence of the high-grade premalignant lesions (CIN, cervical intraepithelial neoplasia) caused by oncogenic HPV infection is a sign of increased risk for developing cervical cancer. These lesions can be detected by routine cervical examination and treated by surgery to prevent progression to cervical cancer. The Papanicolaou test (also called the Pap smear) is a screening test used in gynecology to detect premalignant and malignant cells in cervical swabs. A woman who has a Pap smear with abnormal cells may also be referred for HPV DNA testing by two FDA-approved assays: the Hybrid Capture 2 DNA test, which detects 13 high-risk HPVs (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, and HPV-68) and is available from Qiagen [142], or the Cobas 4800 System HPV test, which detects 14 high-risk HPVs (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-66, and HPV-68) and is available from Roche [143]. A few of the HPV E6/HPV E7 RNA tests also have been introduced. The APTIMA HPV Assay from Hologic was designed to detect




| Primer | Position (nt) | Sequence | Amplicons (bp) |
|---|---|---|---|
| HPV16- Pr106 | 106-125 | 5'-GTTTCAGGACCCACAGGAGC-3' | DNA:(457), |
| HPV16-Pr562 | 545-562 | 5'-TGATTACAGCTGGGTTTC-3' | RNA:E6/E7 unspl. (457), E6*I/E7 (275), E6*II (158) |
| HPV18-Pr121 | 121-140 | 5'-ATCCAACACGGCGACCCTAC-3' | DNA: (730) |
| HPV18-Pr850 | 833-850 | 5'-CTGGAATGCTCGAAGGTC-3' | RNA: E6/E7 unspl. (730), E6*I/E7 (548) |

**Fig. 15** RNA splicing of viral oncogene E6 and E7 transcripts in high-risk human papillomavirus infections. (**a**) Genome structure of high-risk HPV-16 divided by early (genes E1–E7, open boxes) and late (L1–L2, gray boxes) regions and positions of splice sites in the HPV-16 genome. P, promoter; $pA_E$, early polyadenylation site; $pA_L$, late polyadenylation site. (**b**) Alternative splicing of HPV-16 and HPV-18 E6–E7 regions. Open boxes represent E6 and E7 ORFs with their corresponding start and stop codon positions. Transcripts derived from promoter P97 have an intron (dashes) in the E6 and E7 ORF with three alternative 3' splice sites as diagramed. Filled black boxes are exons. Coding potentials for each transcript are showed on the left. Arrows below the transcripts are the primers used for detection of spliced E6E7 transcripts detailed in (C). The diagrams are modified from Zheng and Baker [133] and Wang et al. [141]. (**c**) Sequences and nucleotide positions of two sets of primer pairs in (B) for RT-PCR amplification of alternatively spliced E6–E7 transcripts expressed in HPV-16 and HPV-18 infections [140, 141]. Primer nucleotide positions and sequences are based on corresponding HPV reference strains available on https://pave.niaid.nih.gov/

HPV E6/HPV E7 mRNA from 14 high-risk types (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-66, and HPV-68) [144] with a sensitivity and specificity similar to or better than the Hybrid Capture 2 DNA test [145, 146]. The PreTect HPV-Proofer from PreTect was designed to detect E6 and E7 RNA from HPV types 16, 18, 31, 33, and 45 [147, 148] and is more specific than the HC2 for identifying women with CIN 2+ but has a lower sensitivity [149]. By using the primers detailed in Fig. 15c for RT-PCR assays, the spliced E6/E7 RNAs of HPV-16 and HPV-18 can be easily detected due to an amplicon size smaller than E6/E7 DNA, without worry of any carry-over viral DNA contamination commonly encountered with HPV DNA tests.

## *Herpesviruses*

Herpesviruses are large DNA viruses with a complex life cycle. Their relatively large linear double-stranded DNA (dsDNA) genome (~100–200 kb) is encapsulated in a capsid with icosahedral architecture. The capsid is covered with a heterogeneous layer of viral proteins and RNAs called tegument. Outside of this tegument is a lipid bilayer membrane (envelope) containing several virus-encoded glycoproteins. A hallmark of herpesvirus infection is the establishment of a lifelong "latent" infection in their host following initial infection. Latent virus is often reactivated by various stimuli and causes recurrent infections, which is a typical feature of all herpesviruses.

Currently there are more than 100 known herpesviruses infecting a wide range of animal species. All human herpesviruses belong to the *Herpesviridae* family which is further grouped into four subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, *Gammaherpesvirinae*, and unassigned viruses. Currently, eight herpesvirus species have been isolated from humans; these have been assigned to three subfamilies of *Herpesviridae*. These include (1) herpes simplex virus type 1 [HSV-1, also referred as human herpesvirus 1 (HHV1)], (2) herpes simplex virus type 2 (HSV-2 or HHV2), (3) varicella-zoster virus (VZV or HHV3), (4) Epstein-Barr virus (EBV or HHV4), (5) human cytomegalovirus (CMV or HCMV or HHV5), (6) human herpesvirus 6 (HHV6), (7) human herpesvirus 7 (HHV7), and (8) Kaposi sarcoma-associated herpesvirus (KSHV or HHV8).

After viral entry, the viral genome is translocated to the nucleus of the infected cell where the expression of viral genes and viral genome replication occurs. All herpesviruses have two types of viral life cycle, latent and lytic, with each having a distinctive transcriptional profile. Latent infection is characterized by the expression of a few viral genes (latent transcripts) that maintain the viral genome in latently infected cells. Lytic infection is associated with viral genome replication and production of infectious virions; this generally leads to destruction of the infected cell. In contrast to latent infection, almost all viral lytic genes in the lytic infection are expressed in a timely regulated fashion and are divided, based on their dependence on viral protein expression and viral genome replication, into three kinetic classes:

immediate early, early, and late. In some circumstances, the virus in latently infected cells may be reactivated and proceeds to lytic infection. The mechanisms controlling the establishment of latency and reactivation of herpesviruses are not yet fully understood. The human herpes viral genome encodes up to 100 different genes including a variable number of noncoding genes expressing noncoding RNAs or viral miRNAs [150–152].

Most human herpesviruses are highly prevalent in the general population. Initial infection generally occurs in childhood or early adolescence through body contact and is followed by the establishment of latent infection. Some herpesviruses are sexually transmitted. Blood transfusion, tissue transplantation, and/or congenital transmission are additional mechanisms for acquiring the virus. The primary infection often occurs in epithelia, i.e., the point of entry, followed by establishment of latent infection, which generally occurs in a specialized cell type (neurons or lymphocytes) and serves as a virus reservoir. Recurrence of infection is caused by virus reactivation from its latent state with the virus escaping from the host immunological surveillance. Overall symptoms of herpesvirus infections in healthy individuals are generally mild, but may be life threatening in immunocompromised patients. While it is clear that infections by some herpesviruses such as EBV and KSHV are etiologically linked to the development of several types of cancer, the role of other human herpesviruses in cell transformation remains unknown [153]. Several antiviral compounds are used to treat acute herpesvirus infections. The only vaccine against herpesviruses currently approved for use in clinic is varicella/chickenpox vaccine against VZV.

The infections by herpesviruses are most commonly diagnosed by the presence of specific antibodies and antigens or by detection of viral DNA by PCR. However, without quantification at multiple time points, these techniques are unable to distinguish virus carriers from patients with active virus replication. Detection of viral transcripts associated with virus lytic phase by RT-PCR provides indication of active virus replication, but often leads to a false-positive result due to viral DNA contamination. Such DNA contamination problems could be avoided by selection of an amplicon over the intron in spliced viral transcripts; a specific product of the spliced RNA could be distinguished from its corresponding DNA based on its size. The number of spliced viral transcripts varies from one herpesvirus to another, ranging from only a handful of split genes in HSV-1 to about 30% in KSHV [154]. Both latent and lytic genes may have an intron and sometimes these are alternatively spliced.

### Herpes Simplex Virus Type 1

HSV-1, a member of *Alphaherpesvirinae* subfamily, is a human neurotropic herpesvirus associated with *herpes labialis*, Bell's palsy, and *vestibular neuritis* [155]. After initial infection, HSV-1 establishes a latent infection in sensory neural ganglia from where it can periodically reactivate. The viral genome consists of two unique regions (long and short) flanked with the inverted repeat regions (internal or

terminal) (Fig. 16a). HSV-1 encodes at least 84 genes. Most of these genes are named according to their position within a particular part of the viral genome such as UL1 (unique long region ORF1) or US3 (unique short region ORF3), while others have alternative historical names, such as ICP0 (infected cells protein 0). Only a few HSV-1 transcripts are spliced (LAT, ICP0, UL15, US1, US12/ICP47) (see review [156]), including both latent and lytic transcripts.

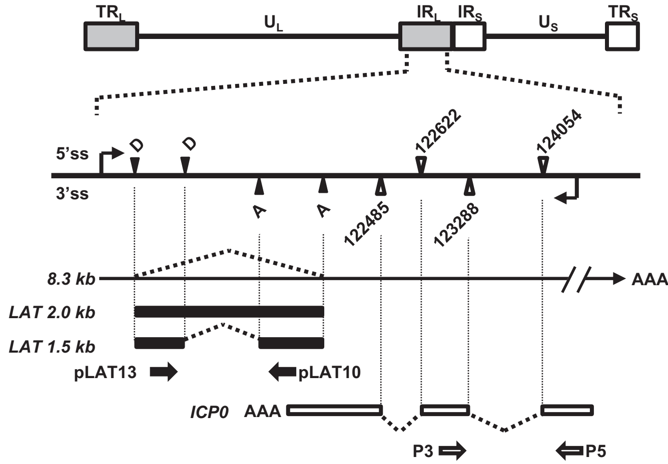
During latency, HSV-1 expresses LAT (latency-associated transcript) RNA using a repeat region of the viral genome called LAT-DNA [157, 158]. Two forms of LAT RNAs are detectable in latently infected neurons. A major 2.0-kb RNA is produced by splicing of a capped and polyadenylated 8.3-kb primary transcript and represents a unique stable intron while the spliced exonic RNA is unstable and quickly degraded [159]. A minor 1.5-kb RNA is generated by further splicing of the 2.0-kb RNA by removal of an internal intron of 559 or 556-bp, depending on the virus strain [160] (Fig. 6a). Both LAT RNAs are uncapped without a poly(A) tail and accumulate in the nucleus of infected cells. HSV-1 LAT RNA is a noncoding regulatory RNA for establishment and maintenance of viral latency by inhibiting the expression of viral lytic genes and thus interfering with the cellular apoptosis pathway [161]. Recent studies have demonstrated that LAT transcript functions as a precursor for the generation of virus-encoded miRNAs [162]. The expression of LAT-DNA also has been observed during lytic infection. Lytic LAT transcripts differ from latent LAT RNA by the presence of a poly(A) tail [163].

ICP0 (IE110) is encoded by a gene located in the viral genome repeat region and partially overlaps with LAT transcripts. Antisense expression of LAT transcripts inhibits the expression of ICP0 during latency. ICP0 is an immediate-early gene expressed in the early stage of lytic infection. ICP0 functions as a non-specific transactivator and a cofactor of another viral transactivator ICP4 [164]. ICP0 initiates lytic replication in both newly infected cells as well as after reactivation in cells with latent infection. ICP0 is transcribed in reverse orientation from viral genome, and its pre-mRNA contains three exons separated by two introns [165] (Fig. 16a). After splicing, the mature mRNA encodes ICP0 protein with 775-aa residues. An alternatively spliced ICP0 transcript retaining intron 2 is detectable in the infected cells [166] and encodes a truncated ICP0R in size of 262-aa residues due to the presence of a stop codon in the intron 2. Thus, both ICP0 and ICP0R have the same aa sequences in the N-terminal part. ICP0R functions as a repressor of viral expression [167].

HSV-2 represents another important human pathogen belonging to the alphaherpesvirus subfamily. Genital infection with HSV-2 causes genital herpes, which is considered to be a sexually transmitted disease. HSV-2 is also neurotropic and establishes latent infection in sacral ganglia. HSV-1 and HSV-2 are two closely related viruses with similar genomes and gene structures, including their LAT and ICP0 regions [168].

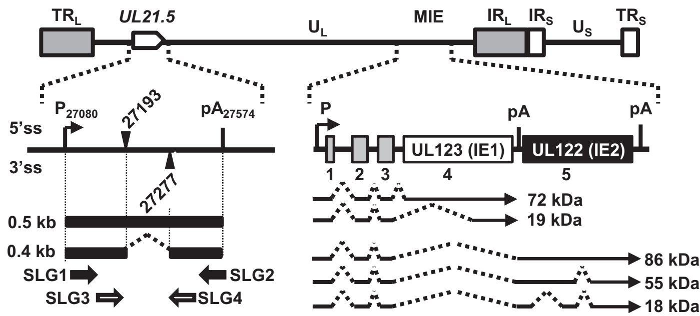
In general, the infection of HSV-1 and HSV-2 is controlled by the host immune system. Thus, initial or recurrent infections are usually associated with only mild symptoms. Infection in immunocompromised patients can cause several severe diseases including encephalitis [169]. Genital infections or reactivation of HSV-2 during pregnancy can lead to congenital infection [170]. Detection of viral DNA may

**A** HSV-1




| Primer | Position (nt) | Sequence | Amplicons (bp) |
|---|---|---|---|
| pLAT10 | 120408-120389 | 5'-ATGGAGCCAGAACCACAGTG-3' | DNA: (947)<br>RNA: LAT 2.0 kb (947),<br>LAT 1.5 kb (297) |
| pLAT13 | 119464 -119484 | 5'-GTAGGTTAGACACCTGCTTCT-3' | |
| P3 | 123214-123233 | 5'-TTCGGTCTCCGCCTGAGAGT-3' | DNA: (922)<br>RNA: ICP0 unspl. (922),<br>ICP0 spl. 1.5 kb (157) |
| P5 | 124116-124135 | 5'-GACCCTCCAGCCGCATACGA-3' | |

**B** HCMV




| Primer | Position (nt) | Sequence | Amplicons (bp) |
|---|---|---|---|
| SLG1 | 27120-27139 | 5'-CTATGGATCTTGAGCTTACT-3' | |
| SLG2 | 27410-27429 | 5'-TCGCTGCCATCTCCGTCTGT-3' | DNA: (258)<br>RNA: unsliced (258)<br>spliced (175) |
| SLG3 | 27144-27163 | 5'-GTGACCTTGACGGTGGCTTT-3' | |
| SLG4 | 27382-27401 | 5'-CGTCATACTCCCCGGAGTAA-3' | |

**Fig. 16** RNA splicing of LAT and ICP0 transcripts in HSV-1 infections and UL21.5, UL122, and UL123 transcripts in HCMV infections. (**a**) Genome of herpes simplex virus type 1 (HSV-1)

not provide sufficient information about virus replication status due to the permanent presence of viral DNA in the infected cells. Detection of viral lytic products, such as spliced ICP0 RNA, may be a better predictor of virus reactivation and may be seen even before the occurrence of clinical symptoms allowing early diagnosis and enabling early treatment. Disappearance of the detectable lytic products could be a sign of treatment efficiency since the viral transcripts disappear earlier than viral DNA.

### Human Cytomegalovirus

Human cytomegalovirus (HCMV) together with HHV-6 and HHV-7 belongs to the *Betaherpesvirinae*. A high prevalence of CMV infection has been noted in 50–80% of the human population. In a majority of healthy individuals, the primary CMV infection occurs asymptomatically but, in some cases, can be associated with sore throat, prolong fever, or a syndrome similar to infectious mononucleosis. After initial infection, the virus usually remains latent in T cells for the rest of the host life without apparent symptoms. In contrast, CMV infections in immunocompromised individuals, such as newborns, transplant recipients, persons with AIDS, or cancer patients, can lead to severe disease and even death. The symptoms include *hepatitis*, *retinitis*, *colitis*, *pneumonia*, *encephalitis*, and others.

CMV has a large genome of about 220 kb capable of encoding approximately 200 genes (reviewed in [171]). While the majority of CMV transcripts do not have introns, the presence of several split genes has been identified in all kinetic classes of the viral genes [172]. A major immediate-early region (MIE) located within a unique long (UL) region of the CMV genome contains several genes that are highly expressed during the early stage of viral lytic infection. These include UL123 (IE1), UL122 (IE2), and UL119-115. MIE transcripts contain multiple introns and undergo complex alternative RNA splicing. MIE transcripts IE1 and IE2 are expressed from the same promoter but are alternatively polyadenylated. These transcripts have five major exons and can be alternatively spliced to express additional isoforms of IE1

**Fig. 16** (continued) consists of two unique regions (long $U_L$ and short $U_S$) flanked with terminal (TR) and internal (IR) repeats. Shown below are two representatives of HSV-1 RNA splicing, latency-associated transcript (*LAT*) and immediate-early *ICP0* transcripts with positions of 5' and 3' splice sites, and primer pairs (arrows below) used for detection of spliced transcripts as detailed in the table further below. Boxes (full for *LAT* and empty for *ICP0*) represent exons divided by introns (dashed lines). The primer pairs in the table were described by Tanaka et al. [214], with nucleotide positions in HSV-1 genome (GenBank Acc. No. X14112.1). (**b**) Genome structure of human cytomegalovirus. See other details in (**a**). Shown below the genome structure are spliced transcripts of late *UL21.5* gene and two immediate-early genes *UL123 (IE1)* and *UL122 (IE2)* expressed from a major immediate-early region (MIE). Boxes or solid lines are exons and dashes are introns or splicing directions. P, promoter; pA, polyadenylation site. Arrows below *UL21.5* transcripts are primer pairs used for detection of spliced *UL21.5* transcripts by nested RT-PCR as described [174] and detailed in the table below, with the nucleotide positions in HCMV genome (strain AD169, GenBank Acc. No. X17403.1)

and IE2 proteins (Fig. 16b). Splicing also was detected in transcripts from other CMV genes such as TRL4, UL89, US3, R160461, and R27080 [172]. Gene UL21.5 (previously named as R27080) is one of the known CMV split late genes (SLG). The UL21.5 transcript that encodes viral glycoprotein is expressed from the UL region posited from nt 27080 to nt 27574 of CMV genome [173] and has a short intron of 83 nts. Removal of this intron leads to production of a mature mRNA in size of ~0.4 kb. Both spliced and unspliced UL21.5 RNAs are easily detectable by RT-PCR from infected cells [174].

Allogenic bone marrow transplant recipients are at high risk for developing CMV diseases. Historically, viremia has been used as an indicator of CMV disease as well as to guide preemptive treatment. Multiple approaches have been developed to detect CMV viremia in circulating lymphocytes by direct virus isolation with cultivation, by detection of viral antigens in polymorphonuclear cells, or by quantitative viral DNA [175, 176]. However, the detection of viremia is not sufficient for disease prediction since many viremic patients never develop symptoms. However, active CMV replication in peripheral blood lymphocytes can be verified by analyzing viral mRNAs [177]. Amplification of spliced viral transcripts has some advantages in comparison to intronless transcripts and is not affected by DNA contamination. Detection of spliced immediate-early transcripts had been reported to have a good correlation with the detection of viral DNA or viral antigen [178–180]. Detection of late gene UL21.5 has a better prediction value and has a significant correlation with disease progression [174, 181, 182].

## Epstein-Barr Virus

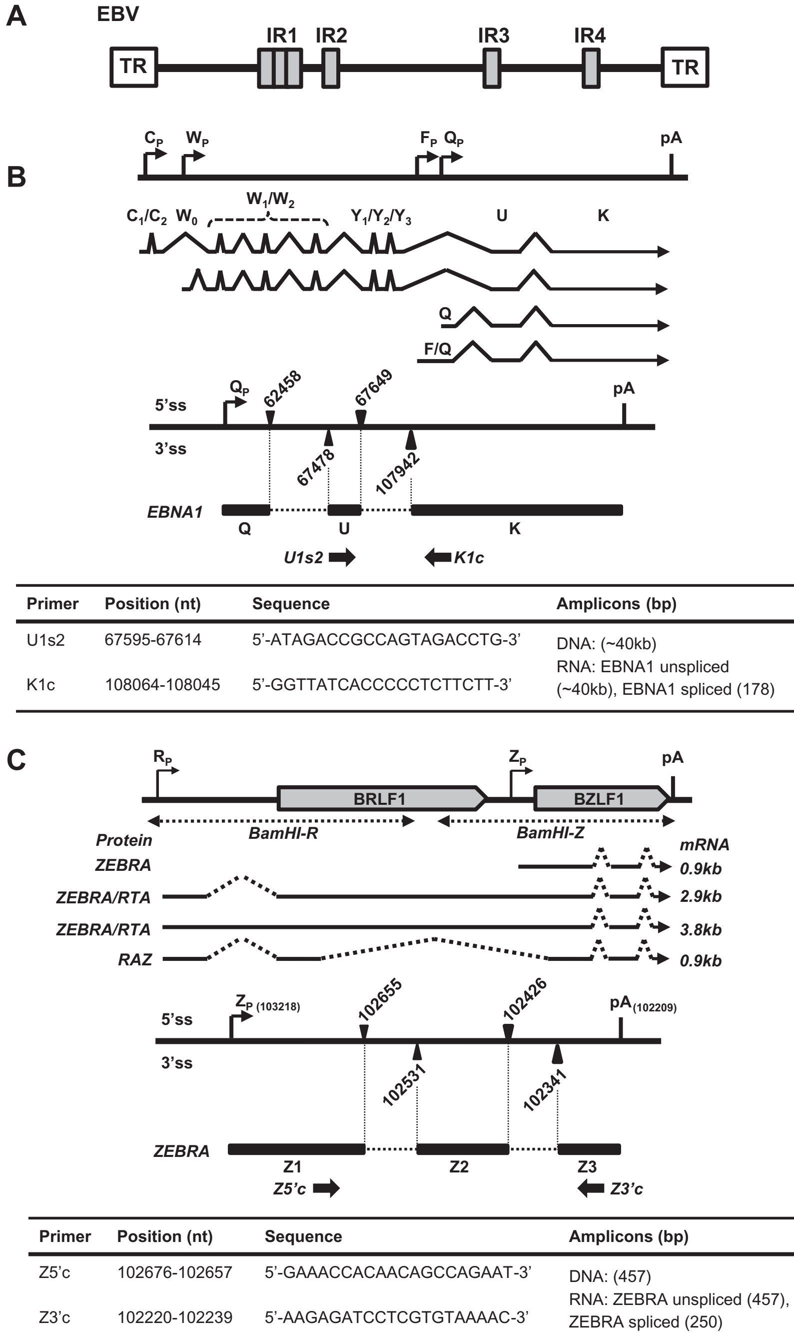
Epstein-Barr virus (EBV), a well-characterized member of the *Gammaherpesvirinae* subfamily, is an important human pathogen. EBV infection is highly prevalent, with more than 95% of the human population becoming seropositive in early life. While primary infection during childhood is usually unremarkable, the virus acquisition in adolescence and adulthood is often associated with the development of the *infectious mononucleosis syndrome*. In healthy individuals, the EBV infection is well controlled by the immune system. However, EBV remains in long-living memory B cells where it establishes a latent infection. EBV is an oncogenic virus capable of transforming the infected B cells [183]. EBV infections have been associated with the development of a number of human malignancies, including *nasopharyngeal carcinoma*, *Burkitt's lymphoma*, *Hodgkin's lymphoma*, *gastric carcinoma*, and others (see review [184]). Active EBV replication due to immunosuppression may cause *posttransplant lymphoproliferative disease* [185]. During latent infection, EBV expresses six nuclear antigens (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP), three latent membrane proteins (LMP-1, LMP-2A, and LMP-2B), and several noncoding transcripts (EBER-1 and EBER-2 and BARTs). Many EBV latent products are defined as oncogenes and are responsible for EBV-mediated cell transformation [115]. Several types of EBV latency have been defined by variable expression of latent genes in a number of malignancies [186].

The EBV genome (~172 kb) is flanked by multiple terminal repeats (TR) and can be divided into a long and a short unique region ($U_L$ and $U_S$) by internal repeat 1 (IR1) (Fig. 17a). EBV encodes at least 80 viral proteins [187] as well as several noncoding RNAs including viral miRNAs [188, 189]. The ORF names are derived from their positions in the *Bam*HI fragment (from A to Z) by orientation (L, left; or R, right) and a digital number representing the frame (F) order (e.g., BZLF1). Other genes retain their historical names based on the gene product function. The number of split genes in EBV is significantly higher than that found in alpha- and beta-herpesviruses. Extensive alternative RNA splicing is prominent, particularly for almost all of the EBV latent transcripts, but the transcripts of many lytic genes are also spliced.

EBNA-1 is a multifunctional viral protein critical for establishing and maintaining EBV latency and for regulation of viral promoter activities [190]. In infected cells, EBNA-1 is expressed from a spliced mRNA derived from a primary transcript of ~100 kb. This transcript originates from one of two alternative promoters, *Cp* or *Wp*, which are named by their localization in different *Bam*HI fragments of the viral genome (Fig. 17b). At the early stage of latent infection, *Wp* is initially used, but EBNA-1 and EBNA-2 expressed from *Wp* transactivate the *Cp* promoter and cause a switch of transcription from *Wp* to *Cp* [191]. Usage of the *Cp* promoter is associated with EBV "latency type III." In Burkitt's lymphoma and Burkitt's lymphoma-derived cell lines, EBNA-1 expression is initiated from the distal *Qp* promoter rather than from *Cp* and *Wp* and is associated with "latency type I" [192]. EBNA-1 is also expressed in the lytic phase from an additional *Fp* promoter located closely upstream to the *Qp* promoter [193].

The establishment of active EBV replication after virus reactivation from latency is dependent on the expression of two immediate-early genes, BZLF1 and BRLF1 [194], and encodes viral transactivators ZEBRA (BZLF1) and RTA (BRLF1). Although BRLF1 and BZLF1 are transcribed separately from a different promoter with the *Rp* for BRLF1 and the *Zp* for BZLF1, both gene transcripts utilize the same polyadenylation site for RNA polyadenylation [195] (Fig. 17c). Thus, the *Zp* promoter-derived transcript is a monocistronic ZEBRA RNA containing two constitutive introns; splicing of these two introns results in production of a 0.9-kb mRNA that encodes ZEBRA protein. Transcription from the *Rp* promoter leads to production of a 3.8-kb bicistronic transcript, ZEBRA/RTA, which contains two additional introns and the ZEBRA RNA. Splicing of the intron 1 in the 5' noncoding region of ZEBRA/RTA transcript leads to production of a 2.9-kb RNA as a major RNA isoform. However, both isoforms of ZEBRA/RTA RNA have the potential to encode ZEBRA and RTA proteins. A third minor isoform of ZEBRA/RTA transcript is derived from the splicing of an additional internal intron spanning from BRLF1 ORF to BZLF1 ORF; this splicing produces a RAZ transcript of ~0.9 kb encoding a RTA-ZEBRA fusion protein, RAZ. RAZ may function as an inhibitor to ZEBRA during EBV infection [196].

Transcripts for EBNA-1 are believed to be expressed in all forms of EBV latent infection, except latently infected nondividing B cells having "latency type 0." This makes detection of EBNA-1 expression a good marker for the presence of EBV in tumors. The expression of ZEBRA during lytic infection could be used to monitor productive EBV infection as well as EBV reactivation.




| Primer | Position (nt) | Sequence | Amplicons (bp) |
|---|---|---|---|
| U1s2 | 67595-67614 | 5'-ATAGACCGCCAGTAGACCTG-3' | DNA: (~40kb) |
| K1c | 108064-108045 | 5'-GGTTATCACCCCCTCTTCTT-3' | RNA: EBNA1 unspliced (~40kb), EBNA1 spliced (178) |

| Primer | Position (nt) | Sequence | Amplicons (bp) |
|---|---|---|---|
| Z5'c | 102676-102657 | 5'-GAAACCACAACAGCCAGAAT-3' | DNA: (457) |
| Z3'c | 102220-102239 | 5'-AAGAGATCCTCGTGTAAAAC-3' | RNA: ZEBRA unspliced (457), ZEBRA spliced (250) |

**Fig. 17** RNA splicing of EBNA1 and ZEBRA transcripts in EBV infections. (**a**) Organization of EBV genome with terminal repeats (TR) and internal repeats (IR1-4). (**b**) Multiple transcripts of

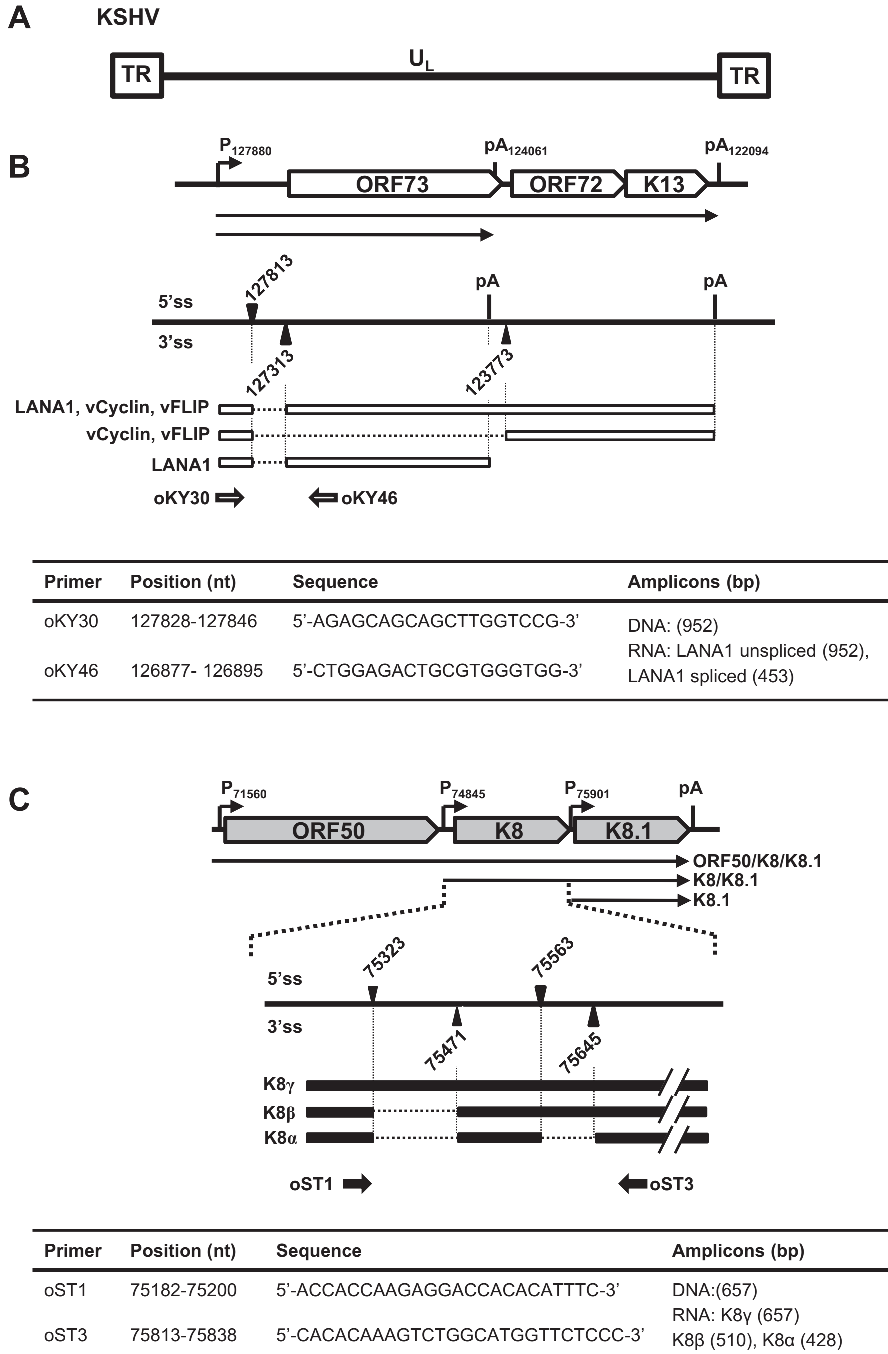
### Kaposi Sarcoma-Associated Herpesvirus

Kaposi sarcoma-associated herpesvirus (KSHV) is the latest human herpesvirus to be discovered [197]. After primary infection, KSHV establishes latent infection in endothelial cells as well as B cells [198]. In healthy individuals, both primary and latent KSHV infections are generally asymptomatic. Suppression of the immune system in KSHV-positive individuals, such as in AIDS patients or tissue transplant recipients, is associated with the development of several cancers, including all forms of *Kaposi sarcoma* (a solid tumor of endothelial origin) or B-cell lymphomas [*primary effusion lymphoma* (PEL) and *multicentric Castleman's disease* (MCD)] (see review [199]). The presence of the viral genome as well as expression of viral-encoded products in all cancer cells strongly suggests the active role of KSHV in cell transformation.

KSHV belongs to the *Gammaherpesvirinae* and has a similar genome organization as does EBV, with a long unique region flanked with terminal repeats (Fig. 18a). The KSHV genome (~165 kb) encodes up to 90 genes that are named by their position in the viral genome from left to right (e.g., ORF47) [200]. Some KSHV unique genes are designated with a digital K number, like K2, while some have alternative names based on their function (ORF57 or MTA-*mRNA transcript accumulation*). KSHV transcripts derived from ~30% of the viral genes, including both latent and lytic genes, undergo RNA splicing [154].

During latency, the KSHV genome expresses a latency-associated nuclear antigen-1 (LANA-1) [201] from ORF73. The gene ORF73 posits along with ORF72 and K13 in a larger latent locus of the virus genome. The latter two genes encode viral homologues of cellular proteins vCyclin (ORF72) and vFLICE (K13). ORF73/72/K13 are transcribed from a single promoter ($P_{127880}$) as a tricistronic RNA containing an intron with two alternative 3' splice sites. Alternative RNA splicing and alternative RNA polyadenylation of the tricistronic pre-mRNA result in production of three mature mRNAs (5.4, 3.3, and 1.7 kb) [202] (Fig. 18b). The 5.4-

**Fig. 17** (continued) EBV latency-associated *EBNA-1* transcribed from several alternative promoters ($C_p$-$Q_p$). EBNA-1 RNA contains multiple exons ($C_1$-$K$, lines) and introns (half triangles). A detailed *EBNA-1* transcript derived from the $Q_p$ promoter is shown below with splice sites (black triangles) and splice directions (dashed lines for introns). A primer pair used to detect the spliced *EBNA-1* transcript from exon *U* to exon *K* [185] is detailed in the table below. (**c**) Gene structures of *BRLF1 (RTA)* and *BZLF1 (ZEBRA)* (two EBV-immediate-early genes) and their spliced RNA products. EBV *RTA* and *ZEBRA* are transcribed by two alternative promoters, $R_P$ and $Z_P$, but polyadenylated by using the same polyadenylation signal downstream of *ZEBRA* ORF. Thus, the bicistronic *RTA* transcript derived from *Rp* promoter contains multiple introns and has potentials to encode *RTA*, *ZEBRA*, and *RAZ* proteins by alternative RNA splicing, whereas the monocistronic *ZEBRA* transcript derived from the *Zp* promoter encodes only *ZEBRA* protein and also contains multiple introns as detailed further below with nucleotide positions of splice sites, exons (*Z1*–*Z3*, black boxes), and introns (dashed lines). Arrows below exons *Z1* and *Z3* are a primer pair used for detection of spliced *ZEBRA* mRNA [185] and detailed in the table, with nucleotide positions in EBV genome (strain B95-8, GenBank Acc. No. V01555.2)




| Primer | Position (nt) | Sequence | Amplicons (bp) |
|---|---|---|---|
| oKY30 | 127828-127846 | 5'-AGAGCAGCAGCTTGGTCCG-3' | DNA: (952)<br>RNA: LANA1 unspliced (952), LANA1 spliced (453) |
| oKY46 | 126877- 126895 | 5'-CTGGAGACTGCGTGGGTGG-3' | |

| Primer | Position (nt) | Sequence | Amplicons (bp) |
|---|---|---|---|
| oST1 | 75182-75200 | 5'-ACCACCAAGAGGACCACACATTTC-3' | DNA:(657)<br>RNA: K8γ (657)<br>K8β (510), K8α (428) |
| oST3 | 75813-75838 | 5'-CACACAAAGTCTGGCATGGTTCTCCC-3' | |

**Fig. 18.** RNA splicing of representative latent transcripts and early transcripts in KSHV infections. (**a**) Genome of KSHV contains a long unique region ($U_L$) flanked by terminal repeats (TR).

kb transcript most likely responsible for LANA-1 expression is produced by usage of the proximal 3' splice site, whereas usage of the distal 3' splice site leads to expression of 1.7-kb transcripts for vCyclin and vFLICE. Both transcripts are polyadenylated at the same distal polyadenylation site. The minor 3.3-kb transcript uses the proximal splice site for RNA splicing but is polyadenylated at a proximal non-canonical polyadenylation signal (Fig. 18b).

KSHV lytic replication is controlled by a major viral transactivator, ORF50 (also referred as Rta) [203, 204]. Like LANA-1, ORF50 posits along with K8 and K8.1 in a larger gene locus (ORF50/K8/K8.1 cluster) (Fig. 18c) and is expressed as an immediately early transcript during lytic virus replication. K8 encoding a viral k-bZIP protein is an early gene and K8.1 encoding a glycoprotein is a late gene. Although each of the three genes bears its own promoter, all of their RNA transcripts use a single polyadenylation site located downstream of K8.1 gene and undergo alternative RNA splicing (see review [154]). Thus, the 3' portion of ORF50 transcript is homologous to K8 and K8.1 and has the same intron and exon structures as seen in the K8 and K8.1 transcripts. The ORF50 transcript is tricistronic, K8 is bicistronic, and K8.1 is monocistronic in nature. The bicistronic K8 transcript is composed of four exons separated by three introns (Fig. 18c). A functional K8α protein is expressed from a fully spliced mRNA, but retention of the intron 2 in K8β mRNA results in the expression of a minor form K8β protein [205]. An unspliced K8 RNA, K8γ, is also detectable, but rare in lytically infected cells.

In summary, LANA-1 expression is a hallmark of KSHV latent infection. Transcripts originated from the ORF73/72/K13 gene cluster are expressed in latently infected, KSHV-transformed cells and are detectable by RT-PCR. Active virus replication is associated with the expression of viral lytic genes. Amplification of the spliced K8 region that detects the expression of both ORF50 tricistronic and K8 bicistronic transcripts could be used to monitor viral lytic replication [206].

**Fig. 18** (continued) (**b**) Gene structure of a latent gene locus containing *ORF73/ORF72/K13* genes. Three genes are transcribed from a single promoter (P) as a polycistronic RNA. This polycistronic primary transcript is processed by alternative RNA splicing and alternative polyadenylation (pA, see arrows below). Nucleotide positions of splice sites (triangles) and polyadenylation site in the KSHV genome (GenBank Acc. No. U75698.1) are diagramed further below with coding potentials of each spliced product on the left. Open boxes are exons and dash lines are introns or splicing directions. oKY30 and oKY46 are a primer pair used to detect spliced LANA transcripts [215] as detailed in the table below. (**c**) Gene structure of a KSHV lytic locus consisting of immediate-early *RTA (ORF50)*, early *K8*, and late *K8.1* genes. Three genes are expressed from three separate promoters (P), but all of their transcripts (full lines with arrows immediately below to the right) are polyadenylated at the same polyadenylation site (pA), resulting in *RTA* as a tricistronic, *K8* as a bicistronic, and *K8.1* as a monocistronic transcript. The enlarged *K8* coding region contains three exons (filled boxes) and two introns (dashed lines), with nucleotide positions of each splice site in the KSHV genome (GenBank Acc. No. U75698.1). Names of three common forms of *K8* transcripts from alternative RNA splicing are shown on the left. oST1 and oST2 are two primers used to detect spliced *K8* RNAs as described [215, 216], and are detailed in the table below

## Conclusion

The major aim of this chapter is to provide readers with knowledge of viral RNA splicing during viral infection as well as how the detection of these spliced viral RNA transcripts can be used as a new approach in diagnostic virology. In the first part, basic information about the mechanisms of RNA splicing and the methodological approaches for specific detection of these spliced RNA molecules is provided. The core of these techniques represents an amplification and detection of nucleic acids. The advantage of nucleic acid-based techniques is the application of the same platform for detection of various viral pathogens, often at the same time, by multiplexing. The rapid setup of these methods is especially important for a rapid response to emerging viruses as has been successfully proven in the case of severe acute respiratory syndrome (SARS), avian influenza, Zika virus, and Ebola virus outbreaks where nucleic acid amplification was rapidly deployed to detect and to confirm these infections. The low material requirement and their simplicity make these detection methods suitable for applications in low resources setting such as laboratories where the first contact is seen as well as field laboratories. Because the genomic sequences for many viruses can be detected by amplification of the nucleic acid molecules as a routine procedure in many diagnostic laboratories, the detection of spliced viral transcripts could be performed simultaneously using already existing methods.

The second part of this chapter summarizes the current knowledge of viral RNA splicing events for the majority of known human viruses. Some unique viral agents, such as human circoviruses and adeno-associated viruses, where a direct link between infection and pathological manifestation remains to be determined, have been included. In addition, examples of each virus, where the detection of spliced viral RNA could bring additional benefit to current techniques to improve the disease prognosis or better monitoring of efficiency of therapeutic intervention, have been provided. Systematic study of RNA splicing events during viral infection is likely to lead to better viral diagnostics and better management of viral therapy and will eventually lead to a better understanding of the pathogenesis of these human viral pathogens.

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# Advanced Molecular Diagnosis of Fungal Infections

**Nathan P. Wiederhold**

## Introduction/Epidemiology of Invasive Fungal Infections

The incidence of invasive infections caused by fungi has been increasing for the last few decades. This is primarily due to the significant increases in the populations of at-risk patients; this includes those receiving immunosuppressive chemotherapy for various malignancies, solid organ transplant recipients, hematopoietic stem cell transplant recipients, those receiving prolonged courses of high-dose corticosteroids, and patients in intensive care units. For example, *Candida* species are now recognized as the fourth most common cause of nosocomial bloodstream infections in the USA [1], and invasive candidiasis is associated with both increased mortality and prolonged lengths of stay [2, 3]. In addition, invasive infections caused by *Aspergillus* species and other filamentous fungi are of major concerns in highly immunocompromised patients; recent surveillance studies have reported higher rates of invasive aspergillosis than that for invasive candidiasis in hematopoietic stem cell transplant recipients [4, 5]. Fortunately, treatment options for patients with invasive fungal infections have also increased over the last 2 decades. However, studies have shown that delays in the initiation of appropriate antifungal therapy result in poor clinical outcomes [6–8]. Delays in appropriate therapy are often caused by difficulties in the diagnosis of invasive fungal infections as well as in the identification of the infecting species, both of which remain challenging. Blood cultures, the gold standard for the diagnosis of many invasive microbial infections, including those caused by yeasts, have significant limitations, including poor sensitivity and lengthy turnaround times for results [9, 10]. Fungal infections that involve specific organs or tissues and do not involve the bloodstream pose additional

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diagnostic challenges. Clinical symptoms, signs, and radiographic findings are often nonspecific, histopathology cannot identify the infecting fungal pathogen to the genus or species levels [11, 12], and available surrogate marker assays may be unable to confirm or exclude invasive fungal infections due to false positives and false negatives in different clinical scenarios [13, 14]. Thus, there has been significant interest in the development of new testing methods, including molecular based assays, for the diagnosis of invasive fungal infections.

## Routine Clinical Methods for Identification of Yeasts

Numerous laboratory tests have been developed for the rapid identification of yeasts. Most of these assays require isolates to be recovered from positive blood cultures or other bodily fluids and tissues and involve biochemical testing and carbohydrate assimilation testing performed on these isolates. Several of these have also been developed as a part of automated systems (e.g., Vitek 2, BD Phoenix, and Microscan Systems), which are capable of providing results in a shortened timeframe [15]. Chromogenic agar assays are also available that can aid in the identification of various *Candida* species. These tests use chromogenic substances that react with an enzyme secreted for a specific species to produce characteristic colors for different species when growth is present on solid media [15]. The L-canavanine glycine bromothymol blue (CGB) agar assay allows for the discrimination between *Cryptococcus neoformans* and *C. gattii* [16]. After a period of incubation of up to 5 days at room temperature, the medium turns a cobalt blue color in the presence of *C. gattii* due to an alkaline pH shift caused by the degradation of creatinine to ammonia. In contrast, the medium remains yellow with *C. neoformans.*

While these assays provide advantages over the sole use of colony and microscopic morphology and other phenotypic characteristics (e.g., temperature studies, evaluation for the ability to grow on selective media such as cycloheximide and benomyl) for species identification, there are limitations. An isolate from a positive culture is required for each of these assays, and incorrect identifications can occur with closely related species. This recently has been highlighted by the emergence of *Candida auris*. Several reports have demonstrated that the biochemical assays commonly used by clinical microbiology laboratories are unable to identify this emerging pathogen and thus provided incorrect identification as a different species, including those that are closely related to this species (e.g., *C. haemulonii*) [17–19].

## Surrogate Marker Diagnostic Assays

Surrogate marker assays that do not require positive fungal cultures are available and are frequently used for the diagnosis of invasive fungal infections. Most of these involve the detection of fungal antigens within biological fluids, such as urine,

serum or plasma, bronchoalveolar lavage (BAL) fluid, and cerebral spinal fluid (CSF). For the diagnosis of cryptococcosis, the detection of the cryptococcal antigen (CRAG) within the serum and CSF has proven particularly useful. Different assay formats are available, including latex agglutination, enzyme immunoassays, and lateral flow immunoassays. Each detects the glucuronoxylomannan component of the capsule surrounding *Cryptococcus* cells. High serum and CSF CRAG titers have been correlated with poor prognosis [20–25]. Overall sensitivity and specificity for these different CRAG detection platforms ranges between 93% and 100% [26–35]. The lateral flow immunoassay (Immuno-Mycologics, Inc.) is available in a point-of-care format and has proven quite useful for the diagnosis of cryptococcal meningitis, especially in resource-poor areas, due to its ease of use, rapid turnaround time for results, and the lack of need for refrigeration or other laboratory equipment for storage and performance [30, 33].

A major and essential component of the cell wall of many fungal species is (1,3)-β-D-glucan, and several assays have been developed for its detection. The Fungitell assay (Associates of Cape Cod) is an FDA-cleared test and is included in the indirect microbiological criteria for the diagnosis of probably invasive fungal infections from the European Organization for the Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) [13]. This test relies on the activation of the horseshoe crab coagulation cascade by (1→3)-β-D-glucan and results in the release of a chromogenic peptide that can be measured using a microplate spectrophotometer [36]. In a meta-analysis of 16 studies that included 299 patients, the pooled sensitivity and specificity for the detection of invasive fungal infections were 76.8–85.3%, respectively [37]. Since many fungal species contain (1→3)-β-D-glucan within their cell walls, this assay serves as a pan-fungal test and cannot distinguish between different species. Important pathogens that are not detected by this assay include the members of the order *Mucorales* (e.g., *Rhizopus*, *Mucor, Cunninghamella, Lichtheimia* species) and *Cryptococcus* species. False positives have been reported for this assay, and these have been caused by substances that have glucan content (e.g., gauze, cellulose membranes used in hemodialysis, and immunoglobulin- and albumin-containing products) [38, 39]. False positives have also been reported in patients with infections caused by various Gram-positive and Gram-negative bacteria infections [40–43].

The detection of galactomannan, and component of the cell wall of *Aspergillus* species, has increasingly played a role in the diagnosis of invasive aspergillosis. The Platelia *Aspergillus* test (Bio-Rad) is a commercially available and FDA-cleared ELISA assay that detects the immunodominant epitope in galactomannan, tetra (1→5)-β-D-galactofuranoside, within plasma or BAL samples [44–46]. Although the specificity of this assay has consistently been reported to be ≥85%, the clinical sensitivity varies considerably between different patient populations at risk for invasive aspergillosis (29%–100%) [47, 48]. Cross-reactivity has also been reported in patients with infections caused by other fungi, including *Penicillium*, *Fusarium,* and *Trichosporon* species*, Histoplasma capsulatum,* and *Blastomyces dermatitidis* [14, 49–51]. The galactomannan assay is also not able to distinguish between different *Aspergillus* species, including cryptic species that may have reduced susceptibility

to certain available antifungals. This may be clinically relevant, as surveillance studies have demonstrated that cryptic species were found in 11% to 14% of cultured clinical *Aspergillus* isolates [52, 53].

## Non–Culture–Based Molecular Assays

Several commercially available molecular assays are now available for the diagnosis of invasive fungal infections. Many of these require growth of the fungal pathogen in blood culture bottle due to issues with analytical sensitivity. However, there are some that are capable of detecting and identifying different fungal species within other direct specimens, including one that is FDA cleared for use in clinical microbiology laboratories.

## PNA Fish

Peptide nucleic acid fluorescence in situ hybridization (PNA FISH) assays were some of the first clinically available and FDA-cleared molecular assays available for the detection and identification of *Candida* species. These assays use dual-labeled fluorescent DNA probes targeting rRNA sequences in *Candida* and detect color differences between species under fluorescence microscopy [15]. Because the nucleic acid probes are not amplified, they are sensitive to the organism burden within the blood. Thus, the assays are performed on samples taken from positive blood culture bottles. Two commercially available assays include the Yeast Traffic Light and the QuickFISH systems (AdvanDx). The Yeast Traffic Light assay can detect the five most common *Candida* species, including *C. albicans*/*C. parapsilosis* (fluoresce green), *C. tropicalis* (fluoresce yellow), and *C. glabrata*/C. *krusei* (fluoresce red). The color groupings are based on typical susceptibilities of these species to fluconazole (green = typically susceptible to fluconazole; red = intrinsic or increased resistance to fluconazole). The turnaround time for the Yeast Traffic Light assay is approximately 90 minutes, and good assay sensitivities (97.5–98.9%) and specificities (98.2–100%) have been reported with the use of positive blood culture bottles [54–56]. The QuickFISH assay is only able to detect three common *Candida* species (*C. albicans* = green, *C. parapsilosis* = yellow, *C. glabrata* = red) but has a faster turnaround time (20–30 min) with similar sensitivity (99.7%) and specificity (98%) [57]. Both assays are limited by the number of species that can be identified (only common *Candida* species) and the need for a fluorescent microscope and personnel appropriately trained in its use.

## *FilmArray*

Another commercially available and FDA-cleared assay for the detection of yeast from positive blood culture bottles is the BioFire FilmArray assay (bioMerieux). This is a closed system that combines DNA extraction and nested multiplex PCR (nmPCR) using non-rRNA regions. The first stage involves a multiplex PCR that produces 200–500 base pair amplicons of each target within the sample, which is then followed by the second stage in which primers are nested within the amplicons to amplify shorter products [58–60]. Post-PCR DNA melt curve analysis is then performed. Two FilmArray assays are able to detect yeast. The blood culture panel is able to detect and identify 5 Candida species, including *C. albicans, C. glabrata, C. krusei, C. parapsilosis*, and *C. tropicalis* species in 100 μl of broth taken from a positive blood culture bottle. In addition to these *Candida* species, this assay is able to detect and identify eight Gram-positive bacteria and ten Gram-negative bacteria species, along with common mechanisms of resistance to antibiotics (i.e., *mecA*, *vanA/B*, and $KPC_{bla}$) [59]. In contrast, the meningitis/encephalitis panel is able to detect and identify *Cryptococcus neoformans* and *C. gattii* in 200 μl of CSF in addition to six bacteria species and seven different viruses [61, 62]. In multicenter study that evaluated the blood culture ID panel, which included both a prospective arm of samples from patients with known invasive candidiasis and a seeded arm in which blood culture bottles were inoculated with whole blood containing known amounts of specific *Candida* species, a high percent positive agreement (99.2%) and negative agreement (99.9%) were reported for the detection and identification of *Candida* species [63]. Two false negatives occurred with the FilmArray assay, one of which was a *C. metapsilosis* isolate, a species that is a member of the *C. parapsilosis* species complex and is not included within the assay. Single-center studies have reported similar results with percent positive agreements with positive blood cultures ranging between 89.4% and 91.6% [58, 64].

There are limited clinical data regarding the FilmArray meningitis/encephalitis assay for the detection and identification of *C. neoformans* and *C. gattii* within CSF. In a study that included 69 patients in Uganda, including 36 with first episode cryptococcal meningitis, the results of the FilmArray assay appeared to be related to the fungal burden within the CSF [62]. The assay sensitivity was 96% when the fungal burden was at or above 100 colony-forming units/ml, while the negative predictive value was 95% when the fungal burden was less than 100 colony-forming units/ml. The authors also reported that, in their experience, the FilmArray assay was able to distinguish between second episode cryptococcal meningitis and immune reconstitution inflammatory syndrome (IRIS), although the number of patients included in this subset analysis was small ($n = 8$). However, there is some concern for false positives with this assay [61], and it does not discriminate between recently described members of the *C. neoformans* and *C. gattii* species complexes [65].

### T2 Magnetic Resonance

One of the disadvantages of the PNA FISH and FilmArray assays for the detection and identification of *Candida* species is the need for testing from a positive blood culture bottle, which can add to the time needed to confirm or rule out infection. T2 *Candida* (T2 Biosystems) is a closed, automated, FDA-cleared assay that is able to detect and identify the five most common *Candida* species (*C. albicans, C. glabrata, C. krusei, C. parapsilosis,* and *C. tropicalis*) directly from whole blood. In this assay, red blood cells are first lysed, the pathogen cells and debris are concentrated, and cells are lysed via mechanical means [66, 67]. *Candida* the DNA is amplified using pan-*Candida* primers directed at the ITS2 region. The amplified DNA then hybridizes to supra-paramagnetic nanoparticles coated with complimentary DNA [68]. The nanoparticles agglomerate into microclusters that produce a signature T2 magnetic resonance signature, which is read and compared to a database to identify the organism. Similar to the PNA FISH Yeast Traffic Light assay, the detection and identification of various species is grouped based on typical antifungal susceptibility patterns, with *C. albicans* grouped with *C. tropicalis* and *C. glabrata* with *C. krusei* [69]. Excellent analytical sensitivity has been reported, with detection ranges between one and three colony-forming units/ml for the species that this assay detects [66, 67, 69]. Excellent clinical specificity has also been reported [67]. In a multicenter study, blood was collected from 1801 patients, the vast majority of whom did not have invasive candidiasis. The overall specificity was 99.4% and was similar for each species grouping (98.9% for *C. albicans*/*C. tropicalis*, 99.3% for *C. parapsilosis*, and 99.9% for *C. krusei*/*C. glabrata*). The mean time to negativity for the T2Candida assay was also significantly shorter than that for traditional blood cultures (4.2 h versus ≥120 h). This suggests that the T2Candida assay may be able to rapidly exclude the possibility of candidemia leading to limited inappropriate use of antifungal agents. The clinical sensitivity of this assay remains unknown, as only six prospectively collected samples were positive for *Candida* species.

### Other Commercially Developed Molecular Assays

Several other molecular assays have been developed for the detection and identification of fungal species associated with invasive fungal infections, but are not currently FDA cleared for this purpose. These include tests that can be performed on cultures, positive culture bottles, and direct specimens. The Luminex xTag system (Luminex Corporation) contains 23 analyte-specific reagents for fungi. The sensitivity and specificity of the assay in positive blood culture bottles and on cultures has been ported to be 100 and 99%, respectively [70, 71]. The LightCycler SeptiFast (Roche) system uses real-time PCR to amplify multi-copy target ITS region of fungal rRNA and highly specific melting curve analysis for species identification.

Fungal species detected and identified by this assay include *Candida* species commonly associated with invasive candidiasis in humans (i.e., *C. albicans, C. glabrata, C. parapsilosis, C. krusei,* and *C. tropicalis*) as well as *A. fumigatus.* The analytical sensitivity has been reported to be between 3 and 100 colony-forming units/mL, depending on the species [15, 59, 72]. However, there are reports of false negatives in positive blood cultures for pathogen covered by the assay [73–75]. A third molecular test for fungal species identification is the PLEX-ID system, which combines multiplex PCR with electrospray ionization-mass spectrometry to amplify and identify organisms based on the mass-to-charge ratio of the PCR amplicons [76, 77]. This system can identify up to 75 fungal species in a single reaction and has been reported to correctly identify cultured fungi at 95.6 and 81.3% to the genus and species levels, respectively [15, 77]. In an analysis of 691 archived BAL samples, genus level and species level identifications by the PLEX-ID system were concordant with those of culture results to the genus level in 87% and species level in 67% [78]. Despite these promising results, the PLEX-ID system has been discontinued by the manufacturer [79].

Commercial assays specific for the detection and identification of *Aspergillus* species have also been developed. These include the AsperGenius assay (PathoNostics) and the MycAssay *Aspergillus* (Myconostica). The AsperGenius assays use multiplex real-time PCR targeting the 28S rRNA to detect and identify *A. fumigatus, A. terreus, A. flavus, A. niger,* and *A. nidulans* directly in BAL fluid. It is also able to identify prevalent mutations in the *CYP51A* gene associated with azole resistance via melt curve analysis [80]. The overall sensitivity and specificity of the assay in different patient populations (i.e., hematology patients and those in intensive care settings) range between 80.0–88.9% and 89.3–93.3%, respectively [81]. The MycAssay *Aspergillus* is a real-time PCR that uses molecular beacons targeted against 18S rRNA to detect and identify 18 different *Aspergillus* species in serum and lower respiratory tract specimens [82, 83]. Improved sensitivity and specificity have been reported when used on BAL versus serum specimens [68, 84]. However, neither the AsperGenius nor the MycAssay is currently FDA cleared for clinical use in the USA.

## Laboratory-Developed Molecular Assays

Numerous molecular-based laboratory-developed tests have been reported in the literature for the diagnosis of invasive fungal infections. These have been performed on various biological tissues and fluids and have included pan-fungal assays as well as those that have primarily focused on the diagnosis of invasive candidiasis, invasive aspergillosis, and mucormycosis due to the frequency and severity of these invasive mycoses. Because they are present in multiple copies, thus improving assay sensitivity, many of these assays have targeted one or more regions rRNA cluster compromising the 18S, 28S, and the ITS regions. Because these regions contain both highly conserved and variable regions, universal primers can be designed

within the conserved regions to amplify DNA from a large number of species, while more specific primers or probes can be designed based on the variable regions for genus or species detection and identification [68].

There are several limitations and unresolved issues with PCR-based methods for the diagnosis of invasive fungal infections. A major limitation is that of false positives, which can occur due to colonization of the tissue or fluid that is sampled or contamination of the specimen or reagents used in these assays. Because laboratory-developed tests are open platforms, it is essential to recognize the potential for error in the pre-analytical and analytical steps that may result in false positives [85]. Strict workflows that eliminate or reduce the potential for contamination as much as possible should be developed and adhered to by laboratory staff, and negative controls should be included [68, 85]. To avoid false positives caused by colonization, some recommend that assays should only be performed on sterile fluids and tissues. Testing on samples from non-sterile sites, including BAL fluid, can also lead to false positives. There is interest in the use of BAL fluid for the diagnosis of invasive fungal infections that primarily affect the lungs, such as invasive aspergillosis, since this fluid is more likely to have a higher burden of organisms therefore improving the sensitivity of the assay. However, a positive result is not confirmative of invasive disease, as molecular-based assays are unable to distinguish between infection and colonization of the airways. However, negative results in BAL fluid may be useful for excluding invasive aspergillosis [68, 86].

The lack of standardization is another limitation. Different specimen types (whole blood, serum or plasma, tissue, BAL, other biological fluids) and volumes, DNA extraction and processing methods, and molecular platforms have been reported in the literature, resulting in a wide range of results. This has prevented the inclusion of PCR-based methods in published and/or guideline criteria for invasive fungal infections [68]. It should be noted that a single positive PCR result may be inadequate for diagnosis. A meta-analysis of 16 studies reported that the use of at least two positive PCR results performed on blood did improve the specificity but not the sensitivity in the diagnosis of invasive aspergillosis [87]. In order to improve the diagnosis of invasive aspergillosis, the European Aspergillus PCR Initiative (EAPCRI) has made recommendations regarding the use of PCR-based platforms [88, 89]. One meta-analysis reported that the use of these recommendations markedly improved both the sensitivity and specificity of these assays for the diagnosis of invasive aspergillosis when at least two positive results were used to define a PCR-positive episode [90]. There is also debate on whether whole blood or serum should be used for these assays. Although more studies have reported the use of whole blood for PCR-based assays, serum is attractive as DNA extraction is easier within this biological fluid, thus standardization may be easier, and there are fewer inhibitors of PCR compared to whole blood. Results from various studies have provided conflicting results [90–95].

Molecular assays have also been developed for the detection and identification of fungi within tissues, including fresh tissue and paraffin-embedded specimens. This may be done in instances where cultures are negative but histopathology results are consistent with a fungal infection. The diagnostic yield reported in the literature for

such cases has been variable, with ranges between 35% and 96% [96–102]. This variability may be due to differences in specimen types and available tissue amounts, DNA extraction methods, amplification platforms, and the DNA targets used (multi-copy versus single copy targets) [96]. It should be remembered that this approach is complimentary to other tests and is most valuable in histopathologically proven infection [96–98, 103, 104]. In contrast, marginal diagnostic yields have been reported for specimens where fungal elements were not observed on histopathology. Thus, some recommend restricting these molecular assays to specimens in which visible fungal elements are seen or for which there are ancillary results (e.g., positive galactomannan or (1→3)-β-D-glucan) in patients at risk for invasive fungal infection [68, 96].

## Identification of Filamentous Fungal Species from Culture

When fungi are cultured from clinical specimens, correct identification to the species level is important, as it may provide information to the clinician regarding appropriate treatment regimens. Some fungal species may be intrinsically resistant or have reduced susceptibility to certain antifungals [105–107]. Clinically relevant examples include observations made in the order *Mucorales* and *Aspergillus* section *Fumigati*. In the *Mucorales*, the causative agents of mucormycosis, different susceptibility patterns have been observed for various genera within this order between posaconazole and isavuconazole, the two azoles that have been used in the treatment of these highly aggressive infections [108, 109]. Similarly, genera-dependent differences have also been reported for amphotericin B, which may have reduced susceptibility against *Cunninghamella* species [108].

Historically, the identification of fungi to the species level has been accomplished by observing morphologic and phenotypic characteristics. These include descriptions of the colony appearance, including color and texture, and reproductive structures observed on microscopy. Other phenotypic characteristics that have been used for species identification include temperature studies, tolerance to cycloheximide and benomyl, tolerance to different concentrations of sodium chloride, nitrate assimilation, growth on bromcresol purple agar, growth on trichophyton agar, and growth on urea agar [110–113]. However, identification based solely on morphologic and phenotypic characteristics may be error prone due to variable features that can be caused by different factors, including the media used for growth and exposure to external stressors, such as antifungal agents prior to recovery from clinical specimens, which can often occur in patients at high risk for invasive fungal infections. In addition, morphologic instability can be observed in closely related species that have very different antifungal susceptibility patterns. For example, *Aspergillus* section *Fumigati* is now recognized to consist of at least 51 distinct species, including several with reduced susceptibility to the azoles that have been documented to cause disease in humans [114–119]. Unfortunately, correct identification to the species level by morphologic/phenotypic characteristics alone is not possible [120,

121], and clinical failures have been reported in cases where the infecting organism was misidentified [105, 115, 118, 120, 122, 123].

The use of molecular tools for species identification of fungi has now become widespread and often includes the analysis of DNA sequences. While this practice has improved the accuracy of species identification, it has also revealed the existence of highly related sibling or cryptic species within morphologically ascribed species [68]. The choice of the target used for DNA sequence analysis can also influence the results. The internal transcribed spacer (ITS) region of rRNA (including ITS1, 5.8S, and ITS2) has been put forth as a potential universal barcode for fungal species identification, as this region has yielded the highest probability of identification for a wide range of species [124]. Indeed, this region has proven useful for the identification of many fungi [125, 126] and, along with the 28S rRNA large subunit region (D1/D2), is recommended for the molecular identification of fungi in clinical cultures [127, 128]. However, this region may not be sufficient to resolve species identification for several clinically relevant species, including (but not limited to) *Aspergillus* species, the *Mucorales*, *Fusarium* species, and several other genera of filamentous fungi [120, 129–133]. Thus, several other targets/genes may be needed for the correct identification of fungi to the species level. These may include actin, β-tubulin, calmodulin, translation elongation factor, RNA polymerase, and glyceraldehyde-3-phosphate dehydrogenase [68, 129]. The choice of targets used for DNA sequence analysis of isolates can be guided by morphologic characteristics.

Once the DNA sequences are obtained, these must be compared to sequences of known organisms in order to obtain the correct identity. Several publically available databases may be used for this purpose; these include GenBank at the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/genbank/), the Westerdijk Fungal Biodiversity Institute (formerly the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre; www.westerdijkinstitute.nl), the International Society of Human and Animal Mycology Barcoding database (ISHAM; its.mycologylab.org), and the Fusarium-ID database (http://isolate.fusariumdb.org). However, it should be remembered that errors within databases exist, as not all fungal deposits have been evaluated for accuracy [134–137]. Thus, comparison of DNA sequences to those obtained for the type species, when available, is preferred in order to prevent misidentifications. When possible, molecular results should also be correlated with morphologic/phenotypic characteristics for species confirmation.

## MALDI-TOF MS

One non-molecular assay that is worth mentioning due to its acceptance into many clinical microbiology laboratories is that of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). In this assay, species identifications are made based on comparisons of mass spectra of ribosomal

proteins that are highly conserved among isolates of the same species but with interspecies variations. Following the processing of specimens, which includes the addition of a sample from a culture to a metal slide followed by the addition of an energy-absorbent compound, desorption and ionization by a laser generates protonated ions that accelerate and separate from each other based on their mass-to-charge ratio. The differences in mass-to-charge ratios are then detected by a mass spectrometer, and the specific mass spectra generated for each isolate is compared to a library of spectra of known species in order for an identification to be made. This method has proven to be a very rapid, accurate, and reproducible means of species identification for bacteria and yeasts [138–141]. Two MALDI-TOF MS systems are currently available for the clinical laboratory use: the Bruker Biotyper and the bioMerieux Vitek MS. In a large study that included 1192 yeast isolates, both MALDI-TOF MS instruments performed similarly with greater than 95% accuracy in species identifications for each [142]. This technology can also discriminate between closely related species with which traditional biochemical assays have difficulty [143, 144]. Studies have also evaluated the ability of this proteomic technology for the identification of filamentous fungi. A recent multicenter study, which included 1519 unique mold isolates, reported that the Vitek MS system, using database v3.0 which was recently cleared by the FDA for clinical use, was able to correctly identify 91% of the isolates to the species level with another 2% to the genus level [145]. One limitation of MALDI-TOF MS technology is the need for accurate reference mass spectra for comparisons within the database being used for comparison and species identification. This was highlighted by the inaccurate results, including both no identifications and misidentifications, reported for both the Biotyper and the Vitek MS systems in identifying *C. auris* prior to accurate reference spectra for this species being included in the respective clinical databases [17]. However, as more laboratories adopt this technology and the availability of reference mass spectra increases, this limitation will decrease, and this technology will become more powerful.

## Conclusions

The diagnosis of invasive fungal infections remains challenging. Numerous molecular assays have been developed to aid in the diagnosis of invasive fungal infections. These include both laboratory-developed and commercially developed tests. Several that can be used for the diagnosis of invasive candidiasis are FDA cleared for clinical use in the USA; these primarily use specimens from positive blood culture bottles, although one that has recently become available can be used with whole blood. Others can also utilize direct specimens or for the detection and identification of fungal species causing various infections. However, much work needs to be done in terms of standardization of the methods used and validation of these assays in order to determine their utility and how they may serve to improve the diagnosis of invasive mycoses.

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# Laboratory Technical Advances in the Diagnosis of *Clostridium difficile*

**Masako Mizusawa and Karen C. Carroll**

## Microbiology and Pathogenesis

*Clostridium difficile* (*C. difficile*) is a spore-forming, anaerobic, gram-positive rod that colonizes the colon of 0−15% of healthy adults and 30–70% of healthy infants [1]. The organism can also be found in a variety of environmental sources including soil, river water, domestic animals, and home and healthcare environments [1]. *C. difficile* acquired its name from the observations by Hall and O'Toole [2] in the difficulty of isolating the organism because of its slow growth (doubling time 40–70 min) compared to other *Clostridium* spp. During logarithmic growth, when vegetative cells predominate, the organism is very aerointolerant. In 1978, prior knowledge of the organism, and the observation that antibiotic-associated diarrhea was associated with a cytotoxin in the hamster model, converged in the work by Bartlett et al. that demonstrated that *C. difficile* caused disease in humans through the elaboration of a cytotoxin [3, 4]. Later it was established that the organism produces two toxins, toxin A, a 308 kDa enterotoxin, and toxin B, a 270 kDa cytotoxin.

Toxin A binds to saccharides in the human glycoprotein receptor gp96 and only recently have the receptors for toxin B been discovered [1, 5, 6]. Both toxins cause disease by glycosylating small guanosine triphosphatases (GTPases) such as Rho, Rac, and Cdc42 when endocytosed into gastrointestinal epithelial cells [1, 5, 7]. Glycosylation of these small proteins disrupts signaling pathways causing irreversible changes in cellular morphology (due to actin filament disassembly) and consequent inhibition of cell division and membrane trafficking, leading to cell death [1, 5–7]. Animal models, initially using the hamster and later mice, and recent phylogenetic analyses offered by new technologies, such as next-generation sequencing,

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have advanced our understanding of the pathogenesis and pathophysiology of *C. difficile* disease caused by its toxins and other virulence factors [1, 4, 7]. For example, it is now clear from animal models that toxin B is required and sufficient for disease—an observation supported by outbreaks of clinical infections caused by toxin A-negative, toxin B-positive strains [1, 7].

The genes that encode toxins A and B, *tcdA* and *tcdB*, are found along with three other genes (*tcdC*, *tcdE*, *tcdR*) on the pathogenicity locus (PaLoc), a conserved 19.6 kb region of the bacterial chromosome [1, 5, 7]. In nontoxigenic strains of *C. difficile*, the PaLoc is replaced by a 115-bp sequence [1, 7]. *tcdE* encodes a protein whose pore-forming activity allows the release of TcdA and TcdB from the cell [1, 7]. *tcdR*, found upstream of *tcdB*, is a positive regulator of *tcdA* and *tcdB* expression [1, 7]. *tcdC* is found downstream of *tcdA*, and this gene has been shown to be a negative regulator of toxin production that prevents transcription of the PaLoc [1, 7–9]. Mutations in many of these various genes have a significant impact on expression of one or both toxins and have been shown to be responsible for the emergence of hypervirulent toxin variant strains (see Epidemiology section).

In addition to toxins A and B, about 6–12% of strains (mostly variant toxinotypes) produce a toxin consisting of two separate components (binary toxin), a *C. difficile* transferase (CDT), encoded by *cdtA* and *cdtB* which are not located on the PaLoc [1, 7, 10]. CdtA is the active component and it ADP-ribosylates actin in eukaryotic cells, destroying the actin cytoskeleton, and CdtB acts by binding to host cells and forms pores that facilitates transfer of CdtA into the cytosol [10]. Binary toxin may contribute to virulence by enhancing cytotoxicity and also by increasing adherence of *C. difficile* in vivo [7, 10]. Some studies have demonstrated an association with higher mortality caused by strains in which it is present [10].

The transmissible form of *C. difficile* is a spore which contributes to survival of the organism in the host and is responsible for recurrence of disease when therapy is withdrawn. Like other bacterial spores, *C. difficile* spores are metabolically dormant, survive for long periods of time, and are resistant to harsh physical or chemical treatments such as 70% ethanol and ultraviolet light [11, 12]. Failure to remove spores from contaminated hospital environments contributes to nosocomial spread in healthcare facilities.

Microbiome research has added another dimension to our understanding of the host resistance to *C. difficile* disease. The microbiota protect the host from *C. difficile* colonization by outcompeting it for space and nutrients, an effect termed colonization resistance [13, 14]. When the microbiota are altered, as in the case of antimicrobial therapy (dysbiosis), research has shown increases in sialic acid and succinate which can be used by *C. difficile* for growth [13, 14]. In addition to these factors, the microbiota are responsible for regulation of primary and secondary bile acids [13, 14]. Primary bile acids are converted to secondary bile acids by the intestinal microbiome [14]. Taurocholate, a primary bile acid, activates spore germinations and permits outgrowth of vegetative cells, whereas the secondary bile acid, chenodeoxycholate, suppresses germination [13, 14]. The former is increased during antimicrobial treatment and the latter is decreased [13, 14]. Studies in mice and fecal transplantation in humans confirm the important roles of human microbiota in

preventing *C. difficile* disease, as well as other enteric pathogens [13, 14]. Finally, the microbiota appear to influence mucosal and other immune responses (reviewed in reference 13) and when disrupted, can impact the severity of *C. difficile* disease [13]. In summary, *C. difficile* disease occurs when a toxin-producing organism is present in a host whose normal microbiome has been disrupted by antimicrobial agents or other factors.

## Epidemiology

*C. difficile* is responsible for 95–100% of cases of pseudomembranous colitis [3] and for 20–25% of antibiotic-associated diarrhea without colitis [15]. Approximately 50% of healthy infants are colonized with *C. difficile*, and the colonization rate decreases to 3% by the age of 1.5 years [16]. These infants rarely develop *C. difficile* infection (CDI) which is thought to be related to the absence of the intestinal receptor that binds *C. difficile* toxin [17]. Among healthy adults, asymptomatic *C. difficile* colonization rate ranges from 0% to 15%, and in the hospital settings, the colonization rate varies between 0% and 51% depending upon the patient population [18]. These asymptomatic carriers are potential sources of disease transmission and may contribute to spread of CDI in the hospitals [19, 20].

There has been a steady increase in the incidence of CDI globally with a significant increase in morbidity and mortality in the early 2000s associated with North American hypervirulent strain BI/NAP1/027 until around 2009 [21]. Recent epidemiological studies in the United States and Europe suggest that the incidence of CDI may have reached a crescendo and is leveling off or slightly declining [19, 22] although it remains historically at high levels. In the United States, it is estimated that there were 453,000 cases of CDI and 29,300 deaths in 2011 [22]. Rates of CDI in Europe have been broadly similar to those reported in North America although a recent pan-Europe survey revealed large discrepancies in the rate of CDI testing across Europe suggesting potential underestimation of the true incidence of CDI [23].

In the molecular epidemiology of CDI, the most commonly used typing systems include restriction endonuclease analysis (REA), pulsed field gel electrophoresis (PFGE), and polymerase chain reaction (PCR) ribotyping [7, 24]. The identification of BI/NAP1/027 strain outbreaks changed the healthcare epidemiology of CDI significantly, as this strain is associated with increased disease severity and mortality. The “wild-type” strain was fluoroquinolone susceptible, but this strain was found to be fluoroquinolone resistant [25]. BI/NAP1/027 strains produce a third toxin (binary toxin) and harbor a point mutation in the tcdC gene, which is thought to be associated with the inability to downregulate tcdA and tcdB transcription leading to increased toxin production [26]. The prevalence of CDI caused by BI/NAP1/027 has significantly decreased in the United Kingdom, while it remains high in the United States [26]. According to the recent Centers for Disease Control and Prevention (CDC) surveillance in which 1364 *C. difficile* isolates were typed,

BI/NAP1/027 was still the most common strain accounting for 30.7% of healthcare-associated CDI and 18.8% of community-associated CDI in the United States [22].

Newer typing methods such as multilocus sequence typing (MLST), multilocus variable-number tandem-repeat analysis (MLVA), and whole genome sequencing (WGS) offer improved data probability and better discriminatory power allowing precise identification of the same strain in relapses even among BI/NAP1/027 strains [7, 24]. A study of WGS typing of initial and recurrent CDI isolates has demonstrated that there is a predominance of the same strain in recurrence meeting the definition of a relapse of infection [27].

There is increasing recognition of CDI cases in the communities and nursing home settings contributing to the global burden of CDI. CDC estimated that nearly 345,400 cases occurred outside of hospitals in 2011, indicating that the prevention of CDI should go beyond hospital settings [22]. In addition, data from the GeoSentinel Global Surveillance Network, a global surveillance network of 59 travel and tropical medicine clinics on 6 continents, showed an increase in reported CDI cases among returning travelers over time [28]. Although this study has many limitations, including significant heterogeneity across sites in diagnosing test methods for CDI, it suggests that returning travelers are a part of at-risk populations for CDI, and *C. difficile* should be considered as a potential cause of traveler's diarrhea [28].

Against this backdrop of evolving strains and emerging epidemiology has been the impetus to implement better and faster diagnostic methods for the detection of *C. difficile*. Although progress has been made in the understanding of disease pathogenesis and new therapies are available, controversy still exists about the optimum method of diagnosis of CDI [29]. Practice guidelines from professional societies have been published to guide the clinical and laboratory approaches to diagnosis [30, 31], and a variety of molecular assays have been approved for diagnosis. This chapter focuses on the rationale for the diagnostic guidelines and the performance of new methods and algorithms for *C. difficile* detection.

## Diagnostic Methods

The Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), as well as the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), have published recommendations regarding the diagnosis and management of CDI. Both sets of practice guidelines emphasize that testing should only be performed on unformed stool specimens in patients at risk for CDI. In both guidelines, toxigenic culture is considered as a reference method and the standard against which other assays are compared [30, 31]. In the ESCMID guidelines, and/or cell culture cytotoxin neutralization assays (CCNA) are considered reference methods and the standard against which other assays are compared studies compared to CCCNA were evaluated separately from those compared to toxigenic culture since the results of these two methods do not

necessarily agree with each other [31]. Currently, there is no single test that can be reliably used to diagnose CDI. Therefore, a two-step test approach is recommended to optimize diagnostic performance in these guidelines. In the case of SHEA/IDSA recent guidelines, the current recommendations are to test unformed stools specimens in patients at risk for CDI using a stool toxin test as as part of a multistep algorithm beginning with an enzyme immunoassay for glutamate dehydrogenase (GDH) or a nucleic acid amplification test (NAAT) followed by toxin testing. Alternatively, in institutions where best practices ensure that only stool samples likely to be from patients with clinical symptoms of CDI are tested, NAAT alone or a mulitstep algorithm as described above are recommended [30]. In the case of the ESCMID, their guidelines recommend empiric testing of all unformed fecal samples submitted to the laboratory except those from children aged under 3 years and the use of an algorithm testing all samples with a first test, either GDH EIA or nucleic acid amplification test (NAAT) followed by a toxin A/B EIA if the first test is positive. If the first test is negative, CDI is unlikely and no further testing is indicated. If the first test is positive and the second test is negative, the case should be evaluated clinically to determine whether the discrepant result is due to CDI with low toxin levels or *C. difficile* colonization. Optionally the third confirmatory test using toxigenic culture (or NAAT if the first test is GDH) can be performed. An alternative algorithm is to test all specimens with both GDH and toxin A/B. If both are negative, CDI is unlikely, and if both are positive, CDI is likely to be present. If GDH is positive and toxin A/B is negative, reflex testing by NAAT can be performed. If GDH is negative and toxin A/B is positive, it is considered as invalid, and the samples should be retested [31]. Table 1 lists the performance characteristics of

**Table 1** Performance characteristics of various test methods for *C. difficile* diagnosis

| | Performance characteristics | |
|---|---|---|
| Methods/assays | Sensitivity (%, range) | Specificity (%, range) |
| Toxigenic culture | Reference | Reference |
| Cell culture cytotoxicity assay[a] | [33–86] | [97–100] |
| Glutamate dehydrogenase[b] | | |
| Microwell EIA | [88–95] | [94–98] |
| Lateral flow membrane EIA | [60–100] | [76–100] |
| ELFA/CLIA | [87–99] | [91–97] |
| Toxin A/B[c] | | |
| Microwell EIA | [41–86] | [91–99] |
| Lateral flow membrane EIA | [29–79] | [89–100] |
| ELFA/CLIA | [41–88] | [89–100] |
| Nucleic acid amplification tests[d] | [62–100] | [89–100] |

*EIA* enzyme immunoassay, *ELFA* enzyme-linked fluorescent assay, *CLIA* chemiluminescent immunoassay

[a]Compiled from [32–38]

[b]Compiled from [37, 39–53]

[c]Compiled from [35, 38–40, 42–66]

[d]Compiled from [33–38, 40, 43–46, 53, 58, 60, 61, 64–89]

test methods currently available for the diagnosis of CDI and their performance characteristics compared to the two accepted reference methods-toxigenic culture and CCCNA. Details of currently available molecular platforms are provided later in the chapter.

## Toxigenic Bacterial Culture

Following the NAP-1 epidemic, many laboratories resurrected bacterial culture for CDI to assist with outbreak investigations. In addition, culture has become the new diagnostic "gold standard" for evaluation of the plethora of available and developing molecular assays for CDI detection. Finally, culture is useful for surveillance of drug resistance and is sometimes helpful in patient management [74]. However, toxigenic culture is too slow and impractical to be used in clinical microbiology laboratories as the primary diagnostic method for CDI.

Toxigenic anaerobic culture requires inoculation of the stool to anaerobic media, incubating the media anaerobically for 2–5 days, and once recovered, determining whether the *C. difficile* isolate is a toxin producer. There is no agreed upon standard method, but a well done culture has been shown to significantly increase the yield of *C. difficile* detection by 15–25% when performed after a negative direct toxin test [74, 90, 91]. Factors to consider when developing a culture method include (1) the need for and type of spore enrichment, (2) the type of media, and (3) the best method for confirming toxin production in the recovered isolate. Each of these factors is briefly addressed.

A variety of media, both selective and nonselective, is available for culturing *C. difficile*. Nonselective anaerobic media have the advantages of being less expensive and more readily available in clinical labs than selective agars or broths, but do not allow for easy presumptive identification of *C. difficile* [92]. The original cycloserine, cefoxitin, fructose agar (CCFA) as described by George et al. contained an egg yolk fructose agar base with 500 mg/ml of cycloserine and 16 mg/ml of cefoxitin [92]. On this medium, the *C. difficile* organisms produced yellow, fluorescent filamentous colonies that were easy to distinguish from other organisms [92].

Over the years incorporation of substances to enhance germination of spores such as horse blood in place of the egg yolk, taurocholate, and lysozyme have been shown to improve recovery [74, 93]. In some studies, CCFA variants with reduced concentrations of the antimicrobial agents were less sensitive compared to the George formulation [94, 95]. However, in the study by Levett et al. using CCFA with cycloserine and cefoxitin at concentrations, half that of the George formulation were less inhibitory to *C. difficile* when combined with a protocol that used alcohol shock for spore enrichment [96]. Whatever medium is chosen, it is important to use prereduced media as the failure to do so can impact the sensitivity of the culture method [95].

More recent studies have examined the utility of broth enrichment compared to direct plating on solid media as well as spore enrichment techniques. In the former

situation, fecal specimens are inoculated to an enrichment broth that contains taurocholate, antibiotics, increased carbohydrates, and/or lysozyme to reduce normal fecal microbiota and enhance recovery of small concentrations of *C. difficile*. Two comprehensive studies have shown enhanced recovery of cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme (CCMB-TAL, Anaerobe Systems, Morgan Hill, CA) compared to direct plating on solid media [97, 98].

Spore enrichment involves treating the fecal specimen with either heat or ethanol to reduce competing normal microbiota. When subjected to heat or ethanol, *C. difficile* develops spores which then germinate when the specimen is planted on anaerobic media. Both heat and alcohol have been shown to reduce competing normal microbiota. With heat shock, a 1 mL aliquot of the stool specimen is incubated in a 80 °C water bath or heat block for 10 min prior to plating [97]. In the case of alcohol shock, 0.5 ml of stool sample is added to an equal volume of ethanol and mixed by vortexing. After standing at room temperature for 1 h, the specimen is then plated on selective *C. difficile* media or other anaerobic media [99].

As is the case for detection of other nosocomial pathogens, a chromogenic medium for *C. difficile* detection is available. chromID *C. difficile* agar (chromID CDIF, bioMerieux, France) has been extensively evaluated in several studies [100–103]. Overall, the agar appears to perform as well as selective *C. difficile* media and produces results in 24 h. It can be used in combination with heat enrichment [102], and one study found that it obviated the need for alcohol shock treatment [101, 103].

Once *C. difficile* is recovered from culture, the isolate should be tested for toxin production, as culture will recover both nontoxigenic as well as toxigenic isolates. At least one study demonstrated that an enzyme immunoassay should not be used to test the isolate for toxin production as it may be too insensitive [104]. A cell culture cytotoxicity neutralization assay (CCCNA) or polymerase chain reaction (PCR) is preferable [104].

In summary, there is no agreed upon standard method for culturing *C. difficile*. A combination of some type of enrichment (whether it be spore enrichment or broth enrichment) and direct plating on solid selective agar seems to provide the most sensitive approach and should be used in circumstances where low quantities of organisms may be present, as may be the case in an epidemiological study. Chromogenic media may be as sensitive as selective media, shortens the time to recovery, and may negate the requirement for enrichment, although more studies are needed regarding the latter point.

## Cell Culture Cytotoxicity Neutralization Assay

The cell culture cytotoxicity neutralization assay (CCCNA) has been used as a reference method against which other assays are compared and considered as the clinical "gold standard" for diagnosis of CDI, while toxigenic culture is the ultimate gold standard for detection of toxigenic *C. difficile*. CCCNA is a labor-intensive,

time-consuming, and costly test which requires technical expertise and maintenance of facilities. The procedure for this assay involves multiple steps, and lack of adherence to these steps can significantly affect test performance.

The fecal sample is first suspended in phosphate-buffered saline (PBS). Then the buffered sample is centrifuged to remove debris and the supernatant is filtered. The diluted filtrate is applied to a cell monolayer usually in a macrotiter or microtiter multiwelled plate. After incubation at 37 °C for 24 h, the plates are assessed for cytopathic effect (CPE) that is characterized by rounding of cells. CPE is neutralized by *C. difficile* or *C. sordellii* antitoxin. If the monolayer does not show CPE, it is reincubated for another 24 h before calling the sample negative. While many different cell types can be used, such as MRC-5, CHO-K1, WI-38, Vero, HEp2, and HFF, HFF cells are preferred in the United States and were the most sensitive cell line in one study [105]. Cells should be fresh (5–14 days old) and of low passage [105]. The specimen should be diluted 1:40 to 1:200 to prevent nonspecific toxicity by substances in fecal samples. Criteria used to interpret CPE can also affect the results. In some instances, CPE can be seen as early as 4 h [105].

Compared to toxigenic culture, the sensitivity of CCCNA is suboptimal with highest reported values being below 90% as shown in Table 17.1. However, CCCNA is very useful for confirmation of toxin production in clinical isolates recovered from anaerobic culture, and there has always been debate as to whether toxigenic culture or CCCNA best defines CDI cases. A recent study in which over 12,000 fecal samples were submitted for *C. difficile* testing from both hospital and community patients showed that patients with a positive toxigenic culture but negative CCCNA had the same case fatality rate as did *C difficile*-negative cases [40]. Many of the current CDI diagnostic tests are more rapid and easier to perform than CCCNA, and this method is now rarely used as a primary diagnostic method. Viral diagnostic methods have shifted to molecular-based assays, and many clinical laboratories have discontinued maintenance of cell culture techniques, which has also limited the availability of CCCNA.

## Toxin Enzyme Immunoassays

Enzyme immunoassays became available in the mid- to late 1980s to replace the more labor-intensive CCCNA. Solid phase microwell formats, which were coated with monoclonal or polyclonal antibodies against toxin A and/or B, allowed for batch testing and the ability to report same-day results. Later, rapid immunoassays in chromatographic cassettes, immunocard, and lateral flow membrane formats became available. More recently, automated systems using enzyme-linked fluorescent assay (ELFA) or chemiluminescent immunoassay (CLIA) to detect toxin A/B have been developed. These assays have variable sensitivity ranging from 44% to 99% when compared to CCCNA and 29% to 88% when compared to toxigenic culture (Table 2). In the studies in which a two-step test algorithm with GDH followed by toxin EIA test was evaluated, sensitivity of toxin EIA tests ranged from

**Table 2** Summary of toxin EIA for detection of *C. difficile*

| Methods/assays | Comparison to CCCNA Sensitivity (%, range) | Comparison to CCCNA Specificity (%, range) | Comparison to TC Sensitivity (%, range) | Comparison to TC Specificity (%, range) |
|---|---|---|---|---|
| Premier toxin A + B[a] | 58–99 | 94–100 | 40–86 | 91–100 |
| TechLab Toxin A/B II[b] | 72–91 | 87–100 | 58–85 | 96–99 |
| Ridascreen Toxin A/B[c] | 57–67 | 95–97 | 52–60 | 96–98 |
| Remel ProSpecT [d] | 90–91 | 93–97 | 82 | 93 |
| Lateral flow membrane EIA | | | | |
| ImmuoCard toxins A/B[e] | 85–96 | 97–99 | 41–69 | 93–99 |
| Tox A/B Quik Chek[f] | 61–84 | 99 | 40–74 | 94–100 |
| Quick Chek complete Tox A/B[g] | 50–73 | 100 | 29–79 | 89–100 |
| Xpect[h] | 44–83 | 99–100 | 48–69 | 95–99 |
| ELFA/CLIA | | | | |
| VIDAS CDAB[i] | 53–98 | 99–100 | 44–80 | 95–100 |
| Liaison *C. difficile* Toxins A&B[j] | 88 | 95 | 69–88 | 95–100 |

*TC* toxigenic culture, *EIA* enzyme immunoassay, *ELFA* enzyme-linked fluorescent assay, *CLIA* chemiluminescent immunoassay
[a]Meridian Bioscience, Inc., Cincinnati, OH [40, 46, 52, 53, 62, 65, 106–109]
[b]TechLab, Blacksburg, VA [39, 40, 53, 57, 106, 107, 110, 111]
[c]R-Biopharm AG, Darmstadt, Germany [53, 112, 113]
[d]Meridian Bioscience, Inc., Cincinnati, OH [44, 53, 55, 62, 106, 114]
[e]TechLab, Blacksburg, VA [42, 53, 63, 64, 115]
[f]Remel, Lenexa, KS [53, 62, 116]
[g]bioMerieux, Durham, NC [42, 53–56, 58–60]
[h]Remel, Lenexa, KS [53, 106]
[i]TechLab, Blacksburg, VA [38, 42–44, 59, 61]
[e]DiaSorin, Saluggia, Italy [52, 57]

38% to 60% when compared to CCCNA [117–119]. In the study by Planche et al., the authors reviewed the literature on the six most commonly used toxin EIAs published from 1994 until November 2007 and only included those reports where the assays were compared to a reference method [120]. The authors defined acceptability criteria as a sensitivity of 90% and false positivity below 3%. Using diagnostic odds ratios (Kruskal-Wallis test) and logistic regression, the authors determined that there was no difference in performance among the various assays [120]. However, none of the assays met the acceptability criteria in this study [120]. In the ESCMID guidelines, the comprehensive review of the literature of the laboratory diagnosis of CDI was conducted, and the meta-analysis of the studies published from 2009 until June 2014 to evaluate commercial assays compared to a reference method showed pooled sensitivities of 83 and 57% when compared to CCCNA and toxigenic culture, respectively [31]. Given the extensive data on EIAs demonstrating inadequate performance, combined with literature that showed that strain variation may impact the performance of these tests [78, 121], professional societies do not recommend

toxin EIAs as stand-alone tests for diagnosis of CDI [30, 31]. That said, there are investigators and epidemiologists who believe, and have demonstrated in recent clinical studies, that the presence of toxin in stools as detected by EIA (or CCCNA) is a more reliable predictor of *C. difficile* disease and more often associated with mortality than other testing methods, especially NAAT [29, 40, 122].

## Glutamate Dehydrogenase Testing

*C. difficile* glutamate dehydrogenase (GDH), also called the common antigen, is present in high levels in all strains of *C. difficile*, both toxigenic and nontoxigenic isolates. Many studies evaluating EIAs for GDH including those combined with toxin A/B assays demonstrated good sensitivity and high negative predictive values [39, 40, 42–46], making the assay a useful first step in screening for the presence of the organism. Recently developed automated GDH assays using ELFA or CLIA appear to have similar sensitivities to those of EIAs [48, 51, 52]. However, there have been a few studies that reported sensitivities below 90% for GDH assays [37, 41, 49, 53]. One of the potential explanations for the variable sensitivities was thought to be associated with ribotype. In a multicenter clinical trial conducted in North America, it was found that GDH was less sensitive than PCR in detecting non-027 ribotype strains and suggested that the sensitivity of GDH may vary according to ribotype [78]. However, in in vitro studies of the previously isolated strains, ribotype seems not to affect detection of GDH by commercial tests [123, 124]. Shetty et al. conducted a meta-analysis including 13 studies that met their selection requirements [125]. Due to significant heterogeneity between studies, the summary receiver operating characteristic analysis was performed, and high diagnostic accuracy for the presence of *C. difficile* was demonstrated with sensitivity and specificity above 90% compared with culture [125]. Recently, another meta-analysis to assess the diagnostic accuracy of GDH was conducted using the hierarchical model. In this study, heterogeneity was low and the summary estimate of sensitivity and specificity was also above 90% [126]. Given the mixed literature on the performance of GDH tests, laboratories that implement it should verify that the assay has acceptable test performance for their patient population.

## Molecular Assays

Molecular tests for detection of *C. difficile* directly from fecal samples were tried in the early 1990s [127, 128]. Most of these assays used conventional polymerase chain reaction techniques and lengthy cumbersome nucleic acid extraction

**Table 3** Features of the FDA-cleared molecular assays available in the United States[a]

| Assay | Manufacturer | Gene Targets | Method | TAT (hrs) | Performance characteristics | | References |
|---|---|---|---|---|---|---|---|
| | | | | | Sens (%) | Spec (%) | |
| Progastro™ Cd | Hologic | *tcdB* | qPCR | 4 | 77–100 | 94–99 | [33, 72, 73] |
| Xpert | Cepheid | *tcdB/tcdC* | qPCR | 1 | 90–100 | 93–99 | [43, 60, 67, 70, 77, 80] |
| Illumigene | Meridian | *tcdA* | LAMP | 1 | 82–100 | 94–100 | [38, 44, 46, 61, 67, 70, 77] |
| Simplexa | Focus | *tcdB* | qPCR | 1 | 87–98 | 99–100 | [67, 71, 80] |
| Verigene | Nanosphere/ Luminex | *tcdA/tcdB tcdC/cdt* | PCR; nanoparticle hybridization | 2 | 91–95 | 93–99 | [53, 80] |
| BD MAX | Becton Dickinson | *tcdB* | qPCR | 2 | 86–98 | 89–100 | [64, 66, 67, 77, 80] |
| Cobas | Roche Molecular | *tcdB* | qPCR | 1.5 | 93 | 99 | [81] |
| Portrait | Great Basin | *tcdB* | bpHDA | 1.5 | 98.2 | 93 | [82, 83] |
| AmpliVue | Quidel | | Helicase dependent amplification | 1.5 | 92–96 | 99–100 | [45, 71, 84] |
| Lyra | Quidel | *tcdA*, *tcdB* | qPCR | 2–3 | 82–89 | 97–99 | [85] |
| IMDx *C. difficile* for Abott m2000 | IMDx | *tcdA*, *tcdB*, *tcdBv* | qPCR | NA | 62–84 | 94–99 | [77, 86] |
| ICEPlex | PrimeraDx | *tcdB* | qPCR | 4 | 90 | 97 | [87] |
| Artus *C. difficile* QS-RGQ | Qiagen | *tcdA*, *tcdB* | qPCR | NA | 100 | 90–100 | [88, 89] |

*qPCR* real-time PCR, *LAMP* loop-mediated amplification, *tcdA* toxin A gene, *tcdB* toxin B gene, *tcdBv* toxin B variant genes, *tcdC* toxin C gene, *cdt* binary toxin gene, bpHDA blocked-primer-mediated helicase-dependent multiplex amplification

[a]As of July 2017. Where available, performance characteristics compared to either toxigenic culture or another molecular assay are provided

methods [127, 128]. Later in the decade, reports of improved fecal extraction methods and success with real-time platforms were published [129]. In 2008, the first qPCR assay, the BD GeneOhm Cdiff assay, received FDA approval. This assay was based upon TaqMan chemistry and targeted conserved regions of *tcdB*. The BD GeneOhm assay was converted to an automated platform, the BDMAX in 2013, the former assay is no longer available [36]. Currently there are 13 FDA-cleared molecular platforms (and several others in clinical trials) that detect either toxin A or toxin B. Table 3 summarizes the chemistry and published performance characteristics where available. From an analytical standpoint, molecular tests are 10 to 100 times more sensitive than cytotoxin assays [61, 114] and are twice the cost of EIAs [29]. To reduce the expense that may be incurred with widespread implementation of these assays, several investigators have adopted three-step algorithms [43, 65, 130, 131]. In these studies, the authors looked at screening with the C diff CHEK Complete assay (GDH plus toxin EIA, see GDH section). If the results are concordantly positive or negative for GDH and toxin, then no additional testing is required. However, if the GDH is positive and the toxin portion of the device is negative, then those specimens (approximately 12% in one study) [130] are tested by a rapid molecular method. Such an algorithm can produce same-day results and potentially save money, but this does require maintenance of multiple test methods, training, and the required proficiency, and raises other regulatory compliance issues such as whether reimbursement is allowed for multiple test methods.

The trend in many clinical laboratories in adoption of molecular methods for detection of *C. difficile* has resulted in a response that ranges from consternation to ready acceptance among clinicians. There is no doubt that in all of the available publications on these tests, molecular assays are more sensitive than EIAs, algorithms that incorporate GDH, and CCCNAs but are not as sensitive as toxigenic culture. There are a few practical concerns that have been raised. One is that these assays do not detect the toxins, but the genes that encode for toxins, raising the issue of clinical specificity. For this reason it is extremely important that physicians not send specimens to the laboratory on patients who do not have diarrhea or otherwise meet a clinical case definition of *C. difficile* disease. Recently a ground swell of "anti-molecular" sentiment, fueled in part by the high rates of *C. difficile* seen with more sensitive tests, and the negative impact this has created because of the laboratory test based National Healthcare Safety Network reporting, has followed the publication of two large prospective studies [40, 122]. Both of these large prospective studies have demonstrated that a positive toxin test correlates better with patient outcomes, such as disease severity and mortality, than a molecular-based assay [40, 122]. These studies were performed among large general populations, and the findings may not be applicable to all patient populations, such as immunocompromised hosts. Several, albeit smaller studies, on patients with cancer, have shown no differences in disease severity or mortality in patients who are toxin positive compared to those who were PCR positive, toxin negative [132–134]. However, amidst this controversy regarding the optimum test methods, it seems prudent, regardless of the testing method employed, to improve test utiliza-

tion and pretest probability by carefully selecting symptomatic patients who are at risk for *C. difficile* disease. In the Dubberke study, it was shown that close to 50% of patients tested either did not have diarrhea or were on a laxative at the time of testing [135].

Laboratories can improve test utilization by rejecting specimens that are not loose or take the shape of the container (Bristol stool charts 5 or 6). In addition, discouraging or preventing testing of patients who have received a laxative within the last 48 h, repeat testing of negative samples within a 7-day period, and "test of cure" on positive patients are other strategies to prevent overutilization [136–139]. Some institutions have established best practice alerts for clinicians using their institutions' electronic medical records [136–139]. If these best practice alerts are associated with "hard stops" at order entry (i.e., the inability to order the test if certain criteria are met), studies have shown a reduction in testing, decrease in empirical vancomycin use, and cost savings [136–139]. In addition, laboratories should monitor positivity rates and assess their environments for contamination even in the era of real-time PCR and automated extraction.

Other questions that surround implementation of molecular testing for CDI include the theoretical concerns of genetic drift in the gene targets, the impact of emerging strain variation on assay performance, and the frequency with which the gene is present but not expressed, among other questions. Other desirable information includes the impact of rapid molecular testing on infection control and patient management. With regard to the former, combined with heightened infection control practices, some institutions have noted a reduction in *C. difficile* transmission after an initial period of seemingly increased rates due to more sensitive test utilization [74, 140].

## Summary

*C. difficile* remains an important cause of antibiotic-associated diarrhea, and data have shown that the incidence has increased over the last two decades. The increase is multifactorial but has largely been driven by the emergence of multidrug-resistant, toxin variant strains and an increasingly susceptible population. The increased frequency of more severe disease and higher mortality rates has forced laboratories to critically evaluate diagnostic testing algorithms. There was a movement away from insensitive toxin A/B EIAs toward more sensitive multistep algorithms and rapid molecular assays. There are currently more than a dozen FDA-cleared molecular assays in the United States, all of which have been shown to have superior analytical performance compared to other methods except toxigenic culture. Overutilization of molecular testing has led to a call for a return to toxin tests or GDH-multistep algorithms. Clinical studies demonstrate a need for improved test utilization regardless of the test employed. Improved and more sensitive toxin assays are needed in conjunction with better institutional practices for selecting symptomatic at-risk patients.

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# Laboratory Diagnosis of HIV-1 Infections: State of the Art

**Nang L. Nguyen, Rodney Arcenas, and Yi-Wei Tang**

## Introduction

In the management of HIV infections, laboratorians/clinical microbiologists typically determine whether a patient is infected with HIV, evaluate the immunologic status of the HIV-infected patient, and monitor antiretroviral therapy. Theoretically, an HIV infection can be diagnosed and monitored by any of five possible ways: (i) direct microscopic examination such as visualization of an HIV virion by electronic microscopy, (ii) cultivation and identification of HIV by suspension lymphocyte culture, (iii) detection of HIV viral antigens, (iv) measurement of HIV-specific immune responses, and (v) detection and quantification of HIV-specific nucleic acids [1, 2]. Practically, the diagnosis and monitoring of HIV infection is done by serologic and molecular methods. Molecular methods were first used by Ou et al. from the US Centers for Disease Control and Prevention in 1988 to directly amplify HIV-1-specific nucleic acids from peripheral blood mononuclear cells of HIV-1-seropositive individuals [3]. In this seminal article, the authors concluded that "the method may therefore be used to complement or replace virus isolation as a routine means of determining HIV-1 infection." This has certainly proven to be correct.

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Indeed, the diagnostic capabilities for HIV infections have improved rapidly and have expanded greatly, thanks to advances in molecular technology. Molecular assays along with serology have become the mainstays for the laboratory diagnosis of HIV infection. HIV-1 plasma viral load assays are routinely used in combination with CD4 cell counts to evaluate therapy and to determine when a regimen is failing (https://aidsinfo.nih.gov/guidelines). Moreover, unlike serologic assays, these assays, especially the qualitative ones, also have been used to diagnose perinatal and acute HIV-1 infections. HIV-1 antiretroviral susceptibility tests, which include a genotyping assay detecting mutations known to confer resistance in viral genomes as well as a phenotypic assay measuring recombinant viral replication in the presence of antiretroviral drugs, have become standard of care for the management of antiretroviral treatment. Assessing host gene polymorphisms and immune responses have emerged in clinical laboratories for better diagnosis and monitoring of HIV-1 infections [4–6]. Point-of-care testing (POCT) based on molecular techniques has been actively developed and will be used for diagnosis and monitoring soon [7–9] (Table 1).

## Qualitative Detection of HIV

Historically, the primary diagnosis of HIV infection usually has been accomplished by first screening for anti-HIV antibody using enzyme immunoassay (EIA); positive results were followed by a confirmatory Western blot (WB) test. As the diagnostic technology has improved, the current standard for an HIV serology assay is an HIV antigen/antibody combination assay; positive results are followed by an antibody-based assay that differentiates between HIV-1 and HIV-2. However, serology may be limited in immunocompromised hosts due to the inability of the host to mount an effective immune response. Moreover, seronegative HIV-1-infected cases have been reported even for common HIV clades [10, 11]. Should there be a discrepancy between the initial antigen-antibody screen and the supplemental assay, the Aptima HIV-1 RNA Qualitative Assay (Hologic Inc., San Diego, CA), the sole nucleic acid test (NAT) currently approved by the FDA for diagnostic NAT testing, should then be employed.

While serology remains the primary method, molecular technologies based on in vitro nucleic acid amplification can be utilized in the diagnosis of acute or primary infection since viral RNA can be detected earlier than the antibody or p24 antigen. Current data indicate that tertiary confirmation of HIV infection by the qualitative molecular method constitutes an effective alternative HIV diagnostic algorithm in certain settings [12]. It shortens the window of non-detection, readily discriminates between HIV-1-infected and -uninfected individuals, and effectively reduces the number of indeterminate results [13].

In addition to aiding in the diagnosis of HIV-1 infection, qualitative NATs including Procleix HIV-1/HCV assay (Gen-Probe, San Diego, CA), which incorporates transcription-mediated amplification technology, also have been developed and are commercially available for screening of donated blood [14, 15]. The qualitative format detects a lower amount of viral RNA than does quantitative testing, i.e., less

**Table 1** Current molecular methods for laboratory diagnosis and monitoring of HIV infections

| Method | Applicability | Turnaround time | Main devices (manufacturer) | Applications | Comments | Exemplary references |
|---|---|---|---|---|---|---|
| Molecular assays, qualitative | B | 1–2 days | Procleix HIV-1/HCV assay (Gen-Probe)<br>Aptima HIV-1 RNA Qualitative Assay (Gen-Probe)<br>Xpert® HIV-1 Qual (Cepheid) | Blood donor screening<br>Acute or primary HIV infection diagnosis | Used for blood donor screening in pooled specimens; test of choice for HIV infections in newborns and infants | [10, 11, 13, 24, 25, 226] |
| Molecular assays, viral load testing | A | 1–2 days | COBAS AmpliPrep/TaqMan HIV-1 (Roche Diagnostics)<br>VERSANT HIV-1 RNA 1.5 Assay (kPCR) (Siemens Healthcare)<br>NucliSens® EASYQ® HIV-1 v2.0 (bioMerieux)<br>Abbott RealTi*me* m2000 HIV-1 (Abbott Molecular)<br>Aptima HIV-1 Quant assay (Hologic Inc.) | Antiretroviral therapy monitoring | Used to guide HAART initiation and monitor the treatment efficacy in conjunction with CD4 counting | [36, 38, 62, 65, 66, 75–78, 227, 228] |
| Genotyping | A | 1–3 days | TruGene HIV-1 genotyping (Siemens)<br>ViroSeq HIV-1 genotyping (Abbott Molecular) | Antiretroviral drug resistance determination | Indirect determination by detecting resistance-related HIV gene mutations; may miss low-level mutations | [88, 90–92, 100] |

(continued)

**Table 1** (continued)

| Method | Applicability | Turnaround time | Main devices (manufacturer) | Applications | Comments | Exemplary references |
|---|---|---|---|---|---|---|
| Phenotyping | B | 3–6 weeks | Antivirogram assay (VIRCO Lab)<br>PhenoSense HIV (Monogram Biosciences)<br>Trofile (Monogram Biosciences) | Antiretroviral drug resistance determination | Direct determination by measuring HIV ability to grow in presence of drugs; time-consuming and expensive tests | [123–126, 191–194, 229–241] |
| Molecular near-the-patient testing | B | 1–4 hours | IsoAmp HIV-1 assay (BioHelix)<br>ExaVir Load assay (Cavidi)<br>Liat HIV Quant assay (IQuum)<br>Xpert® HIV-1 Viral Load (Cepheid) | Diagnosis and monitoring of HIV infection in point of care | Include HIV RNA detection and quantification | [70, 82, 83, 158, 170, 183–186] |

[a]A, molecular assay is widely used for clinical diagnosis and/or monitoring of HIV-1 infections; B, molecular assay is useful under certain circumstances or for the diagnosis of particular populations

than 100 copies of HIV-1 RNA per mL [16, 17]. By using pooled plasma, NATs also allow for rapid and high-volume screening in blood banking. Quantitative HIV RNA assays have been used as an alternative for diagnostic purposes due to the scarcity of FDA-approved qualitative NATs and because HIV viral loads in patients with acute infection are usually high [18–23].

Besides being used for blood donor screening, qualitative molecular assays have become the test of choice for establishing the diagnosis of infection in infants born to HIV-1-infected mothers, particularly those assays that allow detection of HIV proviral DNA [24, 25]. The persistence of maternal antibodies against HIV in exposed infants up to 18 months of age prevents the use of antibody-based assays for early diagnosis of HIV infection in these infants. It is important to promptly establish the infection status of an HIV-exposed infant since the effectiveness of highly active antiretroviral therapy at an early age has been demonstrated. Thus, approaches to early diagnosis of infection in infants lean toward those molecular techniques that amplify target HIV DNA [26], RNA [22, 25], or total nucleic acid [20, 21, 23]. In the diagnosis of HIV in infants, the DNA PCR assays possess sensitivities of ≥95% and even higher specificities [24, 27, 28].

## HIV RNA Viral Load Assays

HIV-1 infection results in lifelong persistence of the virus, despite of advances in antiretroviral treatment. In chronically HIV-1-infected patients, the HIV RNA viral load in plasma in conjunction with the CD4 T-lymphocyte cell numbers is the routine biomarkers utilized by clinicians to guide decisions on the highly active antiretroviral therapy (HAART) as well as to monitor treatment effectiveness and the disease's clinical progression. Characterization of HIV-1 RNA levels as being below the limit of detection indicates HAART adherence and effectiveness [29–34]. Periodic monitoring of HIV-1 viral loads can be performed by either HIV RNA amplification or branched chain DNA (bDNA) tests [35]. Technically, less than a threefold variation (0.5 $\log_{10}$ copies) is considered as intra-assay or biological variabilities; however, a change that is over tenfold (1 $\log_{10}$ copies) is considered clinically significant [36–39]. In the clinical setting, the viral load should fall by at least one log within 1 month of an effective regimen. By 4 to 6 months of effective therapy, the viral load should have fallen below the detection limit of the assay, usually less than 50–75 copies/mL [31, 33, 40].

Sensitive measurement of viral load with a wide dynamic range of detection and enhanced ability to detect broad subtypes of HIV-1 group M virus are two major requirements for the quantitative assay for HIV RNA. While HIV-1 subtype B continues to predominate in Western countries, studies now confirm that the incidence of HIV-1 non-B subtypes is increasing all over the world. The ability of a test to detect a broader range of these genetically diverse viruses is therefore crucial to HIV patient care on a global basis. Given the regional epidemiology, commercial assays in the USA are only approved for HIV-1 quantitation (Table 2). They are licensed for monitoring known HIV-1-infected patients. They are not indicated for

**Table 2** Commercial, FDA-cleared HIV-1 viral load assays

| Assay | Manufacturer | Technology | Genomic target(s) | Dynamic range (copies/mL) | Selected references |
|---|---|---|---|---|---|
| COBAS AmpliPrep/ TaqMan HIV-1 Assay | Roche Diagnostics, Indianapolis, IN | Real-time reverse transcriptase PCR with TaqMan chemistry | *Gag* and LTR | 20–10,000,000 | [36, 38, 42, 43, 62–64, 75, 78, 80, 227, 228] |
| VERSANT HIV-1 RNA 1.5 Assay (kPCR) | Siemens Diagnostics, Tarrytown, NY | Real-time reverse transcriptase PCR with TaqMan chemistry | *Integrase* (of *Pol*) | 37–11,000,000 | [35, 36, 62, 65–69, 78] |
| NucliSens EASYQ® HIV-1 v2.0 | bioMerieux, Durham, NC | Nucleic acid sequence-based amplification | *Gag* | 176–3,470,000 | [36, 62, 69, 71–74] |
| Abbott RealTi*me* m2000 HIV-1 | Abbott Molecular, Des Plaines, IL | Real-time reverse transcriptase PCR with partially double-stranded linear DNA probe | *Integrase* (of *Pol*) | 40–10,000,000 | [35, 42, 43, 75–80] |
| Aptima HIV-1 Quant assay | Hologic Inc., San Diego, CA | Transcription-mediated amplification | *Pol* and LTR | 30–10,000,000 | [81–83] |

use as HIV screening tests nor HIV confirmatory tests, yet they have often been used in this context [19–23, 35, 41]. Performance may vary significantly among HIV viral load assays with respect to subtype(s). HIV viral diversity in patient population must therefore be considered in selecting the viral load platform, and the same format should be kept for routine laboratory services to facilitate the monitoring of HIV-1-infected patients [35, 42, 43]. None of the currently FDA-approved viral load assays detect HIV-2.

Plasma is the main specimen type for HIV-1 viral load testing. Since HIV viral RNA is relatively unstable, plasma needs to be separated from whole blood specimens within 4–6 hours of collection, transferred to a secondary tube before freezing and transportation [44–47]. Non-plasma specimens such as peripheral blood mononuclear cells (PBMCs), saliva, cerebrospinal fluid, seminal fluid, dried plasma, and dried blood spots have been evaluated for HIV-1 viral load testing [41, 48–53]. Dried blood spots have been used at rural and remote healthcare facilities to collect and transport specimens for HIV-1 RNA viral load monitoring [54–58]. When specimens are carefully processed, viral load results are stable and reproducible, and cross-contamination can be avoided [59–61].

The Cobas Amplicor HIV-1 Monitor assay (Roche Diagnostics, Indianapolis, IN) is a reverse transcription (RT)-PCR-based system targeting HIV-1 RNA [36, 38, 62]. Two basic assay platforms were developed, (i) the Amplicor HIV-1 Monitor assay, which is a manual test performed in microwell plates and (ii) the Cobas AmpliPrep/ Cobas TaqMan HIV-1 assay, which provides two-step automated approach: full automation of the nucleic acid extraction followed by real-time PCR amplification. Its first FDA-approved HIV viral load test in 1996 measures viral loads at levels

as low as 400 HIV-1 RNA copies/mL. The Amplicor UltraSensitive test, approved in 1999, uses a slightly different sample processing protocol and measures viral loads down to 50 HIV-1 RNA copies/mL. The current Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 system (version 2) has a reported lower and upper limit of quantification of 20 and 10,000,000 RNA copies/mL, respectively. The system employs probes that target both the *gag* gene and the *LTR* region of the HIV-1 genome, further improving the coverage for all major subtypes of HIV-1 group M and HIV-1 group O [42, 43, 63, 64].

The bDNA-based test known as VERSANT HIV-1 RNA 3.0 assay (Siemens Healthcare, Tarrytown, NY) provides good reproducibility since no amplification variation is expected due to its signal amplification technology [65, 66]. Without extraction steps to isolate HIV-1 RNA, the reproducibility of the bDNA assay has been reported to be a superior test, particularly at the low end of the dynamic range [36, 66]. The influence of inhibitory substances contained in a variety of clinical specimens is much lower in comparison with other methods, and the risk of contamination is reduced as well. This test has good precision across a wide reporting range and can distinguish threefold (0.5 $\log_{10}$) changes across the entire assay range [36, 62, 67]. The bDNA test can also be used to determine quantification of the viral load down to 75 copies/mL [68, 69]. The disadvantages from the intrinsic bDNA technique include the requirement for a large volume of plasma, the absence of an internal quantification standard for each sample tested, and lower specificity compared to target amplification methods. The VERSANT HIV-1 RNA 1.5 assay (kPCR) was the latest offering from Siemens Healthcare before it was discontinued recently. The assay employs TaqMan chemistry for detection and is performed on an open-channel designed system, enabling customization of laboratory-developed and third-party assays. It targets the integrase region of the HIV-1 *pol* gene and yields an improved dynamic range of 37 to 11,000,000 copies/mL per 500uL sample volume [70].

The NucliSens® HIV-1 RNA QT assay (bioMerieux, Inc., Durham, NC) incorporates three key technologies: silica-based nucleic acid extraction, nucleic acid sequence-based amplification (NASBA) for HIV RNA amplification, and electrochemiluminescence detection and quantification of the amplified RNA [62, 71]. The NASBA technology is a sensitive, isothermal amplification method that does not require a thermocycler, so there is no need for heat-stable enzymes. The NucliSens assay is highly sensitive in detecting HIV-1 RNA at low concentrations and reaches a broad linear dynamic ranging from 51 to 5,390,000 copies/mL [72, 73]. The current generation NucliSens EasyQ® HIV-1 v2.0 assay works on both human EDTA plasma and EDTA whole blood spotted on cards (DBS) and consists of nucleic acid amplification combined with a simultaneous detection step. The assay can be used for measuring viral loads at other body sites because the RNA extraction procedure, which is performed on the NucliSENS® easyMAG system, consistently generates RNA products that are free of interfering substances [36, 69, 74, 75]. The isothermal process runs at 41 °C which is lower than the annealing temperature of the primers used, resulting in a lower specificity of the amplification process.

The Abbott RealTi*m*e HIV-1 RNA operated by the *m*2000 system (Abbott Molecular Inc., Des Plaines, IL) consists of two components: the m2000sp module that performs nucleic acid extraction and then loads both the processed samples and

the master mix onto the 96-well optical reaction plate and the m2000rt module that carries out the amplification and detection [75–78]. Designed with a partially double-stranded linear DNA probe [79], this assay offers several advantages over conventional viral load assays, including a broad linear coverage, improved sequence variation tolerance, and decreased risk of carryover contamination. The system offers 2-step automation and can detect a wide variety of HIV-1 genotypes including groups M and its subtypes, group N, and group O viruses [42, 75, 77, 78, 80]. It offers a broad linear range of HIV-1 detection between 40 and 10 million copies/ml, with the lower limit of detection of 40 copies/ml based on the processing of 0.6 ml or 1.0 ml sample volume [35, 43].

Recently, a new trend of combining a single lab test designed for both HIV laboratory diagnosis and treatment monitoring has emerged. The Aptima HIV Quant DX assay developed by Hologic is based on transcription-mediated amplification technology and uses a dual target (*pol* and LTR) approach against highly conserved regions in the HIV genome, ensuring the detectability across HIV-1 groups and subtypes [81–83]. The assay runs on the Panther system, an integrated platform that fully automates all aspects of testing, from sample to result [81]. It substantially reduces hands-on time and provides random and continuous access with rapid turnaround time. The assay has an impressive LLOD of 17.5 copies/mL and a linear range of detection between 30 and $10^7$ copies/mL. At present time, this system is only approved by the FDA for viral load monitoring in the USA for plasma specimens, but studies have been reported on other specimen types [84].

## Antiretroviral Susceptibility Testing

Antiretroviral treatment has become a very effective means of controlling HIV from both the patient and transmission perspectives. One of the inherent difficulties with HIV care is the development of mutations that may confer drug resistance to multiple classes of HIV antivirals. HIV care providers must then monitor for these mutations to ensure treatment efficacy in their patients. Infections with resistant HIV are prevalent with surveys in North America and Europe showing that 8–20% of HIV infections in untreated people contain primary drug resistance mutations [85]. A report from the WHO estimates that in the developed countries of Australia, Japan, and the USA, 10–17% of ARV-naïve individuals are infected with virus that is resistant to at least one antiretroviral drug. Prevalence of drug resistance in low- to middle-income countries increased to 6.3% from 2003 to 2010 [86]. A CDC study showed that prevalence of mutations conferring resistance to any class rose from 15.0% in 2007 to 16.7% in 2010. Prevalence of mutations to a single class rose from 12.6% in 2007 to 14.3% in 2010 [87].

The goal of HAART is to completely suppress viral replication below the detection limit of the most sensitive assay in order to avoid the emergence of drug-resistant virus mutants and to delay clinical progression. Antiretroviral drug resistance is defined as the ability of HIV-1 to replicate in the presence of antiretroviral drugs. In the clinical setting, if a viral load fails to fall adequately, or if it rebounds to a level greater

than 1000 copies/mL, tests for antiretroviral resistance are recommended. Two types of formats are available to determine antiretroviral susceptibilities. Phenotypic resistance assays directly measure viral replication of the patient's virus in the presence of antiretroviral drugs, while genotypic resistance assays detect viral genome mutations known to confer decreased sensitivity to antiretroviral drugs. Both genotypic and phenotypic-based tests are available, but the latter is much more expensive and is usually reserved for patients with prior viral resistance. The clinical utility of HIV susceptibility testing has been evaluated in a number of prospective randomized

**Table 3** Commercial HIV-1 antiretroviral resistance assays

| Device | Manufacturer | Platform | Scope | Additional comments | Selected references |
|---|---|---|---|---|---|
| TruGene HIV-1 genotyping kit | Siemens Diagnostics, Tarrytown, NY | Genotypic | Detection of protease and reverse transcriptase gene mutations | FDA cleared; accuracy varies due to sequence variations of non-B HIV-1 strains | [90, 91, 93, 100, 102] |
| ViroSeq HIV-1 genotyping system | Abbott Molecular, Des Plaines, IL | Genotypic | Detection of protease and reverse transcriptase gene mutations | FDA cleared; accuracy varies due to sequence variations of non-B HIV-1 strains | [93–95, 100, 102] |
| HIV PRT GeneChip assay | Affymetrix, Santa Clara, CA | Genotypic | Detection of protease and reverse transcriptase gene mutations | Potential to detect multiple mutations at lower levels | [109, 110] |
| HIV-1 RT line probe assay | Innogenetics, Ghent, Belgium | Genotypic | Detection of protease and reverse transcriptase gene mutations | Potential to detect multiple mutations at lower levels | [107, 108, 110] |
| PhenoSense, universal, entry, and integrase | Monogram Biosciences, South San Francisco, CA | Phenotypic | Susceptibility determination for reverse transcriptase, protease, entry, and integrase inhibitors | Three separate devices for different drugs | [120–122, 124, 136, 242] |
| Trofile | Monogram Biosciences, South San Francisco, CA | Phenotypic | Susceptibility determination for CCR5 inhibitors | Used prior to initiating therapy | [140–142] |
| Virco TYPE HIV-1 | VIRCO Lab, Inc., Titusville, NJ | Virtual phenotype | Susceptibility determination by using genetic data | Accuracy depends on constant database updates | [128, 129] |

clinical trials [88–95]. The use of HIV-1 susceptibility testing to guide antiretroviral treatment has been reported to be cost-effective (Table 3) [96–98].

Genotypic drug resistance testing has been implemented in clinical guidelines as an important tool to guide therapy changes, overall therapy, and, more recently, initiation of therapy [88, 90–92, 99, 100]. Recommendations from the US Department of Health and Human Services state that initial genotype testing is important for establishing a baseline in a person newly diagnosed with acute HIV [101]. However, the pending genotyping results should not delay the need to start HAART as the regimen can be changed after the genotyping results are available. There are currently two commercial assays available for HIV-1 genotyping: the TruGene HIV-1 genotyping kit and OpenGene DNA Sequencing System (Siemens Healthcare Diagnostics, Tarrytown, NY) and the ViroSeq HIV-1 genotyping system (Abbott Molecular, Des Plaines, IL). Both systems are based on the PCR amplification of reverse transcriptase and protease genes followed by nucleic acid sequencing by the traditional Sanger method. One major difference between the two systems is the sequencing chemistry: ViroSeq uses a four-dye termination system, while the TruGene uses the dye primer system. In addition, 6 samples are needed to analyze patient for ViroSeq, compared with 12 samples for TruGene. The ViroSeq system requires an additional purification step for removal of the dye terminators [102]. A parallel validation revealed that both assays generated an accurate sequence with similarity in overall complexity. While the OpenGene system is limited in throughput, it provides an interpretative report containing information relating mutations to drug resistance [103]. Both systems work well for the USA dominantly circulating HIV-1 groups with varied performance in minor groups. One recent study used the ViroSeq HIV-1 genotyping system to determine drug resistance on a panel of diverse HIV-1 group M isolates circulating in Cameroon. The data revealed that the performance of this assay can be altered by the sequence variation of non-B HIV-1 strains that predominate in African settings [104].

Both systems detect mutations in the reverse transcriptase and protease genes, but do not detect mutations associated with resistance to the fusion inhibitor, integrase inhibitors, and CCR5 inhibitors. Another important limitation of the two genotypic assays is that they are only able to detect mutants that comprise major fractions of the patient's virus; resistant variants must constitute at least 25% of the virus population [99, 105]. Resistant mutations present at low levels missed by standard population-based genotyping assays ultimately can lead to failure of treatment [106]. Two other genotyping formats, the HIV PRT GeneChip assay (Affymetrix, Santa Clara, CA) and the HIV-1 RT line probe assay (Innogenetics, Ghent, Belgium), which have the potential to detect multiple mutations at lower levels, have been reported for the rapid detection of drug resistance-related mutation in HIV genomes [107–110]. Allele-specific PCR [111, 112], single-genome sequencing [106, 111, 113], and ultra-deep sequencing (UDS) [111, 114, 115] have been reported to increase the sensitivity of minority mutation detection. However, due to the innate error rate of reaction enzymes, it has been reported that UDS had limited sensitivity to about 0.5% and may have less utility in treatment-experienced patients with persistent viremia on therapy [116–118]. The majority of low-frequency drug resistance-related mutations

detected using UDS are likely errors inherent to UDS methodology or a consequence of error-prone HIV-1 replication [119].

Major commercial reference laboratories in the USA have also developed their own HIV resistance tests based on the same principles described above. Their testing services are useful for those labs that are unable to afford the costs of implementing and maintaining the testing in-house. Additionally, these molecular-based assays require expertise in molecular testing that may not be readily available in smaller, community-based healthcare institutions.

Phenotyping assays measure the ability of HIV-1 to grow in the presence of various concentrations of antiretroviral agents. Phenotyping assays are considered a molecular method as well since recombinant viruses are generated and used in the testing. Phenotyping uses clinical cutoffs associated with treatment outcome data and estimates the net effect of multiple mutations more directly [120–122]. The current procedure involves recombinant DNA technology. HIV-1 RNA from patient's plasma is extracted, and protease and reverse transcriptase genes are amplified by RT-PCR. The amplified gene fragments from the patient's specimen are then inserted into HIV-1 vectors. The recombinant HIV-1 replication is measured by a reporter gene system [123–126]. Data from these assays are relatively simple to interpret, and a report format of 50% or 90% inhibitory concentrations ($IC_{50}$ or $IC_{90}$) is familiar to clinicians. However, phenotyping requires accurate fold-change clinical cutoff values for prediction of response. The test also requires longer turnaround time as it is labor intensive and technically complex. As with genotypic testing, the phenotypic assays can only detect mutant variants that comprise at least 25% of the viral population. More data needs to be collected for evidence of clinical utility for phenotyping rather than for genotyping [127]. Currently, two HIV-1 phenotyping assays are commercially available: the Antivirogram assay offered by VIRCO Lab (Bridgewater, N.J.) [123] and the PhenoSense® HIV assay by Monogram Biosciences (San Francisco, CA), which is now part of LabCorp [124].

A virtual phenotyping assay has also been described that provides an estimation of the phenotype by averaging viruses with similar genotypes [128, 129]. The GenPherex study showed favorable equivalence in 106 patients when virtual phenotyping results were compared to phenotype testing [130]. Hammer et al. also compared virtual phenotyping to rule-based genotype algorithms and showed that the virtual phenotype baseline was more predictive of virological failure than the genotypic data [131].

As integrase and entry/fusion inhibitors, which block HIV-1 before it enters the human immune cell, have become available as antiretroviral therapy, natural resistance to enfuvirtide among different HIV-1 subtypes and HIV-2 have been reported [140, 141]. As a result, HIV-1 resistance assays have been extended to detect mutations that render viruses resistant to integrase and entry/fusion inhibitor therapies [132–135]. The PhenoSense® Entry and the PhenoSense® Integrase assays (Monogram Biosciences, San Francisco, CA) are used to determine viral phenotype resistance to entry inhibitors and integrase inhibitors, respectively [136, 137].

Another class of antiretroviral drugs, including maraviroc [138], targets the binding of HIV-1 with CCR5, a host immune cell surface marker [139, 140]. Prescreening

with a phenotypic and/or genotypic tropism assay such as the Trofile® assay or its newer version, Trofile® DNA, by Monogram Biosciences (San Francisco, CA) [140–142], or the upgraded SensiTrop II test by Pathway Diagnostics (Malibu, CA) [143, 144], is routinely performed to identify candidate patients infected with exclusively R5 HIV as the viral population that uses CXCR4 would unlikely be affected by the CCR5-targeting therapy. An international HIV-1 coreceptor proficiency panel test results demonstrated that genotypic tropism prediction is a safe procedure for clinical purposes [145].

The utility of next generation sequencing (NGS), also called "massive parallel" or "deep" sequencing, has been applied to many aspects of infectious disease testing for both diagnostic and epidemiologic purposes [146]. NGS offers the ability to further discriminate the presence of HIV subpopulations that may not have been detected with the traditional genotypic and phenotypic methods. NGS is more sensitive than standard Sanger sequencing for detecting minority drug-resistant variants. This ability to detect subpopulations that may harbor resistance mutations is important for the treating healthcare provider. GenoSure Archive® by Monogram Biosciences, for example, is now offered to patients with low or undetectable plasma viral load to analyze archived HIV-1 proviral DNA embedded in host cells during replication, providing resistance data for sustained suppression management. Nevertheless, as with any new technology, NGS needs to overcome a number of technical and operational challenges before becoming a mainstream technology for routine diagnostic care [147, 148]. The development of highly accurate and predictive software algorithms will be a critical component to further support the use of NGS as a tool for monitoring HIV treatment regimens. Technological advances and improvements will certainly transform assays that were once considered only for basic research laboratories into the translational/diagnostic laboratories.

## Point-of-Care Testing

Point-of-care testing (POCT) is defined as testing at a site where the patient is present and is conducted at workplaces, pharmacies, physicians' offices, outpatient clinics, emergency rooms, patient bedsides, home, disaster sites, and remote areas. The need of POCT has been driven by the practitioner's need to make rapid, evidence-based, therapeutic action at or near the site of patient care [149]. This diagnostic approach has also been deployed for infectious diseases testing for several years now. Numerous products are commercially available for the POC diagnosis of viral, bacterial, and parasitic infections [150]. Efforts have been spent in developing POCT devices including HIV antibody and nucleic acid detection as well as CD4 and HIV viral load quantification for the diagnosis and monitoring of HIV infections in resource-limited settings [151]. Since early diagnosis can profoundly impact the healthcare and survival of infected/high-risk individuals and because the time required for conventional testing remains a barrier in many settings, rapid HIV antibody testing has been developed for use at the point of care. An ideal molecular

POCT in the diagnosis of HIV-1 infections should possess relatively good sensitivity with low cost and a very quick turnaround time [152].

Molecular tests are sensitive and can advance the detection period to 8–10 days [153, 154]. At the time of preparation, there are no real molecular POC devices approved by the FDA yet; some nucleic acid detection platforms have potential applications as an "instrument-free" means of HIV nucleic acid amplification and detection. A helicase-dependent amplification (HDA) was developed in which helicases are used to separate DNA strands rather than heat; it simply relies on DNA polymerase to amplify DNA rather than on the combinations of polymerases with other enzymes [155, 156]. This greatly simplifies the enzymology involved in the amplification process, while keeping the advantage of all isothermal amplification technologies. An IsoAmp HIV-1 assay (BioHelix Corp, Beverly, MA) was developed targeting the HIV-1 *gag* gene using the isothermal RT-HDA and a disposable amplicon containment device with an embedded vertical-flow DNA detection strip to detect the presence of HIV-1 amplicons [157]. The vertical-flow DNA detection strip has a control line to validate the performance of the device as well as a test line to detect the analyte. The preliminary limit of detection of the IsoAmp HIV assay is 50 copies of the HIV-1 Armored RNA (Asuragen, Austin TX) that were input into the IsoAmp HIV reaction [158].

In addition to the rapid diagnosis of HIV infections by instrument-free molecular methods, other simple and user-friendly systems have been developed for antiretroviral therapy monitoring near patients. Two smaller flow cytometers (Point-Care and Easy CD4 Analyzer) have been created targeting global health and point-of-care applications with limited functionality [159]. Another simple image cytometer for CD4 enumeration has recently been described and used on HIV-1-infected patients in Thailand [160]. Simple microfluidic approaches merged with rapid detection and counting can capture CD4 cells selectively by fluorescent labeling or label-free techniques [161]. Two groups have reported the development of counting microfluidic chips with lensless imaging to target CD4 cell counts for HIV point-of-care testing in resource-limited settings [162, 163]. Semiconductor quantum dots are integrated into a nanobio chip for enumeration of CD4+ T cell counts at the point of care [164]. A recent study described the use of three POCT devices, including Pima CD4 (Alere Inc., Waltham, Massachusetts, USA) for CD4 counting for accurate CD4 T-cell enumeration and antiretroviral drug toxicity monitoring in primary healthcare clinics in Mozambique. The results indicated that POC diagnostics to monitor antiretroviral therapy at primary healthcare level is technically feasible and should be utilized in efforts to decentralize HIV care and treatment [165].

Each molecular diagnostic device can be divided into nucleic acid extraction, amplification, and detection components [166]. A rapid, point-of-care extraction of HIV-1 proviral DNA from whole blood was reported and used for detection by real-time PCR [167]. Simple and inexpensive molecular assays based on dipstick and zipper technology have been described [168, 169]. The Cepheid Xpert HIV-1 Viral Load assay (Sunnyvale, CA), a single-use sample processing cartridge with integrated multicolor real-time PCR capacity, provides an efficient alternative method for HAART monitoring in clinical management of HIV disease in resource-limited

settings. The rapid test results (<2 h) could help in making an immediate clinical decision [70, 82, 83, 170]. Integration of microfluidics and lensless imaging for point-of-care testing has been reported in HIV-1 point-of-care clinical diagnostics [171]. With incorporation of micro−/nano-fabrications/crystals (e.g., quantum dots), microfluidics and array-based systems will enable the development of more feasible immunological and molecular tests for HIV POCT in resource-limited settings [172–177].

There is an urgent need for low-cost, simple, and accurate HIV-1 viral load monitoring technologies in resource-limited settings, particularly when scaling up first- and second-line highly active antiretroviral therapies [178–180]. The Liat HIV Quant assay (IQuum, Marlborough, MA) is comprised of two components, the Liat Analyzer and the Liat Tube (IQuum), that provide rapid and automated sample-to-result HIV load tests in the near-patient setting within 1.5 hours. The assay demonstrated linearity of 6 logs and a limit of detection of 57 copies/mL and covered HIV-1 group M (clades A-H), group O, and HIV-2 [181, 182]. Besides nucleic acid amplification techniques, ExaVir Load assay (Cavidi, Sweden), which requires simple equipment to perform the modified enzyme-linked immunosorbent assay (ELISA) format to measure viral reverse transcriptase (RT) activity in a simple laboratory environment, correlates with plasma RNA levels [183–187]. Only standard ELISA equipment, together with the ExaVir Load start-up equipment, is required for analysis. The separation equipment used in the current Version 3 makes the procedure less time-consuming, more efficient, and easier to handle than ever. Using a similar principle, the system has been used for HIV phenotypic susceptibility testing (ExaVir Drug assay), which worked well for efavirenz but not for nevirapine [187].

## Host Genetic Testing

Enhanced by the human genome programs, diagnostic virologists envisioned the utilization of genetics beyond HIV-1 genomes to help manage HIV infections [188]. If infections, especially chronic and persistent HIV-1 infections, can be viewed as “horizontally acquired” genetic diseases, it makes sense to view pathogen and host as an integrated system. Increasing evidence indicates that the outcome of HIV infections is influenced by the genetic background of the host [189, 190]. Earlier pioneer studies revealed that resistance to HIV-1 infection, both in vitro and in vivo, has been associated with an internal 32-base-pair deletion in the human chemokine receptor CCR-5 gene [191, 192]. Accordingly, detection of host polymorphisms in the HIV diagnostic field can help identify those at risk of rapid disease progression and help with the timing of the initiation of treatment. Allele frequencies and relative hazard values of CCR5-Δ32, CCR2 64I, CCR5 P1, IL-10 5’A, HLA-B*35, and HLA homozygosity were determined to generate a composite relative hazard of progression to AIDS [193]. Possession of a CCL3L1

copy number lower than the population average is associated with markedly enhanced HIV/AIDS susceptibility, which is enhanced in individuals who possess the CCR5-Δ32 genotype [194]. Genetically, polymorphic profiles in cytochrome P450s and transporters facilitate the optimal chemotherapy for HIV infections; therefore, host genomic testing can be used as a tool to optimize drug therapy for HIV infections [195]. Genome-wide association studies found that HLA-B*5701 allele was associated with severe hypersensitivity reactions to abacavir, and CYP2B6*/6* allele was associated with the central nerve system disorders to efavirenz in patients with HIV-1 infection [196].

Polymorphism analysis can also be performed on hundreds of samples in parallel using a powerful, high-complexity microarray technique [197]. Recent technological advances make it now possible to genotype over one million polymorphisms for thousands of samples by using either the Illumina or Affymetrix system [198, 199]. While more and more HIV infection resistance and disease progression-related host gene polymorphisms have been demonstrated, simple, user-friendly techniques for the detection of such known mutations will soon be adapted into the clinical diagnostic field. Currently used techniques include allele-specific nucleotide amplification [200, 201], single nucleotide primer extension [202], and the oligonucleotide ligation assay [203, 204]. PCR-led amplification technology has been important for these methods since it is either used for the generation of DNA fragments or is part of the detection method. Real-time PCR assays based on TaqMan hydrolysis probes have been used as confirmatory methods, which are very robust but less cost-effective for larger-scale studies [205, 206]. DNA sequencing remains the gold standard and is enhanced by high-throughput processing and deep production scaling and is now considered the most powerful procedure for polymorphism detection [78, 207, 208].

Microarray test, developed by Affymetrix (Santa Clara, CA), has allowed the host transcriptome analyses in individuals with HIV-1 [197, 209]. A comprehensive review of the 34 studies involving HIV-1 and microarrays in the 2000–2006 period concluded that these studies yielded important data on HIV-1-mediated effects on gene expression, providing new insights into the intricate interactions occurring during infection [210]. Several recent studies have demonstrated progress in expanding the pool of target genes and understanding the functional correlates of gene modulation to HIV-1 pathogenesis in vivo [188, 211]. It is predicted that these host transcriptome profiles will be used for the assessment of disease progression and prognosis. The precision of transcriptome analyses will be greatly improved through the added resolution of the RNA-Seq approach, which uses deep-sequencing technologies for transcriptome profiling [212, 213].

In addition to CD4 cell counting, other host responses can be used as a companion diagnosis for monitoring therapy efficacies and side effects in HIV-infected patients receiving antiretroviral therapy [214, 215]. Quantification of T-cell-receptor-chain rearrangement excision circles (TREC) present in naïve T cells is considered to be an accurate measure of thymic function. HIV infection leads to a decrease in thymic function that can be measured in the peripheral blood and lym-

phoid tissues. In adults treated with HAART, there is a rapid and sustained increase in thymic output in most subjects, indicating that the adult thymus can contribute to immune reconstitution following antiretroviral therapy [216–218]. Blockade of the PD-1/PD-1-ligand 1 pathway restores CD8 T-cell function and reduced viral load, indicating a potential option to pursue for development of companion diagnostic devices to improve anti-HIV therapies [4].

Mitochondrial toxicity of antiretroviral drugs, particularly the nucleoside reverse transcriptase inhibitor (NRTI), has been postulated to be responsible for the pathogenesis of many secondary effects of HAART, including hyperlactatemia [219, 220]. During HIV antiretroviral therapy, clinically symptomatic mitochondrial dysfunction has been associated with mitochondrial DNA depletion, and a real-time PCR was developed to determine a mitochondrial DNA versus nuclear DNA ratio as a biomarker of NRTI toxicity [221]. The observed increases in mitochondrial DNA and RNA content during the first year of treatment may represent a restorative trend resulting from suppression of HIV-1 infection, independent of the treatment used. Mitochondrial DNA and RNA content in individual cell subtypes, rather than in peripheral leukocytes, may be better markers of toxicity and deserve further investigation [222]. Other assays, which include mitochondrial RNA quantification by real-time PCR [223] and mitochondrial protein synthesis by Western blot immunoanalysis [224], have been described to measure mitochondrial toxicity-related functional changes. A flow cytometric assay was also developed to gauge mitochondrial function. Flow cytometric quantification of a mitochondrial DNA-encoded mitochondrial protein and a nuclear DNA-encoded mitochondrial protein was optimized and validated, which allows simultaneous detection of mitochondrial DNA and nuclear DNA-encoded proteins at the single cell level, offering a method to monitor for mitochondrial function [225].

## Concluding Remarks

The development and application of molecular diagnostic techniques has resulted in major advances in the diagnosis methodologies and monitoring strategies for infectious diseases. Molecular techniques have quickly become the mainstay for laboratory diagnosis and assessment of HIV-1 infections. Qualitative molecular assays are used as the test of choice to diagnose perinatal and acute HIV-1 infections. HIV-1 viral load assays in combination with CD4 cell counts are routinely used to determine when to initiate therapy and when a regimen is failing. HIV-1 antiretroviral susceptibility testing, which includes a genotyping assay that detects mutations known to confer resistance in viral genomes and a phenotypic assay measuring recombinant viral replication in the presence of antiretroviral drugs, has become an essential part of HIV-positive patient management. Point of care, or near-the-patient molecular assays are being developed with the potential to make rapid, evidence-based, therapeutic action at or near the site of patient care. Assessing host gene

polymorphisms and immune responses have emerged in clinical laboratories as supplementary tools for better monitoring of HIV-1 infections.

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# Multiplex PCR for Detection and Identification of Microbial Pathogens

**Mark A. Poritz and Beth Lingenfelter**

## Introduction

Over the last decade, a number of manufacturers have developed multiplexed in vitro diagnostic (IVD) platforms that can detect the nucleic acid signatures of many of the organisms responsible for infectious disease. Some of these testing platforms offer limited test menus (i.e., influenza A, influenza B, and RSV), while others are designed to detect a more comprehensive set of potential pathogens that can cause a particular infectious disease syndrome (e.g., respiratory, gastrointestinal, sepsis, meningitis) [1–3]. This chapter will describe FDA-cleared and/or CE-marked multiplex assays that are designed to detect a comprehensive set of pathogens associated with a particular infectious disease syndrome (≥10 assays/test). These include the BioFire (Salt Lake City, UT) FilmArray® System [4], the GenMark (Carlsbad, CA) eSensor XT-8® [5] and ePlex® [6], and the Luminex (Austin, TX) xTAG® [7], nxTag® [8], and Verigene® systems [9].

In addition to being comprehensive with respect to pathogens responsible for a particular syndrome, these multiplex panels offer the advantage of superior test sensitivity and specificity. Many of these panels have been designed to be easy-to-use, allowing molecular testing to be performed in moderate or low complexity settings and eliminating barriers that prevented many laboratories from being able to perform molecular assays on-site. Another important benefit of multiplex panel is the fast time to result. Molecular multiplex tests require hours to perform instead of the days required for culture-based methods.

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These systems differ in particular details (the exact turnaround time, sample throughput, cost per sample, and number of target pathogens detected). These differences are due to the underlying technologies used for the detection of the pathogen nucleic acid. However, all these systems share the common attribute that the incremental cost of each additional assay in the test cartridge (in materials, labor, and quality control (QC) testing) is small compared to the total manufacturing costs for the disposable. This has enabled IVD manufacturers to develop broad test panels that include organisms which have not been a part of standard testing protocols because of the technical limitations of existing methods.

Nonetheless the availability of syndromic infectious disease panels poses hard questions for clinicians and the healthcare system overall: Does the wealth of information in a comprehensive test improve the treatment of an individual patient, and how can the economic value of this improved treatment be measured?

## Comparison of Multiplex Nucleic Acid IVD Systems

Commercially available FDA-cleared multiplex nucleic acid-based tests for infectious agents include systems from Luminex, GenMark, and BioFire (now a subsidiary of bioMérieux) (Table 1). At present, all such systems combine the sequential steps of:

1. Nucleic acid purification from the appropriate human sample matrix (e.g., nasal swab, blood or blood culture, stool)
2. cDNA synthesis (reverse transcription) to convert viral RNA to DNA, if necessary
3. Multiplex PCR to amplify molecules of the pathogen nucleic acid
4. Specific detection of the expected amplicons to confirm that the correct target nucleic acids have been identified

The different systems vary mainly in whether the nucleic acid purification steps are integrated into the same cartridge that is used for amplification (Verigene, ePlex, and FilmArray) and in how the specific detection of amplicon is achieved. The Luminex xTag and NxTag systems use a fluorescent signal generated after hybridization of the amplicon to fluorescently encoded bead arrays to detect a specific amplicon [10, 11]. The Verigene system uses hybrid capture of the amplicons on a microarray with detection by gold nanoparticle probes [12]. The GenMark eSensor XT-8 and the ePlex systems use electrochemical detection of the target amplicon hybridized to a specific gold microelectrode [13]. The FilmArray system is described in more detail below.

**Table 1** Multiplex nucleic acid tests: FDA-cleared and/or CE-marked

| Manufacturer | System | Technology | Nucleic acid extraction | Detection method | Panels | CLIA complexity | Hands-on time | Test run time | Samples/run |
|---|---|---|---|---|---|---|---|---|---|
| BioFire Diagnostics | FilmArray | Nested multiplex PCR | Integrated | DNA melt analysis | Respiratory<br>Blood culture<br>Gastrointestinal<br>Meningitis/ encephalitis | Moderate | 2–5 min | 45–65 min | 1 |
| Luminex | xTag<br>NxTag | PCR and liquid phase bead array | External | Fluorescently labeled bead array | Respiratory<br>Gastrointestinal | High | 15 min per 24 samples | 4–8 h | 96 |
| Luminex | Verigene | PCR with low-density nucleotide array | Integrated | Hybridized gold nanoparticle probes | Respiratory<br>Blood culture<br>Gram positive<br>Gram negative<br>Gastrointestinal | Moderate | <5 min | 2–3 h | 1 |
| GenMark | eSensor XT-8 | PCR and hybridization | External | Microarray hybridization and solid-phase electrochemical | Respiratory | High | 55 min | 6 h | 18 |
| GenMark | ePlex | PCR and hybridization | Integrated | Microarray hybridization and solid-phase electrochemical | Respiratory | Moderate | <2 min | 90 min | 3 to 24 |

## The FilmArray Pouch and Chemistry

The FilmArray system performs sample-to-answer multiplex nucleic acid testing for infectious disease. To accomplish this, the FilmArray pouch (Fig. 1) integrates all of the steps of nucleic acid purification, nested multiplex PCR amplification, and automated data analysis into a closed system [4]. The pouch is created by welding two sheets of plastic film together with heat in such a fashion that fluid can move between the working areas of the pouch via channels left in the plastic. A hard plastic fitment attached to the film (indicated in Fig. 1b) provides the enzymes and buffers needed to perform the biochemical reactions in the pouch. Other reagents (#1, #2, and #3 in Fig. 1a) are inserted between the film layers during manufacture of the pouch.

The FilmArray reagents are lyophilized in the pouch, and the pouch is stored under vacuum before use. This benefits the end user in two ways. First the freeze-drying process stabilizes the PCR reagents so that the pouch has a shelf life, at ambient temperature, in excess of 1 year – which simplifies the logistics of acquiring and storing the pouches. Second vacuum storage ensures that the wells of the fitment are also under vacuum. Thus the user does not need to control the volume of hydration fluid or sample that is injected into the pouch – which simplifies the steps needed to load a pouch.

Lysis and homogenization of bacteria, spores, and viruses occur in the FilmArray pouch by means of vigorous agitation in the presence of zirconium beads (#1 in Fig. 1a) and a denaturing buffer. DNA and RNA in the sample are purified by binding to silica magnetic beads (#2 in Fig. 1a), the beads are washed to remove proteins and other PCR inhibitors, and the nucleic acids are eluted into a buffer compatible with reverse transcription and PCR [14].

The eluted material hydrates a pill (#3 in Fig. 1a) that contains all of the primers needed for reverse transcription (for RNA targets) and first stage PCR. The primers in this pill are specific to each pathogen target (assay) contained in the pouch.

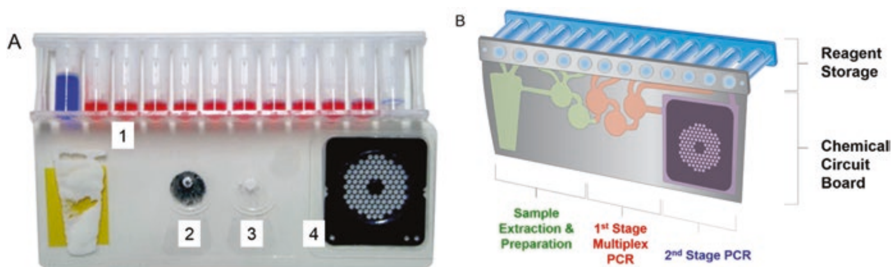


**Fig. 1** The FilmArray Pouch: (**a**) Picture of a pouch hydrated with blue dye (through the left-side sample port) and red dye (through the right-side hydration port). In the film portion of the pouch, white zirconium beads (#1) are located in front of a yellow rectangle of tape which protects the pouch during bead beating. Black silica magnetic beads (#2) bind nucleic acid from the sample. A white pill of PCR primers (#3) is used in the first stage PCR. The black array (#4) contains 102 wells of 1 microliter each for 96 PCR reactions plus control empty wells. (**b**) Schematic of the pouch indicating the different functional sections of the pouch

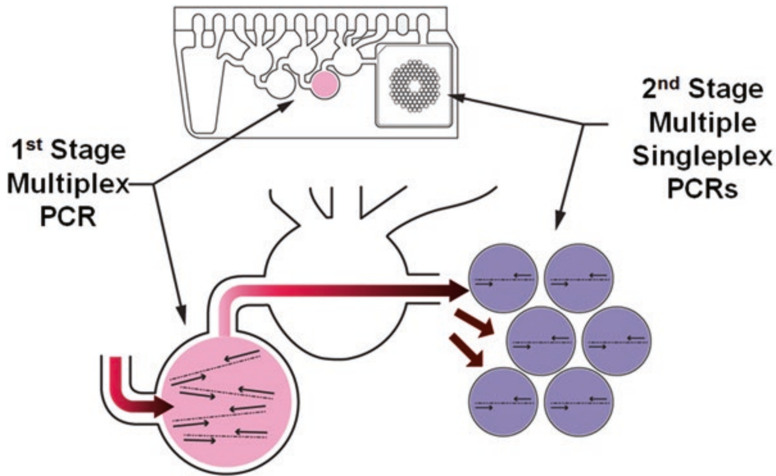


**Fig. 2** Schematic of nested multiplex PCR as performed in the FilmArray pouch. Reverse transcription and the first-stage multiplex PCR occur in a blister of the pouch (shown in pink). Primers spotted into wells of the array (shown in blue) allow singleplex nested PCRs to occur in each well

The FilmArray system performs nested, multiplex PCR in two stages (Fig. 2). Reverse transcription and first stage PCR (PCR1) are performed in the same reaction volume and in a multiplex format, combining primer sets for all assays for a FilmArray panel in one mix. Following PCR1 amplification, the reaction is diluted approximately 100-fold to reduce the concentrations of the outer primers, nonspecific products, and first-stage PCR chemistry. The diluted reaction is then combined with a fresh PCR master mix and flooded over a 102-well array, where a second stage PCR occurs (PCR2). Each well of the array contains assay-specific primers that anneal within the first-stage outer amplicon. At the end of PCR2, a DNA melt curve analysis of the amplicons is performed using a nonspecific DNA-binding dye, LCGreen® Plus [15], that fluoresces in the presence of double-stranded DNA. The presence of a specific melt curve with Tm (melting temperature) in the range predicted for that specific amplicon confirms the presence and identity of the products made in each well.

The mechanics and chemistry of the FilmArray pouch have been described in greater detail elsewhere [4]. Here we highlight some of the features of the system that have contributed to the robustness of the test. The combination of a denaturing lysis buffer and "bead beating" with zirconium beads has been shown to lyse organisms and release intact total nucleic acid (DNA and RNA) from spores [16] as well as from the cerebral spinal fluid (CSF), stool, blood culture media, and whole blood. The nested PCR of the multiplex chemistry makes the FilmArray test remarkably resistant to PCR inhibitors that may remain after nucleic acid purification. Even modest levels of amplification in first-stage PCR can still be detected as an amplicon in the inner, nested product of PCR2. The dilution step following the first-stage amplification reduces the complexity of the input material to the second-stage

reaction enough that, in most cases, the DNA-binding dye detects the presence of a single amplicon in the reaction (the melt curve analysis serves as an additional filter for the correct product).

The FilmArray pouch and instrument contribute to the sensitivity of the overall system in several important ways. First the nucleic acid sample purification starts with a relatively large volume of the sample (100 μl for the RP and GI panels, 60 μl for the BCID), and, nominally, all of the nucleic acid purified from this volume is delivered to the combined reverse transcription – first-stage PCR reaction. Unlike some benchtop protocols, there is no dilution from the purified nucleic acid into an RT step and a second dilution into a PCR. Secondly the pouch is controlled by pneumatic actuators (described below) that can move liquid between blisters of the pouch in seconds. This minimizes the time during which nucleic acid could be degraded or PCR primers could bind to an incorrect DNA or RNA target and thus generate specific "nonspecific" amplicons. To the same end, the FilmArray pouch achieves a true mechanical hot start. In both the first stage and second stage PCRs, the primers do not come in contact with the DNA polymerase/Mg, dNTP mixture until both components are at or above the temperature at which polymerization will take place. This minimizes the formation of primer dimers, and higher-order multiplex primer structures in the first-stage PCR, which compete with the correct amplicons for PCR reagents. This enhances the specificity of the amplification reactions and thus increases the sensitivity of the individual PCR assays.

All FilmArray pouches have at least two internal controls that demonstrate the proper functioning of the pouch. One is a small amount of synthetic DNA spotted onto the second stage PCR array along with primers to amplify this target. This "PCR2" control only monitors the function of the second stage PCR. A more important control for pouch function is generated by freeze-drying a small number of cells of the yeast *Schizosaccharomyces pombe* into the well of the pouch fitment that receives the sample. Nucleic acid purified from these yeast cells must pass through all the steps of the pouch. Outer primers in the first-stage PCR and inner primers spotted onto the second-stage PCR array are designed to amplify a spliced messenger RNA from the *S. pombe* cells (in the FilmArray BCID pouch which does not contain reverse transcriptase, the *S. pombe* target is a genomic DNA sequence). BioFire has shown during the development of several different pouches that artificially induced failure modes that prevent detection of a pathogen organism in a sample also prevent detection of the yeast control.

The FilmArray instrument controls the movement of liquid through the pouch using pneumatically-actuated bladders and seals that force liquid from a pouch blister or prevent liquid from leaving a blister, respectively. The hydrated reagents in the pouch fitment are introduced into the pouch blisters via additional pneumatically actuated pistons.

The amplification reactions are thermocycled using 1 inch square Peltier devices situated adjacent to the first- and second-stage PCRs (#3 and #4 of Fig. 1a, respectively). To detect the melting of the second-stage PCR amplicons, a blue LED illuminates the array, and a camera with a filter to detect green light observes the signal generated by the DNA-binding dye LCGreen® Plus.

## Clinical Utility of Multiplex Panels

Multiplex panels are particularly attractive to clinicians because they provide a comprehensive and accurate test result in a short period of time, and the test panels have been designed to match the clinical syndrome (i.e., respiratory infection, infectious gastroenteritis). However, given the increased cost of these tests, it is important to demonstrate their impact to patient management and their cost-effectiveness. It is intuitive to believe that a rapid, accurate, and comprehensive diagnostic test should improve patient management by shortening the time to the most effective therapy, by preventing inappropriate therapy (especially empiric antimicrobials), by reducing additional diagnostic testing (e.g., imaging studies), by improving the use of infection control measures, and by reducing patient length of stay. Of particular importance, these tests can reduce the unnecessary use of empiric antimicrobials by reducing the time to pathogen-directed therapy. Use of broad-spectrum antibiotics for patients with serious illnesses and the inappropriate use of antibiotics in the outpatient setting "just in case" are important drivers of antibiotic resistance which is one of the major healthcare threats of our time. In this section, we will review what is known about the clinical utility and cost-effectiveness of these multiplex panels. Because the clinical implication for each syndromic panel is different, the discussion is presented by syndrome.

### *Respiratory Multiplex Panels*

Currently there are three vendors with multiplex respiratory panels that are both FDA-cleared and CE marked (BioFire Diagnostics, Salt Lake City, Utah; Luminex, Austin, Texas; and GenMark, San Diego, California) and several more that are CE-marked (Seegene, Seoul South Korea; Curetis, Holzgerlingen, Germany, Fast-Track Diagnostics, Sliema, Malta). These panels include assays for several viral pathogens (e.g., influenza, respiratory syncytial virus (RSV), human rhinovirus, parainfluenza viruses, adenovirus, coronaviruses, etc.) and some also include selected bacterial targets (e.g., *Mycoplasma pneumoniae*, *Bordetell*a spp, *Legionella pneumophila*). All of the FDA-cleared test are limited to testing nasopharyngeal swab (NPS) samples, while several of the CE-marked tests include a larger range of respiratory sample types (e.g., bronchoalveolar lavage, sputum, etc.).

Prior to the availability of multiplex respiratory panels, testing for viral respiratory pathogens relied on viral culture, direct fluorescent antigen (DFA) testing, enzyme immunoassays (EIAs), and traditional PCR assays. While viral culture was the gold standard, it has several limitations, including that it is a complex test requiring highly skilled laboratory workers to both set up the test and interpret the results, it is slow (taking days to complete), and only a limited number of human pathogens can be grown in viral cultures. DFA tests can be performed directly on the patient sample and can have a fast turnaround time; however, these tests are also technically

complex, and the range of pathogens is limited. EIA assays can be performed in a variety of ways and are used for rapid antigen tests. Rapid antigen tests are fast and simple to use but are known to have poor test sensitivity and a limited test menu. Traditional PCR assays are highly sensitive and specific; however, they are complex and require highly trained laboratory staff and specialized laboratory facilities. In addition, each test is ordered independently placing a large burden on the ordering clinician to select the correct test or to order multiple individual tests. Due to their complexity, these tests are commonly sent to specialized reference laboratories which can increase cost and slows the time to result. The introduction of multiplex respiratory panels has allowed for comprehensive testing (ability to test viral pathogens that do not grow in cell culture and the ability to simultaneously test for bacterial and viral pathogens) in a shorter time frame. The easy-to-use systems allow the testing to be performed by laboratory workers without specialized molecular skills and in laboratories without specialized equipment and facilities. These systems allow testing to be performed closer to the patient and therefore further reduce the time to test result by reducing the need to transport samples and eliminating the delays associated with batch testing.

Multiplex respiratory panels have the potential to improve patient management and lower overall healthcare costs by improving use of influenza antivirals, reducing inappropriate use of antibiotics and antivirals, reducing use of healthcare resource (e.g., additional laboratory or imaging procedures), informing appropriate infection control practices, and reducing length of hospital, emergency department, and intensive care unit (ICU) stay.

Several studies have evaluated the effect on patient and healthcare outcomes linked to the use of a multiplex respiratory panel. Xu et al. [17] showed that replacing DFA testing with on-demand use of the FilmArray RP for children presenting to the emergency department (ED) resulted in dramatic reductions in test turnaround time (7 vs 1.4 h), timely (defined as within 3 h of discharge from the emergency department) administration of oseltamivir for 81% of patients testing positive for influenza, effective use of cohorting for admitted patients, and a potential saving of 900 h of ED boarding time. Similarly, in a pre-/post-intervention study of pediatric patients admitted through the ED, Rodgers et al. [18] compared several outcome measures when the FilmArray RP replaced the use of three clinician-ordered traditional PCR tests (Prodesse assays for, FluA/B/RSV, PIV 1,2,3, and hMPV). The study demonstrated that use of the FilmArray RP resulted in a significant increase in the number of patients with positive test results (77.9% vs 59.8%, $p < 0.001$) and a 65% reduction in time to result (6.38 vs 18.65 h, $p < 0.001$) when compared to use of traditional PCR tests. These improvements lead to a mean reduction in length of hospital stay of 0.3 days for patients with positive test results, reduced duration of antibiotics for patients with positive test results (2.7 vs 3.2 days, $p < 0.001$) or when results were reported in <4 h (2.8 vs 3.2 days, $p < 0.001$), and an overall reduction in healthcare costs of $231/patient. Another study of 4779 pediatric patients reported significant reductions in the duration of antibiotic use (4 vs 5 days, $p < 0.01$), use of chest radiographs (59% vs 78%, $p < 0.01$), and an increase in appropriate use of isolation measures [19].

Similar findings have been observed in studies of adult patients. Brendish et al. [20] performed a prospective randomized study of adult patients presenting to the emergency department (ED) with respiratory symptoms over two respiratory seasons. In the control arm, patients were tested for respiratory viruses at the clinician's discretion using nine traditional PCR assays performed at a reference laboratory. In the intervention arm, all patients were tested with the FilmArray RP, and testing was performed in the ED. The study demonstrated the expected increase in pathogen detection (45% vs 15%, $p < 0.0001$) and reduction in time to result (2.3 vs 37.1 h, $p < 0.0001$) but failed to show the expected reduction in the proportion of patients that received antibiotics (84% vs 83%, $p = 0.96$). However, there was an increase in the proportion of patients that received a short course of antibiotics (< 48 h, 17% vs 9%, $p = 0.0047$) especially for patients with positive FilmArray RP test results. Patients in the intervention group also had a mean reduction in hospital length of stay of 1.1 days (5.7 vs 6.8 d, $p = 0.0443$) with the shortest length of stay observed in patients with positive FilmArray RP results. The use of influenza antivirals was the same in both groups (18% vs 14%, $p = 0.16$); however, the intervention group had a significant increase in the number of influenza-positive patients that received influenza antivirals (82% vs 47%, $p = 0.0001$) and a reduction in the use of antivirals for patients that were influenza-negative (18% vs 53%). In another study evaluating adult patients with a positive influenza result on a multiplex respiratory panel, Rappo [21] reported a significantly lower odds ratio for hospital admission ($p = 0.046$), a reduced length of stay ($p = 0.040$), reductions in antimicrobial duration ($p = 0.032$), and a reduction in the number of chest radiographs ($p = 0.005$).

There is currently only one study evaluating the use of multiplex panels in an outpatient setting. Greene et al. evaluated the difference in use of antibiotics and antivirals for adult outpatients tested with the FilmArray RP that were [1] positive for influenza, [2] positive for non-influenza pathogen, or [3] negative for all pathogens [22]. They observed significant increases in the use of influenza antivirals (81.0% vs 5.5–2.5%, $p < 0.001$) and reduced use of the antibiotics (29.5% vs 48.6–49.3%, $p = 0.005$) for individuals with a positive result for influenza. However, detection of non-influenza viral pathogens did not lead to a reduction in the use of antibiotics when compared to those with no pathogen detected (48.6% vs 49.3%). The authors suggest that either influenza testing alone is more cost-effective for this patient population or that additional education and/or antibiotic stewardship is needed to drive appropriate use of antibiotics in the outpatient setting.

## *Blood Culture Panels*

Blood culture panels are designed to test positive blood cultures with the aim to provide a faster time to organism identification. In addition, these panels include assays for selected antibiotic resistance genes providing important information to guide antibiotic therapy. There are currently three vendors with multiplex blood

culture panels that are both FDA-cleared and CE-marked (BioFire Diagnostics; Luminex and Accelerate Diagnostics, Tucson, Arizona), while GenMark (San Diego, California, USA) and Curetis (Holzgerlingen, Germany) have panels that are CE-marked. These panels include assays for gram-positive bacteria, gram-negative bacteria, yeast, and antibiotic resistance markers or, in one case (Accelerate Pheno™ System), antibiotic susceptibility test results. The Curetis Unyvero BCU Blood Culture Application Cartridge® panel is the most comprehensive (with identification of ~100 bacteria and 16 resistance markers) followed by the BioFire FilmArray Blood Culture Identification Panel (identification of ~27 bacteria and 3 resistance markers). The GenMark ePlex and Luminex Verigene systems provide separate panels that are specific to gram-positive bacteria, gram-negative bacteria, or yeast with selection of the appropriate panel determined by blood culture gram stain. All of these tests can be used with a variety of different blood culture media and blood culture systems.

Prior to the availability of multiplex blood culture panels, pathogen identification was performed using classic standard culture-based systems with phenotypic (or MALDI-TOF) identification followed by traditional growth-based antimicrobial susceptibility testing. These tests are the gold standard methods and are very reliable; however, they suffer from a slow time to result (1–3 days after the positive blood culture) and technical complexity. Molecular assays for specific pathogen identification, such as *Staphylococcus aureus* and *Enterococci*, have been in use for some time and have shown improvements in patient outcomes [23–26]. Another method that reduces time to organism identification is using MALDI-TOF to identify bacteria directly from minimally processed blood cultures without the need to subculture to agar plates. The MALDI-TOF identification is very comprehensive, and the reduced time to bacterial identification has also been shown to improve patient outcomes [27, 28].

While individual molecular assays and MALDI-TOF identification have both been shown to improve patient outcomes, they are both technically complex and require specialized skills. As a result, these test methods are typically performed during standard laboratory working hours and are typically not used on night shifts when staffing is limited.

Molecular multiplex panels offer the advantage of a fast time to organism identification along with ease of use. All of the FDA-cleared and CE-marked panels use unprocessed blood culture media and provide identification results within 1–7 h of test initiation. Due to the ease of use, these panels can be used by laboratory staff without specialized molecular biology skills; however, because these panels do not identify all possible pathogens, the results must be interpreted in conjunction with the blood culture gram stain, and traditional culture and sensitives must still be performed. Furthermore, appropriate adjustments to antimicrobials rely on proper test interpretation and a good understanding for the local patterns of antimicrobial resistance (e.g., antibiogram). Treatment adjustments should be based on local guidelines developed by an antimicrobial stewardship program (ASP) team with an in-depth understanding of the capabilities of the test being used, the local antibiogram, and the local patient populations.

As with the individual molecular assays and the MALDI-TOF identification, numerous studies have shown that use of multiplex molecular blood culture panels dramatically reduces the time to organism identification [29–32] which drives more appropriate pathogen-directed therapy. Pathogen-directed therapy includes antibiotic escalation (the addition or change of dose when the current therapy is ineffective against the identified organism) and antibiotic de-escalation (discontinuation of unnecessary empiric antibiotics). A prospective randomized study conducted at the Mayo Clinic showed that antibiotic escalation occurred more quickly with or without real-time ASP oversight (5 h with BCID and ASP, 6 h with BCID only, and 24 h without BCID or ASP, $p = 0.04$); however, optimal antibiotic de-escalation requires oversight by an ASP (21 h with BCID and ASP, 34 h with BCID only, and 38 h without BCID or ASP, $p < 0.001$) [29]. The finding that multiplex molecular blood culture panels paired with an ASP results in a faster time to optimal antibiotic therapy (most narrow effective therapy) has been confirmed in several additional studies [31, 33]. The use of molecular multiplex blood culture panels has also been shown to reduce unnecessary treatment due to contaminated blood cultures [29, 34]. Reducing the use or duration of unnecessary antibiotics is important to reducing the incidence of antibiotic resistance. The cost of molecular multiplex blood culture panel and the fact that they do not replace existing testing have been raised as reasons to not use them; however, studies of the overall healthcare cost prove the panels to be cost neutral [29–31] or to reduce overall healthcare cost [30, 31, 34–37].

While the evidence is strong that multiplex panels dramatically reduce time to organism identification and time to optimal antibiotic therapy, the evidence is inconsistent with regard to reductions in patient mortality and length of hospital stay, mostly likely because the management of patients with sepsis is complex and multifactorial. However, a recent meta-analysis [38] of studies using rapid molecular methods to test positive blood cultures found a small but statistically significant reduction in patient mortality when the results were used as part of an ASP (OR 0.64, 95% CI 0.51–0.79) but not when used outside of an ASP (OR 0.72, 95% CI 0.46–1.12). The improvements were seen for patients with gram-positive (OR 0.73, 95% CI 0.55–0.97) or gram-negative bacteremia (OR 0.51, 95% CI 0.33–0.78), but not for patients with candidemia (OR 0.90 95% CI 0.49–1.67). The study also found that time to effective therapy decreased by a weighted mean difference of −5.03 h (95% CI −8.60 to −1.45) and that length of hospital stay decreased by −2.48 days (95% CI −3.90 to −1.06).

## *GI Panels*

Currently there are three vendors with multiplex gastrointestinal panels that are both FDA-cleared and CE-marked (BioFire Diagnostics; Luminex, and BD, Sparks, Maryland) and several more that are CE-marked (Seegene, Soul, South Korea; Mobidiag, Finland; Serosep, Limerick, Ireland). Some of these panels include assays for bacteria, viruses, and parasites in one test (FilmArray GI Panel, BioFire

Diagnostics; Luminex GGP, Luminex; Verigene Enteric Pathogens Test, Luminex), while others provide separate panels for bacterial, viral, or parasitic pathogens (Allplex™, Seegene; BD Max®, BD; Amplidiag®, Mobidiag; EntericBio® real-time Dx, Serosep). Most tests are performed with raw stool samples or stool in transport media. The use of fecal swabs with transport media is also common, and direct testing of rectal swabs is desirable.

Current testing for infectious gastroenteritis includes many tests (stool culture, ova and parasite examination (O&P), enzyme immunoassays) that are technically complex and suffer from low diagnostic yield. The gold standard for stool pathogen testing is stool culture; however, stool culture has low diagnostic yield, is technical complexity, and has a long time to result. The yield for stool cultures is reported to be between 1.5% and 2.9% with a cost per positive result of $952 to $1200 [39]. Another commonly ordered test is O&P, which requires the collection and testing of three different stool samples. This method is also known to be technically difficult, to have low sensitivity, to be improperly used, and to have a low diagnostic yield of 1.4% [40]. As a result of the low diagnostic yield, clinicians often order multiple tests for the same stool sample, or perform testing sequentially until a causative pathogen is identified. As an example, a study conducted at a children's hospital found that a median of three tests (range 1–10) were ordered per stool sample [41]. To make matters worse, several studies have shown that clinician test ordering practices for gastroenteritis are problematic, in part due to the complexity of which pathogens are covered by what test [40, 42–44].

The use of culture-independent molecular multiplex panels has increased the diagnostic yield for stool testing due both to increased test sensitivity and an expanded test menu. Studies using the FilmArray GI Panel identified a pathogen in 40–50% of stool samples [41, 45, 46]. Some of the assay requires specialized molecular laboratories and personnel (BD Max, Luminex GGP, Allplex); however, some are designed to be simple to use (FilmArray GI Panel, Verigene Enteric Panel) and to provide a fast time to result (as little as 1 h from test initiation).

While these tests have the benefits of offering a comprehensive and accurate result in a relatively fast time period, the impact to patient care is largely unknown; however, one recent pre-/post-implementation study highlighted several important improvements when the FilmArray GI Panel was used to test pediatric and adult inpatients [47]. These included an increase in diagnostic yield from 6.7% to 32.8% and an improved time to result from a mean of 54.75 h to 8.94 h when compared to traditional clinician ordered tests. When compared to a matched historical control group, implementation of the FilmArray GI Panel led to a reduction in the number of additional stool tests (3.02 vs 0.58, $p = 0.001$), a trend toward shorter duration of antibiotics (2.12 days vs 1.54 day, $p = 0.06$), significantly fewer imaging studies (0.39 vs 0.18, $p = 0.0002$), and a reduction in the length of hospital stay after sample collection (3.9 days vs 3.4 days, $p = 0.04$). Reduced length of stay was more pronounced for the adult population (4.3 days vs 3.6 days, $p = 0.01$).

Recent studies have also shown that these tests have important advantages for infection control. In a recent retrospective study, the FilmArray GI Panel was used to test frozen stool samples that had previously been tested for rotavirus and *C. difficile* for infection control purposes [48]. The study showed that 22% of the samples contained

pathogens that should have required infection control measures, including norovirus, rotavirus, and *C. difficile*. Of these patients, 60% were under no or inadequate contact precautions, for a total of 109 patient days. Conversely 24.5% of the patients with negative results by the FilmArray GI Panel were unnecessarily placed under contact precautions for a total of 181 patient days. This study illustrates that without a rapid comprehensive test result, contract precautions are not rationally applied, leading to both an increased risk of nosocomial infections and unnecessary costs associated with inappropriately applied contact precautions. These are important factors when considering the care of patients in hospitals and in long-term care facilities.

Use of the Luminex xTAG gastrointestinal pathogen panel (GPP) has been compared with conventional laboratory testing for hospitalized patients and was found to be cost-effective because the increased cost of the laboratory testing was more than offset by the cost saving for elimination of unneeded contact precautions. Importantly, the cost savings was directly related to the time to test result [49].

The National Institute for Health Care Excellence (NICE) in the UK recently published a very comprehensive health economics assessment of molecular multiplex panels [50]. They reported that there was considerable uncertainty in their models; however, the Luminex GGP Panel was determined to be cost-effective for use in community-acquired and traveler diarrhea and both the FilmArray GI Panel and the Luminex GGP were found to be cost-effective for use in inpatients with diarrhea. These models will be updated as new information (such as the recent study by Beal [47]) becomes available.

## Future Directions

Further clinical utility studies will highlight the importance of the different pathogens detected by the multiplex panels. However, independent of this work, continuing improvements to these IVD systems are likely to increase their value in the clinical infectious disease setting.

### *Real-Time Pathogen-Specific Syndromic Epidemiology*

In contrast to previous generations of infectious disease IVD platforms which used cell culture, microscopy, or immunoassay technology, the current generation of multiplex systems are all highly automated and computerized [4, 6–8, 51]. This opens the possibility of exporting the results of a patient test directly to an internet (or "cloud") database. A pilot version of such a system has been achieved with development of FilmArray Trend [52]. Trend aggregates result from geographically dispersed clinical laboratories and displays the results on a website (www.syndromictrends.com) in close to real time, resulting in a form of infectious disease "weather map."

The pilot project summarized the respiratory pathogen results for the FilmArray RP from >360,000 patient samples acquired over 4 years ending in July 2017 from 20

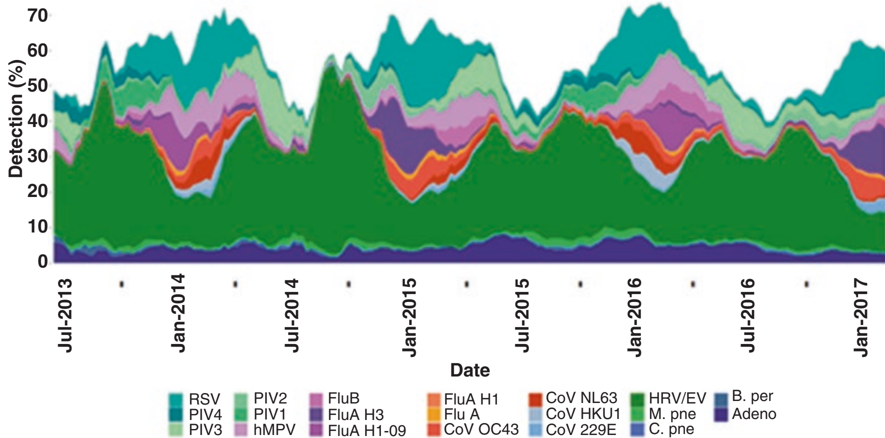


**Fig. 3** Percent detection of each FilmArray RP pathogen in the FilmArray Trend RP dataset displayed as a stacked area graph for the July 2013 to July 2017 time period

clinical laboratories in the United States (Fig. 3). Similar to social media-based disease reporting systems, the data is syndrome-based [53]. However, a unique feature of the Trend dataset is that analysis is pathogen-specific. The FilmArray RP patient test results demonstrate that a number of viruses (RSV, HMPV, PIV, and CoV) show seasonal occurrence that is similar to influenza and thus can be confused with influenza when a symptomatic diagnosis of influenza-like-illness is made. This has important implications for treatment of influenza and for determining the efficacy of influenza vaccination and thus emphasizes the value of multiplex testing for respiratory symptoms. The FilmArray Trend data also show that 7% of the FilmArray RP tests detect the presence of two or three pathogens in a single sample. The importance of this result for patient treatment is not currently clear. Clinical studies that focus on patients presenting with more than one pathogen are needed.

Data from the FilmArray GI panel are also available at the www.syndromictrends.com website. Over time, additional FilmArray IVD panels will be added to Trend, thus enabling the tracking of the pathogens that those panels detect.

## *Moving Multiplex Testing Closer to Point of Care*

In 2017 the US FDA cleared the FilmArray RP-EZ panel [54]. This panel has Clinical Laboratory Improvement Act (CLIA)-waived status and thus can be used in settings close to the patient including low-complexity outpatient settings. In other work, the time to result for the RP panel has been decreased from 63 min for RP v1.7 to 45 min for the RP2 panel [55]. Speed, ease of use, and a comprehensive test menu are all critical features if the possibilities of point-of-care syndromic testing are to be fully realized [56].

In the longer term, additional simplification of the test setup procedure as well as reductions in the time to result should further expand the number of outpatient settings able to use multiplex testing. The data from Farrar and Wittwer [57] on "extreme" PCR conditions (cycle times below 1 s) suggest that reductions in the time to result are limited by the instrument and not by the chemistry and that substantial improvements are still possible.

## *Technology Improvements and the Utility of Multiplex Nucleic Acid Testing*

As noted earlier, the cost of multiplex diagnostic systems is not driven by that of the primers needed for each assay, so the number of assays in a test is limited by assay format and the ability to separate the signal for each analyte. The depth of multiplex achieved in PCR multiplexes for infectious disease has steadily increased from the earliest, manually performed multiplexes, compared to the those being developed for the automated systems of today. The point will soon be reached in which the feasible number of assays in a test begins to exceed the number of distinct pathogens that need to be tested for in any particular syndrome. However, there are situations (e.g., HIV drug resistance testing or HPV serotyping) where the ability to detect a limited number of point mutations would add great value to the test result.

There is also great interest in the direct detection of sepsis pathogens from blood because the hours saved by not having to culture the bacteria reduces the risk of incorrect antibiotic treatment. With a combination of direct enrichment of the bacteria from the blood and careful attention to removing endogenous bacteria from the test manufacturing process, it should be possible to achieve the necessary sensitivity to detect bacterial pathogens directly from blood, without the time and labor of culturing the sample.

An Infectious Disease Society of America policy paper from 2013 [58] made a number of recommendations for the key characteristics of future infectious disease IVD tests. The current generation of multiplex tests already meets many of these goals (direct testing from easily accessible sample types, able to rule out infection with high certainty, based on clinical syndromes). The technical improvements described above suggest that meeting many of the remaining goals (rapid testing, point-of-care syndromic testing, improved outbreak detection) will be accomplished in the next decade.

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# Molecular Diagnosis and Monitoring of Human Papillomavirus Infections

**Bruce K. Patterson**

## Introduction

Though the incidence of cervical cancer is low in the USA, cervical cancer is still the second leading cause of cancer and cancer-related deaths worldwide [1]. The Papanicolaou (Pap) smear has been the hallmark of cervical cancer screening since 1949. Following the discovery that HPV was the causative agent in >99% of cervical cancers [2], the detection of high-risk HPV types (HRHPV) that cause cervical cancer in cervical samples became an important adjunct in the cervical cancer screening algorithm [3, 4]. HPV DNA testing first became integrated in cervical cancer screening as a co-test to the Pap test and as a reflex test for cytology samples determined to be atypical squamous cells of undetermined significance (ASCUS) [5]. Both of these recommendations were only in women over 30 years old (>30). Recently, HRHPV was approved as a primary screen for cervical cancer though the field remains divided about eliminating cervical cytology from screening algorithms.

Though HRHPV types were universally found in cervical cancer, infection by HRHPV types results in cervical cancer in a very small percentage of infections [6]. More recently, new- or second-generation cervical cancer diagnostics targeting different aspects of the mechanism of cervical cancer pathogenesis have been brought into clinical use and added much needed specificity to the cervical cancer screening algorithm [7]. These newer diagnostics include genotyping for HPV 16, HPV 18, and in some assays HPV 45. In addition, HPV E6 and E7 mRNA with and without proliferation, p16 with and without the proliferation marker Ki67, and DNA methylation have shown increases in utility as a reflex to the high-sensitivity/low-specificity screening assays based on HRHPV detection.

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The purpose of this chapter is to understand the utility of HPV/cervical cancer diagnostics, especially at a time when therapeutic approaches to cancer are changing dramatically with the understanding of the immune response and immuno-oncology. On one level, the discussion focuses on the relationship between the diagnostic target and the pathogenesis of cervical cancer as the field attempts to direct diagnostics toward detection of lesions requiring treatment and minimize the number of women sent to unnecessary, invasive procedures. In other words, cervical cancer diagnostics to date have focused on the mere presence of HRHPV and the associated risk of developing cervical cancer rather than the HPV-driven mechanism by which HPV causes cervical cancer. Another level of diagnostic utility is companion diagnostics and immuno-oncology. New therapies including therapeutic vaccines, antiviral drugs, and immune checkpoint inhibitors are all in various stages of development and attempt to treat pre-cervical cancer without removing the cervix or portions of it. Last, consideration is also given to other uses of HPV diagnostics including anal and head and neck cancers. As a field, diagnosticians and pharmaceutic companies are beginning to approach cervical, anal, and head/neck cancers collectively as squamous cell diseases with the intent of using consolidated diagnostic and therapeutic approaches to all three organ sites.

## Specimen Collection for HPV Diagnostics

Collection of cervical cytology specimens changed dramatically in the mid-1990s as new technologies were introduced that allowed the cervical cytology sample collected by spatula or brush to be placed in a liquid preservative [8, 9]. Liquid cytology preservatives are typically alcohol based (ethanol or methanol) and, in general, contain compounds that dissolve mucus and disaggregate cells. All technologies create a slide that contains a monolayer of representative cells from the ecto- and endocervix stained by the method of Pap. SurePath liquid (Becton Dickinson, Burlington, NC) is used for collection of cervical cytology specimens and involves pressing cell suspensions through the small orifice of a syringe which disaggregates cell clusters. Isolation of epithelial cells is accomplished by removing interfering debris and inflammatory cells by centrifugation through a density gradient. After centrifugation, the tubes including cell pellets are placed on the PrepStain System for staining using the Pap stains. ThinPrep (Hologic, Marlborough, MA) uses a filter pressed through a cell suspension until a monolayer is created on the filter membrane. The filter is then pressed onto a treated slide and stained by the method of Pap. These technologies improved the quality and uniformity of cells on slides and reduced but did not eliminate unsatisfactory slides [8, 9]. A third liquid-based cervical cytology solution, LiquiPrep (LP), has also been used extensively around the world. LiquiPrep was the first LBC technology not requiring a dedicated processor. Through the use of its proprietary separation chemistry, this LBC system can be run on any swing-bucket centrifuge allowing for broad adoption. The LP Preservative solution contains no hazmat chemicals which allows for lower shipping costs and simple disposal, the first truly “green” LBC system. The LP Preservative also does

not cause morphology changes as seen with the first-generation system utilizing high concentration of methanol. Through the use of LP Cell Base, the cells are affixed to the slide maintaining classic morphology which allows labs to quickly adopt reading of the technology. To maximize cost-effectiveness, the LP system offers the selective use of LP Cleaner to remove mucus in those samples where inflammatory cells are an issue. To provide further savings, no special coated slides are required, so any lab slide can be used.

As is discussed in subsequent sections, liquid-based cervical cytology has significantly aided the use of automated screening devices and the automation of advanced molecular and proteomic assays. Some studies suggest that liquid cytology preservatives can preserve RNA for up to 12 months [10]. This has led to the use of liquid-based cervical cytology samples as well as swabs for HPV diagnostics that target either DNA or RNA.

## HPV and Cervical Carcinogenesis

In this section, the role of HPV in cervical carcinogenesis is discussed with an overlay of the commercially available diagnostics that detect different phases of HPV infection through transformation leading to cancer (Fig. 1). In HRHPV-associated lesions, HPV DNA exists as an episome in the cytoplasm of epithelial cells

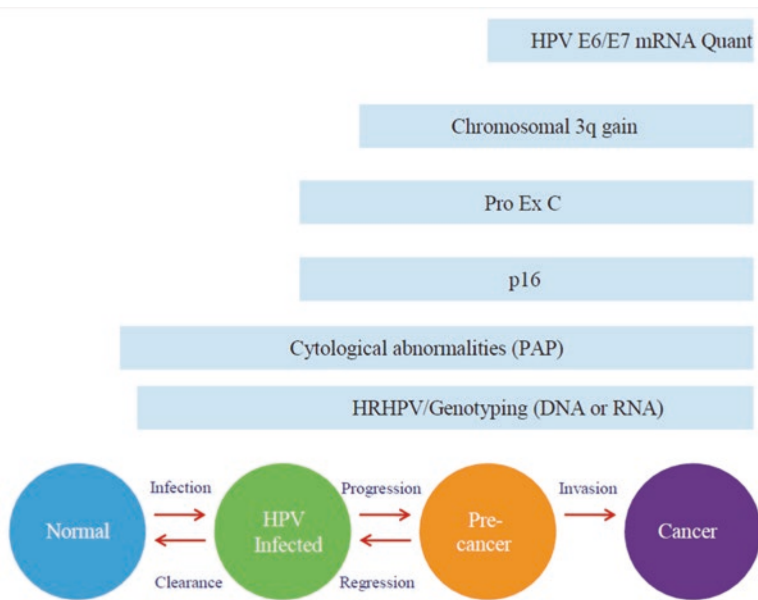


**Fig. 1** Overlay of cervical cancer pathogenesis and diagnostic tests. This figure illustrates the detection of various stages of disease and where in the disease process various diagnostic tests detect changes

(diagnostic correlate: Hybrid Capture 2 (Qiagen), Cervista (Hologic), cobas 4800 HPV test (Roche), INFINITI (AutoGenomics), CLART (Genomica), RealTime High Risk HPV test (Abbott), Xpert HPV (Cepheid)). Under certain circumstances HPV DNA linearizes and integrates into the host cell chromosome [11]. During integration, the E2 gene, which is a negative regulator of E6 and E7, (diagnostic correlate: Aptima (Hologic), NucliSENS EasyQ (BioMerieux), PreTect HPV-Proofer (NorChip), Onclarity (Becton Dickinson)) is deleted leading to overexpression of E6 and E7 mRNA (diagnostic correlate: HPV OncoTect (IncellDx)) [12–15]. This derepression of E6 and E7 mRNA is different from the small amounts of E6 and E7 that are required for replication and expressed during a "normal life cycle" [16]. E6 and E7 and certain host factors are clearly involved in the transformation of cells leading to cell cycle abnormalities and proliferation (diagnostic correlate: HPV OncoTect 3Dx (IncellDx), CINtec PLUS (Ventana), ProEx C (Becton Dickinson), and eventually cancer) [17] (Fig. 1). Mechanistically, the E6 protein causes degradation of p53, BAK, and activation of SRC family kinases in addition to the activation of telomerase (diagnostic correlate: 3q addition (Ikonisys)). Similarly and synergistically, the E7 protein degrades the RB releasing the transcription factor E2F from inhibition and upregulating the cellular protein p16INK4A (diagnostic correlate: CINtec p16 (Ventana)) [17–28]. Several reports have shown not only the ubiquitous presence of E6 and E7 mRNA regardless of the high-risk type in cervical cancer but also a quantitative difference in the overexpression of E6/E7 depending on the severity of the cervical lesion, CIN 0, 18%; CIN 1, 58%; CIN 2, 77%; and CIN 3, 84%, and 100% of cervical cancers [29, 30]. More recently, diagnostics aimed at host factors have also gained entry into the marketplace. These include assays of tumor suppressor gene DNA methylation (diagnostic correlate: QIAsure (Qiagen)). DNA methylation is a late event in cervical carcinogenesis as these assays have only 67% sensitivity for high-grade cervical precancer (CIN 3) but virtually 100% sensitivity for cancer. It remains to be seen whether these assays may detect lesions too late for intervention.

## HPV and Cervical Cancer Diagnostics

As discussed above, the indication for HPV testing in the cervical cancer algorithm is for co-testing with cervical cytology and for ASCUS reflex testing. The metric by which cervical cancer diagnostics are measured is histology. In particular, high-grade dysplasia at the level of cervical intraepithelial neoplasia 2 (CIN 2) or, worse, CIN 3 and squamous cell carcinoma is considered the "gold standard." For several reasons this standard is not perfect because it is subject to sampling bias, and the reproducibility of calling a lesion CIN 2 is poor relative to pathologist's ability to call a lesion CIN 1 or CIN 3 [31]. The CIN 2 or higher (CIN 2+) standard was also chosen since it was an actionable metric. In other words, clinicians generally treat a lesion of CIN2+ severity though recently the diagnosis and immediate treatment of CIN 2 are debated. A summary of the performance of current commercially available tests follows, and the attributes of each test are summarized in Table 1.

**Table 1** Summary of HPV/cervical cancer test attributes by diagnostic category

| | Pap | P16 | HPV DNA | | L1 genotyping | | | E6 and E7 genotyping | | E6 and E7 mRNA quantification |
|---|---|---|---|---|---|---|---|---|---|---|
| | Hologic BD | Ventana CINtec | Qiagen Digene HC2 | Cepheid | Roche cobas | Genomica | AutoGenomics | Hologic | BD | IncellDx |
| *Performance attributes* | | | | | | | | | | |
| Technology | CytoStain | ICC/IHC | Hybrid capture | PCR | PCR | Array | Array | TMA | PCR | SUSHI |
| Specific (by biopsy) | 90% | 60% | 30% | 80% | 40% | 40% | 40% | 30–40% | 50% | 90% |
| Sensitivity (by biopsy) | 50% | 80% | 95% | 95% | 90% | 90% | 90% | 80–95% | 95% | 93% |
| Sample controls | No | No | No | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Cell type differentiation | Yes | Yes | No | No | No | No | No | No | No | Yes |
| Women <30 effective | Yes | Yes | No | No | No | No | No | No | No | Yes |
| *Laboratory workflow attributes* | | | | | | | | | | |
| Subjectivity | Yes | Yes | No | No | No | No | No | No | No | No |
| Use of slides | Yes | Yes | No | No | No | No | No | No | No | No |
| Nucleic acid Extraction | No | No | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| Assay completion time | 1–2 days | 6–24 h | 6 h | 6 h | 6 h | 8 h | 8 h | 6 h | 6 h | 3.5 h |
| Automated | Yes | No | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Reagent stability | Ambient | 4–8 °C | 4–8 °C | −20 °C | 4–8 °C | −20 °C | Ambient | 4–8 °C | Ambient | Ambient |

## HPV DNA Tests (Consensus)

The first test on the market as an adjunct to cervical cytology was the Digene (now Qiagen) Hybrid Capture (HC2) technology which is a liquid hybridization assay that detects 13 high-risk strains of HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). The performance of HC2 for the detection of high-grade disease (CIN 2+) is well documented, but in general the sensitivity is >95%, and the specificity is <30% [32]. Similarly, the negative predictive value (NPV) is 99%, and the positive predictive value is 15–25%. Though not yet approved by the US FDA, newer versions of the Qiagen offering will reflex to HPV 16, 18, and 45 genotyping. The Cervista test, like HC2, is approved by the FDA in the USA and detects the same strains of HPV as HC2 in addition to HPV 66, now considered to be a high-risk type. Cervista also includes an HPV 16 and 18 reflex component, which is discussed under HPV genotyping. In general, Cervista has an identical performance profile as HC2 though some publications have indicated that the positivity rate of Cervista on normal cytology specimens may be as high as 18% [33].

## HPV DNA (Genotyping)

Many HPV genotyping assays exist in the market worldwide including INFINITI (AutoGenomics), CLART (Genomica), PapilloCheck (Greiner Bio-One), RealTime High Risk HPV test (Abbott), and cobas 4800 HPV test (Roche) which were recently FDA approved for cervical cancer screening in the USA. All of these tests will report at least 15 high-risk types (16, 18, 31, 33, 35, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68). These assays are important from an epidemiologic standpoint, and more recent evidence has suggested that detection of HPV 16 and 18 in particular increases the risk of a high-grade lesion (CIN 3+) from approximately 5% to 11% [34, 35]. In a study with >40,000 women enrolled with an ASCUS diagnosis, the cobas 4800 HPV test demonstrated 93.5% sensitivity, 69.3% specificity, 8.4% PPV, and 99.7% NPV. By comparison, HC2 showed 91.3% sensitivity, 70% specificity, 8.5% PPV, and 99.6% NPV [34, 35].

## HPV E6 and E7 mRNA (Qualitative/Genotyping)

Aptima (Hologic), NucliSENS EasyQ HPV (BioMerieux), and PreTect HPV Proofer (NorChip) are E6 and E7 mRNA assays that provide a genotype based on qualitative detection of genotypic sequences within the E6 and E7 genes. The Aptima test detects genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, and the other two tests detect 16, 18, 31, 33, and 45. Since expression of E6 and E7 mRNA is an event that would indicate cellular transformation, the intent of this approach was to improve on the low specificity and low PPV of HPV

DNA assays by increasing the specificity for CIN2+ lesions. Interestingly, these two tests have very different performance profiles. The Hologic Aptima assay has a performance profile similar to HPV DNA assays with sensitivity for CIN2+ >95% and specificity of 40% and a PPV of 40% [32]. The other two assays that detect only five high-risk types have sensitivities of 70–75% and specificities of 70–75% with a PPV of 55% [32].

## HPV E6 and E7 mRNA (Quantitative)

HPV OncoTect is a quantitative assay that uses flow or image cytometry to quantify both the overexpression of E6 and E7 mRNA on a cell-by-cell basis, which is the hallmark of cellular transformation, and the quantity of cells overexpressing E6 and E7 mRNA in a liquid-based cervical cytology specimen [36]. Because flow cytometry has high analytic sensitivity and can analyze thousands of cells with high throughput, HPV OncoTect has a unique performance profile with both a high sensitivity of 90–93% and a high specificity of 80–90% [37]. The PPV of HPV OncoTect is between 60% and 80% depending on the age of the woman [38].

## Other HPV-Related Diagnostics

As described above, the cervical cancer screening field is moving toward more specific markers that indicate that HPV is actually causing disease. The CINtec p16 marker is a protein that is upregulated following E7 overexpression [39]. Reactivity for the p16 marker was 68% in CIN 2 and 82% in CIN 3 in a meta-analysis of clinical studies. Because p16 staining is a slide-based test, the authors concluded that subjectivity affected reproducibility [39]. The ProEx C test detects the markers mtm2 and Top2a that are produced as a result of cell cycle dysregulation [18], and the 3q addition cytogenetics test reflects the activation of telomerase as a result of HPV transformation [40].

## Next-Generation Cervical Cancer Diagnostics: Cervical Cancer Staging, Companion Diagnostics, and New Therapeutic Approaches

The cervical cancer field is rapidly evolving with new therapeutic approaches driving new test development and companion diagnostics. Antiviral drugs, therapeutic vaccines, and even immunotherapies are being developed and reported in the field. The recurring theme of these approaches is the need to more clearly define the difference between HPV infection and its inherent risk and actual

markers of disease that can both be utilized for patient stratification to therapies but also for monitoring the efficacy of these therapeutic approaches. In the past, persistence as defined by continued detection of HRHPV DNA has been used as a surrogate for disease that goes beyond a transient infection of HRHPV DNA. The problem is that persistence, as suggested by the term, takes time to define and is not readily actionable at the time of patient presentation. As such, new markers especially related to the impact of transcriptionally active HPV infections are being used to actually stage cervical disease and identify the earliest possible transition from infection to actual disease. As Fig. 2 shows, a series of events drive infection with HPV, in particular HRHPV, through oncogene overexpression, tumor suppressor gene inactivation, cell cycle dysregulation, proliferation, and progression. Cell cycle is a particularly intriguing marker as it embodies multiple stages of cervical disease. Proliferation of cervical epithelium can be seen early in

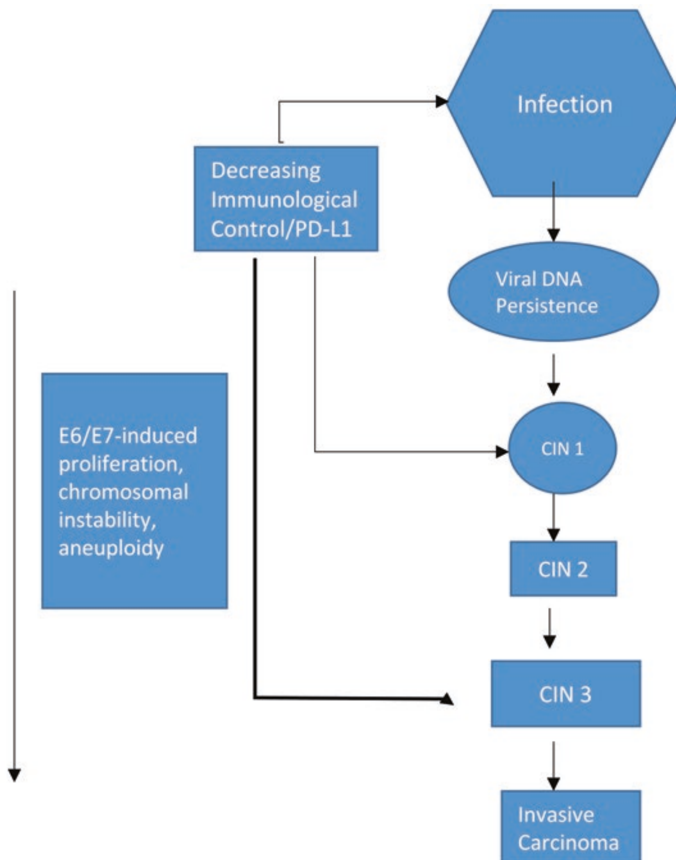


**Fig. 2** Schematic of HPV-induced cervical cancer. The progression to cancer is accompanied by oncogene (E6, E7) overexpression followed by tumor suppressor gene inactivation, genomic instability, cell cycle dysregulation, proliferation, and progression

infection as part of a normal process then later driven by the events that result from oncogene overexpression. Recently published work on cells from the cervix demonstrated that the combination of oncogene overexpression with proliferation accompanied by reduced mean corpuscular volume (MCV) defined high-grade lesions of the cervix, while low-grade lesions had reduced proliferation and higher MCV (Fig. 3). Normal epithelium had the highest MCV with some proliferation and, of course, no oncogene overexpression.

Similarly, the pattern of HPV gene expression has been suggested as a way to stage cervical disease. Of interest, expression of both HPV 16 and HPV 18 E1–E4 in proliferating epithelial cells causes a dramatic arrest in G2 which would account for the decrease in % G2-M in low-grade lesions discussed above. In short, E1^E4 and cell cycle may give the field approaches in defining the transition from infection to disease. These diagnostic capabilities are necessary as new therapies are being evaluated.

Last, the cervical cancer field has always posited that the immune system is responsible for approximately 60% regression of CIN 1, 50% regression of CIN 2, and 20–30% regression of even CIN 3. The recent explosion of data in immuno-oncology especially as it relates to squamous cell biology will undoubtedly be the next frontier in cervical cancer monitoring and therapy. Squamous cell carcinoma of the oropharynx is a precursor to approaches that will eventually target

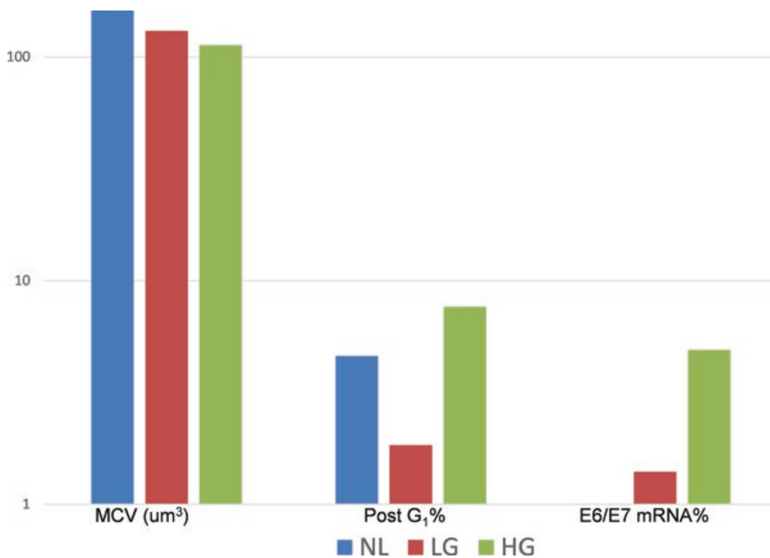


**Fig. 3** Staging cervical precancer using a combination of cell volume (MCV), cell cycle (post-G1%), and oncogene overexpression (E6, E7 mRNA). Normal cervical epithelium is characterized by large cells, proliferation, and no oncogene overexpression. Low-grade lesions are characterized by smaller cells (relative to normal), loss of normal proliferation, and low levels of oncogene overexpression. High-grade lesions are characterized by very small cells and high proliferation driven by oncogene overexpression [49]. Bar graph log scale

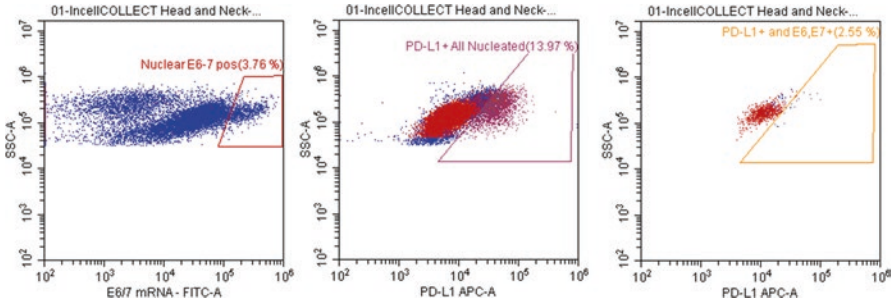


**Fig. 4** Flow cytometric analysis of a head and neck squamous cell carcinoma that overexpresses HPV E6 and E7 mRNA (left plot) and expresses PD-L1 (middle plot). Of interest, the cells over-expressing E6 and E7 mRNA have minimal expression of PD-L1 (right plot) relative to the overall squamous cell population (middle plot) [50]

cervical and anal neoplasia. Several PD-L1 pathway drugs have been approved for head and neck cancer including nivolumab (Bristol-Myers Squibb) and pembrolizumab (Merck). In addition, the role of HPV in head and neck cancer has been extensively investigated. Diagnostics detecting either HPV status or PD-L1 status or preferably both (Fig. 4) are now critical in managing patients with this cancer.

## Comments, Future Approaches, and Summary

Because HPV has been implicated in a number of other epithelial cancers including anal cancer, head and neck cancers, and even breast cancer, HPV diagnostics are gaining importance in sites other than the cervix. Both HPV DNA assays such as HC2 and PCR (Roche Linear Array) and HPV RNA (HPV OncoTect) have been used to screen for anal intraepithelial neoplasia (AIN) in a similar manner to cervical cancer screening. The results vary with sensitivities for AIN 2 and 3 between 50% (Roche Linear Array) and 75% (HC2 and HPV OncoTect) [41, 42]. The specificity of HPV DNA for AIN 2 and 3 is similar to the specificity for cervical disease around 30–40%, and as expected the specificity of HPV RNA by HPV OncoTect was higher at 60–70%. Other studies have suggested that HPV RNA may be a better marker for anal screening because of the potential reservoir of HPV in the anus that does not cause disease [43].

For head and neck squamous cell carcinomas, molecular markers that differentiate HPV-positive tumors from HPV-negative tumors correlate with tumor prognosis with increased survival in transcriptionally active HPV infections [44, 45]. For example, oropharyngeal squamous cell carcinomas that have p16 expression exhibit a 79% 5-year survival, compared with 20% 5-year survival in individuals with HPV-negative tumors and 18% 5-year survival in persons with HPV-16-positive tumors with no evidence of transcriptional activity [46].

In summary, the trend toward more specific HPV/cervical cancer tests either singly or in combination suggests a movement of the field to replace the Pap

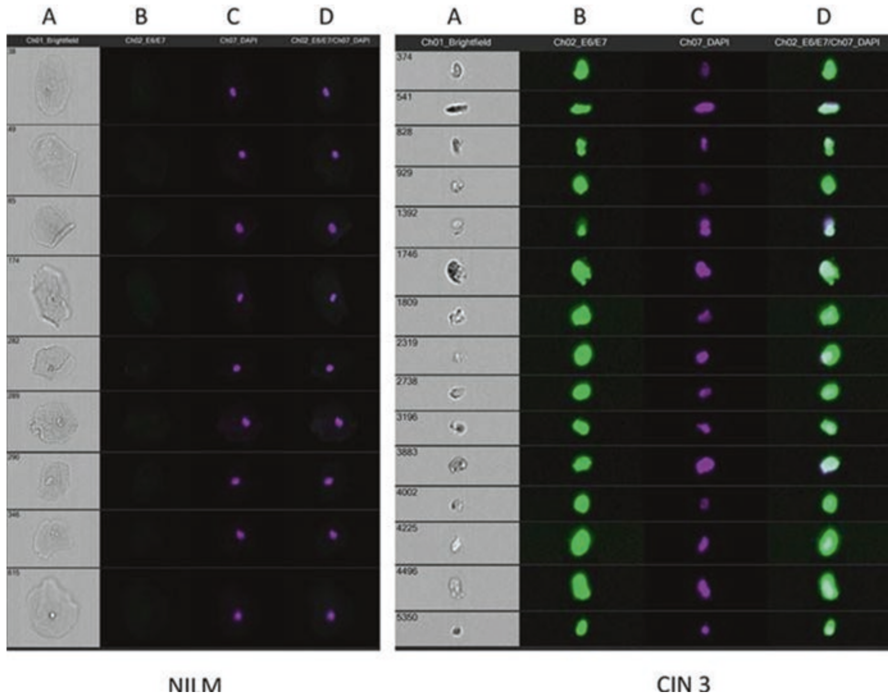


**Fig. 5** Simultaneous morphology using nuclear to (**a**) cytoplasmic ratio (N/C), (**b**) E6 and E7 mRNA expression (green), (**c**) cell cycle/nuclear pleomorphism (purple), and (**d**) overlay on cervical cytology cells in suspension (HPV OncoTect 3Dx)

smear—so-called primary screening. Now that HRHPV DNA has been approved for cervical cancer screening [47], the field is moving once again toward walkaway solutions that combine high sensitivity with high specificity. In fact a new technology, HPV OncoTect 3Dx technology, allows for simultaneous E6 and E7 mRNA quantification, cell cycle (ploidy analysis), and imaging of the cells while still in suspension for high-throughput analysis without ever producing a slide (Fig. 5) [48]. Using this technology, studies showed that ASCUS cytology specimens clearly fell into normal, LSIL or rarely, HSIL diagnostic categories, thus demonstrating the potential of eliminating this ambiguous category [48].

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# Molecular Strategies for the Laboratory Diagnosis of Sepsis

**Diana R. Hernandez and Donna M. Wolk**

## Introduction

Bloodstream infections are among the top causes of death in the USA [1]. Several studies have documented that substantial mortality can be attributed to delays in identification of the clinical signs of sepsis [2–5]. More recently, delays in the determination of the microbial cause(s) and selection of the appropriate antibiotic also have been shown to contribute to increased mortality [6–11]. In this review, the basic attributes of bacteremia and sepsis as well as human and financial impacts of sepsis, predisposing factors, symptoms, and common modes of bacterial pathogenesis are summarized. Important clinical and laboratory criteria for the diagnosis of sepsis as well as key aspects of the Surviving Sepsis Campaign Guidelines are detailed as they relate to the diagnosis, therapy, and resuscitation of the septic event [5]. Clinical laboratories must expand their understanding of the complexities related to diagnosing and treating sepsis and expand their role as productive members of interdisciplinary healthcare teams that are focused on improving sepsis survival and limiting the fiscal impact that sepsis imparts on healthcare systems [12].

In the USA, septicemia is among the top 20 causes of mortality in the general population and among the top 10 for infants, killing nearly 600 people per day [1]. Many septic patients are treated in emergency medicine departments (EMDs) or intensive care units (ICUs), settings in which rapid administration of antibiotics with a focus on targeted antibiotic therapy drastically reduces mortality [3, 13].

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Unfortunately, current microbiology laboratory methods often are too slow to support rapid interventions, some requiring over 24 h to detect the presence of blood-borne pathogens and at least 3–5 days for antimicrobial susceptibility test results, which guide the selection of the most appropriate antimicrobial therapy. Moreover, cultures from septic patients are often falsely negative due to empiric antimicrobial therapy, the presence of fastidious organisms, or microbes that are present in low density [14]. Broad-spectrum empiric antimicrobial therapy is commonly used, which is a costly approach that may fail to effectively target the correct microbe, may inadvertently harm patients via antimicrobial toxicity, and may contribute to the evolution of drug-resistant microbes. New rapid molecular methods that enhance the laboratory's ability to quickly identify the pathogens in bloodstream infections are becoming the new standard of care for clinical microbiology laboratories.

## The Impact of Sepsis

### *Morbidity and Mortality*

Bloodstream infections can lead to sepsis, which is recognized as a "life-threatening organ dysfunction caused by a dysregulated host response to infection" [15]. Determination of the incidence of sepsis and sepsis-related mortality has been controversial because it is a syndrome without an internationally accepted definition, without a validated criterion, and without standard diagnostic test methods; all of these points result in major variations for the reported incidence and mortality rates [16]. This chapter previously noted that there were over 750,000 episodes each year in the USA with a mortality rate exceeding 50%, based on the report by Angus et al. [17]. Since then, a number of reports have published different numbers, depending on the method of data abstraction used: death certificates, International Classification of Diseases (ICD) codes for sepsis, or combined ICD-9 (or, recently, ICD-10) codes for organ dysfunction and sepsis [16]. Depending on the method of data abstraction, the incidence of sepsis in the USA ranges from 894,013 to 3,110,630 cases per year with a mortality ranging from 14.7% to 29.9% [16, 18]. According to the most recent National Vital Statistics Reports [1], there were 38,940 deaths due to septicemia in 2014 in the USA, which comprises 1.5% of all causes of death and is the #11 cause of death in this country [1]. The impact of sepsis worldwide continues to grow, increasing from an estimate of 18 million cases in 2001 to 31.5 million cases of sepsis in 2014, thus creating an increasing number of challenges for healthcare systems worldwide [1]. Annually 19.4 million cases of severe sepsis occur, with potentially as many as 5.3 million deaths [19, 20].

### *Economic Burden of Sepsis*

Sepsis occurs in over 3% of all hospitalizations and in 75% of intensive care patients, resulting in an enormous concomitant social and economic burden [21, 22]. In the USA, the current per capita incidence is at least 270–300 patients per 100,000 people, and estimated US healthcare costs exceed $25 billion dollars per year, a 57% increase from 2003 to 2007 [19, 22]. EMDs are a common interface with septic patients, who account for approximately 571,000 EMD visits per year [12, 17, 23]. Sepsis is commonly associated with a prolonged length of stay both in the hospital and in the ICU, up to 9 days longer, according to Lagu et al., *MEDPAR Hospital Discharge Databases 2004 through 2005*, and additional critical care reviews [17, 22, 24]. Due to approximately 2.26 cases of sepsis per 100 hospital discharges, the costs for caring for septic patients impart a large economic burden, averaging $22,100– $40,890 per case [12, 17, 25]. Costs in the ICU include staffing requirements (46.4–56.1%); medications, including antibiotics (15.6–21.7%); and diagnostic procedures and testing (17.9–20.4%) [26]. Bacteremia is a common precursor to sepsis and, when acquired in the ICU, is associated with an increased mortality, a longer ICU stay, and a 25% increase in the cost of hospital care ($85,137 vs. $67,879, for bacteremic and non-bacteremic patients, respectively) [27].

## The Attributes of Risk

### *Predisposing Factors and Underlying Diseases Increase Risks for Sepsis*

Contributing factors for sepsis are varied and include the following:

- *Critical care and surgery*: Sepsis can lead to a fatal outcome for postsurgical patients in a variety of clinical settings including transplantation, wound surgery, splenectomy, intra-abdominal surgery, and cancer surgery [28–33].
- *Cancer*: An estimated 5% of cancer patients acquire sepsis. Hospitalized patients with cancer are more than 5 times more likely to die (37.8%) than cancer patients without sepsis. Patients with hematologic cancers are 15 times more likely than the average person to suffer from sepsis. The use of cytotoxic agents is largely responsible for immune suppression in these patients, which predisposes them to sepsis. In addition, necrotic neoplasms can provide entry for bacteria into the bloodstream [34–36].
- *Age*: People over 65 years old account for only 1/8 of the US population but account for 2/3 of all sepsis cases. Age over 40 is a risk factor for sepsis, with the poorest outcomes in patients over age 85, whose mortality rate exceeds 38.4% [2, 17, 37]. At the other end of the age spectrum, infants also have increased mortality rates, especially premature infants with a very low birth weight [24, 38].

- *Other:* Rheumatic or congenital heart disease, septic abortion, pelvic infection, intravenous drug abuse, other infections such as severe community-acquired pneumonia (CAP), abdominal infection, and urinary tract infection all present risks of acquiring bacteremia/sepsis. In addition, alcoholism, meningitis, cellulitis, and chronic diseases (including diabetes, heart failure, chronic renal failure, and COPD), surgery, or cirrhosis are all risk factors for sepsis [39]. Immunocompromised status due to HIV/AIDS increases risks, as do other immunosuppressive states. Indeed, due to hospital associated drug-resistant infections, the simple act of hospitalization increases the risk for bloodstream infections and sepsis [39].

## *The Pathophysiology of Sepsis*

Bloodstream infections can lead to sepsis, in the following manner. At the onset of bacteremia, a bacterial cell can trigger a host immune response. For Gram-negative blood-borne pathogens, the bacterial cell wall contains endotoxin, a lipopolysaccharide from the outer cell membrane; endotoxin is a potent initiator of the human inflammatory response. For Gram-positive blood-borne pathogens, cell wall components such as lipoteichoic acid and peptidoglycan, as well as extracellular products (exotoxins such as TSST-1), trigger the immune response. Once the trigger occurs, a systemic inflammatory response follows, resulting in multiple host responses, including vascular, cellular, and chemical responses. These immune responses are designed to reduce injury and include edema, which dilutes toxins, and increased phagocytosis, which removes bacteria and cell debris [40].

The pathogenesis of sepsis involves pro-inflammatory mediators, anti-inflammatory mediators, and vaso-inflammatory mediators. There are several inflammatory responses of importance; these are linked to human cells that respond to bacterial invasion: (1) phagocytes (monocytes/macrophages, neutrophils, eosinophils); (2) mast cells, which are induced by lipopolysaccharide (LPS) and complement components (C3a and C5a) to release immune mediators; and (3) natural killer cells, which cause lysis of pathogens and production of cytokines like IFN-γ and TNF-α in a process called cell-mediated cytotoxicity. Other physiological changes include reduced protein C activity, microvascular thrombosis, cellular necrosis (ischemic injury), inhibition of fibrinolysis, apoptosis, leukocyte-mediated tissue injury, endothelial dysfunction, and cytopathic hypoxia [40].

While some patients may die of infection due to their lack of ability to mount an effective immune response, in other cases sepsis and death can result from a robust human immune response to infection; this immune response goes awry and out of control (Fig. 1). Patient survival is dependent on whether or not physicians can impact the patient's immune response to microbial pathogens and return the patient's immune systems to homeostasis.

Due to the widespread inflammatory response, disease symptoms can be markedly variable and include fever, chills, hypotension, neutrophilic leukocytosis

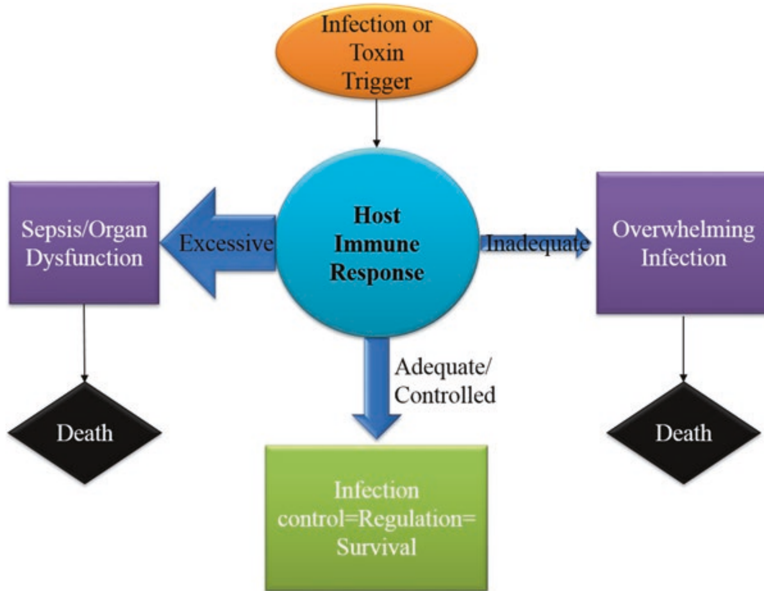


**Fig. 1** Inflammation responses that can occur in response to bacterial bloodstream infection (not drawn to scale)

or neutropenia, hypothermia (especially in the elderly), diaphoresis, apprehension, change in mental status, tachypnea, tachycardia, hyperventilation, respiratory alkalosis, reduced vascular tone, and ultimately organ dysfunction. Hematologic findings are also extremely important – the septic patient can present with thrombocytopenia, toxic granulations of neutrophils, or disseminated intravascular coagulation (DIC). Renal and gastrointestinal signs include acute tubular necrosis, oliguria, anuria, upper GI bleeding, cholestatic jaundice, increased transaminase levels, or hypoglycemia [37].

There are some consensus definitions that define the serial stages of sepsis, a progression of disease detailed below, but these are not universally accepted. Bacteremia is generally defined as the presence of viable bacteria in blood. Further disease progression has been characterized in "The Third International Consensus Definitions for Sepsis and Septic Shock" (Sepsis-3); these definitions are described below [2, 15].

1. Sepsis: "A life-threatening organ dysfunction caused by a dysregulated host response to infection" [15].
2. Septic shock is a subset of sepsis with circulatory and cellular/metabolic dysfunction associated with a higher risk of mortality.

Note: The third revision of the Surviving Sepsis Campaign removed severe sepsis from the official list of definitions [15].

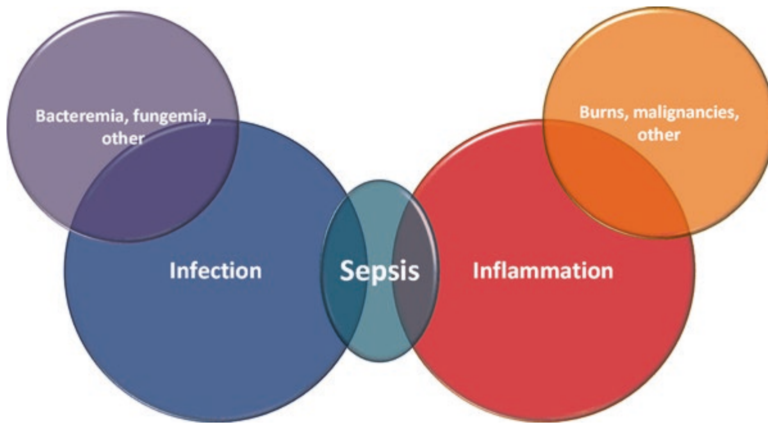


**Fig. 2** Substantial overlap between infection, inflammatory response, and sepsis mingles with noninfectious causes of inflammation, which must be eliminated before treatment for microbial sepsis (not drawn to scale)

Figure 2 illustrates the substantial overlap of this disease gradient that can be seen with bacteremia or another microbial infection and ends with septic shock. Nonspecific causes of inflammation must be considered in assessment of patients who present with inflammatory symptoms.

## Diagnostic Approach to the Septic Patient

Unfortunately, despite the enormous human and financial impact of sepsis, the diagnosis of sepsis remains largely a clinical one, due to the limited number of rapid, sensitive, and specific laboratory tests to detect the causative pathogens directly from clinical samples [41]. To provide a more accurate diagnosis, there is a significant need to improve the speed and diagnostic breadth of laboratory detection methods for blood-borne pathogens, bloodstream infections, and sepsis in general.

As with all complex diseases, the diagnostic approach to sepsis is multi-faceted; therefore, laboratory collaboration with the EMD and ICU is essential. Laboratories can participate with efforts from the entire healthcare team by setting goals to provide rapid laboratory testing to maximize effectiveness of early goal-directed therapy, improve targeted antibiotic therapy, shorten antibiotic treatment duration, and avoid development of antibiotic resistance and side effects, decrease mortality and morbidity, decrease length of stay, and decrease overall hospital costs. The clinical microbiology laboratory must help drive antibiotic intervention in partnership with pharmacists and physicians [14, 42, 43].

Upon presentation of a patient with symptoms of infection, physicians will seek the primary site of infection and attempt to direct therapy to that primary site; this process is known as “source control.” A full patient history is important to define the potential source and risks. For instance, important factors include the source of inflammation, community- or hospital-acquired status, prior or current medications received, recent manipulations or surgery, underlying or chronic diseases, and travel history [44].

Combining a variety of clinical assessments using symptoms and signs with selected laboratory tests from clinical microbiology, hematology, chemistry, point-of-care testing, and blood gas laboratories is an important aspect required for the optimum care and treatment of septic patients. In addition, there are several standardized classification systems for ICU patients, created to assess severity of illness; one such scoring system in common use is the APACHE II score (Acute Physiology and Chronic Health Evaluation). After admission of a patient to an ICU, an integer score from 0 to 71 is computed based on several measurements, including assessment of temperature, arterial blood gas pH, mean arterial pressure (MAP), serum sodium, serum creatinine, heart rate, hematocrit, respiratory rate, white blood cell counts, oxygenation parameters, and the Glasgow Coma Score. Higher scores imply a more severe disease and a higher risk of death [45].

The most recent scoring system for sepsis is published in the Sepsis-3 Consensus Definitions [5], which use the sequential [sepsis-related] organ failure assessment (SOFA) score as a means of prognosis after the clinical assessment of septic patients [46]. The higher the SOFA score, the higher the probability of mortality. The SOFA score grades abnormality by organ system dysfunction. Organ dysfunction adds 2 points or more in the SOFA score, which is associated with an in-hospital mortality greater than 10%; however, laboratory variables, namely, $PaO_2$, platelet count, creatinine level, and bilirubin level, are needed for full computation of the score [46]. Despite the fact that selection of variables and cutoff values were developed by consensus, SOFA is not well known or widely used outside the critical care community.

Patients with septic shock also can be clinically identified by the vasopressor requirement for maintaining a mean arterial pressure of 65 mm Hg or greater as well as a serum lactate level greater than 2 mmol/L (>18 mg/dL) in the absence of hypovolemia. This combination of clinical criteria is associated with hospital mortality rates greater than 40% [5].

In the Sepsis-3 definitions for an out-of-hospital, emergency department, or general hospital ward setting, adult patients with suspected bloodstream infection can be rapidly identified as more likely to have a poor outcome and progress to sepsis if they have at least two of the following clinical criteria: respiratory rate of 22/min or greater, altered mental status, or systolic blood pressure of 100 mm Hg or less. Together, these criteria constitute a new clinical score called quickSOFA (i.e., qSOFA) score [5].

## Evidence-Based Sepsis Guidelines

The Surviving Sepsis Campaign is a worldwide consortium of healthcare providers committed to improving the outcomes for patients with sepsis [2, 3, 15]. All components of the "Surviving Sepsis Campaign Guidelines" are focused on reducing mortality by using standardized criteria for patient assessment and treatment. The guidelines, aspects of which are also referred to as "Early Goal-Directed Therapy" (EGDT), often rely on laboratory data for optimal use. EGDT is a combination of prompt recognition of the symptoms of sepsis and septic shock, early antibiotic administration and aggressive, protocol-driven resuscitation interventions, and subsequent continuous monitoring of patients [15, 47].

River's landmark paper on EGDT demonstrated a 16% absolute reduction in mortality in patients treated using EGDT compared to standard therapy [47]. Used appropriately, EGDT has been shown to reduce mortality from 46.5% to 30.5% [47, 48]. However, despite its success, implementing EGDT is both costly and resource intensive. Physicians must place invasive lines and monitor resuscitation of patients closely. Nurses must manage multiple medications (including vasopressors) and tailor prescribed therapy based upon a number of parameters – some of which may be measured continuously and some of which require frequent or periodic blood draws by phlebotomists. In light of the resource and cost intensity of EGDT, there is concern that overly sensitive and insufficiently specific criteria for identifying patients with true bacterial sepsis may cause overuse of this intervention. Overly sensitive criteria may also lead to over-administration of antibiotics, increasing bacterial resistance and putting patients at risk of experiencing side effects from allergic reactions to organ toxicity. To focus EGDT at the most appropriate patients, EMD and CCU services may use a "Sepsis Team" comprised of experts whose focus is implementation and follow-up of practices called "sepsis bundles," as detailed below.

### *Sepsis Resuscitation Bundle*

Bundles of diagnostic tests, coupled with treatment interventions, are often triggered in the EMD or ICU in what is sometimes referred to as a "Code Sepsis" response [49]. The Surviving Sepsis Campaign Guidelines differ from those recently defined in the new SEP-1 core measures (circa 2015) as proposed by the Centers for Medicare and Medicaid Services and The Joint Commission; these guidelines and treatment parameters are somewhat controversial and are currently in revision. The Surviving Sepsis Campaign Guidelines define a series of processes, called bundles, to optimize care. Bundles are split into two sets, based on their timing. One is to be accomplished within 3 h of patient triage in the EMD to document symptoms – this is commonly called the "resuscitation bundle" and actions include:

1. Measuring a serum lactate level
2. Obtaining blood cultures prior to administration of antibiotics
3. Administering broad spectrum antibiotics
4. Administering 30 ml/kg crystalloid for hypotension or lactate ≥4 mmol/L

Further actions are required within 6 h of presentation and include:

5. Applying vasopressors (for hypotension that does not respond to initial fluid resuscitation) to maintain a MAP ≥65 mmHg
6. Reassessing volume status and tissue perfusion and documenting findings in the event of persistent hypotension after initial fluid administration (MAP <65 mm Hg) or if initial lactate was ≥4 mmol/L
7. Remeasuring lactate if initial lactate elevated

Other follow-up processes can include:

1. Steroid replacement: Low-dose steroids administered for septic shock that is poorly responsive to adequate fluid resuscitation and vasopressors in accordance with a standardized ICU policy
2. Glycemic control: Glucose maintenance approximating 150 mg/dl (8.3 mmol/L) (nutritional support and insulin control are often included in this focus) (Note: The recommendations for this intervention are still controversial)
3. Inspiratory plateau pressures maintained <30 cm $H_2O$ for mechanically ventilated patients

### *Rapid Antibiotic Therapy Saves More Lives than Any Other Intervention*

In cases of sepsis, rapid intervention with appropriate antimicrobial therapy can be critical to patient survival [47, 50]. For aerobes, anaerobes, and fungi, appropriate antibiotic therapy can increase survival by approximately 25–45% [4, 51, 52]. Eliminating delays in appropriate antibiotic administration has been shown to increase survival by approximately 7–10% per hour [4, 52]. According to a review of more than 2600 cases from 15 ICUs in 5 US and Canadian cities, the risk of death from sepsis increases by 6–10% per hour, starting from the onset of septic shock to the start of effective antimicrobial therapy [4]. Optimized antibiotic care requires intravenous (IV) broad-spectrum antibiotics with daily reevaluation to optimize efficacy, prevent resistance, avoid toxicity, and minimize costs; the goal of such therapy is to discontinue broad-spectrum coverage within 3–5 days and continue antibiotics that are targeted to the causative pathogen [44].

## Historical Laboratory Methods for Blood Cultures

Conventional laboratory methods for the identification and susceptibility testing of blood-borne pathogens may take several days to produce useful results and may be ineffective for detection of some pathogens [53]. Rapid detection of bloodstream infections in the critically ill, followed by appropriate antimicrobial therapy, can have a lifesaving impact. Thus, the development of rapid, sensitive, and accurate diagnostic laboratory methods to identify bacterial and fungal blood-borne pathogens and characterize associated antimicrobial resistance determinants and/or immune response will markedly benefit the diagnosis and therapy for septic patients and save many lives. A variety of historical methods have been grandfathered into laboratory protocols prior to CLIA regulations. New methods currently are being implemented by validating them as laboratory-defined tests (LDTs); the clinical utility and accuracy of these off-label assays must be reestablished in each laboratory where the method is used [54–68].

Despite attempts to rapidly identify pathogens and their associated resistance determinants, these techniques are not yet a common practice; thus patients, and their physicians, continue to wait for confirmatory results from blood cultures. Blood cultures remain the reference standard for the diagnosis of bacterial sepsis; these cultures can take several days to isolate a pathogen, limiting their usefulness in the management of the acutely ill patient. Moreover, blood cultures can frequently remain negative even in severe cases of sepsis [69]. For example, routine methods are relatively ineffective for detection of certain pathogens, such as *Coccidioides* spp. and *Brucella* spp., causes of culture-negative fungemia and bacterial endocarditis, respectively [69].

### *Resin Blood Culture Bottles*

Automated blood culture incubation systems have made some progress in reducing the time to flag for positive samples, commonly called time to positivity or time to detection. There are three blood culture systems currently available in the USA; the BD BACTEC FX (Becton Dickinson, Franklin Lakes, NJ), the BacT/ALERT 3D and Virtuo (bioMérieux, Durham, NC), and the VersaTREK (ThermoFisher Scientific, Waltham, MA). Charcoal-containing blood culture bottles have been reformulated in favor of resin particles that have increased capacity for antibiotic capture, lower volume requirements, and better lytic agents for phagocytes – all contributing to faster incubation time and improved microorganism recovery [70, 71]. For example, Kirn et al. have demonstrated that the BacT/Alert FA Plus bottles, containing resin, had an improved total recovery and faster detection time, when compared to the charcoal BacT/Alert FA bottles using spiked blood [70]. Similar results were found when comparing the BACTEC FX Plus bottles, containing resin, versus the BacT/Alert 3D FAN bottles, containing charcoal [71].

## Molecular Methods for the Diagnosis of Sepsis and Bacteremia

In order to provide a more rapid and accurate diagnosis, there is a significant need to improve the speed and diagnostic breadth of laboratory detection methods for bloodstream infections and sepsis. Routine diagnostic methods, including routine cultures for bacteria, fungi, and, rarely, viruses, almost all require subsequent subculture for organism identification, and the entire process can take days to weeks to produce a final result. Because of associated multiple-organ dysfunction in septic patients, death can occur rapidly, so improvements in diagnosis and treatment require both rapid identification/susceptibility testing and characterization of complex host symptoms [3, 47, 48]. Microbiology detection methods to characterize microbial pathogens and host response patterns are historically too slow, too insensitive, or too nonspecific to support differential diagnosis for EGDT strategies and do not fully assess the complexities of the host immune response during sepsis, which appears critical to the understanding of associated multiple-organ dysfunction and death.

Because of the complex nature of sepsis, there are no single laboratory tests that can be combined with clinical information to assess health outcomes or describe a time course for certain key biomarkers. Such a multi-component test will be the key to unraveling the parallel and complex processes in sepsis and to providing clinicians with a tool for detection, prognosis, and therapeutic monitoring of sepsis.

Clearly, historical immunological and molecular methods are impractical for detecting the complex patterns of immune responses seen in sepsis and the wide variety of blood-borne pathogens that can cause disease; therefore, clinical microbiology laboratories will need to consider the approach of "sepsis diagnostic panels," to combine detection of bloodstream pathogens and key aspects of host's innate and systemic immune response. In addition, sensitive genotypic and phenotypic predictors of antibiotic resistance and pharmacogenomic markers for potential drug toxicity will play a role in the not-too-distant future. Clearly, it will be critical to offset the costs of these new rapid methods with an overall reduction of hospital costs. For the laboratory to adapt and implement these changes, a team approach is needed; this approach relies on the interaction of the laboratory with pharmacists, physicians, and other healthcare staff so as to determine the most judicious use of these methods. One approach may include selective testing on only high-risk patients, which may benefit most from rapid testing.

Discussion with healthcare finance and reimbursement teams as well as antimicrobial utilization teams are critical for the proper test utilization decisions to be made. Considering the complexity and urgency of the diagnostic challenges we face, this review summarizes the most recent published developments in the diagnosis of sepsis and bacteremia, which impact clinical microbiology laboratories.

## *FDA-Approved Molecular Methods for Blood Culture Bottles*

### Peptide Nucleic Acid Fluorescent In Situ Hybridization (PNA FISH)

Peptide nucleic acid fluorescence in situ hybridization (PNA FISH) was essentially the first rapid diagnostic method whose utility was well documented for bloodstream infections [6, 8]. PNA FISH is a technology to test for pathogens on blood smears made directly from positive blood culture bottles. PNA FISH provides results in 30 min, with the QuickFISH version of the test, to 1.5 h with the traditional methods. PNA FISH probes target rRNA in microbial chromosomes, offering advantages of a small subunit rRNA (ssRNA) target; (1) sequences are known and unique between species; (2) ssRNA targets are highly abundant target, multi-copy; and (3) PNA probes, due to their small size, can bind in highly conserved regions that are not accessible to larger DNA probes [72]. The probes are commercially available from OpGen, Gaithersburg, MD (previously AdvanDx, Woburn, MA), for the direct identification of *S. aureus*/CoNS (coagulase-negative *Staphylococcus*), *Candida* spp., Gram-negative bacilli, GNB, (*E. coli*, *P. aeruginosa*, and *K. pneumoniae*), and *E. faecalis*/other enterococcus from positive blood cultures. Probe kit utilization is driven by the Gram stain result; thus Gram-positive cocci in clusters would utilize the *S. aureus* probe, yeast would utilize the *Candida* probe, and Gram-positive cocci in pairs and chains would utilize the dual *E. faecalis*/other enterococci probe.

PNA molecules contain the same nucleotide bases as DNA and follow standard base-pairing rules for hybridization for the following base pairs: adenine (A), thymine (T), cytosine (C), and guanine (G). The difference between DNA hybridization probes and PNA hybridization probes are that the negatively charged sugar-phosphate backbone of DNA is replaced with a non-charged polyamide or “peptide” backbone for the PNA probes. This replacement confers several advantages over conventional nucleic acid probes: (1) the strength of the FISH binding is greater than the DNA probe; PNA chemistry creates very small dye-labeled probes, approximately 12–20mers in size; therefore they are easy to hybridize to the target of interest; (2) because of the neutral charge, there is no natural repulsion with charged backbones; therefore they bind tightly; and (3) there is exquisite base discrimination, allowing for more specific binding to target DNA or RNA. In addition, the PNA probes confer very low background, which allows the fluorescent signal to be visualized and confers greater sensitivity [72].

Martinez et al. evaluated the performance of the four available *Quick*FISH assays: *S. aureus*/CNS, GNB, *Candida* (not FDA-approved), and *Enterococcus*. The sensitivity of all the assays, in this study, was 100%, compared to standard identification methods ($n = 159$) [73].

**PNA FISH Algorithms used for Intervention** PNA FISH algorithms were the first to focus on impact of escalation or de-escalation of antimicrobials based on PNA FISH results and by doing so, set the stage for some of the current antimicrobial stewardship algorithms based on rapid diagnostics, most notably for

*Enterococcus* spp. and *Candida* spp. The PNA algorithms, used by Forrest et al. [8, 74–76], were a historic shift to the concept that laboratories can provide actionable microbiology results and thus assist the provider's ability to impact care. Considering the historic shift that occurred after documentation of impact via PNA FISH, the clinical care pathways created by its use and some personal experience with the assays will be reviewed and discussed in detail.

The following review details studies that have applied PNA FISH as an adjunct to the Gram stain from positive blood culture bottles. When used appropriately with a team approach that involves the laboratory, pharmacy, as well as physicians, PNA FISH not only can direct therapy but can also drive down antibiotic usage/costs and hospital costs, and save lives [8, 74–76]. Optimal outcomes for rapid intervention with PNA FISH have been best documented when there is a strong collaborative effort between the laboratory and pharmacy. Holtzman et al., for example, found no impact when their pharmacy was not involved [77]. Others have noted success with physician intervention as detailed here. Wolk and colleagues found substantial decreases in both mortality and hospital expenditures using a multidisciplinary team of laboratorians, pharmacists, and physicians [6]. In a quasi-experimental study (pre- and post-intervention periods), the clinical utility was retrospectively assessed in an academic medical center. Laboratory scientists performed testing according to manufacturer's instructions for the *E. faecalis*/OE PNA FISH and *C. albicans/C. glabrata* PNA FISH. Results were phoned to pharmacists (once per shift) or on-call physicians (8PM–8AM) for use in antimicrobial assessments. A total of 683 patients were tested by PNA FISH. Total laboratory costs for reagents, controls, and supplies were $63,047. For *E. faecalis*/OE PNA (Gram-positive cocci in pairs/chains (GPCPC) identification), turn around time (TAT) was reduced from 4.4 d to 1.1 d and for *Candida* spp. from 6.7 d to 2.1 d ($p < 0.0001$). For GPCPC ($n = 460$), reporting PNA FISH was associated with reduction in all-cause mortality, from 13.1% to 8.0% ($p = 0.09$). In logistic regression analysis (adjusted for age, gender, and DRG code), mortality was significantly reduced ($p = 0.04$). For ICU patients, mortality significantly decreased from 34.6% to 18.3% ($p = 0.04$). Cost avoidance exceeded $2.6 million/year, and ICU costs were significantly reduced ($p = 0.01$). For yeast ($n = 125$), all-cause mortality decreased from 26.8% to 14.5% ($p = 0.14$). Logistic regression revealed that for 24/7 test performance ICU mortality was significantly reduced from 41.7% to 5.7% ($p = 0.02$). Cost avoidance for the yeast intervention exceeded $2.2 million/year. Overall cost avoidance for an academic healthcare system exceeded $4.7 million US dollars per year [6].

Using a targeted algorithm directed by the *E. faecalis* PNA FISH probes, Toombs et al. demonstrated that appropriate treatment for VRE and non-VRE can be directed for bloodstream infections [8]. Use of the PNA FISH probes significantly reduced the number of days required to reach appropriate therapy, from 2.5 to 1.4 days ($p < 0.05$). In turn, there was a trend toward less mortality (36% vs. 14%) ($p < 0.05$). Reduction in mortality was also observed in a quasi-experimental study performed by Forrest et al. in which PNA FISH was added to a treatment algorithm for hospital-acquired enterococcal bacteremia [76]. In this study, the primary outcome assessed

was defined as the "time from blood culture draw to implementation of effective antimicrobial therapy." Comparing 129 patients in the pre-intervention arm vs. 95 in the post-intervention arm, using PNA FISH, the investigators identified *E. faecalis* in 1.1 days vs. 4.1 days without PNA FISH, $p < 0.001$. For *E. faecium*, results were obtained in 1.1 vs. 3.4 days, $p < 0.001$. Comparisons between the pre- and post-intervention period revealed a decreased 30 d mortality (26% vs. 45%; $p = 0.04$) and overall hospital savings of $20,000/year [76].

Ly et al. studied 202 patients with Gram-positive cocci in clusters in the blood culture Gram stain [7]. In the intervention group, results and general organism information from the PNA FISH were relayed to the treating clinician. In the control group, no call was made. For patients, whose physicians received a PNA FISH result, an 80% reduction in ICU-related mortality was observed for bloodstream infections due to *S. aureus*. A median hospital cost savings of $19,441 per patient was observed, as was a 61% reduction in antibiotics for CNS, when deemed a blood culture contaminant [7].

Testing a high percentage of skin contaminants with PNA FISH can be costly and labor intensive; therefore, it is prudent to limit the percentage of skin contaminants recovered from blood culture bottles. Skin and line antisepsis is critical to prevent blood culture contamination. A < 3% contamination rate is considered a benchmark of good blood culture collection practice.

*Candida* species are the fourth most common cause of nosocomial bloodstream infections, commonly in the immunocompromised host population. Of all the *Candida* species, *C. albicans* is the most common isolate, accounting for 55% of all candidemias [78]. Guidelines defined by the Infectious Diseases Society of America (IDSA) promote the use of fluconazole as the initial therapy for *C. albicans* without prior azole use. Alternatively, guidelines suggest that broad-spectrum agents should be considered for non-*C. albicans* because of the risk of possible fluconazole resistance [79]. To support rapid adherence to those guidelines, PNA FISH testing provides direct identification from positive blood cultures in 2.5 h, as opposed to 1–5 days by conventional culture methods.

The ability of PNA FISH to support appropriate antifungal selection has been evaluated [74, 78, 79]. In one key study, the cost savings achieved with the *C. albicans* PNA FISH was $1729–1837 per patient. The majority of the cost savings were from antifungal expenditures [78]. Reporting of *C. albicans* by PNA FISH led to early switch to generic fluconazole without compromising patient safety.

## DNA Probes

**Luminex VERIGENE** The VERIGENE® platform (previously Nanosphere, now Luminex, Austin, TX) utilizes multiplex PCR coupled with nanoparticle technology for microorganism identification. There are two FDA-approved tests on this platform: the BC-GN, Gram-negative blood culture test, and the BC-GP, Gram-positive

blood culture test. The BC-GN identifies 8 pathogens and 6 antibiotic resistance markers, and the BC-GP tests for 11 pathogens and 3 antibiotic resistance markers. Accuracy studies report overall sensitivity of 92.6–100% and 85.9–93.8% for BC-GP and BC-GN, respectively [80, 81]. Clinical outcomes such as implementation of targeted therapy, length of ICU stay, 30-day mortality, and mortality associated to drug-resistant pathogens have been improved by implementing the VERIGENE BC-GN assay [43].

**GeneXpert®, Xpert® MRSA/SA BC Assay** In a multicenter preclinical evaluation, Wolk et al. evaluated the performance of two Xpert MRSA/SA (Cepheid, Sunnyvale, CA) assays for detection of methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) [82]. Using an integrated DNA extraction process coupled to real-time PCR, MSSA and MRSA were identified directly from positive blood culture bottles or wound swabs in <1 h. A total of 114 wound specimens and 406 blood culture bottles were tested from study sites in the USA and Europe, to characterize assay performance in a clinical setting. The primers and probes in the Xpert MRSA/SA assays detect sequences within the staphylococcal protein A (*spa*) gene, the gene for methicillin resistance (*mecA*), and the staphylococcal cassette chromosome (SCC*mec*) inserted into the SA chromosomal *att*B insertion site. Inclusion of both the *att*B insertion site and the *mecA* gene targets enables the assays to identify the presence of SCC*mec* cassette variants with *mecA* gene deletions, thus reducing false-positive results that occur in molecular tests that only target the SCC*mec* cassette [83, 84]. For the Xpert MRSA/SA assay performed on Cepheid GeneXpert system, sensitivity was 97.1% and 98.3% for MRSA in wound and blood culture specimens, respectively. Sensitivity was 100% for *S. aureus* from both specimen types [82]. The impact of implementation of the Xpert MRSA/SA assay was assessed by Emonet et al. in a randomized clinical trial [85]. The control group had blood cultures with Gram-positive cocci in clusters identified by conventional methods; the intervention group had MRSA/SA identified by the Xpert MRSA/SA assay. No significant effects were observed on clinical outcomes; however, 85.4% of patients in the intervention group received appropriate targeted therapy sooner, based on the Xpert results [85].

In 2010, Bauer et al. [86] used rapid organism detection of *S. aureus* bacteremia and communication to clinicians to expedite antibiotic optimization. They evaluated outcomes of the rapid polymerase chain reaction methicillin-resistant *S. aureus*/*S. aureus* blood culture test (GeneXpert, Cepheid, Sunnyvale, CA). Multivariable regression assessed outcomes from 156 patients. The average time to switch from empiric vancomycin to cefazolin or nafcillin in patients with methicillin-susceptible *S. aureus* bacteremia was 1.7 days shorter post-PCR ($p = 0.002$). Although not statistically significant, in the post-PCR methicillin-susceptible and methicillin-resistant *S. aureus* groups, the mean length of stay was 6.2 days shorter ($p = 0.07$), and the mean hospital costs were $21,387 less ($p = 0.02$).

## *Multi-Target PCR for Positive Blood Cultures*

**Portrait Staph ID/R Blood Culture Panel** A new multiplex PCR for the identification of *S. aureus*, *S. lugdunensis*, other staphylococci, and the *mecA* gene, in positive blood cultures, was approved by the FDA in 2017; the Portrait Staph ID/R blood culture panel (Great Basin Scientific, Salt Lake City, UT). Multicenter performance evaluation of the panel determined a positive percent agreement of 98.6% (211/214) for *S. aureus*, 100% (3/3) for *S. lugdunensis*, and 98.9% (444/449) for other *Staphylococcus* species, when compared to traditional identification methods. When using the cefoxitin disk diffusion test for the detection of methicillin resistance, the Portrait Staph ID/R blood culture panel showed a positive agreement of 96% (72/75) for *S. aureus* and 93.9% (247/263) for other *Staphylococcus* species. Negative agreement ranged from 99.4% to 99.6% for species identification and 96.4 to 100% for the *mecA* gene [87].

**BioFire FilmArray BCID** In 2012 BioFire (bioMérieux, Salt Lake City, UT) introduced a new test for the identification of microorganisms in positive blood cultures: FilmArray BCID. The assay is based on nested multiplex PCR, performed in a pouch, and provides results in 1 h. The original panel contained primers for >25 pathogens and 4 antibiotic resistance genes [88]. This panel was later revised and launched with 19 bacteria, 4 yeasts, and 3 antibiotic resistance gene targets. The assay was initially evaluated and determined to have a positive agreement of 91.6% for samples with monomicrobial growth and 71% when more than one microorganism was found by conventional methods [89]. Southern et al. found the panel to have a 94.6% sensitivity and 100% specificity, when considering on-panel organisms [90]. The clinical and economic impact of the FilmArray BCID on patients with Gram-positive cocci or *Candida* in blood was evaluated in a retrospective study. Utilization of the panel was associated with significant reductions in mortality, ICU days, ICU costs, and total costs. Results from the FilmArray BCID panel also had a significant impact on vancomycin treatment, earlier targeted therapy for vancomycin-resistant enterococcal bacteremia, and less use of the antibiotic for MSSA or coagulase-negative *Staphylococcus* (CNS) bacteremia [91].

**GenMark ePlex™** The ePlex respiratory pathogen (RP)™ was the first FDA-approved multiplex panel for the ePlex system (GenMark Diagnostics, Carlsbad, CA). The ePlex features a modular, scalable system with integrated data analytics, short hands-on time for setup, and time to result of 2 h [92]. Among the development pipeline for the system are three new panels for blood culture microorganism and antimicrobial resistance detection, as well as unique markers that allow for cross-detection of organisms not featured in the panel. The Gram-positive cartridge detects 20 bacteria, 4 markers for antibiotic resistance, and 2 markers that indicate the presence of Gram-negative bacteria and/or *Candida* in the sample. The Gram-negative panel detects 21 bacteria and 6 resistance genes and also features detection for Gram-positive organisms and/or *Candida*. Lastly, the fungal cartridge has the

ability to detect ten species of *Candida* and six other fungi genre/species [93]. GenMark plans to complete the clinical trials for the blood culture panels by the end of 2018.

## Emerging Methods for Bloodstream Infection Diagnosis

The future of clinical diagnostics is anticipated to include a variety of rapid, multiplex, and direct-from-blood methods. A review of all technologies is beyond the scope of this review; however, several will be discussed, including multiplex-PCR, DNA sequencing, liquid microarrays, and whole genome sequencing. It is known that blood culture bottles, positive for bacteria/fungi, do not always support cultivation of the pathogen to agar [53, 94]; therefore, new molecular methods may allow microbiology laboratories to identify fastidious pathogens or those damaged by antibiotics more efficiently. More detailed information about the following techniques may be found in other reviews [95, 96].

### *Direct-From-Blood Tests*

To reduce the time to detection in cases of sepsis and septic shock, several bioindustry teams have developed, or are in the process of developing, assays that bypass the need for blood cultures. Among them, the SepsiTest and the T2Dx have been evaluated and published and are discussed below.

Assays still in development include the Q-linea ASTar™ (Uppsala, Sweden), which will include both identification and rapid AST from direct blood; Seegene's Magicplex™ (Seoul, Korea), a direct-from-blood multiplex PCR; and the Qvella™ FAST™ (Richmond Hill, ON, Canada) assay that uses electrical lysis instead of DNA extraction, coupled with PCR for organism identification.

#### Molzym SepsiTest™

A PCR/pyrosequencing method is based on the detection of conserved sequences in whole blood for rapid diagnosis of bacteremia and fungemia. The SepsiTest (Molzym Molecular Diagnostics, Bremen, Germany) is not commercially available in the USA. The test uses a universal PCR from the 16S and 18S rRNA genes with subsequent identification of bacteria and fungi, respectively, from positive samples by sequence analysis of amplicons. The SepsiTest was evaluated in a prospective, multicenter study of 342 blood samples from 187 patients with systemic inflammatory response syndrome, sepsis, or neutropenic fever. Compared to blood culture, the diagnostic sensitivity and specificity of the PCR were 87.0% and 85.8%, respectively, achieving improved results in accuracy over the SeptiFast and yielding results

in a few hours. The concordance of PCR and traditional methods for both positive and negative samples was 86.0% despite the indispensability of blood culture diagnostics [97, 98].

In the USA, SepsiTest was evaluated with whole blood extracted from 1130 samples from 913 patients with suspected bacteremia. Compared to traditional blood culture, the SepsiTest performed with a sensitivity of 77.8%, specificity of 99.3%, positive predictive value of 93.3%, and negative predictive value of 97.2%. SepsiTest accurately identified bacteria in 77.8% (98/126) of culture-confirmed sepsis samples. A reduction in time to results from 81.6 ± 24.0 h when using traditional blood culture methods to 7.5 h when using SepsiTest from whole blood was also observed [99].

#### T2Biosystems®

The T2Dx® instrument uses magnetic resonance technology for microorganism and biomarker identification from whole blood. The T2Candida® Panel is FDA-approved and identifies five Candida species, in three categories: *C. albicans*/*C. tropicalis*, *C. parapsilosis*, and *C. glabrata*/*C. krusei* [100]. The performance of the assay was evaluated in a multicenter clinical trial resulting in an overall sensitivity of 91.1% and specificity of 99.4% (*n* = 1501 fresh and 250 spiked samples). The limit of detection of the assay ranges from 1 to 3 CFU/ml depending on the *Candida* species. A reduction of up to 125 h in time to detection was observed during the study. It is to be noted, however, that the assay shows a high number of invalid results and technical errors, *n* = 245/2264 [101]. A model on the economic impact of the T2Candida panel estimates savings of ~$25 K/patient with candidemia, in hospitals admitting >5000 high-risk patients, with ~60% reduction in mortality and length of stay [102].

In May 2018, the T2Bacteria™ panel gained FDA clearance; however, results from the accuracy study have not been published. The company intents on releasing a Lyme disease diagnostic test in the future, as well as panels for hemostasis determination.

### *Direct from Blood Culture Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)*

In the past decade, wide implementation of MALDI-TOF MS for microbial identification has revolutionized the clinical microbiology laboratory workflow. Two systems are FDA-approved for use as microbiology diagnostic tools: the VITEK® MS (bioMérieux Inc.) and the MALDI Biotyper CA System (Bruker Daltonics Inc.). Both systems are cleared for bacterial identification, and the VITEK MS is also approved for yeast [103]. MALDI-TOF MS as approved requires growth of

microorganisms on agar plates from which colonies are picked and spotted onto target plates with the matrix that allows ionization and analysis [104]. Even though this approach saves time to identification, it is not optimal for blood cultures. Several procedures have been developed to be able to use blood culture media directly on target plates. Laboratory-developed extraction methods include the use of separator tubes, several washing steps, and diverse types of lysis solutions to remove red blood cells previous to spotting onto the target plates [105–109]. Bruker Daltonics (Billerica, MS) developed an extraction kit for positive blood cultures, the Sepsityper. Coupling the Sepsityper to the Bruker Biotyper instrument, Buchan et al. found 97.6% concordance at genus and 94.1% at species level compared to routine identification methods. The time to result was 23–83 h faster for Gram-positives and 34–51 h for Gram-negatives using the Sepsityper/Biotyper procedure [110]. The Sepsityper kit can also be used to transfer samples, when laboratories do not have the ability to perform MALDI-TOF in house [111].

Studies evaluating the impact of direct-from-blood culture MALDI-TOF are still limited. Vlek et al. found that using a laboratory-developed lysis buffer, followed by MALDI-TOF MS, from positive blood cultures lead to an 11.3% increase in the proportion of patients receiving appropriate antimicrobial treatment [112]. Integrating the implementation of MALDI-TOF MS to a strong antimicrobial stewardship program reduces the time to optimal therapy by more than 30 h for positive blood cultures as well as cultures detecting contaminant organisms [113]. Overall length of stay, days in ICU, and length of antimicrobial therapy were also reduced when MALDI-TOF MS was coupled with antimicrobial stewardship intervention [113].

### *Nucleic Acid Sequencing*

Pyrosequencing® (Qiagen, Germantown, MD) is a rapid method for sequencing based on the detection of pyrophosphate released during DNA synthesis. These methods' advantages include speed and ease of use in comparison with traditional sequencing technology; disadvantages include the short lengths of sequences that can be currently analyzed. Pyrosequencing® provides short sequence information roughly 30–50 bases; it is useful for short-read DNA and mutation/SNP analysis. It is ideally suited for applied genomics research including molecular applications for disease diagnosis, clinical prognosis, and pharmacogenomics testing. After PCR amplification and amplicon cleanup, run times approach 1 h for 96 samples, with approximately 30–45 min for sequence analysis applications [114].

Jordan et al. evaluated pyrosequencing directly from blood culture bottles to assess its potential to differentiate between bacteria commonly associated with neonatal sepsis [114]. An informative 15 bases within the 380-bp amplicon was targeted for pyrosequencing following enrichment culture and PCR amplification. A total of 643 bacterial isolates commonly associated with neonatal sepsis and 15 PCR-positive, culture-positive neonatal whole blood samples were analyzed by pyrosequencing. Results of DNA sequencing and culture identification were compared and

were successful at using PCR and pyrosequencing together to accurately differentiate between several bacterial groups, both Gram-positive and Gram-negative. The system had some difficulty with viridians group strep and *S. pyogenes* [114]. The same group evaluated specimens from isolates from neonatal sepsis events, to support species identification that could lead to rapid de-escalation or targeting of antibiotic therapy. A total of 643 bacterial isolates and 15 PCR-positive, culture-positive neonatal whole blood samples were analyzed by pyrosequencing. Pyrosequencing was able to provide useful information on the identity of species based on the amplicon generated by PCR [114]. Quiles-Melero et al. tested a unique paper-based DNA preservation method prior to pyrosequencing of *Candida* species and found 100% concordance to species identification for 48 positive blood cultures, containing 47 yeast and 1 filamentous fungus. Primers for *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, *C. krusei*, and *A. niger* were included [97].

#### Whole-Genome Sequencing

Traditionally, whole-genome sequencing (WGS) has been used primarily as an epidemiologic instrument; however, advances in the technology to make it affordable, fast, and user-friendly allow for microbiologists to start thinking about it as a diagnostic tool. Coll et al. created a genomic library to identify antibiotic resistance markers in cultured *M. tuberculosis*, a test that can take weeks to perform, in a matter of hours, using the Illumina platform for WGS [115]. The same platform has been used for surveillance of carbapenem resistance in clinical isolates with >99% concordance with phenotypic assays [116]. Oxford Nanopore Technologies (Oxford, UK) had developed a portable, real-time device for DNA and RNA sequencing: MinION™. This technology has great potential for clinical diagnosis and is being used primarily in remote locations where access to full-size equipment is very limited. An Indonesian team developed and validated an assay coupling reverse transcription loop-mediated isothermal amplification with MinION sequencing of the product to determine dengue virus serotypes in serum [117]. The platform also proved to be very useful in the Ebola outbreak of 2014–2015 allowing researchers to sequence the virus directly from patient's blood [118]. Uses of this technology in the clinical laboratory can greatly improve detection of unculturable organisms. Further development and evaluations are necessary before it becomes an integral part of the clinical microbiology laboratory workflow.

### *Accelerate Pheno™ System*

Bacterial immobilization coupled with automated microscopy allows for real-time, live measurement of growth rate. Proof-of-concept papers for the use of this technology for bacterial identification and rapid AST were published in 2013–2014.

Metzger et al. used the technology to identify *S. aureus* and *P. aeruginosa* in bronchoalveolar samples, whereas Burnham et al. used it for rapid classification of *K. pneumoniae* for carbapenem resistance [119, 120]. Since then, the technology has been adapted, by Accelerate Diagnostics™ (AXDX, Tucson, AZ), to an automated system called the Accelerate Pheno™ that uses FISH probes and a diverse panel of antibiotics to perform microorganism identification in 1.5 h and AST in less than 7 h. Results for AST are provided as minimum inhibitory concentrations. During 2016 the Accelerate PhenoTest™ BC kit for microorganism identification and rapid AST from positive blood cultures was evaluated in a multicenter clinical trial, and the results were submitted to the FDA for approval. Both the Accelerate Pheno™ System and the Accelerate PhenoTest™ BC kit gained de novo FDA clearance in February of 2017. The Accelerate PhenoTest™ BC kit is approved to test for six Gram-positive (two at genus level), eight Gram-negative (four at genus level), and two *Candida* species. The system was also approved for a unique *monomicrobial* designation; the combination of specific FISH probes with a universal nucleic acid stain allows the system to confidently make a call for a single organism in the sample.

Full text publications on the evaluation of the system/kit are very limited; Marschal et al. utilized the kit to identify Gram-negative bacteria and perform rapid AST and compared the results to traditional methods [121]. The study included 115 samples from patients with bloodstream infections, 88.7% of all the infections were correctly identified by the Accelerate Pheno™ system, and 97.1% of all isolates were included in the Accelerate PhenoTest BC kit. Categorical agreement between the Accelerate PhenoTest BC kit and culture-based AST was 96.4% [121]. In a late-breaker poster presented at the ASMicrobe 2017 meeting in New Orleans (June 03, 2017), Hernandez et al. found that the Accelerate PhenoTest BC kit performed with an overall sensitivity of 98.2% and a specificity of 99.8% in a combination of fresh positive blood cultures and spiked samples. The monomicrobial call was made for 75% of the samples tested in the study, with a positive predictive value of 100%. For AST, the overall agreement was 96.1% for essential and 94.2% for categorical agreements, compared to Vitek2 [122]. The multicenter evaluation of the Accelerate Pheno system that led to its clearance by the FDA found that the system accurately identified 14 common bacterial pathogens and 2 *Candida* spp. with sensitivities ranging from 94.6% to 100% [123]. The positive predictive value for the monomicrobial call was 97.3%, and the call was made for 89% of the fresh positive blood cultures. For AST, the overall essential and categorical agreements, for Gram-positive cocci, were 97.6% and 97.9%, respectively. For Gram-negative rods the overall essential agreement for AST was 95.4%, and the categorical agreement was 94.3%. Very major errors were found for 0.5–1% of samples tested, whereas major and minor errors ranged from 0.7% to 4.8%. For the antibiotic resistance markers for cefoxitin and MLSb, the Accelerate PhenoTest BC kit showed >96% agreement [123].

## Molecular Testing for Drug Resistance

Rapid and accurate determination of microbial drug susceptibility is actually the most critical challenge; it is essential to facilitate successful antimicrobial therapy of any person and particularly useful for those with immune function impairment. Rapid testing for genetic resistance markers is an emerging clinical practice that can not only identify the potential for drug resistance but also help distinguish ambiguous breakpoints associated with susceptibility testing. Future methods should facilitate educated choices for therapy, which can be initiated early in diagnosis to impact patient outcomes. Evidence-based studies will be key to the adoption of these new testing antimicrobial testing paradigms.

Targets for genetic testing require relative genetic stability of the target sequence, a requirement that demands national and international efforts for monitoring genetic mutations in these target regions. Together, MRSA and VRE are the two most important resistant bacterial pathogens in US hospitals, and their rapid detection remains a critical necessity as antimicrobial resistance continues to increase in the USA and worldwide [124]. Among bacteria, other useful antimicrobial resistance targets include resistance genes for β-lactams, aminocylitols, aminoglycosides, chloramphenicol, fluoroquinolones, glycopeptides, isoniazids, macrolides, mupicurin, rifampin, sulfonamides, tetracyclines, and trimethoprim [125–127]. For fungi, rapid detection of antifungal resistance is useful, primarily due to the increase in fungal infections among immunocompromised patients. Current antifungal assays rely on fungal susceptibility testing which is dependent on growth. The practical application of antifungal molecular testing is yet to be seen, as there is still much to learn about the genetic markers, which mediate resistance. The genetic information needed to examine fungal resistance at the molecular level is complex; a review of molecular mechanisms of antifungal resistance has been published [128].

The full potential of molecular diagnostics for drug resistance testing in microbiology has not reached its full potential – its application is still in its infancy. As the molecular mechanisms of antimicrobial resistance are described, newer technologies may enhance the utility of such an approach. Furthermore, microarray technology has the promise to impact the rapid and accurate detection of multiple mutations associated with resistant bacteria, mycobacterium, viruses, and fungi. Until the full potential of drug-resistant markers is understood, rapid molecular antimicrobial testing must still be combined with traditional microbial cultivation [124].

Some multiplex assays like the BioFire FilmArray and the Luminex VERIGENE include limited testing for antibiotic resistance markers. Other, stand-alone multiplex PCRs are also available. Streck (Omaha, NE), a molecular technology company, produces the ARM-D® kits for β-lactamases and *ampC* identification. The β-lactamase kit includes primers for five carbapenemases, two ESBLs, and two AmpCs; and the *ampC* kit tests for six gene families for the resistance markers. Strains previously characterized by phenotypic means were tested with the ARM-D

β-lactamase kit to prove its utility. The kit, coupled with Streck's Philisa Thermal Cycler, provided results in 18 min and showed 92% sensitivity and 100% specificity [129]. Utilization of this multiplex PCR on positive blood cultures and whole blood is under investigation. Check-Points, another company that produces multiplex for antibiotic resistance markers, based in the Netherlands. Their products are only approved for use in Europe and range from carbapenemase detection to confirmation of ESBLs and AmpC status on Gram-negative bacteria [130–132]. The performance of the kits has been evaluated using fecal swab samples where the carbapenemase kit (Check-Direct CPE®) showed 100% sensitivity and 94% specificity, when compared to selective culture [131]; on isolates, the CT103XL kit for β-lactamase detection performed with 94.2% accuracy [130]. Lastly, a proof-of-concept study was published by Juiz et al. [133] demonstrating the feasibility of using the Check-MDR CT102 ESBL–carbapenemase microarray directly from positive blood cultures.

## Biomarkers for the Diagnosis of Sepsis

Biological substrates related to immune reactions, the coagulation cascade, vascular endothelial damage, and others have been used as surrogate markers for the prognosis of outcomes of severe infections and sepsis. Over 170 interleukins, chemokines, inflammatory proteins, tumor necrosis factors, toll-like receptors, etc. have all been evaluated as markers for sepsis and septic shock with different degrees of success [134]. Procalcitonin (PCT) emerged as a diagnostic biomarker for sepsis in the early 2000s, and it has been extensively studied. Despite the fact that it is used clinically, its sensitivity/specificity to differentiate sepsis from other inflammatory responses ranges in the 67–76% which makes it non-reliable as a stand-alone test [135]. To increase the diagnostic utility of sepsis biomarkers, several studies have been performed using combinations or substrates. Bauer et al. evaluated a combination of PCT, C-reactive protein (CRP), and cellular immune markers in a prospective observational study. The authors found that the combination of PCT, CRP, and neutrophil CD64, after adjustment for APACHE IV score, is a significant predictor of sepsis (area under the curve, AUC = 0.90) [136]. Immunexpress® (Brisbane, Australia) has developed a panel that tests for four RNA biomarkers: CEACAM4, LAMP1, PLA2G7, and PLAC8. The SeptiCyte™ LAB is FDA-approved for the differentiation of sepsis from infection-negative SIRS. The panel performed with AUCs in the 0.89–0.95 range in a multicenter clinical trial [137]. Studies evaluating the clinical utility of the panel and its impact on critically ill patients are still pending.

## Other Technologies in Development for Sepsis Diagnosis

Specific™, a company located in Mountain View, CA, has designed a sensor array system that detects volatile organic compounds to determine microbial identity based on colorimetric assays. The system includes a variation on the blood culture incubators and bottles. The Specific bottles have the sensor array integrated on the cap and can only be used in the Specific incubator. The first proof-of-concept paper for the technology showed that the rapID Dx™ is able to detect 18 species of bacteria with an overall accuracy of 91.9%, 2 h faster than the BacT/Alert system [138]. A follow-up paper determined that the rapID Dx can detect yeast at an inoculum load of 1.7 CFU/ml, 6.8 h faster than the BacT/Alert platform, with an overall sensitivity of 74% at detection. The sensitivity increased with time, to almost 95% at 4 h after detection; however, leaving bottles incubating that long would effectively nullify the time saved with the Specific system, compared to BacT/Alert [139]. It is to be noted that both of these studies used spiked samples; the system has not been tested on clinical samples. The company is planning clinical trials sometime in 2018–2019. They also have a rapid AST assay in development.

Another assay that might be developed for its use in clinical microbiology laboratories is being developed by ZEUS Scientific (Branchburg, NJ) in collaboration with St. Luke's University Health Network, located in Bethlehem, PA. The assay measures the activity of the microbial DNA polymerase during logarithmic growth, potentially reducing time to detection in blood cultures [140]. The assay is based on manual quantitative PCR and performed with sensitivity and specificity of 70.3% and 99.3%, respectively, for microbial detection, on a prospective study with matched cultures processed with traditional methods [141].

## Summary

Bacteremia and sepsis are critically important clinical syndromes with a high mortality, morbidity, and associated costs. Bloodstream infections and sepsis are among the top causes of mortality in the USA, killing over 600 people per day. Many septic patients are treated in emergency medicine departments or critical care units, settings in which rapid administration of targeted antibiotic therapy drastically reduces mortality. Unfortunately, routine blood cultures are too slow to support rapid therapeutic interventions. As a result, empiric, broad-spectrum treatment is common – a costly approach that may fail to effectively target the correct microbe, may inadvertently harm patients via antimicrobial toxicity, and may contribute to the evolution of drug-resistant microbes. To meet these diagnostic challenges, laboratories must understand the complexity of diagnosing and treating septic patients, in order to focus on creating algorithms that help direct targeted antibiotic therapy and synergize with existing EMD and ICU clinical practices put forth in the Surviving Sepsis Campaign Guidelines.

Several methods for rapid molecular identification of pathogens from blood cultures bottles are available. Labs can integrate with overall care to support local Surviving Sepsis Campaigns by providing rapid testing to facilitate targeted therapeutic interventions for infections with common blood-borne pathogens. As a result, empiric, broad-spectrum, antibiotic therapy can be shortened to improve survival, reduce healthcare costs, and decrease antibiotic resistance. More evidence and clinical utility studies are needed to justify the added expense of molecular methods, to determine an appropriate niche in patient populations for which the cost benefit would be favorable. Multiplex and PNA FISH assays are now part of the microbiology laboratory workflow in large healthcare centers and have proven to be cost-effective and clinically useful. Nucleic acid sequencing has proven accuracy and awaits practical adaption to routine microbiology laboratories. MALDI-TOF MS shows real promise and is currently being adapted to rapid identification of pathogen isolates and potentially to blood cultures.

The development of molecular diagnostic assays for detection of single pathogens from blood culture bottles have already shown to have impact on reducing mortality and costs. In the future, tests for multiple pathogens that could characterize Gram-positive, Gram-negative, and fungal infections would enable more rapid and targeted antimicrobial interventions for those with severe disease. Targeted drug resistance gene testing of blood culture bottles will enable risk assessment and guide treatment options for sepsis. Ultimately, early intervention by molecular detection of bacteria and fungi directly from whole blood would provide the most patient benefit and contribute to a tailored antibiotic coverage of the patient early in the course of the disease, allowing for more effective treatment and better outcomes of patients with sepsis and septic shock.

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# Advanced Pathology Techniques for Detecting Emerging Infectious Disease Pathogens

**Wun-Ju Shieh**

## Introduction

Detection and surveillance for emerging and reemerging pathogens require a multidisciplinary approach. The intertwining complexity of these pathogens with their diverse tissue tropisms, direct effects on host cells, multiphasic immunological responses, and additional influence of superimposed secondary agents is beyond the expertise of any single discipline in modern medicine. A combined evaluation of patient's history, clinical manifestations, and physical examination may suggest a list of differential diagnosis, but it is often insufficient to determine the specific infectious etiology. Laboratory methods are essential to identify an etiologic agent from testing clinical samples, such as blood, serum, nasopharyngeal swab, etc. These methods, including traditional microbiological techniques, conventional immunological assays, and modern molecular methods, remain the mainstay in today's practice of clinical microbiology and infectious disease medicine. Nevertheless, there are technical and logistic issues associated with these methods, and the test results often lack a clinicopathologic correlation that can confound the interpretation of their clinical significance. For example, microbiological culture may fail to grow a causative organism, while the organism isolated by the laboratory in vitro may arise from contamination and does not represent the actual infective agent in vivo.

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Pathology plays a key role as a bridging subspecialty in such multidisciplinary approach. Pathologic examination, if available, can establish a more specific diagnosis correlated with clinical manifestations. Although general practice of pathology is largely oriented toward diagnosis of neoplastic diseases, pathologists have been increasingly called upon to make diagnoses from tissue samples collected by cytology, biopsy, and autopsy procedures in response to the challenge of emerging infections [1–4]. Using these tissue samples as the source for laboratory workup, pathologists have made various contributions to our understanding of emerging infectious diseases in diagnostics, pathogenesis, epidemiology, and clinical aspects of these diseases (Table 1). In addition, results from pathologic studies can help design better strategies for control and prevention of these emerging infectious diseases, especially when they occur as an outbreak [5, 6]. Furthermore, pathologic studies also play an essential role in identifying the effects of secondary pathogens that commonly complicate the primary disease syndrome [7, 8].

Recent advances in molecular biology have revolutionized the practice of medicine, especially in the arena of diagnostic pathology and laboratory medicine [9–11]. The practice of pathology has evolved from using morphologic pattern recognition as the main tool to a sophisticated medical subspecialty by applying a wide array of advanced immunologic and molecular techniques on top of the traditional methods. The so-called traditional methods include routine hematoxylin and eosin (H&E) stain, histochemical (special) stain, and electron microscopy (EM). The more commonly used advanced techniques include immunohistochemistry (IHC), in situ hybridization (ISH), polymerase chain reaction assay (PCR), and tissue microarrays. Other advanced techniques that are less standardized as diagnostic utilities for infectious diseases include confocal microscopy, proteomics, laser capture microdissection (LCM), in situ PCR, pyrosequencing, and next-generation sequencing (NGS). The results from these techniques provide different information regarding the infectious agents in the organ systems they involve (Table 2). Each technique has its respective advantages and limitations, and there is no single technique that can stand alone as the only method for etiologic diagnosis. The advanced techniques complement the traditional methods to confirm the diagnosis; therefore, it is always necessary to apply these techniques as an integrated laboratory utility to take full advantage of the pathology approach. A good example to illustrate such approach is the identification of a novel coronavirus during the global epidemic of severe acute respiratory syndrome (SARS) in 2003 [12–17]. By using traditional culture (Fig. 1a) and EM examinations (Fig. 1b) on clinical samples and tissue specimens, the morphologic evidence of coronavirus leads to subsequent anatomic localization of this novel virus in lung tissues by using a combination of IHC (Fig. 1c), ISH (Fig. 1d), and PCR. Ultimately, correlations of these data with serological and clinical findings confirmed the SARS-associated coronavirus (SARS-CoV) as the etiologic pathogen of the outbreak. This is a prime example of the contributions made by infectious disease pathology as part of a multidisciplinary approach to investigate emerging infections and disease outbreaks.

**Table 1** Examples of outbreaks caused by emerging pathogens initially identified or confirmed by pathologic studies

| Year(s) | Disease outbreak | Country or geopolitical region |
|---|---|---|
| 1993 | Hantavirus pulmonary syndrome | USA |
| 1995 | Ebola hemorrhagic fever | Zaire |
| 1995 | Leptospirosis associated with pulmonary hemorrhage | Nicaragua |
| 1996 | Lassa hemorrhagic fever | Sierra Leone |
| 1997 | Enterovirus 71 hand-foot-and-mouth disease with encephalitis | Malaysia |
| 1997 | H5N1 influenza | Hong Kong |
| 1998 | Enterovirus 71 hand-foot-and-mouth disease with encephalitis | Taiwan |
| 1998–1999 | Marburg hemorrhagic fever | Democratic Republic of the Congo |
| 1999 | Nipah virus encephalitis | Malaysia |
| 1999 | West Nile encephalitis | USA |
| 2000 | Rift Valley fever | Saudi Arabia/Yemen |
| 2000 | Ebola hemorrhagic fever | Uganda |
| 2001 | Inhalational and cutaneous anthrax | USA |
| 2002 | Transplant-associated West Nile encephalitis | USA |
| 2003 | Sever acute respiratory syndrome | Global |
| 2003 | Monkeypox | USA |
| 2003, 2005, 2007, 2010 | Transplant-associated lymphocytic choriomeningitis virus | USA |
| 2004 | Transplant-associated rabies | USA |
| 2006/07 | Rift Valley fever | Kenya/Somalia |
| 2008 | Lujo virus hemorrhagic fever | Zambia/South Africa |
| 2009 | H1N1 pandemic Influenza | Global |
| 2009 | Transplant-associated Balamuthia mandrillaris meningoencephalitis | USA |
| 2010 | Dengue hemorrhagic fever | Puerto Rico |
| 2011 | Leptospirosis | Puerto Rico |
| 2012 | Transplant-associated microsporidia infection | USA |
| 2012 | Multistate steroid injection-associated fungal meningitis | USA |
| 2013 | Ferret-badger rabies | Taiwan |
| 2014–2015 | Chikungunya virus fatal cases | Puerto Rico |
| 2015–2016 | Congenital Zika syndrome | Brazil/Colombia/Puerto Rico/Caribbean |

**Table 2** Pathology techniques and their utilities for infectious disease diagnosis

| Technique | Main utility | Remarks |
|---|---|---|
| Hematoxylin and eosin Stain (H&E) | Shows histopathologic features of infectious process | Illustrates the evidence of a microbial infection and provides guidance to subsequent laboratory testing<br>Does not highlight the pathogen per se<br>Can only suggest certain infections and not a specific etiologic organism |
| Histochemical stain (special stain) | Highlights organisms | More useful for bacterial, mycobacterial, and fungal organisms<br>Only categorizes organisms within a broad classification but not a specific species<br>Can be difficult to interpret |
| Electron microscopy (EM) | Illustrates microbial ultrastructure | The most direct evidence to show an infectious agent<br>Timeconsuming and limited to small areas of interest |
| Immunohistochemistry (IHC) | Localizes microbial antigens | Demonstrates antigens regardless the organism is intact or not<br>Provides histomorphologic correlation of infectious process<br>Many commercially available antibodies for common pathogens<br>Antibodies of novel pathogens may not be readily available<br>Formalin fixation may decrease sensitivity |
| In situ hybridization (ISH) | Localizes microbial nucleic acids | Probes can be synthesized in house with known sequence<br>Provides histomorphologic correlation of infectious process<br>Usually more specific but less sensitive than IHC<br>Formalin fixation may decrease sensitivity |
| Polymerase chain reaction assay (PCR) | Amplifies small amount of microbial nucleic acids | Usually more sensitive than IHC and ISH<br>Contamination issues frequently encountered<br>Does not provide histomorphologic correlation of infectious process<br>Formalin fixation may decrease sensitivity |
| Tissue microarray | Detects multiple microbial nucleic acids | Facilitate sequence analysis and pathogen identification<br>Can detect microbes and assess related host responses simultaneously<br>Biosafety concerns using frozen tissues<br>Less sensitive than conventional PCR |
| Next-generation sequencing (NGS) | Analyze individual genome and large-scale sequencing | Can analyze individual near-complete exome or genome to assist in the diagnosis<br>Reduce the cost of large-scale sequencing<br>Current limitations, including nonstandardized platforms, long turnaround time, and the need of powerful bioinformatics to analyze large amount of data |

(continued)

**Table 2** (continued)

| Technique | Main utility | Remarks |
|---|---|---|
| Confocal microscopy | Increases morphologic dimension | Provides wider spectrum for histopathologic or cytologic interpretation<br>Limited diagnostic utility for emerging pathogens |
| Laser capture microdissection (LCM) | Dissect special target cells for PCR or proteomic studies | Useful in studies of pathogenesis<br>Limited diagnostic utility for emerging pathogens |
| In situ polymerase chain reaction assay | Localizes microbial nucleic acids with amplification process | Combines amplification and in situ localization methods<br>Inherent technical issues with nonstandardized protocols<br>Formalin fixation may decrease sensitivity<br>Limited diagnostic utility for emerging pathogens |
| Proteomics | Detects microbial and host peptides | Useful in studies of pathogenesis<br>Formalin fixation may decrease sensitivity<br>Limited diagnostic utility for emerging pathogens |

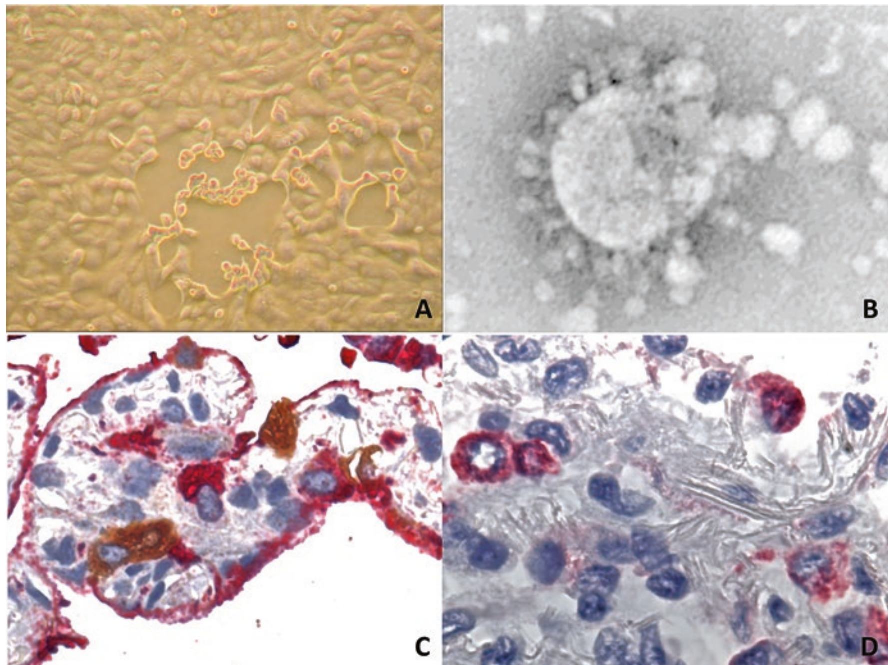


**Fig. 1** (**a**) Vero E6 cells show early cytopathic effect with coronavirus isolates from patients with SARS (Courtesy of Dr. Thomas G. Ksiazek). (**b**) Negative-stain (methylamine tungstate stain) electron microscopy shows coronavirus particle with an internal helical nucleocapsid-like structure and club-shaped surface projections (Courtesy of Dr. Charles D. Humphrey). (**c**) Double-stain IHC (immunoalkaline phosphatase polymer and peroxidase polymer) shows SARS-CoV (red) and surfactant antigens (brown) in type II pneumocytes. (**d**) ISH shows SARS-CoV nucleic acids in pneumocytes

## Highlights of Techniques

### *Hematoxylin and Eosin Stain*

Any pathology laboratory dealing with clinical diagnosis routinely performs hematoxylin and eosin (H&E) stain. It demonstrates the histologic and cytologic features in a tissue section and allows the pathologists to examine the microscopic changes related to infectious processes. Although it cannot highlight the pathogen per se, microscopic examination of H&E-stained slides is the most unequivocal method to illustrate the evidence of a microbial infection and its consequence in the tissue. For example, the presence of abundant neutrophils in the pulmonary alveoli is indicative of pneumonia (Fig. 2a), while neutrophils in the meninges support the diagnosis of meningitis. In some instances, the histopathologic features may be suggestive of infection caused by a specific pathogen; for example, the presence of smudge cells with necrotizing pneumonitis is indicative of an adenovirus infection in the lung. Nevertheless, most of the histopathologic findings shown by H&E stain are not specific because they can be caused by a variety of organisms. Their importance is to pave the first step leading to further laboratory assays for detecting the causative agent.

### *Histochemical Stains (Special Stains)*

Many histochemical stains have been developed to highlight a variety of microbial organisms. Some of the common ones are tissue Gram stain (for bacteria), Grocott's methenamine silver stain (for fungi), acid-fast stain (for mycobacteria), periodic acid-Schiff stain (for organisms with high content of carbohydrate macromolecules), Warthin-Starry silver stain, or Steiner's silver stain (for spirochetes and other bacteria). Interpretation of these special stains performed on tissue sections is usually more difficult than those performed on cultures because the coexistence of host tissue responses and accompanied histopathologic changes in the sections can confound the interpretation. It needs more expertise and effort to examine these special stains and usually requires a trained pathologist to carry out such examination. For example, *Streptococcus pneumoniae* can appear as gram-negative cocci in tissue sections because the host inflammatory responses, antibiotic treatment, or autolysin produced by the bacteria per se can damage the bacterial cell wall and render the Gram stain appear negative. Even when these special stains properly highlight organisms of interest, they can only categorize them within a broad classification but not a specific species. For example, gram-positive cocci demonstrated by tissue Gram stain in a lung section (Fig. 2b) could represent different species of *Streptococci* or *Staphylococci*, and further testing with more specific assays is needed to reveal the true identity of these cocci.

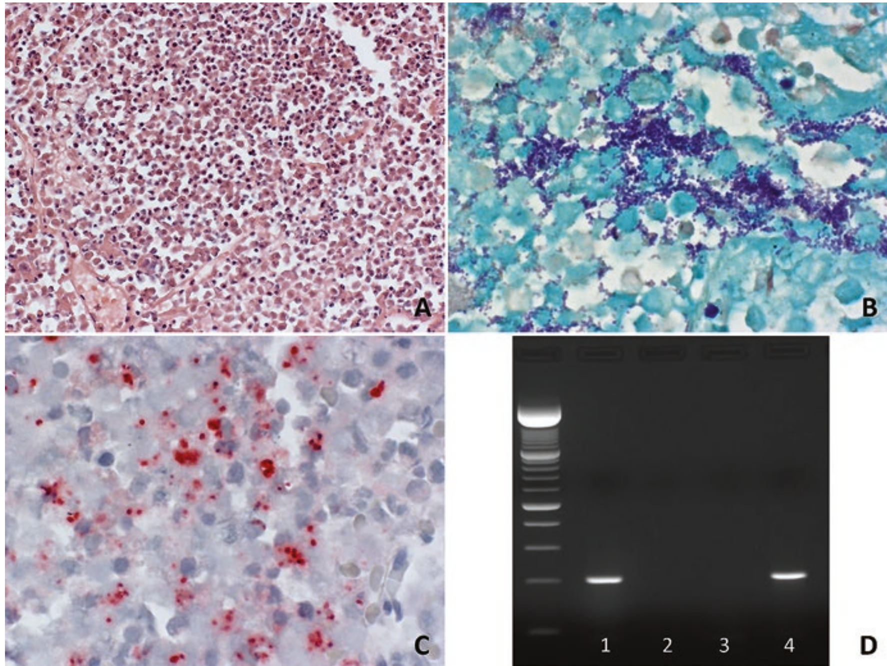


**Fig. 2** (**a**) H&E stain shows abundant polymorphonuclear inflammatory cells in alveoli indicative of an acute pneumonia. (**b**) Gram stain highlights numerous gram-positive cocci mixed with inflammatory cells. (**c**) IHC with anti-*S. pneumoniae* antibody shows abundant extracellular and intracellular bacterial antigens. (**d**) PCR targeting pneumolysin gene of *S. pneumonia* shows positive amplicon (lane 1, positive control; lane 2, negative control; lane 3, water control; lane 4, lung sample tested)

## *Electron Microscopy*

Four decades ago, electron microscopy (EM) was the only ancillary technique available to the pathologists when routine H&E and special stains failed to reveal diagnostic features in histopathology [18]. EM examination provides a direct visualization of microbial organisms at a high magnification. Ultrastructural finding is the most direct evidence to show the presence of an infectious agent in clinical specimens. Thin section and negative stain are two common EM methods used to study pathogen morphology and morphogenesis of the microorganisms with recognition of their cytoplasmic organelles and matrix constituents. Therefore, correlation of light and electron microscopic findings not only improves pathologist's diagnostic acumen but also allows for a more coherent explanation of the pathogenesis. Since the advent of immunohistochemical and molecular techniques, EM has been less often used for identifying infectious agents. However, EM still played an essential role in determining the specific family of the pathogen involved in several

outbreaks caused by novel viruses, such as Sin Nombre virus [19, 20], Nipah virus [21, 22], SARS-CoV [12, 23], and monkeypox virus [24]. In these outbreak investigations, negative stain of virus isolated from tissue culture and thin-section preparation of tissue specimen facilitated the ultrastructural examination. The determination of etiologic agents guided subsequent laboratory, clinical, and epidemiologic investigations. Advanced EM methods, such as immuno-EM or EM in situ hybridization using colloidal gold labels, have been developed for a more specific ultrastructural diagnosis.

## *Immunohistochemistry*

Immunohistochemistry (IHC) has been widely used in all aspects of pathology diagnosis in the past three decades [25–27]. A large number of IHC are available that can be helpful in the identification of microorganisms. By using a variety of antibodies, IHC can detect the presence of microbial antigens in tissue specimens, whether they represent the intact or degraded pathogens and whether they are intracellular or extracellular (Fig. 2c). Therefore, IHC has become a powerful technique used by pathologists for tissue diagnosis of infectious diseases. There are many ways to visualize an antibody-antigen interaction. The most common method is to apply an antibody conjugated to an enzyme, such as peroxidase [28–30] or alkaline phosphatase [31, 32], which can further catalyze a reaction for colorimetric detection. The antibodies used for specific detection can be polyclonal or monoclonal. Polyclonal antibodies are a heterogeneous mixture of antibodies that recognize several epitopes of a specific organism or more commonly, a group of related organisms. Monoclonal antibodies are generated against a single epitope and hence more specific to the target antigen than polyclonal antibodies. Many of these antibodies are commercially available and are widely used in diagnostic pathology laboratories. Others, especially those antibodies for detecting novel emerging pathogens, are available only at highly specialized centers such as the Centers for Disease Control and Prevention. Development of new IHC is a worthwhile but usually labor-intensive task. Similar to all other laboratory assays, the sensitivity and specificity of any IHC always need a careful evaluation before establishing its status as a diagnostic assay.

Detection of two or more target antigens on one slide can be achieved with multiple staining IHC assays [33–35]. These assays can expand the information obtained from each slide and reduce turnaround time compared to single staining or sequential staining methods. It is possible to assess the topographic relationship of the targets by using multiple staining IHC assays for determining the cellular tropism of viral infection with antibodies raised against virus and specific cellular markers, respectively (Fig. 1c). These multiple staining methods not only help confirm the immunolocalization of pathogens but also enhance further understanding of pathogenesis in many emerging infections [7, 16, 19, 36].

There are many advantages of using formalin-fixed tissues and IHC to detect etiologic pathogens. It is particular useful in detecting those fastidious or slow-

growing organisms, such as mycobacteria [37, 38] or *Tropheryma whipplei* [39], and can improve the speed, sensitivity, and specificity of microbial diagnosis. It is also valuable for characterizing emerging infections, whose causes are initially unknown, such as those caused by Nipah virus [21], SARS-CoV [12], or Zika virus [40]. Immunolocalization of antigens by IHC provides histomorphologic correlation between the infectious pathogen and host tissue responses, which is not only crucial for diagnosis but also important to study the pathogenesis of those emerging infections [19, 21, 41, 42]. Additionally, IHC performed on fixed tissues can minimize laboratory worker's potential risk of exposure to infectious agents because of the deactivation of pathogens by formalin fixation. Another advantage of using IHC is its capability of detecting well-preserved microbial antigens in archived formalin-fixed, paraffin-embedded (FFPE) tissues, which allows retrospective studies of many emerging pathogens even after decades of archive [43, 44].

### *In Situ Hybridization*

In situ hybridization (ISH) is a technique that uses fluorescent or radiolabeled nucleic acid probes comprising complementary DNA or RNA strand to localize specific sequences in tissue sections [45, 46]. It has been applied in many medical diagnostics, such as gene expression profiling, chromosomal integrity, and karyotyping, etc. There are also many ways to perform ISH in diagnosis of infectious pathogens with a variety of probes [47–52], including double-stranded DNA (dsDNA) probes, single-stranded DNA (ssDNA) probes, RNA probes (riboprobes), and synthetic oligonucleotides (oligoprobes). ISH can localize nucleic acids of microorganisms in tissues and provides histomorphologic correlation between the infectious pathogen and host tissue responses (Fig. 1d). ISH can utilize in-house probes synthesized in a well-equipped laboratory with known sequences of the target nucleic acids, minimizing the need to depend on commercial resources. The advantages of using formalin-fixed tissues and ISH to detect etiologic pathogens are similar to IHC, except it is usually less sensitive than IHC because of the potential fragmentation of target nucleic acids by formalin fixation [53, 54]. Therefore, clinical utility of ISH for infectious disease diagnosis is much more limited than IHC, especially for in situ RNA analysis. This disparity is particularly notable when considering the abundance of RNA biomarkers discovered through whole-genome expression profiling. The reasons are mainly due to the high degree of technical complexity and insufficient sensitivity and specificity of conventional RNA ISH techniques. A novel RNA ISH technology, RNAscope, has been developed and utilized in the past few years. The technology applies a unique probe design strategy that allows simultaneous signal amplification and background suppression to achieve single-molecule visualization while preserving tissue morphology. RNAscope can be applied on routine formalin-fixed, paraffin-embedded tissue specimens and can use either conventional chromogenic dyes for bright-field microscopy or fluorescent dyes for multiplex analysis. Unlike conventional RNA

analysis methods such as real-time RT-PCR, RNAscope brings the benefits of in situ analysis to RNA biomarkers and may enable rapid development of RNA ISH-based molecular diagnostic assays [55, 56].

### *Polymerase Chain Reaction Assay*

Polymerase chain reaction (PCR) assay amplification undoubtedly is the most sensitive method available to detect microbial organisms in tissue specimens and has become a common practice in many pathology laboratories. PCR can be performed on FFPE samples [57–60]; therefore, diagnoses can be made even if cultures were not obtained initially from biopsy or autopsy at the time of processing. In addition, molecular identification can accelerate definitive diagnosis of fastidious organisms that either grow slowly or do not grow at all with culture methods. When combined with other techniques mentioned above, PCR has markedly improved the capabilities of providing rapid and accurate detection of many emerging and reemerging pathogens [61] as well as pathogens commonly encountered in medical practice [58, 59].

PCR requires the isolation of nucleic acids from microorganisms in clinical samples and needs to apply adjunct techniques with restriction endonuclease enzymes, gel electrophoresis (Fig. 2d), and other nucleic acid hybridization methods. Degenerate primers can be employed in PCR assays at reduced stringency to facilitate detection of related but unknown organisms [12, 62, 63]. A vast number of PCR-based techniques have been developed in the past two decades and have been increasingly applied to clinical samples. For instance, multiplex PCR has been shown to increase the diagnostic yield in acute respiratory tract infections and contribute to overall improved outcome in patient care [64, 65]. New platforms such as real-time polymerase chain reaction (rt-PCR) combine nucleic acid amplification and fluorescent detection of the amplified product in the same closed system, resulting in an excellent technique that can diagnose a wide spectrum of infectious pathogens with tremendous flexibility, rapidity, and accuracy [59, 64, 66–68]. Nucleic acid sequence analysis has become highly automated and is now practical for use in many diagnostic and reference laboratories for the identification of a large number of microorganisms, whether they are cultivatable or not.

One particularly prevalent utility of PCR is the usage of the wide-range pan-eubacterial 16S ribosomal RNA (16S rRNA) PCR for detecting unknown bacterial organisms in tissue specimens. 16S rRNA is 1542 nucleotides in length and is a component of the 30S subunit of prokaryotic ribosomes. The 16S rRNA gene in bacteria contains well-conserved sequences that can be used as binding sites for universal PCR primers adjacent to variable sequences [69–71]. Subsequent analyses and comparisons of the sequences from amplicons to databases of known sequences can provide valuable information for etiologic diagnosis and further speciation. A set of broad-range PCR primers directed against conserved regions in the 16S rRNA gene was designed to specifically amplify either gram-positive or gram-negative

bacteria [72]. These differential 16S rRNA gene PCR assays provide more specific information regarding the bacteria identity, which are very useful for detecting bacterial pathogens in tissue samples in conjunction with histopathologic evaluation, special stains, and IHC.

Despite their high sensitivity, PCR techniques often face challenges from potential contamination issues. Processing of tissue samples, especially autopsy tissues, is often performed under a rather lax sterile condition and may enhance the chance of contamination. Many infectious pathogens can be present in the environment as commensals and their clinical relevance from PCR testing results can be confounded by such nature. Therefore, the PCR results should always be evaluated within the context of other diagnostic criteria. Moreover, any PCR testing of formalin-fixed tissues may be compromised by damage to DNA caused by the fixative. It is also important to know that identification to the species level may not be rigorous because the target gene may contain limited amount of sequence data available for comparison.

## ***Microarrays***

Microarrays can be performed on frozen tissue samples and may be helpful when multiplex PCR or other nucleic acid methods fail [73–75]. However, the sensitivity is generally lower than those multiplex PCR methods. Viral microarrays can be roughly divided into those targeting 10–100 agents and those designed for detection of thousands of agents, including unknown pathogens. Arrays designed to address a limited number of agents may employ multiplex consensus PCR to amplify specific genetic targets. Oligonucleotide microarrays with probes of up to 70 nt can offer a considerable advantage for detection of rapidly evolving targets, such as RNA viruses because these arrays are less likely to be confounded by minor sequence variation. Viral microarrays can facilitate sequence analysis and pathogen identification [73, 76–78]. Additionally, both microbial and host gene targets can be incorporated in these high-density arrays, thus allowing an opportunity to detect microbes and assess related host responses simultaneously for pathogenic features consistent with various classes of infectious agents.

## ***Next-Generation Sequencing***

Next-generation sequencing (NGS) is a powerful technique that can be applied on the FFPE tissue samples and has been rapidly spreading in the clinical and research arena [79, 80]. NGS can significantly reduce the cost of large-scale sequencing and is feasible to analyze an individual's near-complete exome or genome to assist in the diagnosis of a wide array of clinical scenarios. It can also facilitate further advances in therapeutic decision-making and disease prediction for at-risk patients. Currently,

there are still multiple factors that limit the diagnostic use of NGS in clinical laboratories, such as nonstandardized platforms, long turnaround time, large amount of data, and the need of powerful bioinformatics for data analysis. Targeted NGS investigates specific areas of interest rather than an entire gene or exon and thus produces smaller, more manageable datasets, reduces turnaround time, and decreases sequencing costs. Also, as it focuses on specific regions of interest, it leads to greater depth of coverage and increases the confidence of detecting a low-level variant in clinical samples [81, 82].

### *Other Advanced Techniques*

Other advanced pathology techniques, such as confocal microscopy [83], proteomics [84–86], laser capture microdissection (LCM) [87, 88], in situ PCR [89], and pyrosequencing [90, 91], have been used sparingly for detecting novel pathogens in a few specialized laboratories. Although they can become potentially powerful tools for diagnosis of emerging infections, most of them remain as pilot utilities and need further optimization to gain wide acceptance as mainstream techniques in practice of infectious diseases pathology.

## General Guidelines of Using Pathology Techniques

Appropriate clinical specimen collection, transport, and processing are crucial to establish an accurate laboratory diagnosis of infectious diseases. Similarly, adequate tissue sampling is the first and the most important step to obtain an organism-specific diagnosis of infectious diseases by using pathology techniques. The pathology laboratory must have practical guidelines for optimal specimen collection and handling and should communicate this information to the clinical staff and patient care sites. It is prudent to obtain biopsy or surgical samples from the precise site of infection and preferably before initiation of therapy to minimize the impact of treatment on subsequent diagnostic tests. This is particularly true for bacterial or fungal infections. Tissue specimens obtained surgically are acquired at great expense and pose considerable risk to the patient; therefore, they should be procured with an amount of material adequate for both histopathologic and microbiological examination. Swabs are rarely adequate for this purpose. Representative samples from all major organs should be collected in autopsy cases, especially those unexplained fatal cases due to infectious causes.

Etiologic pathogens may be focally or sparsely present in involved organs, and only a complete postmortem examination can attentively localize the causative organisms, as well as the full spectrum of their pathologic effects. In addition, the predilection site for infection may vary among different organisms. For example, herpes simplex virus tends to involve the temporal lobe in the brain more frequently,

**Table 3** Tissue sample collection with syndrome-based approach

| Target system (syndrome) | Representative tissue sample collection |
|---|---|
| Central nervous system (meningitis, encephalitis, myelitis) | Cerebral cortex (frontal, parietal, temporal, and occipital), brain stem (midbrain, pons, medulla), spinal cord, cerebellum, basal ganglia, thalamus, hypothalamus, hippocampus, and meninges |
| Respiratory system (laryngitis, tracheitis, bronchitis, pneumonia, pulmonary hemorrhage) | Larynx, trachea, left and right main bronchi, hilar lung with segmental bronchi, and peripheral pulmonary parenchyma from both lungs |
| Cardiovascular system (myocarditis, endocarditis, pericarditis) | Ventricles, interventricular septa, and atria, including endocardium, epicardium, and pericardium |
| Hepatobiliary system (hepatitis, cholecystitis, hepatic failure) | Different areas of the liver, gall bladder |
| Gastrointestinal system (gastritis, enteritis, intestinal perforation, intussusception) | Esophagus, stomach, small intestine, large intestine, appendix, and mesenteric lymph nodes |
| Urinary system (nephritis, cystitis, renal failure) | Renal cortex and medulla, urinary bladder, and adrenal gland |
| Reproductive system (cervicitis, endometritis, pelvic inflammatory diseases, funisitis, chorioamnionitis, orchitis) | Cervix, uterus (endometrium and myometrium), ovary, fallopian tube, umbilical cord, placenta, testicles |
| Cutaneous system (skin rashes, including macule, papule, vesicle, pustule, ulceration, and eschar) | Minimally, a 3 mm punch, deep shave, or excisional biopsy specimen from the representative rash lesion. Multiple biopsies should be obtained if multiple stages or forms of cutaneous lesions are identified |

while West Nile virus usually causes more severe infection in the brain stem and spinal cord. Moreover, since multiple organs can be involved in the context of systemic diseases, collecting multiple representative portions of target organs with syndrome-based approach (Table 3) and tissue samples from any other organ system with findings suggestive of infection ensures the best chance of detecting the causative agent. Influenza-associated myocarditis is a good example to show the difficulty of identifying influenza virus in the heart tissue even with prominent histopathologic changes of myocarditis, while the evidence of infection is usually present in the respiratory tissues [92].

FFPE tissue samples are usually adequate for routine H&E stain, special stains, IHC, and ISH assays. However, prolonged formalin fixation can cause cross-linking of proteins and nucleic acids in tissues and hence decrease the sensitivity of IHC, ISH, or PCR assays. In general, antigens and nucleic acids in tissue samples can be well preserved in paraffin-embedded blocks if formalin fixation does not exceed 2 weeks. It is highly recommended to embed tissue samples in paraffin no longer than 72 h after adequate formalin fixation. Although FFPE blocks can also be used for ultrastructural examination, it is preferably to dissect tissue samples into small thin pieces (1 $mm^3$), placed in glutaraldehyde fixative, and stored in a refrigerator for optimal EM studies.

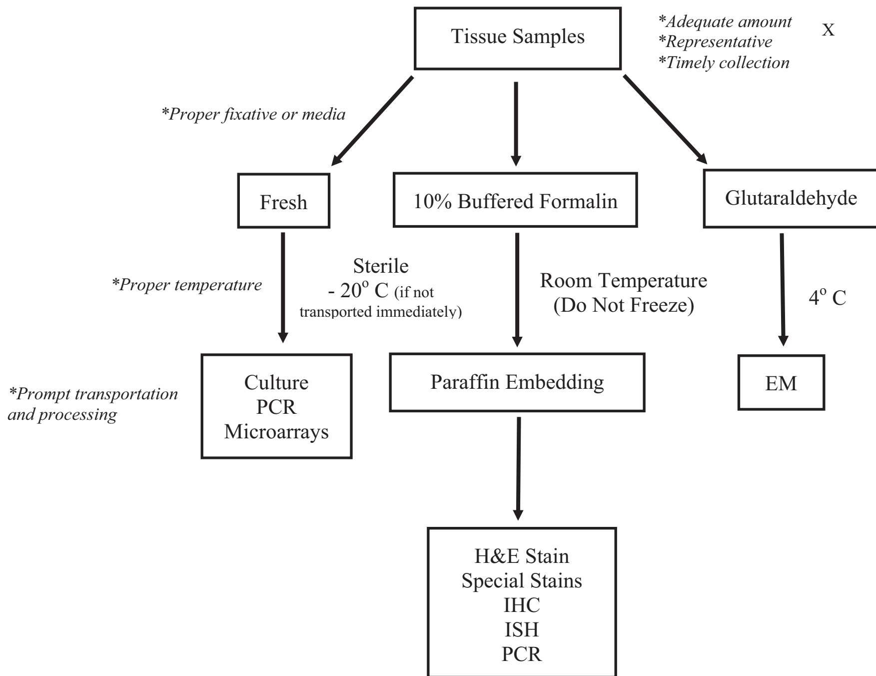


**Fig. 3** Optimal tissue collections for pathologic studies

Sterile techniques are mandatory to obtain target tissue samples for microbiologic culture and PCR assays. While biopsy procedure is usually performed under a stringent sterile condition, autopsy is not. In addition, delay of postmortem examination will facilitate colonization by normal flora or contamination by environmental organisms and interfere subsequent diagnostic assays. Therefore, autopsy should be performed as soon as possible (preferably within 12 h after death) to minimize these postmortem confounding factors. Representative tissue samples for potential PCR assay should be obtained with sterile technique and frozen at –20° C. It is noteworthy that FFPE can also be used for PCR testing if frozen samples are not readily available, but the sensitivity is usually lower because of the chemical property of formalin fixative mentioned earlier.

A diagram of optimal tissue collection for pathologic studies is shown in Fig. 3.

## Summary

Diagnosis with pathologic techniques provides histomorphologic correlation for a specific infectious agent with the disease it causes and is essential for identifying the cause of death. It helps identify or confirm the etiology of an outbreak caused by a

novel pathogen, especially from severe or fatal cases. It is crucial for the management of the clinical patient with unknown etiology of infection, control, and prevention for emerging disease outbreak, epidemiologic surveillance, and study of pathogenesis. Tissue samples, especially postmortem specimens, should be collected adequately and promptly. They should be preserved in proper media and processed in a timely fashion. The histopathologic features identified in the tissue specimens in conjunction with relevant clinical and epidemiologic information should determine the performance of specific IHC, ultrastructural, molecular, or other assays.

There are limitations of using pathologic techniques despite the advantages. Because immune mechanisms can greatly amplify the host response, the actual numbers of pathogens present in tissues can be relatively small. This means that many sections may need to be examined before a pathogen is identified. Topographic issues related to tissue sampling can also affect the outcome of tests. If the tissue specimens are not obtained from relevant lesions or areas with histopathologic changes, the subsequent tests performed on such specimens can all result in false-negative outcomes. Timing of tissue sampling, as mentioned earlier, is another crucial element that can affect test results. Delayed autopsy procedure increases the chance of tissue autolysis and postmortem contamination, which can significantly interfere with histopathologic evaluation and all related pathologic tests. Technical issues, such as sensitivity and specificity, are universally present for each IHC, ISH, or PCR testing. A negative result cannot exclude the possibility of an infection caused by certain organisms because duration of illness, modalities of treatment, tissue sampling, and fixation may affect the outcome of these assays. Therefore, a correlation of the test results with clinical history, epidemiological information, and other laboratory assays is highly recommended for a more accurate interpretation involving inpatient care and public health management.

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# Diagnosis and Assessment of Microbial Infections with Host and Microbial MicroRNA Profiles

**Lunbiao Cui, Athina Markou, Charles W. Stratton, and Evi Lianidou**

## Introduction

Biomarkers are continuously being sought in the field of diagnostic microbiology for the laboratory diagnosis and assessment of microbial infections. These have evolved from a simple clinical index [1, 2] using nonspecific screening tests such as the white blood cell with leukocyte differential, the erythrocyte sedimentation rate, and the C-reactive protein to the use of pro-inflammatory cytokines/chemokines [3, 4] to most recent use of microRNA (miRNA) molecules [5–7], the last of which have the greatest potential for predicting infection. A set of clinical and laboratory criteria necessary for an ideal diagnostic marker of infection have previously been proposed by Ng and his colleagues [8]. According these criteria, an ideal biomarker should possess at a minimum the following characteristics: (a) biochemically, a biomarker should be stable and remain significantly deregulated in the body fluid compartment for at least 12–24 h even after commencement of appropriate treatment that may allow an adequate time window for specimen collection or storage without significant decomposition of the active compound until laboratory processing; (b) its concentration should be determined quantitatively, and the method of measurement should be automatic, rapid, easy, and inexpensive; (c) the collection of a specimen should be minimally invasive and require a small volume (e.g., <0.5 mL blood).

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Numerous biomarkers have been found and tested in clinical practice [1–4]. Currently, microRNA (miRNA) molecules are without a doubt the biomarkers with the greatest potential capacities in the diagnostic microbiology field.

The first miRNA, lin-4, was inadvertently discovered in *Caenorhabditis elegans* in 1993 by Lee and colleagues [9]. However, recognition of the miRNA let-7 and its ability to regulate lin-14 by Ruvkun and colleagues [10] in 2000 resulted in the establishment of this new class of regulatory nucleic acids and their potential value in diagnostic microbiology.

The majority of the characterized miRNA is intergenic and is located in introns [11]. Primary miRNA transcripts, i.e., pri-miRNAs, are transcribed by RNA polymerase II or polymerase III. In the nucleus, ribonuclease Drosha cleaves pri-miRNAs and releases 60–80 nt stem-loop intermediate structures named pre-miRNAs. The pre-miRNAs are transported to cytoplasm by exportin-5 protein and then are cleaved by Dicer RNase III to form a mature double-stranded miRNA. One strand of the miRNA duplex is subsequently unwound and then incorporated into an effector protein complex termed RNA-induced silencing complex (RISC), which is responsible for the gene silencing in a posttranscriptional manner [12–14]. Mature miRNAs are single-stranded RNA molecules of about 19–25 nucleotides in length. Through partial homology to the 3′-untranscribed region (UTR) in target mRNAs, miRNAs control of gene expression via repression of translation as well as reducing mRNA levels directly.

A large number of miRNAs have been found in various animal and plant tissues [15]. According to miRBase 21.0 (http://microrna.sanger.org/), which is a collective registry of currently known miRNA sequences and targets hosted by the Sanger Institute, there are currently 2588 recognized miRNA sequences in the *Homo sapiens* genome; this number is constantly growing as new miRNA sequences are discovered. Importantly, miRNAs have been detected in human body fluids, including peripheral blood plasma; these circulating miRNAs are found as extracellular nuclease-resistant entities that are strikingly stable in blood plasma [16]. Such miRNAs have been found circulating not only in serum and plasma but also in other body fluids such as saliva, tears, and urine [17]. Some of these miRNAs appear to be enriched in specific fluids [17, 18]. These circulating miRNAs subsequently have become the focus of ongoing research. The properties, origin, function, and relationship with disease of circulating miRNAs have been intensively investigated [16–19]. A number of important observations have been noted. Circulating miRNAs are present in a stable form that is protected from endogenous RNase activity [16, 19, 20]. Most extracellular circulating miRNAs in plasma completely pass through 0.22 micron filters but remain in the supernatant after ultracentrifugation at 110,000 g, which indicates the non-vesicular origin of these miRNAs [16]. Finally, circulating miRNAs have been shown to co-immunoprecipitate with the 96 kDa Ago2 protein; this Ago2 protein is part of an RNA-induced silencing complex [16] and might account for the high stability of this comlex [16]. Some groups also reported a higher stability of miRNAs compared to mRNA in samples obtained

from formalin-fixed paraffin-embedded tissues [21–23]. The expression level of miRNAs has been noted as consistent among individuals of the same species [19]. Expression alteration of circulating miRNAs has been reported to be associated with pathophysiological states including various cancers, heart disease, pregnancy, and diabetes [24, 25]. Needless to say, serum, plasma, and other body fluid specimens are generally available for clinical testing. Profiling hundreds of miRNA requires only 200 ul of sera [20]. Thus, these unique and stable characteristics of circulating miRNAs potentially make them extremely useful biomarkers for disease diagnosis and prognosis. The potential use of tissue and/or circulating miRNAs for diagnosing cancer was quickly recognized [12, 13, 18–20, 22]. The discovery of virus-encoded miRNAs and the recognition that such miRNAs played multiple roles in virus infections has resulted in their use in diagnosing viral infections [5, 26–28].

## Virus-Encoded miRNAs

Virus-encoded miRNAs were recognized as having many functions in viral infections, including controls for viral replication and thus potentially limiting antiviral responses, inhibition of apoptosis, and stimulation of cellular growth [26]. Moreover, unique host cell miRNAs expression profiles have been revealed in response to various microbial infections [27, 28]. Host miRNA thus appears to play an important role in viral replication and may be used by host cells to control viral infection. The first virus-encoded miRNA was described by Pfeffer and his colleagues in 2004 when they identified five EBV-encoded pre-miRNAs [29]. Since then, hundreds of virus-encoded miRNAs have been described in humans, animals, and plants. Examples of human virus-encoded miRNA are shown in Table 1. Bewilderingly, more than 95% of the virus-encoded miRNAs known today are of herpesvirus origin [30]. Herpesvirus miRNAs were initially determined to not be required for lytic replication, but were thought to strongly enhance viral pathogenesis, including oncogenesis, and also to promote latently infected cells [30]. Subsequently, the role of herpesvirus miRNAs in virus latency and persistence has been confirmed, with specific cellular miRNAs being identified as inhibiting reactivation of herpesviruses, thereby promoting latent infections [31, 32]. Interestingly, almost all virus-encoded miRNAs are encoded by DNA viruses except those encoded by retroviruses, which reverse-transcribe and integrate their genetic material into host DNA. Aberrantly expressed circulating miRNAs have been explored for the diagnosis and prognosis of several infectious diseases, including sepsis [33, 34], HBV [35], and HCV [36].

The interactions between viral and cellular miRNAs in viral diseases and virus-associated cancers are complex [37]. Viral miRNAs target perfectly complementary viral mRNAs as well as imperfectly complementary viral and/or cellular mRNAs.

**Table 1** Virus-encoded miRNAs related to human infection

| Name of virus | Number of precursors | Number of mature |
|---|---|---|
| Bovine foamy virus | 2 | 4 |
| Bovine herpesvirus 1 | 10 | 12 |
| Bovine herpesvirus 5 | 5 | 5 |
| BK polyomavirus | 1 | 2 |
| Bovine leukemia virus | 5 | 10 |
| Bandicoot papillomatosis carcinomatosis virus type 1 | 1 | 1 |
| Bandicoot papillomatosis carcinomatosis virus type 2 | 1 | 1 |
| Duck enteritis virus | 24 | 33 |
| Epstein-Barr virus | 25 | 44 |
| Herpes B virus | 12 | 15 |
| Human cytomegalovirus | 15 | 26 |
| Human herpesvirus 6B | 4 | 8 |
| Human immunodeficiency virus 1 | 3 | 4 |
| Herpes simplex virus 1 | 18 | 27 |
| Herpes simplex virus 2 | 18 | 24 |
| Herpesvirus saimiri strain A11 | 3 | 6 |
| Herpesvirus of turkeys | 17 | 28 |
| Infectious laryngotracheitis virus | 7 | 10 |
| JC polyomavirus | 1 | 2 |
| Kaposi sarcoma-associated herpesvirus | 13 | 25 |
| Mouse cytomegalovirus | 18 | 29 |
| Merkel cell polyomavirus | 1 | 2 |

Adapted with permission from miRBase 21.0 (http://microrna.sanger.org/)

Viral miRNAs modulate expression of host gene involved in cell proliferation and survival, stress responses, and antiviral defense pathways, which are pivotal for viral replication [26–28, 37–39]. Another primary function of virus miRNAs is to regulate the latent-lytic switch. During latency, the host cell maintains the viral genome, and only a limited portion of virus genome is expressed. Viral gene expression is restricted, but virus miRNAs and their precursors are regularly detected [38]. There is an accumulating amount of evidence that has demonstrated that virus-encoded miRNAs mediate evolutionarily conserved functions (e.g., immune evasion, cell cycle control, promotion of latency, etc.) [5, 26–28, 37–39]. The miRNAs themselves show poorly primary sequence conservation [39]. These phenomena raise an important question: can viral miRNAs be used as the detection maker for virus infection during the latent infection phase despite the fact that little or no viral protein is being produced? Further studies are needed to evaluate this hypothesis.

## Host miRNA Response in Relation to Microbial Infection

Microbial infections are known to down-modulate at least some cellular mRNAs and thereby exert physiological effects [40]. Microbial infections induce changes in the host miRNA expression profile, which may also have a profound effect on the outcome of infection [6, 41, 42]. Host miRNA may directly or indirectly affect virus replication and pathogenesis. For example, liver-specific miR-122 is required for HCV replication [43, 44]. Moreover, miR-28, miR-125b, miR-150, miR-223, and miR-382 are over-expressed in resting CD4+ T lymphocytes compared to their activated counterparts [45]. These miRNAs are able to target sequences near the 3′ portion of HIV-1 mRNA. This finding suggests that miRNAs may contribute to viral latency [45]. However, it is unclear whether these miRNAs are actively inhibited by viral factors or whether their deregulation is due to host responses. Host miRNAs expression profiles have been noted to represent specific pathophysiological states [24, 25]. Theoretically, a characteristic profile should be potential biomarkers for disease diagnosis and prognosis. A number of studies have been conducted to demonstrate this theory, and promising results have been seen in a number of altered physiological states including various cancers, heart disease, pregnancy, diabetes, injury, and infection. The use of such miRNA profiles in specific infection diseases will be discussed in the next sections.

**HIV–1 and Other Human Retroviruses**

The roles of microRNAs in HIV-1 replication and latency are being intensely investigated in order to provide new approaches to clear the viral reservoir [46, 47]. It has become apparent that cellular miRNAs may play crucial roles in controlling HIV-1 infection and replication [47]. Houzet and colleagues have profiled miRNAs in peripheral blood mononuclear cells (PBMCs) from HIV-1-infected patients [48]. They found the T cell-abundant miRNAs (miR-223, miR-150, miR-146, miR-16, and miR-191) were downregulated three- to ninefold compared to cells from uninfected controls, depending on the disease stage of the patient [48]. Triloubet et al. reported increased expression of 11 miRNAs including miR-122, miR-370, miR-373*, and miR-297 in HIV-1-infected Jurkat cells, whereas expression of the polycistronic miRNA cluster miR-17/miR-92 (comprises miR-17-(5p/3p), miR-18, miR-19a, miR-20a, miR-19b-1, and miR-92-1) was strongly decreased [49]. Two cellular miRNAs, miR-196b and miR-1290, have been found to contribute to HIV-1 latency [50]. Like HIV-1, human T cell leukemia virus type 1 (HTLV-1) also infects CD4+ T cells. As seen with HIV-1, miRNAs also play an important role in the pathogenesis of HTLV-1 infection and transformation [51, 52]. Two miRNA profiling studies have been performed in infected cell lines and ATL (adult T cell leukemia) cells [53, 54]. The studies find two common miRNAs that are consistently downregulated in the context of HTLV-1 infection. For both HIV-1 and HTLV-1, it is clear the miRNAs play a role in latency; inhibitors of these miRNA could be used to activate latent retroviruses in order to assist in clearing the reservoirs of virus.

**Respiratory Viruses**
The emergence of Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 following the earlier severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002–2003 marked two instances in which a highly pathogenic coronavirus was introduced into the human population in the twenty-first century [55]. Along with highly pathogenic avian influenza viruses [56], these respiratory viruses are notable due to their potential to cause pandemics [57]. The potential role of miRNAs to diagnose these respiratory tract infections is under investigation. For example, the miRNA expression profile in bronchoalveolar stem cells (BASCs) infected with SARS coronavirus (CoV) has been determined using miRNA microarray [58]. A total of 116 miRNAs were found differentially expressed. Upregulated BASC miRNAs-17*, miRNAs-574-5p, and miRNAs-214 are co-opted by SARS-CoV to suppress its own replication and evade immune elimination until successful transmission takes place. In contrast, viral nucleocapsid and spike protein targets seem to co-opt downregulated miR-223 and miR-98, respectively [58]. The miRNA expression of avian influenza strains has also been investigated. Differentially expressed miRNAs in chicken lung and trachea infected with a low pathogenic strain of H5N3 avian influenza virus were analyzed by a deep sequencing approach [59]. A total of 73 and 36 miRNAs are differentially expressed in lungs and trachea upon virus infection, respectively [59]. Lung cellular "microRNAome" of mice infected by reconstructed 1918 influenza virus was compared with that of mice infected by a nonlethal seasonal influenza virus, A/Texas/36/91 [60]. A group of microRNAs, including miR-200a and miR-223, were differentially expressed in response to influenza virus infection, and infection by these two influenza viruses induced distinct microRNA expression profiles [60]. Finally, a novel avian-origin influenza A (H7N9) caused 137 human infection cases with a 32.8% mortality rate; characterization of the miRNA profile in response to infection by this strain revealed significant alterations in serum miRNA expression following virus infection in comparison with controls [61]. This study confirmed the potential for using serum miRNA expression for the diagnosis of viral respiratory diseases.

**Adenovirus**
Human adenoviruses are DNA viruses that cause infections in both immunocompetent and immunosuppressed patients [62]. Adenovirus infections are associated with viral persistence and reactivation and continue to provide clinical challenges in terms of diagnosis and treatment. Adenoviruses express large amounts of noncoding virus-associated RNAs able to saturate key factors of the RNA interference processing pathway, including Exportin 5 and Dicer [63]. Moreover, a proportion of the noncoding virus-associated RNA is cleaved by Dicer in viral miRNAs. This cleaved RNA results in miRNAs that can saturate Argonaute, which is an essential protein for miRNA function that engages in transcriptional silencing processes in the nucleus [63, 64]. Therefore, processing and function of cellular miRNAs are blocked in cells infected by adenovirus [63]. Of note is that the cellular silencing machinery is active early after infection and can be used to control the adenovirus cell cycle [63]. It is also important to realize that miRNA expression has been found to

fluctuate during the course of an adenovirus type 2 infection in human lung fibroblasts [65]. The miRNA expression profiles from adenovirus type 3 (AD3)-infected human laryngeal epithelial (Hep2) cells have been analyzed using a SOLiD deep sequencing [66]. A total of 44 miRNAs demonstrated high expression, and 36 miRNAs showed lower expression in the AD3-infected cells than in control cells [66]. The role of miRNAs in adenovirus-infected cells is relevant because of the past and future use of recombinant adenoviruses as vectors for gene therapy [67]. Manipulation of the viral genome allows the use of these vectors to express therapeutic miRNAs or to be silenced by the RNAi machinery leading to safer vectors with specific tropisms. Adenovirus is known to interact with a number of different extracellular, intracellular, and membrane-bound innate immune sensing systems [68] such as Toll-like receptor 4 [68, 69]. The investigation of miRNAs expression during adenovirus infection is likely to provide important new insights into the scope and mechanisms of these cellular defensive responses [70].

**Human Herpesviruses**

Among the human herpesviruses, cytomegalovirus (CMV) is an important human pathogen that has the potential to disseminate via the bloodstream to all organs, but only produces overt clinical disease if the viral load achieves high levels [71]. Normally there is a strong immune response such that the infected individual typically remains asymptomatic [71]. Over time, this immune response wains and infected individuals can become symptomatic due to a higher viral load. For this reason, miRNA expression in latent and symptomatic infections is being studied [72]. Wang et al. monitored the time course of cellular miRNA expression in human cytomegalovirus (CMV)-infected cells using miRNA microarrays and found that 49 miRNAs significantly changed on at least 1 time point [73]. There were no global unidirectional changes, with changes for these miRNAs sometimes being transient. Fu and colleagues noted similar results in a human cytomegalovirus latent infection cell model using THP-1 cells [74]. The miR-199a/miR-214 cluster (miR-199a-5p, miR-199a-3p, and miR-214) was recently found to be downregulated in CMV-infected cells [75]. Human cytomegalovirus miRNA miR-US25-1-5p has been shown to inhibit viral replication by targeting multiple cellular genes during infection [75]. Clearly additional studies are needed, but the use of miRNAs will undoubtedly increase our understanding of the pathogenesis of cytomegalovirus.

**Herpes Simplex Viruses (HSV)**

Herpes simplex viruses (HSV) are evolutionarily ancient viruses that are ubiquitous, having a worldwide prevalence [76]. There are two serotypes, HSV-1 and HSV-2, both of which primarily infect humans through epithelial cells. HSV infections are extremely common; seropositivity occurs in 50–90% of adult populations [76]. The success of HSV-1 and HSV-2 as human pathogens is due to the virus first infecting epithelial cells and then entering sensory neurons via nerve termini [76, 77]. Latency within long-lived neuronal cell bodies and subsequent mucocutaneous shedding is central to the survival of this neurotrophic virus [77]. The generally mild sequelae of HSV infection reflects a balance between the host and the virus in most immunocompetent persons [76, 77]. However, HSV infections of the central

nervous system are recognized, but relatively rare complications of this infection [78]. The miRNAs of HSV are of particular interest due to the latency of this virus as well as its role in CNS infections [79]. Infection of human primary neural cells with a high phenotypic reactivator HSV-1 (17syn+) can induce upregulation of a brain-enriched microRNA (miRNA)-146a [80]. Both miR-101 and miR-132 are also found to be highly upregulated after HSV-1 [81, 82].

Another factor in terms of the involvement of HSV in CNS infections is the fact that miRNAs are key regulators of neuroinflammation [83]. Several miRNAs have been found to play an important role in the microglia-mediated inflammatory response including miR-155 and miR-146a [83]. Another miRNA, miR-125, plays a critical role in the adaptation of microglia and macrophages to the CNS microenvironment [84]. Traumatic brain injury has been shown to produce profound and lasting neuroinflammation; microRNAs have been implicated in the regulation of inflammation after traumatic brain injury [85]. In particular, miR-155 is induced after traumatic brain injury and is thought to play an important role in the regulation of the IFN response and neurodegeneration following brain injuries [85]. The net sum of this regulation is thought to be neuroprotective, which in turn may predispose the brain to viral infections such as herpes simplex encephalitis [86].

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) is the etiological agent of KS; this virus is also known as human herpesvirus 8 (HHV-8) [87]. Kaposi's sarcoma is a mesenchymal tumor with poorly understood molecular and cytogenetic changes. The predicted target genes for differentially expressed miRNAs include genes that are involved in cellular processes such as angiogenesis and apoptosis, which suggests a role for these miRNAs in the pathogenesis of Kaposi's sarcoma [88]. KS tumor cells are latently infected with KSHV, which express only a subset of viral genes, among them 12 miRNAs [89]. The metabolic properties of KSHV-infected cells are similar to those of cancer cell and display features of lymphatic endothelial differentiation [89]. The M type K15 protein of KSHV induces the expression of microRNAs miR-21 and miR-31 via this conserved motif [90], while K13 strongly stimulated upregulation of miR-146a [91]. KSHV miRNAs expression decrease mitochondrial biogenesis and induce aerobic glycolysis; this metabolic shift favors latency and offers a growth advantage [89].

**Epstein–Barr Virus (EBV)**

Epstein-Barr virus (EBV) is a gammaherpesvirus that infects most humans during their lifetime [92, 93]; these infections are usually asymptomatic but result in a lifelong latent infection [94] that is controlled by the host's immune system [95]. EBV also is an oncogenic herpesvirus [96] that is endemic in humans and is found in about 15% of patients with diffuse large B-cell lymphoma (DLBCL) [97]. Multiple cellular functions are mediated by the miRNAs of EBV [98]. For example, EBV de novo infection of primary cultured human B-cells results in a dramatic downregulation of cellular miRNA expression, with 99.5% of the miRNAs detected being downregulated, with an average downregulation of 19.92-fold [99]. Imig et al. found that expression of hsa-miR-424, hsa-miR-223, hsa-miR-199a-3p, hsa-miR-199a-5p, hsa-miR-27b, hsa-miR-378, hsa-miR-26b, hsa-miR-23a, and

hsa-miR-23b were upregulated and those of hsa-miR-155, hsa-miR-20b, hsa-miR-221, hsa-miR-151-3p, hsa-miR-222, hsa-miR-29b/c, and hsa-miR-106a were downregulated more than twofold due to EBV-infection of DLBCL [100]. Cameron et al. demonstrated differential expression of cellular miRNAs in type III versus type I EBV latency including elevated expression of miR-21, miR-23a, miR-24, miR-27a, miR-34a, miR-146a and b, and miR-155. In contrast, miR-28 expression was found to be lower in type III latency [101].

**Bacterial Infections**

The role of miRNAs in mammalian host signaling and defense against bacterial pathogens has been recognized [102] and will provide both insights and diagnostic opportunities. The use of miRNA patterns for diagnosing bacterial infections is evolving [6]. *Helicobacter pylori* is a bacterium that utilizes multiple colonization factors and virulence factors to persist in the human stomach for life [103]. This persistent colonization of the gastric mucosa results in an inflammatory process that may remain asymptomatic for decades or progress to a more serious disease such as gastric carcinoma [104]. The host immune response along with the *H. pylori* gene expression and miRNAs is involved in this process; specific miRNA patterns may prove to be useful for detecting a shift from asymptomatic carriage to gastric carcinoma [104]. In vitro infection assays have revealed that *H. pylori* infection can affect miRNA expression profiles: specifically, miRNAs such as miRNA-155, miRNA-16, and miRNA-146a are significantly upregulated in human gastric epithelial cells during infection [105]. More recently, expression patterns of miRNA in gastric mucosa infected with *H. pylori* using endoscopic biopsy specimens were determined by microarray. There were 31 differentially expressed miRNAs between the *H. pylori*-infected and *H. pylori*-uninfected mucosa (more than twofold), and miRNA expression profiling could distinguish *H. pylori* status, with the eight miRNAs yielding acceptable sensitivity and specificity [106]. Overexpression of miR-223 has been described in *H. pylori*-associated gastric cancer and appears to contribute to cancer cell proliferation and migration [107]. Muscle-specific miRNAs miR-1 and miR-133 were significantly downregulated in the stomachs after long-term infection with *H. pylori* in mouse model [108].

*Salmonellae* cause a wide range of human infections, including gastroenteritis, bacteremia, enteric fever, and focal infections such as osteomyelitis [109]. *Salmonellae* have been shown to render human host cells more susceptible to infection by controlling host cell cycle progression through the active modulation of host cell miRNAs [110]. Schulte et al. identified differentially regulated miRNAs by comparative deep sequencing of a total of 14 cDNA libraries prepared from the small RNA population of host cells before or after *Salmonella* infection, or in mock-treated cells [111]. In murine RAW 264.7 cells, upregulation of miR-21, miR-146a/b, and miR-155 was observed after infection; they also observed significant downregulation of several let-7 family members, namely, let-7a/let-7c/let-7d/let-7f/let-7 g/let-7i and miR-98. In HeLa cells, a significant upregulation of miRNAs by *Salmonella* was limited to miR-1308. In contrast, miR-21, miR-146a/b, or miR-155 remained unaffected. Intriguingly, downregulation of let-7 miRNAs also occurred in HeLa cells [111].

**Other Microbial Agents**

*Mycobacterium avium* subspecies hominissuis is an opportunistic pathogen of immunocompromised individuals [112]. Sharbati et al. performed miRNA as well as mRNA expression analysis of human monocyte-derived macrophages infected with several *Mycobacterium avium hominissuis* strains using microarrays as well as RT-qPCR [113]. They that found expressions of let-7e, miR-29a, and miR-886-5p were increased in response to mycobacterial infection at 48 h [113].

The human papillomavirus (HPV) is associated with a number of oral, genital, and cutaneous conditions that may be benign or malignant [114, 115]. The association of HPV and cervical cancer is well-known because most cervical cancers contain HPV DNA, notably HPV types 16 and 18 [116]. The contribution of HPV to anogenital, oral, and oropharyngeal cancers is less clear [115]. The altered expression of miRNAs in these HPV-associated cancers has been investigated as a marker for possible diagnosis and therapy [117]. Expressions of miR-23b, miR-34a, and miR-218 are significantly reduced by HPV E6 infection, while HPV E7 infection downregulates expression of miR-15a/miR-16-1 and miR-203 [118].

## Methods of miRNA Detection

Accurate determination of miRNA expression levels in a specific cell, tissue, or fluids is prerequisite to assess their biological, pathological, and clinical roles in health and disease. Theoretically, all mRNA detection methods should be useful for miRNA analysis. However, the following characteristics of miRNA sequences make quantification of miRNAs expression a technical challenge. Firstly, mature miRNA is short (only 19–25 nucleotides; nts), and miRNAs within the same family may differ by a single nucleotide, which makes it difficult to design specific primers and probes and to reliably amplify or label each miRNA without introducing signal bias. Secondly, miRNAs are heterogeneous in their GC content, which results in melting temperatures (Tm) of these nucleic acid duplexes that vary widely. Finally, the target sequence is present in the primary miRNA transcript (pri-miRNA), the precursor miRNA (pre-miRNA), and the mature miRNA. It is therefore important to ensure that the non-active pri-miRNA and pre-miRNA precursor species do not contribute to the detection signal [119]. Several standard methods for quantification of mRNA levels have been successfully adapted to miRNA including northern blotting, cloning, in situ hybridization, RT-PCR, and microarrays. In addition, emerging techniques based on colorimetric, fluorescence, bioluminescence, enzyme, and electrochemical hold immense promise for the future of miRNA detection. However, technical issues must be addressed before they are included among the current standard methods [120].

**Northern Blotting**

Northern blotting was the first technique used to detect miRNAs and is considered the “gold standard” for characterizing miRNA expression. The basic procedures of miRNA northern blotting are similar to traditional blotting and are done as follows:

(a) the small RNA molecules are separated by using high-percentage denaturing urea-acrylamide gels rather than the usual agarose electrophoresis gels; (b) the small RNA molecules are transferred from the gel onto a membrane; (c) the miRNA molecules are fixed on the membrane through various cross-linking procedures; and (d) the membrane is hybridized with radiolabeled oligonucleotide probes. Northern blotting methods are able to determine the absolute amount of miRNA in a sample by blotting a dilution series of synthetic oligo miRNA molecules of known concentrations in parallel with the sample. The concentration of miRNA in the sample can be calculated by a standard curve obtained from the dilution series. However, short length as well as low prevalence of mature miRNA molecules can lead to poor sensitivity of such routine northern analysis. It requires a large amount of total RNA for each sample (generally, more than 5 μg). Other disadvantages of this method include low throughput and potential environment hazards of radiolabeling. Several technical modifications have been used to improve detection sensitivity. LNA (locked nucleic acid)-modified oligonucleotide probes increase the affinity between LNA probes and target miRNA which results to at least tenfold increase of sensitivity [121]. Using soluble carbodiimide cross-link method increases the efficiency of miRNA that are fixed on the membrane, which can increase by 25–50-fold miRNA detection sensitivity compared to the traditional UV cross-linking method [122].

**RT-qPCR**

The most widely used method for detection and qualification of miRNA appears to be real-time quantitative RT-PCR (RT-qPCR). The small size of the mature miRNA sequences as well as sequence homology between the mature and precursor miRNA forms limits the direct application of conventional RT-PCR protocols to miRNA detection. To solve these problems, innovative solutions have been applied for each step of RT-qPCR used for the quantitative analysis of miRNAs. The first step in RT-qPCR of miRNAs is the accurate and complete conversion of miRNA into cDNA. Two different approaches for reverse transcription of miRNAs have been reported. In the first approach, miRNAs are reverse transcribed individually by using miRNAs-specific reverse transcription primers. Both stem-loop (Applied Biosystems Co, Fig. 1a) and a linear primer (Exiqon, Fig. 1b) containing partial complementary sequence of 3′-end of miRNA can be annealed to miRNA in order to prime the reverse transcription. The double-stranded structure of the stem-loop primer prevents its nonspecific binding to pre- and pri-miRNAs, thereby increasing the specificity of the assay. Unlike stem-loop primer, the design of the linear primer is simpler. But the linear primer cannot discriminate mature miRNA from their precursors.

Another approach is done as follows: miRNAs are first tailed with adenosine nucleotides at 3′-end of miRNA with poly(A) polymerase (Fig. 1c) or linker adaptor with T4 RNA Ligase 1 (Fig. 1d). Then, a primer consisting of an oligo(dT) sequence with a universal primer-binding sequence at its 5′-end or a universal primer complementary to the 3′-end of the linker is used to prime reverse transcription [119]. The universal reverse primer sequence is introduced into the cDNA during reverse transcription. The design of the miRNA-specific forward primer is critical for the specificity and sensitivity of the RT-qPCR assay. LNA modification is a widely used

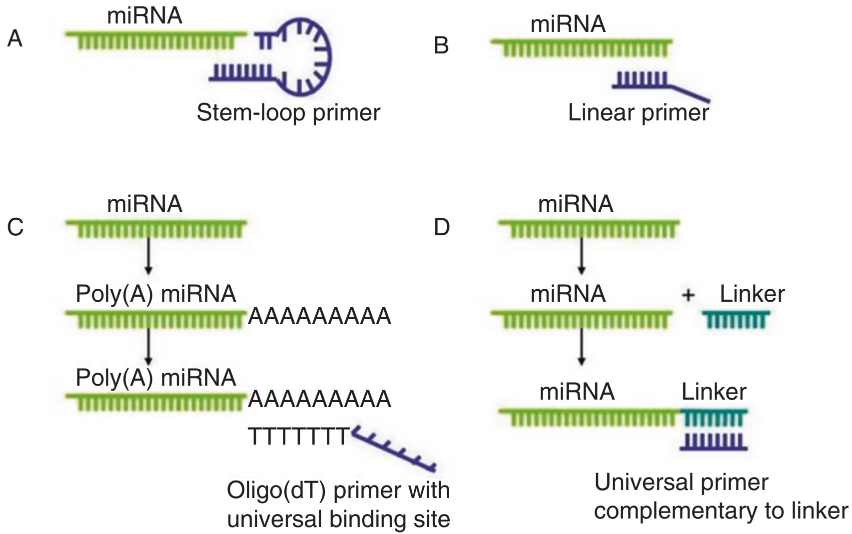


**Fig. 1** Schematic representation of reverse transcription methods used to generate cDNA. Reverse transcription of individual mature miRNAs is done using stem-loop (**a**) or linear (**b**) primer. miRNAs are first tailed with adenosine nucleotides at 3′-end of miRNA with poly (**a**) polymerase (**c**) or linker adaptor with T4 RNA Ligase 1 (**d**). Then, a primer consisting of an oligo(dT) sequence with a universal primer-binding sequence at its 5′-end (**c**) or a universal primer complementary to the 3′-end of the linker (**d**) was used to prime reverse transcription

method for increasing the Tm and the specificity of primer. Each incorporated LNA monomer increases the Tm up to 2–8 °C, depending upon the position of the LNA moiety in the oligonucleotide primer [123].

There are two approaches available for detection of RT-qPCR products. One approach uses SYBR green dye whose fluorescence increases approximately 100 times upon intercalating into dsDNA chain; this property is used to monitor real-time amplification products as they accumulate during the PCR reaction. One limitation of the SYBR green-based method is that target PCR products and nonspecific products cannot be discriminated. Therefore, a melting point analysis is usually required following the PCR amplification, which can be reached by using a dual-labeled hydrolysis TaqMan probe. The basis for this type of TaqMan probe detection has been reviewed by Benes [119]. The characteristics of TaqMan miRNA assays make them ideally suited for detection of mature miRNAs. Moreover, these TaqMan miRNA assays can discriminate related miRNAs that differ only one nucleotide. However, the cost of the TaqMan probes is higher than the SYBR green method, which limits its routine use in most laboratories.

The RT-qPCR method has demonstrated a high sensitivity and specificity with ability to accurately detect miRNAs in a single stem cell [124, 125]. Only low amounts of starting material (in the range of nanograms of total RNA) are needed, and quantitative results can be acquired within 3 h. This method also has a considerably larger dynamic range compared to microarray analysis. Recently, the RT-qPCR

technique has been adapted for increased throughput by developing a miRNA PCR array that can detect hundreds of miRNAs at one reaction tube. Examples include the TaqMan Low density microRNA Array (TLDA, Applied Biosystems) and the RT [2] miRNA PCR Arrays (SABioscience, a Qiagen Company).

**Microarrays**

Another widely used high-throughput technique for analysis of the expression level of miRNA molecules is microarray technology. The microarray technology was firstly applied to miRNA studies in 2003 [126]. Since then, numerous approaches based on different microarray platforms have been developed for miRNA quantification. The basic procedures of miRNA quantification using a microarray platform include (a) miRNA probe design and array preparation, (b) isolation of miRNA and labeling, and (c) hybridization and signal detection. A schematic flow chart of the miRNA profiling microarray is shown in Fig. 2. The design of the microRNA probes, isolation of microRNA from samples, and the labeling of miRNAs are the most critical procedures in the miRNA microarray assay.

The short length of miRNAs makes design of the probes more difficult because the design of the probe is almost exclusively determined by sequence of the miRNA itself. Sequence of the miRNA determines the wider Tm distribution of the probes. Therefore, Tm normalization of the full set of probes is absolutely required since the hybridization is usually carried out at one temperature. To solve this problem, two techniques, LNA utilization or adjusting the lengths of the probes, have been successfully used to normalize Tm value [127, 128]. Quantity of miRNA is also very important for miRNA microarray assay since the abundance of miRNAs in total RNA is very low and a relatively large amount of miRNA is needed in comparison to a RT-PCR assay. Conventional denaturing urea-acrylamide gel electrophoresis

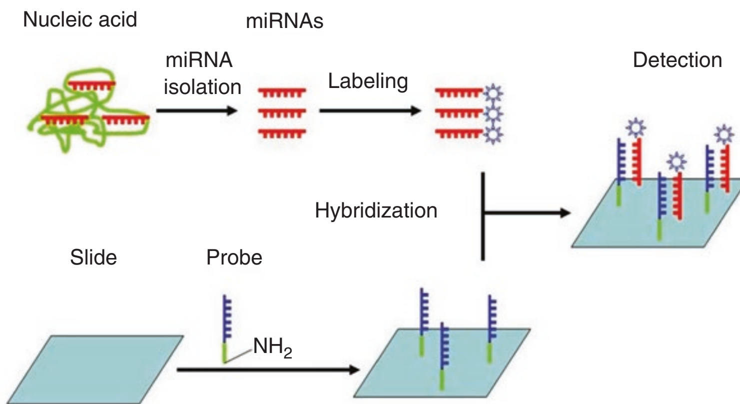


**Fig. 2** Schematic flow chart of the microRNA profiling microarray. A miRNA microarray prepared by amine-modified microRNA probes that consist of “linker” sequences (yellow) and capture sequences (blue) spotting to amine-reactive glass slides. After miRNAs are isolated from samples and labeled with fluorescence dye, they are hybridized with microarray and then the expression signal can be detected by fluorescence detector

combined with a commercialized kit (e.g., the mirVana™ microRNA Isolation Kit of Ambion, Inc. and the PureLink™ microRNA Isolation Kit of Invitrogen Co) has demonstrated excellent efficiency in isolating miRNA.

Labeling of miRNA is an absolutely key step for the overall sensitivity of the microarray. Many methods have been developed to label miRNA, and these can be classified into two main categories: direct labeling and indirect labeling. Direct labeling with fluorescent dye can be accomplished enzymatically. Currently used methods include labeling through poly(A) polymerase, labeling through T4 RNA ligase, guanine labeling, and labeling microRNA through a RNA-primed array-based Klenow enzyme assay (RAKE). Fluorescent dye also can conjugate with adjacent 3'-OH of mature miRNAs using a chemical reagent [129, 130]. Indirect labeling methods include labeling through miRNA reverse transcript, the RT-PCR product of miRNA, or the in vitro transcript of miRNA. The advantages of indirect labeling are obvious since the reverse transcription product of miRNAs is more stable and easy to preserve. In addition, miRNA can be amplified and labeled synchronously through PCR or in vitro transcription, which is very useful for low-abundance miRNAs. Although direct labeling is simple, this method has inherent problems including the fact that guanine labeling is not suitable for miRNAs lacking G residues, that T4 ligase labeling can introduce base bias, and that the procedure of chemical labeling is somewhat complicated. Though indirect labeling is more sensitive, this method may introduce artificial errors during the ligation and PCR amplification procedures.

Another microarray technique, which uses biotin-labeled miRNAs to hybridize with LAN probes, has been coupled to xMAP suspension microspheres (Luminex Co) to offer more rapid and reproducible results than does solid planar array due to its favorable reaction kinetics in liquid phase. Using this technique, a single nt difference can be discriminated [131]. Microarray technology has proven to be standard technique for profiling miRNA expression. However, due to relatively low specificity and reduced dynamic range compared to other methods, the results obtained from microarray often require the validation via RT-qPCR.

**Next-Generation Sequencing (NGS)**

Next-generation sequencing (NGS) is also called massively parallel or deep sequencing and is becoming the most effective method for miRNA analysis. Sequence throughput of NGS is unapproachable by other miRNA analysis technique since they lay DNA fragments on a single chip and simultaneous sequencing up to millions of these fragments in parallel. The principle of NGS has been described in other chapter of this book and elsewhere [132, 133]. Currently, three main platforms are in widespread use for miRNA profiling and discovery: the Roche (454) GS FLX sequencer, the Illumina Genome Analyzer, and the Applied Biosystems SOLiD sequencer. The methodologies of the NGS for miRNA analysis are similar and include sRNA isolation, library preparation, sequencing, and data analysis. In addition to increased throughput, NGS technique significantly reduces cloning biases observed with traditional capillary sequencing since sequence reads are generated from fragment libraries that don't need to be cloned and amplified.

Another key advantage of NGS over microarrays in miRNA study is that it can profile unknown genes since no sequence-specific probes are needed for detection. Currently, most novel miRNAs have been discovered and characterized through NGS. NGS can also gauge miRNA expression level by counting clone frequencies, which has demonstrated more sensitivity than microarrays [132]. One disadvantage of NGS to comprehensively profile mRNA expression is that this technique is rather expensive compared to microarray though its cost has significantly decreased with the development of commercial platforms. Another limitation of NGS is that the read length is relatively short (35–500 bp) compared to traditional capillary sequencing (1000–1200 bp). This limits their use for de novo assembly of complete genomes but makes them become the ideal instruments for miRNA profiling since the length of miRNAs (21–35 nucleotides) is shorter than the read length of NGS.

As described above, each technique for miRNA detection has its advantages and disadvantages. The method used should best fit the research goal and experimental conditions. For example, for new miRNA discovery and identification, in addition to sequencing technique, cloning of miRNA may be the simplest method. In situ hybridization is more suitable for location of miRNA in tissue. Northern blotting is very sensitive, but it is very time-consuming and not practical in large clinical studies for routine detection of the expression of hundreds of miRNAs. RT-qPCR is able to detect low copy numbers with high sensitivity and specificity. When studying the expression levels of multi-miRNAs simultaneously, microarray and sequencing may be the best choice. Another important problem is that although each platform is relatively stable in terms of its own microRNA profiling intra-reproducibility, the inter-platform reproducibility among different platforms is low [134]. An "industry standard" for analysis of miRNA expression awaits further advances in both technology and computation [135].

**Quantification of miRNAs in Biological Specimens: Normalization Approaches**

The growing interest in developing circulating miRNAs as blood-based biomarkers in the diagnostic microbiology field necessitates very careful consideration of the effects of various pre-analytical and analytical parameters on their quantification. To ensure that miRNA quantification is not affected by the technical variability that may be introduced at the multiple different analysis steps and to minimize any other potential effect of non-biological variation in the quantification results, it is important to select and identify stable miRNAs as normalizers and to choose the right normalization approaches.

The correct quantification of miRNA transcripts in clinical samples should include data normalization using both endogenous and exogenous control miRNAs [136–138]. The selection of endogenous control miRNAs is necessary to avoid false negative results due to a bad sample quality, but it is difficult. In this case a miRNA gene that is expected to be stably expressed in all analyzed samples should be selected as an endogenous control. Preferably target mRNA levels should be normalized using as miRNA normalizers control genes belonging to the same RNA class [139]. Based on the same concept, normalization of miRNA levels should be

based on endogenous control genes that belong to the small noncoding RNA family (ncRNA) of RNAs, such as small nuclear RNAs (snRNA). It is important to note that the endogenous miRNAs should meet the following characteristics in order to be used as miRNA normalizer: (a) the miRNA normalizer should be highly expressed in most samples, (b) the miRNA normalizer should show invariable expression across the test sample, and (c) the miRNA normalizer should have equivalent extraction and quantification efficiency with the target miRNAs. In order to avoid misinterpreted data and to identify true changes in miRNA expression levels, it is important to select the correct endogenous miRNA normalizer. Different algorithms can be used to select the best endogenous miRNA normalizer including geNorm [140], NormFinder [141], and BestKeeper [142]. In most cases reported so far, researchers select their endogenous reference genes for miRNA quantification according to reports in the literature or based on distinguishable low standard deviations (SD) in miRNA microarrays data. In the majority of studies, a relative quantification (RQ) step is included to compare the expression levels of target miRNA gene with the expression of an endogenous reference gene, based on the ΔΔCq approach as described by Livak and Schmittgen [143]. Therefore, in each case different miRNA normalizers should be first evaluated and then established for different sample types. The combination of several normalizers might be more appropriate than a single universal normalizer [144]. It is crucial to mention that the selection of a gene as a miRNA normalizer should always follow validation screening tests on a subset of samples under analysis.

The inclusion of synthetic miRNAs as exogenous controls added to samples prior to any analysis step is also very important for miRNA quantification. This is the only way to correct the different recovery rates for each sample during the various steps of miRNA isolation and PCR amplification between individual clinical samples. Exogenous synthetic miRNAs have been used as external controls for data normalization of sample-to-sample variations in RNA isolation [136, 137, 145]. The synthetic miRNA is added to all the plasma aliquots as an exogenous miRNA spiked-in control after the addition of the denaturating solution to avoid differences in template quality and warrant efficiency of the reverse transcription reaction. Several synthetic miRNAs have been used so far, including *C. elegans miRNA cel-miR-39* which is the almost widely used [136, 146], *miRNAs Quanto EC1 and Quanto EC2* [147], and the *simian virus gene SV40* [148] . We have to point out that using only spike-in miRNA controls for the quantification of miRNA expression in clinical samples is not correct since in this case only the handling of experiments is considered, but not the sample quality. Several studies have shown that normalization should be based on a combination of an endogenous and an exogenous control miRNA, since in this case differences in miRNA recovery and differences in cDNA synthesis between samples are compensated [136, 137, 145].

Concerning miRNA profiling by using microarrays technology, the normalization methods have not been investigated in detail so far. One of the most widely used normalization methods in this case is based on the mean expression value of all miRNAs [149]; however, quantile normalization is also a popular method for large-scale mRNA array expression [150–152], while rank-invariant set normalization [153]

has been also used. The first normalization methods that were used with miRNA array data employed centering to median values [154, 155] or scaling based on total array intensities [156]. Variance stabilizing normalization (VSN) methods have also been applied to miRNA array data [157, 158]. Normalization procedures based on the set of invariants and quantile were the most robust over all experimental conditions tested. Suo et al. and Pradervand et al. evaluated the effectiveness of these methods by comparing the normalized microarray data to qPCR data. The correlation between the microarray and qPCR data tended to be low [157, 158].

## Application of Circulating miRNAs for Diagnosis and Prognosis of Microbial Infection

Although altered miRNA expression profiles have been detected in various tissues or cells following microbial infection, these profiles have not yet been used as biomarkers in clinical practice because obtaining infected tissues and cells is difficult without invasive procedures. As described above, circulating miRNAs in serum/plasma seem more suitable for biomarkers that can be easily used for the diagnosis or prognosis of these infections. Some potential uses will be discussed in the next section.

### *Virus-Encoded miRNAs*

#### Epstein-Barr Virus

Chronic active Epstein-Barr virus (EBV) infection has high mortality and morbidity [92–94]. To explore the biomarkers for disease severity and prognosis, Kawano et al. assessed the 12 plasma miRNA expression levels encoded by EBV [159]. They found that virus-encoded miR-BART1-5p, miR-BART2-5p, miR-BART5, and miR-BART22 levels in patients with chronic active EBV infection were significantly greater than those in patients with infectious mononucleosis and in controls. Plasma miR-BART2-5p, miR-BART4, miR-BART7, miR-BART13, miR-BART15, and miR-BART22 levels were significantly increased in the patients with systemic symptoms, compared with levels in patients without systemic symptoms. The levels of miR-BART2-5p, 13, and 15 showed clinical cutoff values associated with specific clinical conditions, in contrast to plasma EBV loads which can serve as the potentially biomarkers of disease severity or progress [159].

#### JC and BK Polyomavirus

Polyomaviruses are ubiquitous, species-specific viruses that belong to the Papovaviridae family [160]. JC and BK polyomaviruses were first described in the 1970s and are the two most commonly recognized human polyomaviruses [160]. JC polyomavirus causes a fatal central nervous system demyelinating disease known as

progressive multifocal leukoencephalopathy (PML) in immunocompromised individuals or individuals being treated with potent immunosuppressive therapies [161, 162]. JC polyomavirus is a DNA tumor virus that has a double-stranded DNA genome encoding a well-studied oncogene, large T antigen [163]. The expression of the JC polyomavirus miRNAs has been investigated after infection in vitro [164]. The JC polyomavirus expressed several miRNAs, JC-miRNA-3p and JC-miRNA-5p [164]. JC polyomavirus also encodes another microRNA, jcv-miR-J1. The expression of jcv-miR-J1-5p and its variant jcv-miR-J1a-5p in 50 healthy subjects was investigated [165]. The overall detection rate of JCPyV miRNA was 74% (37/50) in plasma and 62% (31/50) in urine. The detection rate was 86% (12/14) and 57% (8/14) of plasma and urine samples in seronegative subjects, while the detection rate was 69% (25/36) and 64% (23/36) in seropositive subjects. Furthermore, in seropositive subjects shedding virus in urine, higher levels of urinary viral miRNAs were observed, compared to non-shedding seropositive subjects [165].

BK polyomavirus is the cause of nephritis in renal transplant patients and often results in graft loss [160, 166]. BK polyomavirus is latent in the urogenital tract and is able to reactivate and replicate in the nucleus of renal epithelial tubular cells of the transplanted kidney [160, 167]. BK polyomavirus-specific bkv-miR-B1-5p, JC polyomavirus-specific jcv-miR-J1-5p, and bkv-miR-B1-3p/jcv-miR-J1-3p, sharing identical sequences between the two viruses, were analyzed from body fluids diagnosed with, or suspected of, a severe polyomavirus-associated disease [167]. The miRNAs frequently amplified from human plasma, urine, and cerebrospinal fluid samples. Bkv-miR-B1-5p was amplified from one-third of the samples which often contained high viral DNA loads. Their diagnosis and management significances in severe polyomavirus-associated diseases need further clinical evaluation [167].

## *Host-Encoded miRNAs*

**Sepsis**

Diagnosis and monitoring of sepsis can be difficult because many of its signs and symptoms can be caused by other noninfectious disorders [1, 2, 4, 8]. The current gold standard for diagnosing septicemia is the blood culture, which generally takes several days or longer. Other early biomarkers of sepsis are being investigated; these include acute phase proteins (C-reactive protein), cytokines (IL-1, IL-6, IL-10 and TNF-a), chemokines (IL-8, MCP-1 and G-CSF), procalcitonin, and metabonomic [1, 2, 4, 8]. To date, these biomarkers have not demonstrated sufficient sensitivity and/or specificity to guide clinical management. Host miRNA expression profiles have been intensively studied using both in vitro or in vivo models of inflammation [33, 34, 168, 169]; some of these studies use *Escherichia coli* lipopolysaccharide (LPS) stimulation as a trigger [170]. For example, expression of miR-146, miR-155, and miR-132 increased in human acute monocytic leukemia cell line THP-1 in response to LPS stimulation [171]. Upregulation of miR-155, miR-223, and miR-146a and downregulation of miR-125b, miR-144, and miR-142–5p have been

observed in human monocyte-derived dendritic cells by Ceppi et al. [172]. Schmidt et al. screened for differentially expressed miRNAs in circulating leukocytes using an in vivo model of acute inflammation also triggered by LPS [170]. They found that four miRNAs were downregulated (miR-146b, miR-150, miR-342, and let-7 g) and one was upregulated (miR-143).

Vasilescu et al. profiled genome-wide miRNAs by microarray in peripheral blood leukocytes of sepsis patients and found that miR-150, miR-182, miR-342-5p, and miR-486 expression profiles differentiated sepsis patients from healthy controls [33]. Moreover, miR-150 levels were significantly reduced in plasma samples of sepsis patients and correlated with the level of disease severity. Finally, these investigators noted that the plasma levels ratio for miR-150/interleukin-18 can be used for assessing the severity of the sepsis. More recently, Wang et al. have analyzed seven miRNAs expression levels in patients diagnosed with sepsis, systemic inflammatory response syndrome (SIRS), and healthy controls using a RT-qPCR assay [34]. They determined serum miR-146a and miR-223 were significantly reduced in septic patients compared with SIRS patients and healthy controls. The areas under the receiver operating characteristic curve of miR-146a, miR-223, and IL-6 were 0.858, 0.804, and 0.785, respectively.

**Pulmonary Tuberculosis**

With approximately nine million new cases of tuberculosis (TB) each year, tuberculosis remains a global scourge [173, 174]. Moreover, the emergence and increase in highly resistant strains as well as the emergence of functionally untreatable TB have made the diagnosis and control of TB particularly important [175]. The gold standard for the diagnosis of TB continues to be the growth of *Mycobacterium tuberculosis* in selective media, but this culture in clinical specimens requires long incubation time (3–12 weeks) due to the slow growth of *M. tuberculosis* [173]. The diagnostic usefulness of interferon-gamma-releasing assays has been reviewed, the T-SPOT. TB assay has proven to be a helpful adjunct test for diagnosing TB [176]. Additional accurate, tuberculosis-specific biomarkers are needed [177]. Human and mycobacterial miRNAs are being evaluated for their usefulness as tuberculosis-specific biomarkers [7, 178, 179]. Studies have shown that miR-155 and miR-155* in peripheral blood mononuclear cells (PBMCs) isolated from active TB (ATB) patients exhibited characteristic expression under purified protein derivative (PPD) challenge [180]. MiRNA expression profiles have been shown to be different in PBMCs from patients with active TB, latent TB infection (LTB), and healthy controls [181]. Differences in miRNA expression of whole blood between TB and sarcoidosis (SARC) were also detected [182]. The expression levels of miRNAs in serum samples from 30 patients with active tuberculosis have been profiled [183]. Ninety-seven miRNAs were differentially expressed in pulmonary TB patient sera compared with healthy controls (90 upregulated and 7 downregulated). Following RT-qPCR confirmation and receiver operating characteristic (ROC) curve analysis, three miRNAs (miR-361-5p, miR-889, and miR-576-3p) were shown to distinguish TB-infected patients from healthy controls and other microbial infections with moderate sensitivity and specificity (area under curve (AUC) value range,

0.711–0.848). Multiple logistic regression analysis of a combination of these three miRNAs showed an enhanced ability to discriminate between these two groups with an AUC value of 0.863 [184]. Fu et al. also explore the potential roles of circulating miRNAs in active pulmonary tuberculosis infection. They found that 59 miRNAs were downregulated and 33 miRNAs were upregulated in the TB serum compared to their levels in the control serum. Interestingly, only two differentially expressed miRNAs were increased not only in the serum but also in the sputum of patients with active pulmonary tuberculosis compared to the levels for the healthy controls. Their results indicated that upregulated miR-29a could discriminate TB patients from healthy controls with reasonable sensitivity and specificity [185]. Zhang et al. employed qPCR assay to detect the expression level of miR-183 in blood from TB patients and healthy individuals. Expression level of miR-183 was found to be increased in serum samples from TB patients, compared with healthy controls. Further analysis revealed that miR-183 level is positively associated with the activity of macrophages from TB patients [186]. References 158–163 don't make sense in that the topics in these references are not about TB.

**Pertussis**

Pertussis, also known as whooping cough, is caused by *Bordetella pertussis* (*B. pertussis*) [187]. Despite high levels of vaccination, *B. pertussis* continues to circulate in Asia, Europe, the United States, Australia, and other countries, making pertussis a reemerging disease [188–192]. It is clear that the diagnosis of pertussis is still relevant despite ongoing efforts to improve pertussis vaccines [192]. The serum miRNA profile in pertussis patients was investigated in order to explore its potential as a novel diagnostic biomarker for pertussis [193]. Serum miRNA profile in pertussis patients was analyzed using a miRNA array; 50 miRNAs were overexpressed, and 81 were under-expressed in the serum of pertussis patients [193]. Expression levels of seven candidate miRNAs were further evaluated by real-time RT-qPCR. A panel of five miRNAs (miR-202, miR-342-5p, miR-206, miR-487b, miR-576-5p) was confirmed as being overexpressed in pertussis patients [193]. Risk score and receiver operating characteristic (ROC) curve analysis showed that the area under the curve of the five-member miRNA profile was 0.980. At an optimal cutoff value (0.707), this panel of miRNAs yielded a sensitivity of 97.4% and a specificity of 94.3%. These data suggest that this five-member serum miRNA profile may serve as a new biomarker for pertussis diagnosis with high specificity and sensitivity [193].

**Varicella**

Varicella, also called chickenpox, is a highly contagious disease caused by varicella-zoster virus [194–196]. Although varicella vaccination has become routine for all children at 12–15 months of age in the United States, Germany, Australia, and Korea [196], outbreaks of varicella are still seen in the community [194]. Expression levels of miRNAs in serum samples from 29 patients with varicella were analyzed using TLDA [197]. The array results showed that 247 miRNAs were differentially expressed in sera of the varicella patients compared with healthy controls (215 upregulated and 32 downregulated). Through the following RT-qPCR confirmation

and receiver operating characteristic (ROC) curve analysis, five miRNAs (miR-197, miR-629, miR-363, miR-132, and miR-122) were shown to distinguish varicella patients from healthy controls and other microbial infections with moderate sensitivity and specificity [197]. Li et al. found that six miRNAs, including miR-190b, miR-571, miR-1276, miR-1303, miR-943, and miR-661, exhibited significant higher expression levels (more than fourfold) in herpes zoster (HZ) patients, compared with those of healthy controls and herpes simplex virus (HSV) patients [197]. The altered miRNA could be potentially used as biomarkers to test for latent HZ infection [198, 199].

**Avian Influenza A (H7N9) Virus**

Novel human influenza A virus strains continue to emerge and evolve from avian influenza strains and result in yearly epidemics and occasional pandemics [200]. The latest of these zoonotic avian influenza A strains to infection humans is the H7N9 avian influenza strain [201, 202]. MiRNA regulates host immune response and pathogenesis during influenza A infection and modulated viral replication [203]. Serum miRNA profile in response to H7N9 virus infection has been characterized using TLDA [61]. Upon infection, a total of 395 miRNAs were expressed in the serum pool of patients, far beyond the 221 in healthy controls. Among the 187 commonly expressed miRNAs, 146 were upregulated and only 7 were downregulated in patients. Further analysis by quantitative RT-PCR revealed that the serum levels of miR-17, miR-20a, miR-106a, and miR-376c were significantly elevated in patients compared with healthy individuals. ROC curves were constructed to show that each miRNA could discriminate H7N9 patients from controls with AUC values ranging from 0.622 to 0.898, whereas a combination of miR-17, miR-20a, miR-106a, and miR-376c obtained a higher discriminating ability with an AUC value of 0.96. These findings reveal significant alterations in serum miRNA expression following influenza virus infection [61] and confirm the great potential of circulating miRNAs for the diagnosis of influenza and other viral diseases [5, 203].

**HIV**

Infection with HIV-1 leads to a systemic destruction of T cells and diminished cell-mediated immunity resulting in a wide range of opportunistic infections as well as cancers [204]. Although treatment with antiretroviral therapy increases the survival of HIV-infected individuals, it does not result in eradication of infection [205]. Moreover, efforts to vaccinate against HIV-1 have not been successful [206]. HIV-1 is known to persist in resting T cells and also may persist in different cell types [205]. Understanding the role that miRNAs may play in the pathogenesis of HIV-1 may allow different approaches to both antiretroviral therapy and vaccine development [45–47, 207]. Studies have shown the different expression pattern of miRNAs in peripheral blood mononuclear cells (PBMCs), $CD4^+$ T cells, $CD8^+$ T cells, and monocytes from HIV-1-infected subjects [207]. For instance, Wang et al. have reported that four miRNAs (miRNA-28, miRNA-150, miRNA-223, and miRNA-382) showed different expression levels between monocytes and macrophages in HIV infection [50]. A cohort of 128 plasma samples from HIV-1-infected subjects and 37 samples from healthy donors have been analyzed in the light of

HIV-1-infected patients with low (<200 cell/μL), medium (200–350 cell/μL), and high (>350 cell/μL) CD4+ T cell count (LTC, MTC, and HTC). Of the 754 host miRNAs (excluding endogenous controls) incorporated in the array, 232, 346, 316, and 258 miRNAs were detected in plasma of healthy controls, LTC, MTC, and HTC groups, respectively. A total of 297 miRNAs differentially expressed in LTC subjects, of which 273 were upregulated and 24 were downregulated compared to healthy controls. Similarly, a total of 257 miRNAs (236 upregulated and 21 downregulated) were differentially regulated in MTC subjects compared to healthy controls. However, in the HTC group, only 127 miRNAs (85 upregulated and 42 downregulated) were differentially regulated compared to healthy controls. Fifteen miRNAs (miR-29a, miR-223, miR-27a, miR-19b, miR-151-3p, miR-28-5p, miR-766, miR-30a-3p, miR-136*, miR-125b, miR-18a, miR-769, miR-942, miR-1197, and miR-518b) were randomly selected for further analysis. Among these, seven miRNAs (miR-1197, miR-766, miR-136*, miR-151-3p, miR-518b, miR-769, and miR-942) were commonly dysregulated in all three groups. The other eight miRNAs were upregulated expression in LTC, and MTC groups compared controls. A combination of nine miRNAs (miR-29a, miR-223, miR-27a, miR-19b, miR-151-3p, miR-28-5p, miR-766, miR-30a-3p, and miR-136*) were found to distinguish the HIV-1-infected patients from healthy controls with sensitivity of 96.1% and specificity of 97.3% and AUC = 0.994 [208]. Seven of them were significantly associated with $CD4^{+}$ T cell count and thus have a great potential to serve as biomarkers for monitoring the HIV immune status.

**Viral Hepatitis B**

Hepatitis B virus (HPV) infects the liver and causes acute and/or chronic liver diseases that may progress to cirrhosis of the liver or to hepatocellular carcinoma [209]. HBV infection is also known to modulate the expression of host cellular miRNAs, which then participate in development of HBV-related liver diseases [210, 211]. The miRNA profiles in chronic hepatitis B patient tissues or in HBV-expressing cells have been reviewed by Liu et al. [212]. Li et al. profiled serum miRNAs of healthy controls, HBV-, HCV-, and HBV-positive hepatocellular carcinoma (HCC)-affected individuals by Solexa sequencing followed by validation with quantitative RT-PCR assay [34]. These investigators successfully identified 13 miRNAs that are differentially expressed in HBV serum. This 13-miRNA-based biomarker accurately discriminated not only HBV cases from controls and HCV cases but also HBV-positive HCC cases from control and HBV cases. For example, when using four markers (miR-375, miR-10a, miR-223, and miR-423) to separate the control and HBV groups, the AUC was 99.9 ± 0.1% (sensitivity, 99.3%; specificity, 98.8%). Similarly, two markers (miR-92a and miR-423) could separate the control and HCV groups with a high specificity and sensitivity (AUC, 99.6 ± 0.4%; sensitivity, 97.9%; specificity, 99.4%). The control and HBV-positive HCC group could be clearly separated by five markers (miR-23b, miR-423, miR-375, miR- 23a, and miR-342-3p; AUC, 99.9 ± 0.1%; sensitivity, 96.9%; specificity, 99.4%). Similarly, the HBV and the HBV-positive HCC group could be separated by two markers (miR-10a and miR-125b; AUC, 99.2 ± 0.6%; sensitivity, 98.5%; specificity, 98.5%) [34].

Another study profiled miRNA expression on pooled sera obtained from identified groups of chronic asymptomatic carriers (ASC), patients with chronic hepatitis B (CHB) and HBV-associated acute-on-chronic liver failure (ACLF), as well as healthy controls (HC) using Applied Biosystems TaqmanArray assay [213]. A total of 37 miRNAs were amplified from HC, whereas 77, 101, and 135 were amplified from ASC, CHB, and ACLF, respectively. The expression levels of most miRNAs were also upregulated in HBV-infected patients when compared to HC. Furthermore, the level of miRNAs in the CHB serum was upregulated most in hepatitis B e antigen-positive patients. The expression of MiR-122, the most abundant miRNA in liver tissue, was significantly higher in HBV-infected groups than in HC. The expression of miR-223 was similar between HC and ASC but increased significantly in CHB and ACLF. The expression levels of miR-122 and miR-194 correlated negatively with the age of patients with CHB or ACLF.

**Viral Hepatitis C**

Hepatitis C virus (HCV) also infects the liver and causes hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [214]. miRNAs are thought to regulate multiple aspects of HCV live cycles; certain miRNAs appear to serve as essential mediators for interferon-based antiviral therapy [215, 216]. In an in vitro acute HCV infection model, 108 human miRNAs were identified whose expression levels changed for more than 2.0-fold in response to HCV infection [217]. Marquez et al. measured miR-122 and miR-21 levels in HCV-infected human liver biopsies relative to uninfected human livers and correlated these with clinical patient data [218]. They found that miR-21 expression correlated with viral load, fibrosis, and serum liver transaminase levels, while miR-122 expression inversely correlated with fibrosis, liver transaminase levels, and patient age. Morita et al. has described hepatic miR-122 expression that was weakly and positively correlated with the serum HCV load but was not correlated with HCV load in the human liver [219].

Bihrer et al. found that sera from patients with chronic HCV infection contained higher levels of miR-122 than sera from healthy controls [36]. Serum miR-122 levels correlated well with markers of liver inflammatory activity, that is, the serum levels of alanine leucine transaminase (ALT) and aspartate transaminase, and the histologic activity index (HAI) score. In patients with persistently normal ALT levels, serum miR-122 levels did not differ from healthy controls. There was no correlation of serum miR-122 levels with serum albumin, international normalized ratio, liver fibrosis, or serum HCV RNA. Thus, serum miR-122 appears to act as a biomarker of necroinflammation in patients with chronic hepatitis C infection.

**Enteroviral Infections**

Enteroviruses are common causes of human infections with a diverse array of clinical features ranging from gastroenteritis to meningoencephalitis and myocarditis to pleuritis [220]. The role of miRNAs in the pathogenesis of enterovirus infections are becoming appreciated although not yet fully understood [221]. A comprehensive miRNA profiling in EV71-infected Hep2 cells using deep sequencing has been performed [222]. A total of 64 miRNAs were found whose expression levels

changed for more than twofold in response to EV71 infection [222]. Ho et al. found that upregulation of miR-141 upon enterovirus infection can facilitate viral propagation by expediting the translational switch [223]. Host serum miRNA levels in patients with hand-foot-and-mouth disease caused by enterovirus 71 (EV71) and coxsackievirus 16 (CVA16) as well as in other microbial infections and in healthy individuals have been compared [224]. Among 664 different miRNAs analyzed using a miRNA array, 102 were upregulated and 26 were downregulated in sera of patients with enteroviral infections. Expression levels of ten candidate miRNAs were further evaluated by quantitative real-time PCR assays. A receiver operating characteristic (ROC) curve analysis revealed that six miRNAs (miR-148a, miR-143, miR-324-3p, miR-628-3p, miR-140-5p, and miR-362-3p) were able to discriminate patients with enterovirus infections from healthy controls with area under curve (AUC) values ranged from 0.828 to 0.934. The combined six miRNA using multiple logistic regression analysis provided not only a sensitivity of 97.1% and a specificity of 92.7% but also a unique profile that differentiated enteroviral infections from other microbial infections. Expression levels of five miRNAs (miR-148a, miR-143, miR-324-3p, miR-545, and miR-140-5p) were significantly increased in patients with CVA16 versus those with EV71 ($p < 0.05$). Combination of miR-545, miR-324-3p, and miR-143 possessed a moderate ability to discrimination between CVA16 and EV71 with an AUC value of 0.761. These data indicate that sera from patients with different subtypes of enteroviral infection express unique miRNA profiles. Serum miRNA expression profiles may provide supplemental biomarkers for diagnosing and subtyping enteroviral hand-foot-and-mouth disease infections [224].

**Cytomegalovirus**

Cytomegalovirus (CMV) is an important human pathogen that is often asymptomatic until the infected individual becomes immunosuppressed [71]. CMV miRNA is currently under investigation [72]. Plasma levels of 11 human- and 3 CMV-encoded miRNAs were quantitated by real-time PCR in 13 infants with congenital CMV infection. The levels of miR-183-5p and miR-210-3p were significantly higher in patients with congenital CMV infection than in control infants. The results indicated that plasma miRNAs could be associated with the pathogenesis of congenital CMV infection and could be used as disease biomarkers [225].

**Ebola Virus**

Ebola virus (EBOV) is a filovirus that initially infects dendritic cells and macrophages, which leads to lethal infections in humans and primates [226]. EBOV miRNAs have been identified and may serve as biomarkers for the diagnosis and therapy of Ebola viral infections [227]. EBOV-induced changes in circulating miRNA populations of nonhuman primates and humans have been investigated [228]. Eight miRNAs, including hsa-miR-146a-5p, hsa-miR-18b-5p, hsa-miR-21-3p, hsa-miR-22-3p, hsa-miR-29a-3p, hsa-miR-432-5p, hsa-miR-511-5p, and hsa-miR-596, can correctly categorize infection status in 64/74 (86%) human and nonhuman primates samples [228].

**Dengue Virus Type 1**

Dengue is currently regarded as the most prevalent and rapidly spreading mosquito-borne virus [229]. Efforts have been made at increasing our understanding of the pathogenesis and immunology of this viral infection [230, 231]. Among these advances in knowledge are the roles of miRNAs in dengue [232]. Expression levels of miRNAs in serum samples from three patients with dengue virus type 1 (DENV-1) and three healthy volunteers were separately analyzed using miRNA PCR arrays [210]. The expressions of the five selected miRNAs were verified by RT-qPCR. SerummiR-21-5p, miR-146a-5p, miR-590-5p, miR-188-5p, and miR-152-3p were identified as promising serum indicators for dengue infection [233].

**Parasitic Infections**

Parasitic infections continue to have high morbidity and mortality rates on humans. Newly recognized insight has resulted from genetic studies [210] and microRNA studies [234–236]. The expression levels of circulating miRNAs were also analyzed in filarial-, *Toxoplasma gondii*-, and *Plasmodium vivax*-infected patients [237–239]. These studies proved that altered plasma or serum miRNAs were useful as the biomarker for the detection of parasite infection. For example, serum miR-223 could serve as a potential new biomarker for the detection of schistosome infection and the assessment of the response to chemotherapy. Plasma miR-451 and miR-16 are relevant biomarkers for malaria infection. miR-71 and miR-34 discriminated *Onchocerca volvulus*-infected samples from uninfected samples.

## Concluding Remarks

Circulating miRNAs have been investigated as the diagnosis or prognosis marker for microbial infections. Studies on host miRNA profiles for microbial infections are underway. Host miRNA profiles have a considerable way to go before they will be ready for use in clinical practice. Several issues remain to be clarified in this field: (A) source material should be fixed. Plasma, serum, whole blood, and isolated exosomes or microvesicles have been used as the source for miRNA profiling. Advantages and disadvantages of each source should be considered prior to selecting the source. (B) Though differences in circulating miRNAs between males and females have not been found with the exception of differences associated with pregnancy [240, 241], miRNA levels in plasma, and serum from a large number of normal individuals of both genders and various ages, even the same individual over time should be extensively studied. (C) No acknowledged reference genes have been found in serum/plasma. Commonly used endogenous controls, such as miR-16, are dysregulated in some diseases; RNU6B is degraded in serum. Spiking into RNA isolation processes with synthetic exogenous miRNA only acts as a normalizers for differences in recovery between samples. (D) The methods of miRNA quantification including RNA isolation should be standardized since inter-platform reproducibility among different platforms is low. (E) It seems to lack of specificity using single miRNA as biomarker since the miRNA commonly regulated in various disease. A panel of miRNAs would be a best choice.

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# Quantitative Approach in Clinical Microbiology: A Paradigm Shift Toward Culture-Free Methods

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## Introduction

The human microbiome represents the entire ecological community of microorganisms that colonize the human body including bacteria, fungi, archaea, and viruses. From birth to adulthood, the human microbiome establishes symbiotic relationships with the host, becoming involved in critical biochemical processes and pathways [1]. It is now accepted that bacteria not only cause infectious diseases as proposed by Koch in the 1800s, but when the microbiome is unbalanced, it predisposes the host to a wide spectrum of diseases, such as cancer, autoimmune disease, and neurodegenerative and psychiatric illnesses [2–4].

There are two main specimen types associated with microbial diagnostics in a pathology laboratory: (1) clinical isolates, where microbes are grown as pure clonal isolates on a suitable media, and (2) human clinical specimens, where any pathogens may be present in a complex environment, potentially with commensal organisms or in the presence of host cells. Culturing techniques have several limitations: they depend on the bacteria being able to reproduce in a culture medium,

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they often take several days before a conclusive result is obtained, and they require that clinicians suspect a causative infectious disease agent in order to set up pathogen-specific culturing conditions.

DNA and RNA sequencing has now become indispensable for the study of microbiology. Comprehensive new genome sequencing technology instruments and reagents have emerged over the last decade, following the successful sequencing of the human genome in 2001 [5]. High-throughput sequencing (HTS), also known as next-generation sequencing, is the catchall term used to describe a number of different modern sequencing technologies which allow us to sequence genetic material much more quickly and cheaply than the previously imagined. These technologies have been applied to bacteria, viruses, and fungi, and microbial reference genomes are now available to researchers and clinicians [6]. Genetic inventories of human microbial samples using HTS are now routinely completed by researchers investigating the association between the human microbiome and disease [7]. The application of sequencing technology in the clinical setting is inevitable given its dominance in microbial identification in the research setting.

This chapter discusses (1) the application of quantitative HTS in microbial diagnostics and (2) HTS implementation in a clinical laboratory setting. The benefits and challenges associated with the application of HTS technology in a clinical setting are discussed.

## *Microorganism Identification*

The main clinical application of HTS is in microorganism detection and identification. Identification of bacteria and viruses in biological samples using physiological, biochemical, and serological measurements has relied largely on cell culturing and, more recently, by molecular characterization [8]. While culture-based methods have proven critical to the accurate diagnosis of infectious illnesses, there are disadvantages. Firstly, many microorganisms do not grow well in culture, and thus an inherent bias exists in a polymicrobial sample as to what microorganism can survive and flourish. This was the principal reason as to why there was a lack of appreciation as to the extent of the microbiome.

Secondly, culture-based methods require a working understanding of the type of culture method needed at the pretest stage. For instance, the clinical team must take the sample at the correct clinical time (e.g., at the point of fever in infective endocarditis) and also select the most appropriate culture bottles (e.g., bacterial, viral, fungal). In addition, the microbiologist must choose the most relevant media in which to grow the sample. Thirdly, turnaround time is usually 1–2 days for most samples but can be longer for samples with a limited number of pathogenic organisms such as blood, cerebrospinal fluid, or urine [9, 10]. The accurate detection of non-culturable or difficult-to-culture organisms, including slow-growing organisms, fastidious bacteria and anaerobes, and possible biothreat agents, has been another early

application of microbial HTS. For example, *Mycobacterium tuberculosis*, the causative agent of tuberculosis, typically takes 2–4 weeks on average to get a primary culture, and antibiotic susceptibility is determined after an additional 2–4 weeks [11]. For these microorganisms, culture-free technologies could provide an alternative mechanism for microorganism identification. Additionally, in the presence of coinfection, population-based methods eliminate the need to isolate and culture individual microbial species as multiple pathogenic bacteria can be sequenced and identified simultaneously.

Traditional polymerase chain reaction (PCR) and, more recently, microarray assays can be used to analyze a pathogen's genetic profile, rather than the morphological, phenotypic, and biochemical features that standard culture methods utilize [12]. PCR assays are quick, specific, and cheap; however, they rely on the inherent bias of pre-analytic selection for primers for target sequences [12]. Microarrays have a wider detection than PCR but require updating as new microbial genomes are sequence, which can be time-consuming and expensive [13].

In HTS, DNA and RNA are sequenced from clinical samples and matched to reference databases to identify the microbes present in a sample. The advantage of HTS is that the method of microbial detection is unbiased; all of the metagenome present is assessed within the interrogated sample [14]. Secondly, it is culture-independent. It should be noted however that currently sequencing might not overcome the issue of detecting small colony species or low levels of genetic content. Current population-based techniques can match the 48-h turnaround time of culture methods, but the gene level analysis currently takes longer than the culture methods, which typically take 1–2 days. However, if current trends continue, higher coverage with a shorter run time and lower cost will be achievable as the technology improves [15]. Table 1 summarizes the characteristics of the taxonomic identification methods.

## *Quantitative Microbial Assessment*

The data analysis protocol in population-based microbial diagnostics follows a quantitative logic, whereby nucleic acid sequences are used to describe the presence and abundance of microbial species in a sample. Taxonomic assignment of the sequence data is the first step in quantitative microbial analysis, which determines how many kinds of taxa are in a sample (alpha diversity) and how taxa are shared between samples (beta diversity) rather than presence-absence data produced by microbial culturing [20]. This is referred to as phylogenetic profiling, and the output is a contingency table of taxonomic units per sample.

The next step in population-based analysis is to combine the taxonomic observation data with other patient information such as clinical, metabolomic, or environmental factors. With integrated representation of the data, it is easy to use microbial species information to explain clinical presentations [21].

**Table 1** Characteristics of taxonomic identification methods

| Technique | Speed (days) | Cost | Accuracy | High-throughput | Multiple species | Novel pathogens | Antibiotic resistance | Genome reference database |
|---|---|---|---|---|---|---|---|---|
| Culture | 2–14 | $ | Genus/species | No | No | No | Yes | None |
| PCR | 2 | $ | Genus/species | No | No | No | No | None |
| 16S | 1.5–2 | $$ | Genus/species | Yes | Yes | Yes | No | Ribosomal RNA databases available, e.g., Greengenes, SILVA, or the ribosomal database project [RDP] [16] |
| Metagenomic | 2 | $$$ | Species/strain | Yes | Yes | Yes | Yes | EBI Metagenomics [17], KEGG [18], PFAM [19] |

The census of the inhabitants of microbiome samples tends to vary greatly between individuals, with some dominant taxa in one person being present in low numbers in another person [20]. Considering the present opportunistic pathogens belong to the normal human microbiome community, thresholds to determine whether a pathogen is clinically relevant will have to be defined [7].

Established methods in clinical microbiology laboratories designed to detect and identify single pathogens could be replaced by high-throughput but low-cost sequencing technologies to allow the detection of specific pathogens for the diagnosis of infectious diseases as well as profiling the entire microbial population for microbiome diseases or polypathogenic diseases [22].

## Applications of Quantitative Microbiology in Disease Diagnosis

This section presents the clinical applications of quantitative microbiology in disease diagnosis, as summarized in Fig. 1.

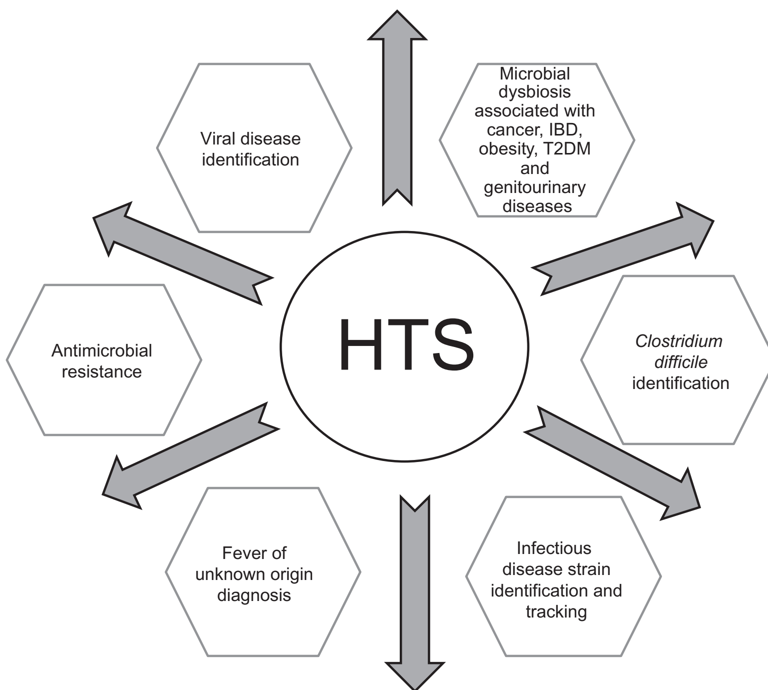


**Fig. 1** Clinical applications of quantitative microbiology

## *Fever of Unknown Origin*

Fever of unknown origin is a diagnostic challenge that clinicians encounter when a patient exhibits a recurrent fever (>38.3 °C) for longer than 3 weeks that has no clear source [23]. The initial presentation requires a systematic clinical workup by the clinician to identify the source of infection. In adults, blood and urine cultures are usually taken and a chest radiograph performed. In young children, cerebrospinal fluid can be sent for culture. Acquisition of a quick diagnosis in cases of sepsis and septic shock is critical to obtain quick specific treatment and prevent death and subsequent comorbidities when empirical therapies fail [24]. HTS use has led to improved pathogen detection compared to existing cultures, serologic tests, and pathogen-specific PCR assays.

David Relman was one of the first researchers to demonstrate the application of DNA sequencing technology in microbiology when, in 1992, he identified a previously uncultured *Bacillus* associated with Whipple's disease [25]. Following this, the technique was used to classify a new *Hantavirus* responsible for an outbreak of an acute respiratory illness [26]. Over the past few decades, DNA sequencing has been increasingly used to characterize pathogens associated with infectious diseases.

In 2008, Nakamura et al. identified the pathogen *Campylobacter jejuni* from patient's feces during a diarrheal illness using HTS [27]. Several cases in the literature have successfully employed HTS to diagnose rare, novel, or atypical infectious etiologies for encephalitis, including cases of infection by *Leptospira* [28], Astrovirus [29, 30], and Bornavirus [31]. In the case described by Wilson et al. [28], 38 different diagnostic tests had been conducted and failed to yield an actionable answer before HTS analysis was performed, which identified the pathogen.

## *Epidemiological Typing of Infectious Bacteria*

From a field epidemiology perspective, HTS use allows the drawing of more accurate epidemiological outbreak maps and the deciphering of both the evolutionary history and the genetic makeup of particular outbreak isolates. For example, the *Mycobacterium* genus is responsible for several diseases in humans including tuberculosis which is linked to *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. canettii*, *M. caprae*, *M. microti*, and *M. pinnipedii*, leprosy caused by *M. leprae*, and declining lung function in individuals with cystic fibrosis associated with non-tuberculous mycobacteria. Established culture and metabolic detection techniques are time-consuming and difficult because most pathogenic mycobacteria are slow-growing and they do not allow for proper identification of mycobacterial species and subspecies [32]. HTS analysis both at the species [33, 34] and the gene level [32, 35] has been successfully used to identify mycobacterium.

HTS use in outbreak monitoring has been applied with huge success. HTS was used to identify the origin of the *Vibrio cholerae* isolate that devastated Haiti after the 2010 earthquake. It was revealed that the epidemic isolate was closely related to isolates from Asia rather than circulating South American isolates and likely introduced by human activity [36]. HTS has been also been applied to analyze *S. aureus* epidemics in hospitals revealing the dynamics of outbreaks in single hospital wards [37, 38] and transmission between the hospital and community settings [39, 40]. Perhaps one of the most striking applications of sequencing in the field was its use during the 2014 Ebola outbreak, when European Mobile Laboratories in Guinea were able to monitor the transmission history and evolution of the Ebola virus as the outbreak unfolded [41]. HTS analysis will provide real-time, longitudinal analysis of outbreaks in progress.

## *Microbial Characterization/Antimicrobial Resistance/Marker Detection for Guided Therapy*

The progression and outcome of infectious disease are determined by the dynamics of host–pathogen interactions, and recent studies employing HTS have offered novel insights into the evolution of bacterial pathogens during the course of colonization and infection. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Mycobacterium abscessus*, *Mycobacterium tuberculosis*, and *Burkholderia dolosa* have been demonstrated to undergo considerable diversification during infection, resulting in “clouds of diversity” that originated from a single or closely related group of infecting bacteria [39, 42–45].

The microbiome plays a crucial role in medication response by altering drug responses and tolerance, but to date it has been under-explored. The application of HTS in antimicrobial susceptibility testing is limited, given that the sensitivity and robustness of phenotypic susceptibility testing are not reached currently by HTS technology, in part due to incomplete data linkage of genotype to phenotype. In addition, phenotypic antimicrobial susceptibility testing is inexpensive and has been highly automated and fully established in numerous clinical laboratories. However, there are certain immediate applications for the use of HTS technology for antimicrobial resistance: (i) where phenotypic testing is prohibitively slow, e.g., as mentioned previously, the genotypic antimicrobial susceptibility testing for microbes that are difficult to grow [11], and (ii) where drug resistance traits are not caused by multiple genetic components but instead linked to point mutations or small indels in a single gene, e.g., rifampin resistance in *M. tuberculosis*, methicillin resistance in *S. aureus*, and trimethoprim-sulfamethoxazole resistance in *T. whipplei* [46]. In addition, if the overall HTS turnaround time, including molecular and bioinformatics methods, can be reduced to 1 day, then HTS could complement phenotypic testing to rule in resistance for certain antibiotics where

known drug resistance mutations or genes are found before phenotypic results become available [37] and time and resources could be redirected to detect resistance encoded by novel mechanisms.

Many bacteria express toxins that can cause severe disease, for example, toxic shock syndrome caused by *Streptococcus pyogenes* [47]. Traditionally, detection of these virulence factors involved using bacterial serotyping or PCR-based techniques; however, these assays can give false-negative results if the toxin-encoding gene has been mutated. In this situation, HTS could provide an alternative mechanism that would allow the sensitive detection of bacterial virulence factors even in the presence of mutations.

## *Viral Disease Identification*

HTS technologies can provide advanced molecular surveillance of viruses to help monitor transmission and provide new opportunities to reduce virulence of pathogens. Fisher et al. performed HTS analyses to successfully detect multiple minor variant drug resistance mutations in HIV-1 reverse transcriptase in infants where prevention of mother-to-child transmission failed [48]. Guan et al. screened cerebrospinal fluid for viral DNA to diagnose the cause of four cases of suspected viral meningoencephalitis. Herpes simplex virus (HSV) 1, HSV 2, or herpes virus type 3 were detected in the different patients, and the results were confirmed with PCR [49].

Genomic sequencing of group A rotavirus (RVA) strains was used to record the evolving virus population post vaccine introduction in the United States. RVA are double-stranded RNA viruses that are a significant cause of acute pediatric gastroenteritis. The genetic information is being collected to identify possible mechanisms of immune escape, which result in RVA gastroenteritis in vaccinated individuals [50].

Hepatitis C virus (HCV) is classified into 7 major genotypes and 67 subtypes. HCV exploits complex molecular mechanisms, which result in a high degree of intrahost genetic heterogeneity. This high degree of variability represents a challenge for the accurate establishment of genetic relatedness between cases and complicates the identification of sources of infection [51]. A recent study has shown that in HCV subtyping, using HTS can be a useful alternative to current methods if adequate sequence depth can be achieved [52].

## **Clostridium difficile** *Colonization and Diagnosis*

*Clostridium difficile* infection (CDI) is the leading cause of infectious diarrhea in hospitalized patients in the Western world [53]. As new strains of infection emerge, there is a growing challenge for laboratories, and to adapt to increasing rates of

infection and detection does not always equate with disease [54]. Enzyme immunoassays for *C. difficile* toxin(s) and molecular assays are currently used to detect the pathogen. Laboratory tests available for the detection of *C. difficile* in stool specimens include culture, toxin antigen detection, and detection of toxin genes by nucleic acid amplification tests (NAATs) [55]. While culture for toxin-producing *C. difficile* is considered the gold standard, this test is ill suited to the clinical laboratory, as it is technically demanding and requires, at minimum, 3 days to perform. In contrast, enzyme immunoassays (EIA) for toxins A and/or B in stool have been widely used by clinical laboratories in the United States as a rapid method by which to detect *C. difficile*. However, the sensitivity of these EIAs is poor compared to culture, ranging from 33% to 65% [56, 57]. One main disadvantage of NAATs is that they do not detect the presence of biologically active toxin in stool specimens [55].

HTS was used to study the transmission of *C. difficile* whereby sequencing was performed on 486 samples from cases documented over 4 years in the United Kingdom [58]. In another pilot study, HTS was performed in almost real time to evaluate a *C. difficile* outbreak; samples from all cases identified over a 6-week period in one hospital were sequenced and compared with local strain sequences from the previous 3 years. Analysis of these strains illustrated that HTS could provide early outbreak detection and also suggested community transmission, which was not previously suspected. Improvements in HTS technology are expected to increase its use in *C. difficile* infections.

## *Microbiome and Cancer Risk*

It has been increasingly recognized that bacteria and viruses are involved in the pathogenesis of cancer not only through their pathogenic activities but also their metabolites as commensal bacteria [59]. It is estimated that 15% of worldwide cancer is of an infectious nature, with human papillomavirus, hepatitis B virus, hepatitis C virus, human herpesvirus-8, and *Helicobacter pylori* recognized as the definitive cause of cervical cancer, hepatocellular carcinoma, Kaposi's sarcoma, and gastric cancer/lymphoma, respectively [60].

Several case-control studies have demonstrated that enrichment and depletion of several bacterial populations are associated with colorectal cancer (CRC) [61–63]. HTS of bacteria in stool samples has the potential to be a useful prognostic biomarker for CRC [64]. Two studies have demonstrated that HTS of bacteria in stool samples are useful prognostic biomarkers for CRC by focusing on quantification of abundance of specific species. Wei et al. [65] selected 3 species: *F. nucleatum*, *B. fragilis*, and *F. prausnitzii*, while the second selected 22 CRC marker species [63]. Tahara et al. [63] found the prediction of CRC with the metagenomic classifier was similar to the standard fecal occult blood test (FOBT), and when both approaches were combined, sensitivity improved >45% relative to the FOBT while maintaining its specificity.

### *Inflammatory Bowel Disease Risk*

Genome-wide association studies have linked inflammatory bowel disease (IBD), which comprises both Crohn's disease and ulcerative colitis, with loci that implicate an abnormal immune response to the intestinal microbiota [66]. Microbiome profiling studies of the intestinal microbiome have associated the pathology of IBD with characteristic shifts in the composition of the intestinal microbiome. Reduced taxa richness, a decreased representation of several taxa within the *Firmicutes* phylum [67], and an increase in *Fusobacterium* have been found in IBD patients [68]. Microbiome dysbiosis, quantified using HTS, may be a potential screening/diagnostic tool for IBD [69].

### *Obesity and Type 2 Diabetes Mellitus (T2DM) Risk*

There is a growing body of evidence suggesting that the gut microbiota has a role in the regulation of the energy homeostasis associated with various metabolic disorders, including T2D and obesity. There was an overall decrease in gut microbiome diversity at both the phylogenetic level (i.e., reduced number of distinct species) [70] and metagenomic gene count level in the obese (i.e., reduced number of distinct genes) in HTS-based studies [71]. Sequencing of the gut microbiome showed that patients with T2DM were characterized by a decrease in the abundance of some universal butyrate-producing bacteria, in particular, *Faecalibacterium prausnitzii* and *Bifidobacterium* species, and an increase in various opportunistic pathogens, as well as an enrichment of other microbial functions conferring sulfate reduction and oxidative stress resistance [72, 73]. Studies have also demonstrated the composition of the gut microbiome is correlated with insulin resistance [74], and the ratio of *Bacteroidetes* to *Firmicutes* species is linked to fasting blood sugar. Researchers are suggesting that microbial changes that occur prior to the onset of T2D and obesity may potentially be used for early diagnosis and intervention by HTS analysis of fecal stool samples [75]. Applications of HTS in describing metabolic disorders are increasingly expected.

### *Microbiome and Genitourinary Disease Risk*

HTS of bacterial communities inhabiting the human urinary tract through urine specimen analysis is another potential application of the technology. Colonization with *O. formigenes* has recently been associated with a 70% reduction in the risk for being a recurrent calcium oxalate stone formation [76]. Furthermore, the urinary microbiome and its relationship to urinary tract conditions such as urge incontinence and interstitial cystitis are currently under investigation [76, 77]. Profiling of the urinary tract microbiome for detection of certain urinary diseases will involve HTS technology.

## Incorporating HTS into the Clinical Laboratory: Opportunities and Challenges

Despite the burgeoning clinical potential of the microbiome, its application in the clinical laboratory requires navigating some obstacles.

### *Cost-Effectiveness*

Whole genome sequencing via HTS is still a relatively new method, therefore not yet cost-effective. For the identification of pathogens, it is unlikely to replace the established automated culturing systems (e.g., BP Phoenix) or newer mass spectrometry systems because of cost and sensitivity comparisons alone. Given the dramatic reduction in cost of sequencing over the last decade, HTS is likely to become a cost-effectiveness option for some clinical applications [6].

### *Validation*

Every pathology test must undergo a process of validation by the FDA to ensure that the test is performing correctly. HTS applications in microbiology must be characterized in terms of accuracy (sensitivity and specificity), precision, reference range, and reportable range in order to be FDA approved [78]. Laboratory-developed tests will also need to be evaluated for their analytical sensitivity or lower limit of detection as well as analytical specificity, which includes things like cross-reactivity with other targets and also interferences from components that may be part of the specimen. It is a complex process, carried out in conjunction with the laboratory accreditation agency for specific requirements that requires thorough planning that will lead to efficient use of specimens, reagents, and technologists' time [79].

### *Data Processing and Bioinformatics*

HTS generates a tremendous amount of raw data that requires a significant level of computational time/power to analyze the data outputs. For the conversion of raw sequences to actionable information for patient treatment, steps such as genome annotation, genome assembly, manual closure, and genome finishing will need to be automated in a streamlined process. Currently noncommercial sequence analysis software provided as online services such as the Galaxy platform [80] and the Rapid Annotation using Subsystem Technology (RAST) server [81] for bacterial genome annotation are popular among researchers. As genome sequencing by clinical microbiology laboratories becomes commonplace, complete, and thoroughly sorted, clinical microbiology software packages must become widely available.

Staff training to use the new equipment will be essential. The extent to which the implementation of HTS technology affects the need for certain types of laboratory professionals is thus far lacking consensus. While some reports suggest increases in laboratory productivity without incurring higher labor costs, other sources have said that while new laboratory technologies potentially decreased the need for a large staff, workload has increased [82].

### *Reference Databases*

Beyond the data processing stage, the clinical interpretation of the significance of a specific bacterial species or virus subtype can be unique to the laboratory that performed the testing (i.e., it is not reproducible among laboratories). Variability in interpretation for sequence variants is due, in part, to the lack of professionally curated information to support clinical decision-making, combined with the amount of information typically generated by such analyses [83].

Currently, HTS-produced sequences are identified using reference databases. Investigation of multiple databases can be required to assess the potential significance of HTS output, and that is a cumbersome, time-consuming, and unfeasible process because of the clinical environment [84–86]. Adding to that complexity, not all databases contain accurate information, and a single database may have variability in the quality of its information for different variants. “Clinical grade” databases—that is, the HTS results generated under clinical quality standards, which can be used to characterize the microbiome and infer risk of disease, guide diagnosis, predict prognosis, and/or to indicate a potential therapeutic target—are needed for broad and effective clinical use of HTS in clinical laboratories [83].

### *Data Storage and Integration*

One of the major challenges of implementing HTS in the clinical laboratory is managing the large amount of data generated. As described above, HTS methods rely heavily on the use of reference databases. Widespread clinical use of HTS will generate a substantial amount of new microbial sequencing data, which will in turn need to be integrated back into the reference databases. Central data storage strategies would offer economic advantages for data management and are likely to be developed [87].

### *Clinician Acceptability*

In the same way, clinicians have been reticent to use the results of genomic information; it is likely microbiome data will be analogous, because of uncertainties on its importance and lack of understanding. The College of American Pathologists has

produced a checklist for the application of HTS technology in clinical testing [88]. As this is a rapidly developing field, the practices are still largely under development. Demonstrating the application of HTS for conditions where there is currently an unmet clinical need will be important to gain acceptance by the clinical community and encourage up-skilling and adoption of new practices.

## Conclusion

Through DNA and RNA sequencing, researchers have obtained comprehensive genomic information on the human microbiome and demonstrated a range of diagnostic applications of the technology from infectious disease identification to disease surveillance and disease risk quantification. The progress sequencing technology has been rapid. The cost, time, and labor for sequencing have been greatly reduced, and this trend will likely continue for the foreseeable future. Therefore, the population-based quantitative technology may soon be adopted as the main method for examining microorganisms in clinical laboratories.

In order for the microbiome data from HTS to be successfully incorporated into the clinic, crucial steps will include defining a specific clinical "intended use" for unmet clinical needs, conducting clinical trials to demonstrate clinical utilities in order to obtain regulatory approval and gain acceptance by the clinical community, and streamlining of data processing and storage. The wide ranges of applications of this rapidly advancing technology make it an exciting time for clinical microbiology.

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# Metagenomic Next-Generation Sequencing for Pathogen Detection and Identification

**Steve Miller and Charles Chiu**

## Introduction

The development of high-throughput next-generation sequencing methods has enabled a number of metagenomic investigations focused on the identification and in-depth characterization of microbial populations within diverse ecological niches. From a human health perspective, a key sequencing application in diagnostic microbiology is the detection of pathogenic organisms causing infectious disease. Traditional approaches involving culture, serology, and specific molecular amplification techniques are able to detect only a limited number of organisms or organism types (i.e., culturable bacteria or fungi, specific viruses with targeted PCR, etc.). In contrast, metagenomic next-generation sequencing (mNGS) offers the potential to detect a wide range of organisms with the use of targeted enrichment strategies or all organism types simultaneously, in an unbiased fashion, on the basis of their genomic sequence (DNA and/or RNA). This approach represents a major paradigm shift in which a single test is able to theoretically identify or rule out infection by any known pathogen.

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## General mNGS Approaches

### *Targeted Sequencing*

Targeted mNGS uses a selection or enrichment approach to enhance the detection of organisms of particular interest. Common methods to perform target enrichment are hybridization capture probes, which can target relatively long sequence regions and tolerate mismatches, and primer amplification methods, which typically rely on short oligonucleotide primers to hybridize specifically to microbial regions of interest [1–3]. Amplification of conserved regions, such as the 16S ribosomal RNA gene, using so-called "universal" primers is commonly used for microbiome analysis and can be applied to mNGS for pathogen detection [4], but any individual primer set is inherently somewhat biased and will not amplify all species [5]. An enrichment technique using a panel of short primers can be applied to design a sensitive targeted assay for organisms associated with particular infection types (sepsis, pneumonia, meningitis, etc.) while still allowing for unbiased organism detection [6].

### *Unbiased Sequencing*

High-throughput metagenomic or unbiased sequencing of total nucleic acid, both DNA and RNA, can potentially yield genomic data from any organism present. This approach is promising for diagnostic microbiology because of the enormous breadth of detection in a single test, encompassing viruses, bacteria, fungi, and parasites, as well as the ability to identify uncommon or novel infectious agents, such as emerging viruses [6]. The potential of this approach is illustrated in its use to diagnose a case of neuroleptospirosis in a critically ill 14-year-old boy, leading to appropriate treatment and prompt recovery [7]. However, this approach has some limitations. In cases of suspected infection, clinical samples may harbor large numbers of host inflammatory cells, resulting in high levels of human host background that significantly decrease the proportion of nonhuman reads and, thus, the sensitivity of pathogen detection. Increasing sequencing depth, or total number of reads generated, can be used to attempt to overcome this problem, but may not be feasible given the cost and sequencing yield of currently available instruments. Thus, some amount of general microbial enrichment is often applied to unbiased mNGS approaches.

A number of methods to either deplete human host reads or enrich for microbial (nonhuman) reads from metagenomic sequencing libraries have been described in the literature. For encapsidated organisms such as viruses, centrifugation, filtration, and pre-extraction nuclease treatment can enrich for the population of interest [8]. Pathogens with RNA genomes and RNA transcripts can be enriched through the use of DNase treatment [9] or removal of typically abundant host ribosomal RNA [10]. Because mammalian DNA is heavily methylated relative to most microbial species

DNA, depletion of human host methylated DNA can enrich pathogen levels [11, 12], as can selective enrichment of non-methylated CpG motifs in microbial genomes [13]. Differential lysis approaches can be used to deplete human relative to microbial DNA [14]. Additionally, specific depletion of targeted sequences can be done through methods such as Cas9 cleavage on the library [15].

## Diagnostic Considerations

While the potential for mNGS pathogen diagnostics remains high, there are a number of practical limitations to its application in clinical infectious disease. These fall into three main categories as follows: [1] sensitive and accurate detection, [2] contamination, and [3] interpretation and clinical relevance. While technological advances in wet lab library preparation and computational bioinformatics are rapidly addressing many of these issues, there are inherent considerations relevant to mNGS that must be kept in mind when considering the diagnostic potential of these assays. Many of these relate to the difference between molecular detection and microbial cultures (i.e., detection of dead organisms and amplicon contamination), which are discussed in other chapters. However, some are unique to mNGS (i.e., database completeness/accuracy and differentiation of related species) or are amplified in scope relative to specific molecular methods (i.e., the large variety of potential sources and types of contamination and clinical relevance of detection). Thorough studies to determine the analytic performance characteristics and clinical utility of mNGS assays are needed prior to widespread deployment of these comprehensive assays for routine clinical diagnosis.

### *Sensitive and Accurate Organism Detection by mNGS*

There are a number of factors that influence the ability of mNGS assays to detect organisms, including library preparation methods, bioinformatic analysis, and host background. Specific targeting or enrichment methods may be necessary for sensitive organism detection and can bias the assay for some pathogen types. Different computational pipelines for data analysis will have varying ability to sensitively and specifically identify sequences from various organisms, depending on the sequence matching algorithms and thresholds used, database accuracy and completeness, and ability to distinguish between conserved and unique sequence regions of pathogens. High levels of host nucleic acid in patient samples can essentially overwhelm the library, so that few or no pathogen reads are present, reducing the sensitivity of detection. For these reasons, each step in assay performance and their combination must be carefully evaluated to ensure sensitive organism detection while avoiding false-positive findings due to database bias or other factors.

### *Contamination Control*

Microbial contamination is a major concern for mNGS assays, since the presence of nucleic acid from any contaminant species will not only affect test sensitivity but can lead to inadvertent or misleading positive findings. Substantial microbial contamination in laboratory reagents has been reported [16, 17], and this contamination must be taken into consideration when interpreting mNGS assay results. Laboratories performing mNGS assays should have an understanding of expected background contamination components and levels, as well as potential sources of contamination so that quality control investigations can be appropriately performed. Results of mNGS assays may sometimes be confirmed using specific detection methods such as PCR, although this can be laborious, will also detect true contaminating sequences present in the library, and can fail to detect low-titer organisms near the limits of detection or sequence variants (unless the primers are designed directly from mNGS reads). Although many commonly detected microbial contaminants are environmental organisms that are not typically relevant to human infections and might be safely ignored, clinicians must keep in mind that immunocompromised patients can be infected with unusual environmental species that are normally thought to have little to no pathogenic potential. Thus, differentiating positive mNGS findings due to contamination versus those due to clinical infection remains challenging, requiring both well-designed laboratory controls and clinical interpretation.

### *Interpretation and Clinical Relevance*

Due to the spectrum of organisms that are broadly detected using mNGS, determination of their clinical relevance in cases of suspected infection can be difficult. Specimen contamination at the point of collection or laboratory contamination will lead to analytically true- but clinically false-positive findings, such as flora found at the site of collection or environmental bacteria and fungi. Normal flora will be present at reasonably high levels in non-sterile body sites (e.g., skin, respiratory secretions, and stool), but even typically sterile compartments may contain endogenous flora, such as anelloviruses in blood [18] or transient flora from adjacent or remote body sites. Known pathogens can also translocate between body sites. For example, patients with respiratory infections due to influenza may have detectable virus in CSF, but the clinical relevance of these viruses in central nervous system tissue is far from clear [19]. Other viral agents including human herpesvirus 6 (HHV-6) and Epstein-Barr virus can be pathogenic, particularly in immunocompromised patients, but might also be detected due to asymptomatic viral reactivation or latent infection in host lymphocytes [20]. Clinical presentation and likely exposure assessment are needed to classify mNGS findings as consistent with causing infection (likely pathogen), inconsistent with causing infection (unlikely pathogen), or uncertain to cause infection (uncertain pathogen). Multidisciplinary case discussions can be

helpful in interpreting the significance of the mNGS pathogen detection and appropriate responses with respect to diagnostic follow-up and clinical management.

## mNGS Methods

### *Sample Types*

The ultimate goal of mNGS analysis is the identification of genomic sequence from infecting pathogens in clinical samples (Fig. 1). While nearly any sample type can be analyzed by mNGS, the interpretation of findings is generally more straightforward from sterile body sites, such as cerebrospinal fluid and blood. In these cases, the detection of a putative infectious agent that is not thought to comprise part of normal flora would yield the diagnosis. On the other hand, the complex microbial profiles ("microbiome") associated with non-sterile body sites may also help pinpoint the etiology of the associated illness. Lower microbial diversity in respiratory

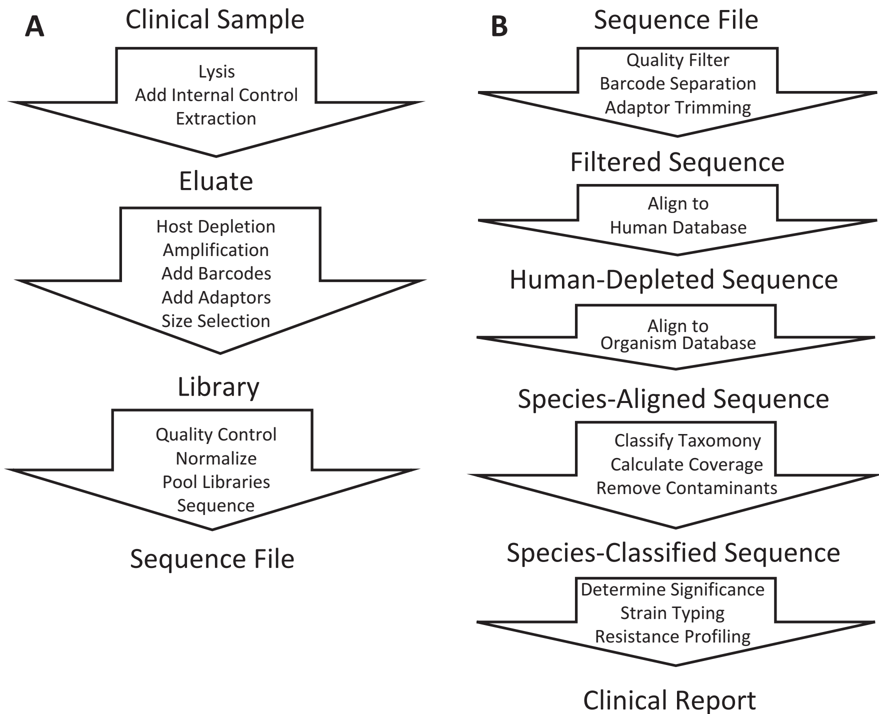


**Fig. 1** Clinical workflow for mNGS testing. (**a**) Wet laboratory steps including sample preparation, nucleic acid extraction, library preparation, and sequencing. (**b**) Bioinformatics analysis including quality filtering, human subtraction, organism alignment, read classification, and result interpretation

secretions and stool in the setting of respiratory and diarrheal illness, respectively, can indicate disruption of the microbiome by a potential pathogen [21]. However, the utility of analyzing microbiome and associated host sequencing data for clinical diagnostics has yet to be demonstrated.

For processing, liquid or dispersed samples are easier to manipulate than tissues, although the latter can be ground up (homogenized) and subject to nucleic acid extraction methods. Formalin-fixed and decalcified tissues pose additional difficulties, as the nucleic acid present is cross-linked or degraded due to processing and only relatively small DNA fragments are generally able to be recovered from these tissues [22, 23]. If the resulting library consists only of very short DNA fragments, the sequence reads could be too short for accurate species classification. Additionally, pathology laboratories performing formalin fixation and paraffin embedding generally do not handle tissues in a sterile fashion, which increases the risk of inadvertent contamination.

### *Nucleic Acid Purification*

Isolation of purified nucleic acid from clinical samples is an essential step in library preparation for mNGS assays. In general, the approach taken by mNGS is similar to that used for other molecular tests. However, mNGS must take into account a large variety of organism types and genome compositions (DNA or RNA, single- or double-stranded). A number of microbial types, including Gram-positive bacteria, molds, and parasites, harbor rigid cell walls that are difficult to lyse, resulting in inefficient extraction of the genomic material; pre-extraction mechanical and/or enzymatic lysis steps may be needed. Extraction methods are selected to yield optimal recovery of pathogen genomes, which may include RNA, DNA, or total nucleic acid methods; for metagenomic sequencing, universal extraction methods able to recover nucleic acid from all types of organisms are needed.

### *Library Preparation*

Preparation of mNGS libraries from extracted nucleic acid first involves a series of pre-enrichment steps designed to remove host sequences or enrich for pathogen sequences. As described in detail above, commonly employed enrichment steps include DNase treatment of RNA libraries, depletion of methyl-DNA sequences, primer-based amplification of microbial sequences, and removal of human sequences by hybridization methods. Following these steps, RNA is reverse transcribed to cDNA. Sequencing and barcode adapters required for nearly all sequencing technologies are then added to the library using a variety of methods, including end ligation and PCR [24].

For mNGS assays for pathogen detection, relevant considerations for generating sequencing libraries include choosing a technique that maximizes the yield of non-human (pathogen) reads and decreases, to the extent possible, the proportion of background reads corresponding to the host genome. Size selection is important to yield a library of the proper size for efficient sequencing. Library amplicons need to be long enough to permit classification of sequenced reads to the species level. The variation in library amplicon size can also be important; for instance, larger variations in size can affect cluster density on an Illumina sequencing instrument [25]. Often, there are trade-offs between sequencing read length, number of sequence reads, cost, speed, and other factors that should be considered and optimized for each mNGS assay.

Libraries are typically barcoded to allow for multiplexing of many samples in a single sequencing run. The barcodes essentially allow for each sequenced molecule to be assigned to each individual sample library. Because of the potential for sequencing errors in the barcode, it is preferable to use barcodes that differ by as many nucleotides as possible; an example is the use of Hamming or Levenshtein barcodes [26, 27]. However, barcode switching between libraries can occur due to sequencing or clustering errors or cross-contamination during library preparation, and it is recommended that different barcodes be used at each end of a bidirectionally sequenced molecule to ensure accurate sample assignment for all reads ("dual-index" barcodes). Barcode adaptors can also be seen in subsequent runs due to instrument carryover or cross-contamination, and rotating regularly among a large set of unique barcodes can help to avoid inadvertent misassignment of sequence reads to the wrong sample library.

## *Sequencing*

Currently, a variety of next-generation sequencing instruments are commercially available, allowing for up to hundreds of millions of individually sequenced DNA molecules per run [28]. While Illumina (MiSeq™, HiSeq™, NextSeq™, MiniSeq™) and Thermo Fisher (Ion Torrent™, Ion PGM™, Ion Proton™) instruments are used most commonly today in clinical laboratories, other systems such as PacBio(Sequel, RSII™) and Oxford Nanopore Technologies (MinION™, PromethION™) have the potential to yield longer sequence reads and, for the MinION, a smaller instrument footprint [29–31]. Regardless of the sequencing technology used, sufficient sequencing depth and quality are necessary for confidence in pathogen detection and identification.

### *Bioinformatics Analysis*

Raw sequence reads are analyzed using bioinformatics pipelines that perform a series of sequential analysis steps for pathogen detection. Published algorithms include SURPI, Pathosphere, Taxonomer, MetaGenSense, and others [32–35]. These different pipelines share many similar features, although they differ in the specific algorithms and databases used. Initial quality control checks are performed to ensure sequence quality and length are sufficient for downstream analysis. Low-quality, low-complexity, and primer/adapter sequences are removed in a preprocessing step. Host reads (corresponding to human reads for clinical samples) are then subtracted from the preprocessed data by alignment to the human genome. The remaining nonhost reads are then aligned to custom databases containing known microbial genomes or to the complete the National Center for Biotechnology Information (NCBI) GenBank database, and aligned reads are checked for specificity. This alignment is typically done at the nucleotide level, but additional translated amino acid alignment to protein reference databases can be performed to evaluate for organisms with significant nucleotide sequence divergence but conserved protein structures, such as RNA viruses. The pathogen-aligned reads are then tabulated and then made available for interpretation.

Additional graphical analysis tools may be used to support interpretation for pathogen detection (Fig. 2). Color-coded interactive heat maps are useful for viewing species, genus, or family-level hits corresponding to individual barcoded samples from a given run. Reads aligning to organism genomes can be presented as coverage maps showing specific genome locations covered and sequence identity, which can assist in determining the correct species call for related organisms [32]. Taxonomy viewers can present the reads aligning to known levels of taxonomy [36], as reads may be family- or genus-specific, thus allowing definitive identification only to a given taxonomic level.

Assembly of individual sequence reads to form longer contigs ("contiguous sequences") can be done pre- or post-alignment and can add to the specificity of organism identification due to much longer coverage of genome sequence. Additionally, the remaining unaligned reads can be assembled de novo to form contigs that can reveal the presence of an organism which is divergent from those represented in the existing database and thus can be used for novel pathogen discovery. Assembled contigs or mapped reads can also be checked against databases containing pathogenic or resistance determinants, such as the Comprehensive Antibiotic Resistance Database, to provide a molecular prediction of antimicrobial susceptibility or likely virulence factors [37]. They can also be uploaded to web-based tools to predict phenotype drug resistance, such as for HIV and HCV [38, 39]. Further analysis of the human-matched reads to evaluate the host transcriptome is an emerging tool to further classify organism detections as likely pathogenic or nonpathogenic, based on the level of host immune response elements seen in the metagenomic dataset [40]. Analysis of human mRNA reads in host transcriptome libraries can identify host profiles associated with clinical illnesses such as Lyme disease [41] and chronic fatigue syndrome [42].

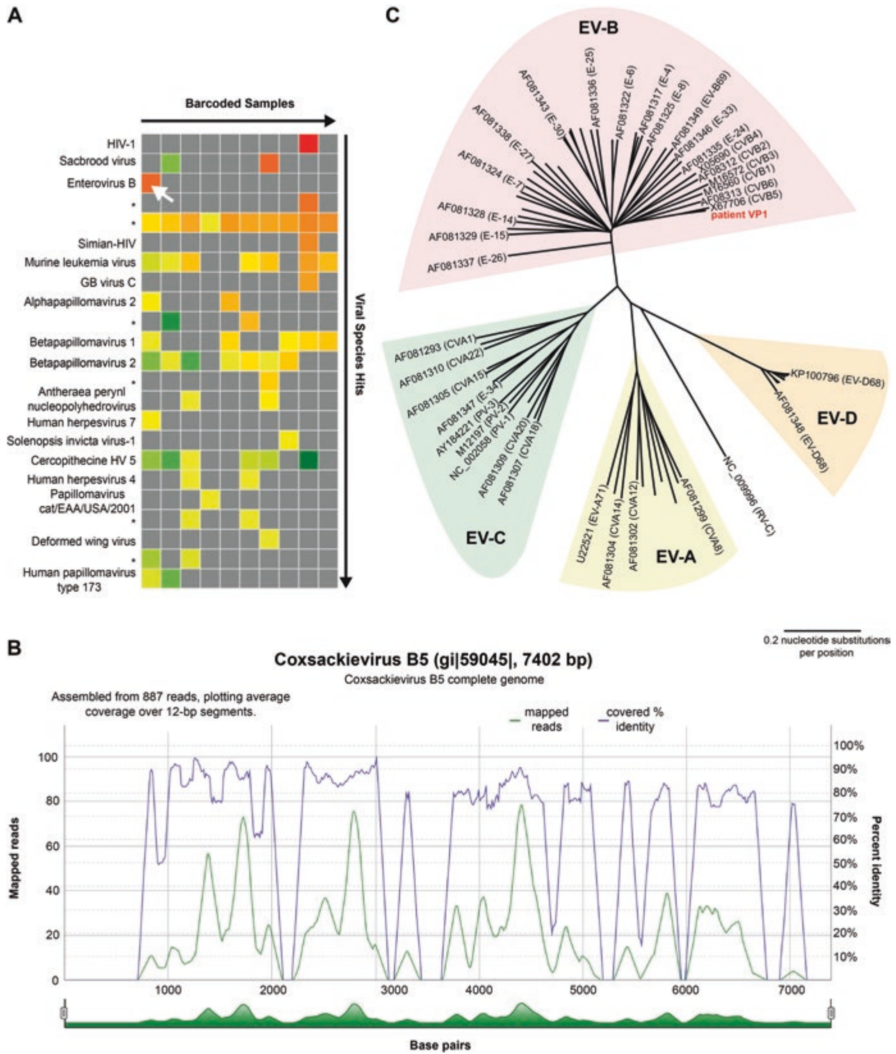


**Fig. 2** Sequence data visualization using SURPIviz™ software and phylogenetic tree analysis. (**a**) Heat map showing all viral matched reads from RNA library preparation with at least 10 reads in any sample for each identified taxonomic classification. Samples are shown in columns, and taxonomic identifications are shown in rows. Sequence hits that are not species-specific are declassified to a higher taxonomic level, represented by an asterisk (*). Identified human viral pathogens include enterovirus B in a patient CSF sample (column 1, white arrow) and human immunodeficiency virus in a positive control sample (column 9, red square). Other viral hits represent known viral flora (i.e., papillomaviruses), environmental contaminants (i.e., sacbrood virus), clinically insignificant viruses (i.e., GB virus C), misclassified viral reads within a detected genus (i.e., simian-human immunodeficiency virus), or DNA viruses with reads seen in RNA library that are better analyzed from the DNA library data (i.e., human herpesvirus 4, human herpesvirus 7). Abbreviations: HIV, human immunodeficiency virus; HV, herpesvirus. (**b**) Coverage map automatically generated during SURPI+™ analysis for reads matching to enterovirus B showing coverage statistics. Bottom graph shows sequence coverage within an adjustable window for viewing. Viral subtyping as coxsackievirus B5 was confirmed based on consensus sequence of all reads mapping to VP1 region. (**c**) Phylogenetic tree view of enterovirus VP1 sequence for the consensus sequence from a patient CSF sample compared to 79 VP1 sequences representing all enterovirus strains, showing closest match to coxsackievirus B5

## *Clinical Interpretation of mNGS Results*

The bioinformatics pipeline will typically generate a list of database matches or "hits," whose significance must then be determined. Background contaminants that are present in reagents and samples tested are screened out and not reported. Similarly, normal microbial flora needs to be defined a priori and removed to avoid misinterpretation of these organisms as pathogens. Flora commonly seen in human samples, even from sterile sites, include endogenous retroviruses, anelloviruses, and human pegivirus 1 (formerly known as GB virus), viruses that have no known clinical significance and should not be reported as pathogens.

Contamination and normal flora can be screened at the organism level, and it can be helpful to maintain databases of these hits in order to properly interpret future analyses. The availability of such a database can also assist with troubleshooting suspected anomalous results by tracking the hits associated with particular reagent lots and aliquots. Remaining bona fide organism hits are analyzed for relevant metrics including genomic coverage, pairwise percent identity (to the closest matching sequence in the reference database), and assigned taxonomy. For organisms seen in negative or no-template control samples, the relative number of sequence reads aligning to that organism can be used to establish a threshold level for detection, using a normalized cutoff or by Z-score analysis. This approach for detection above background levels is particularly useful for organisms that are commonly detected in sequencing libraries as a result of background contamination or because they constitute normal flora, such as *Escherichia coli* or *Propionibacterium acnes*, and can increase the specificity of detection [43].

Finally, the clinical significance of the organism hits is determined, based on the identity and the number/diversity of detected microbes, with the assessment reported as part of a result summary. For example, some samples may have multiple bacterial or fungal genera detected; polymicrobial findings from a sterile site may be deemed more likely due sample or laboratory contamination rather than a true infection. Other organisms may have uncertain pathogenic potential when detected from a given sample type, such as human herpesvirus 6 detection in CSF [44], or may be nonhuman pathogens (i.e., insect or plant viruses) that are unlikely to cause human infection and thus signify likely contamination. Other findings may be of public health significance (i.e., hantavirus, Ebola), requiring follow-up testing and notification of the appropriate authorities. The laboratory director overseeing the mNGS assay must consider every scenario and develop a protocol for dealing with each of these situations. In many cases, a subsequent discussion with clinical providers is advisable to consider the clinical importance of both positive and negative mNGS results and to determine optimal diagnostic and management approaches.

### *Validation and Controls*

Clinical laboratories must meet stringent validation and quality assurance requirements in order to successfully implement mNGS testing for patient care. The broad and potentially comprehensive ability of mNGS testing to detect infecting pathogens is not compatible with traditional analyte-specific approaches to test validation, and alternative strategies are required. These include the use of representative organism types to establish assay performance characteristics, bioinformatics assessment, and error-based risk assessment approaches [43, 45, 46].

Quality control is required at multiple steps due to the complicated workflow and processing of mNGS assays and should include external positive and negative controls, internal controls, library quality assessment, and sequencing quality assessment. Each of these may have multiple parameters designed to detect errors or failures in sample preparation or analysis, with quantitative means and variability established over time. Successful performance of mNGS quality control metrics increases the confidence in result accuracy and is needed to determine the diagnostic utility of results [43, 47, 48].

## Clinical Utility

### *Patient Diagnosis*

One of the clearest indications of the clinical utility of mNGS pathogen detection is the identification of known but atypical or unexpected pathogens, which can then be confirmed using alternative means. Notable examples of this include diagnosis of *Leptospira* meningitis in an immunocompromised pediatric patient [7], *Balamuthia mandrillaris* encephalitis [49], hepatitis E meningoencephalitis [48], St. Louis encephalitis virus meningoencephalitis [50], and *Abiotrophia defectiva* endocarditis [51]. There are also examples of novel pathogen discovery using mNGS, such as a novel rhabdovirus associated with acute hemorrhagic fever in Africa [52] and a neuroinvasive astrovirus [53]. There are numerous examples of application of mNGS to other infectious diseases, such as intraocular infections [54], respiratory infections [55], arboviral infections [56], pediatric fever [57], diarrhea [58], and acute liver failure [59], just to name a few.

Due to the potentially comprehensive microbial assessment using mNGS pathogen detection assays, the ability of a negative result to essentially rule out infection is of great interest to clinical providers. An unbiased mNGS assay with high sensitivity for all relevant pathogen types would have a high negative predictive value, and patients with negative results may be treated more rapidly and expeditiously for noninfectious causes of their illness, including autoimmune, metabolic, and neoplastic conditions. Under this model, having a negative mNGS result could enable

more rapid institution of appropriate therapy, such as immunosuppressive agents and corticosteroids for autoimmune disease, with potentially improved clinical outcomes.

### *Epidemiologic Investigations*

Community or patient cohort investigations have shown the ability of mNGS assays to investigate outbreaks of influenza or determine the prevalence of human papillomavirus types [60, 61]. Metagenomic sequencing can more fully describe the epidemiology of certain infection types, such as respiratory infections [62]. Novel pathogen-disease associations are also amenable to mNGS investigations, such as enterovirus strain D-68 and acute flaccid myelitis [63]. Point-of-care technologies such as nanopore sequencing can potentially facilitate the application of metagenomic sequencing in remote, low-resource field settings [64]. The use of mNGS, coupled with targeted enrichment approaches, has also been demonstrated to be useful for tracking outbreaks from emerging viruses such as Ebola [65] and Zika virus [66, 67]. These studies aim to provide more comprehensive information than possible using traditional targeted methods and are becoming more common as mNGS technology and computational tools are more accessible.

### *Strain Typing and Pathogenic and Antibiotic Resistance Determinants*

In addition to pathogen detection, metagenomic approaches can simultaneously yield information on strain types and pathogenic and antibiotic resistance determinants. Sequencing of sputum from cystic fibrosis patients yielded information on strain types utilizing existing multi-locus sequence typing databases [68]. As an example of differentiating pathogenic from nonpathogenic strains, *Escherichia coli* strains with sequence markers associated with necrotizing enterocolitis were found among infants with urinary tract colonization [69]. Addition of antibiotic resistance databases to the mNGS analysis pipeline allows detection of resistance genes that can be used to optimize treatment regimens [70, 71].

## Future Directions

As high-throughput sequencing becomes more cost-effective with simpler and more automated laboratory protocols, more clinical laboratories will begin to offer these mNGS assays for clinical use. Bioinformatics development remains challenging,

and while several data analysis pipelines are now available, significant expertise is still needed to maintain these and adapt to new knowledge and database changes. The development of clinical-grade reference databases will streamline the analysis tools needed; a notable effort in this area is the FDA-ARGOS database (https://www.fda.gov/MedicalDevices/ScienceandResearch/DatabaseforReferenceGradeMicrobialSequences/default.htm). Clinical validation requires extensive and ongoing effort, particularly as improved protocols and bioinformatics algorithms are developed. Significant computational resources are needed for data transfer, analysis, and storage.

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# Host Immune Repertoire and Infection

**Dongni Hou, Jieming Qu, and Yuanlin Song**

## Introduction

The adaptive immune response to microbial infections relies on the recognition of antigens by specific lymphocyte cell surface receptors or by specific antibodies. This specificity of these lymphocytes/antibodies is determined by the diverse nature of T (TCRs) and B cell receptors (BCRs) known as the complementarity-determining regions (CDRs). To protect humans from a near infinite number of different external antigens, the diversity and dynamics of this lymphocyte pool is critical. Thus, the investigation of the immune repertoire, portrayed as the antigen-specific information within lymphocytes, has been a key to understanding adaptive immune responses during infection.

In the past two decades, high-throughput sequencing (HTS) of TCR/BCR has resulted in major advances in our understanding of the immune repertoires involved in various diseases, including infection, cancer, autoimmune diseases, and graft-versus-host disease. Traditional sequencing methods (i.e., Sanger sequencing) have been used to determine the cDNA segments encoding variable regions of immunoglobulin (or TCRs) [1, 2], but these sequencing methods have not been able to provide sufficient detailed information of TCR/BCR. In contrast, HTS data, along with related bioinformatic and statistical tools, have provided a new approach that appears to be capable of analyzing the immune repertoire at a single sequence level. These HTS methods have achieved an unprecedentedly high-resolution analysis of the immune repertoire without the limitations of previous sequencing methods [3].

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Considering the extremely important role of the adaptive immune system in defending against infectious agents, the availability of massive parallel sequencing data of TCR/BCR has great potential for providing a more comprehensive understanding of the interaction between the human host and microbes, as well as the capability for discovering novel anti-infectious agents, antibodies, or vaccines. This chapter focuses on the implementation of high-throughput sequencing data of the human immune repertoires involved in infectious diseases. Specifically, this chapter will review the associated bioinformatic tools required for data processing and analysis of CDRs as well as the promise of this burgeoning field to facilitate the exploration of infection-related immune repertoires for applications involving the clinical diagnosis, treatment, and prevention of infectious diseases.

## Basis of Immune Repertoire Generation

Diversity is the most important property of the human immune repertoire and directly reflects the responsive status and potential of T/B cell pools. This immense diversity is generated by a complex series of genetic events [4]. TCR/BCR consist of both conserved regions and variable regions, while the latter is divided into three complementarity-determining regions (CDRs) and four framework regions (FRs) according to their variability and function (Fig. 1). CDRs are the variable portion of the receptors that bind with the peptide-MHC complex and thus determine the antigen specificity. While CDR1 and CDR2 are formed by variable (V) genes, CDR3 is generated by selection and recombination of variable (V), diversity (D), and joining (J) gene segment in the heavy chain (V and J region gene segment in the light chain) [5, 6]. Thus, CDR3 is the most diverse component of a receptor and is the vital determinant of antigen specificity; consequently, CDR3 is the most intensively studied component in immune repertoire analysis. Construction of the TCR/BCR with an alpha (light) chain and a beta (heavy) chain is yet another variable process that contributes to receptor diversity.

In addition to constant formation and renewal, the diverse T/B cell lymphocyte receptor pool is continuously being remodeled by ongoing adaptive immune responses. The dynamics of each lymphocyte clone depends on its antigen specificity as well as the history of antigen exposure. When encountering exogenous antigens, T cells that express receptors capable of binding to a specifically compatible peptide-MHC (pMHC) complex will expand, thus resulting in a large population of antigen-specific T cells that initiate the adaptive immune response to this particular antigen [7–11]. Although these CD4+ and CD8+ lymphocytes show comparable protein expression, proliferation rate, and transcriptome features after 2 days of non-infective stimulation, subsequent division of these T cells differently depends on the continuous existence of self-pMHC complexes. CD4+ T cells proliferate in a limited pattern, and the subsequent response of these cells requires persistent stimulation from antigen-presenting cells. In contrast, CD8+ T cell starts extensive expansion after a short stimulation, which continues even when transferred into antigen free hosts [12].

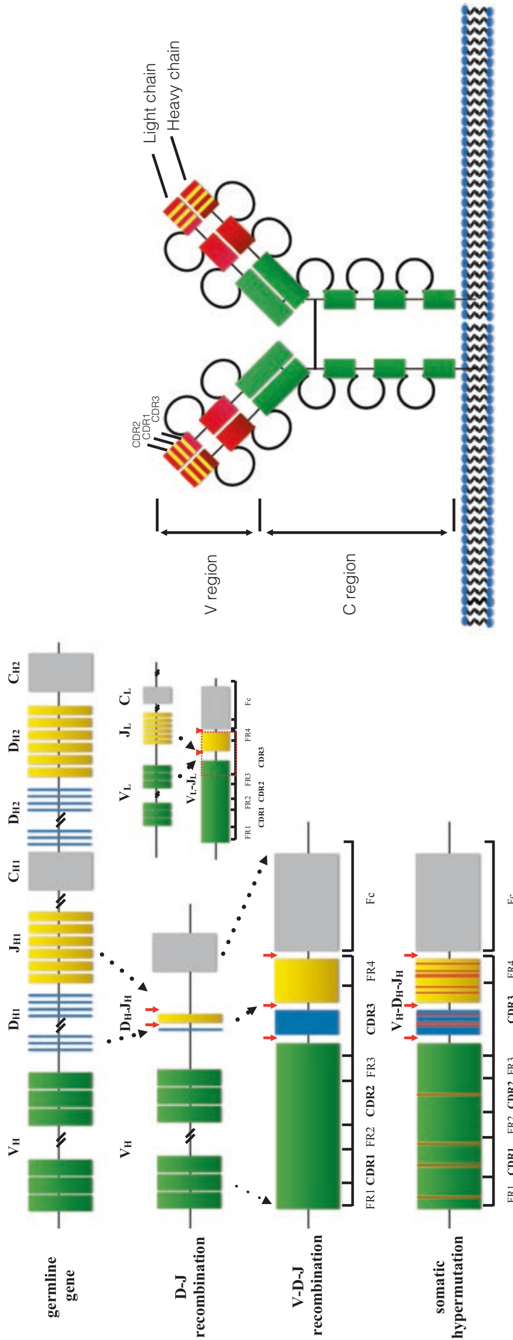


**Fig. 1** Generation of a diverse B cell repertoire. (**a**) Steps of the B cell receptor gene formation. The V, D, and J gene segments were selected and rearranged with insertions and deletions at the junctions in the heavy chain. Light chains are constructed only by V and J gene segments. Paring of heavy and light chain also contributes to diversity. When stimulated by specific antigen, somatic hyper-mutations occur in complementarity-determining region (CDR) and frame region, which provides further diversity for B cell repertoire. (**b**) Structure of B cell receptor. (Modified from [25] with permission)

Stimulation of naïve B cells is followed by additional processes that further contribute to the diversification of the B cell repertoire, including somatic hypermutation and class-switch recombination [6]. Somatic hyper-mutation refers to point mutations at CDR1, CDR2, CDR3, and FR3 that produce highly diverse intermediate sequences, from which clones with high affinity are selected and expanded; this process is called affinity maturation. Moreover, in class-switch recombination, the gene loci encoding the C region of BCRs is excised and replaced by a series of new constant gene segments, resulting in functional differences of IgG, IgE, or IgA that participate in different immune response mechanisms during pathogen elimination.

## Exploration of the T/B Lymphocyte Repertoires by Sequence Analysis

### *Limitations of Traditional Strategies*

Prior to high-throughput sequencing, many other strategies were developed to explore the post-infection immune repertoires [13]. For example, immunoscope spectratyping has been used to investigate TCR/BCR receptor repertoires since the 1990s [5, 14]. In this technique, using one (for B cell) or more (for T cell) V or J gene-specific primer pairs, the length of CDR3 can be determined [15]. The distribution of CDR3 length and V/J gene fraction roughly reflects status of the repertoire in healthy individuals and patients with different diseases [16–20]. However, the bases for existence of alterations in clones with the same CDR3 length or V/J gene remain unclear.

Traditional DNA sequencing techniques have provided detailed nucleotide sequences of the CDR3 region with low throughput, which requires other complementary techniques for screening the potentially functional lymphocytes in order to narrow down the cell population; these techniques include flow cytometry, antigen-binding tests, and CDR3 spectratyping [21, 22]. Recently, single cell sequencing of B cells has provided high sensitivity for identifying rare B cell clones that produce monoclonal antibodies specific to certain virus. This technique contributes greatly to the ability to analyze genetic features of antibodies during the process of antibody discovery [23, 24]. Although these strategies are well designed for targeted investigation of antibody specificity, they are insufficient for creating a high-resolution picture of the human immune repertoire.

### *Exploration of the T/B Lymphocyte Repertoires by Sequence Analysis*

High-throughput sequencing has recently become a novel and powerful tool for investigating the human immune repertoire and provides greater depth and comprehensiveness than previous investigative methods.

Establishing a lymphocyte repertoire database begins with sample collection using carefully selected populations and isolation of specific T cell or B cell subgroups of interest. Carefully designed serial sample collection is useful as well as required for reducing the complexity of data interpretation due to the well-acknowledged fact that the TCR/BCR between individuals have a high heterogeneity. In addition, tracking the dynamic alterations of T/B cell clones is critical in investigating the specific processes of the adaptive immune response and identifying the evolution pathway for particular antibody clones. Classification of subgroups of T cell and B cells, e.g., naïve and memory T/B cells and CD4+ and CD8+ T cells, is required if the distinct behavior of each of these subgroups is to be considered in detail.

PCR is a key step in sample preparation, because proper selection of the primers will determine the reliability of the ultimate immune repertoire data (Table 1). Due to differences of the V and J gene segments, no shared sequences are sufficient for binding of a universal primer. Thus multiplex primers must be utilized to amplify multiple loci simultaneously. Despite cautious selection of primers with minimized variation in predicted binding affinity, bias will be introduced in this process due to different amplification efficiency resulting from non-specific amplification, primer-dimer formation, and uneven reaction conditions. More precise and quantitative multiplex PCR results may be achieved through primer concentration adjustment and bias filtering using amplification bias among the templates as controls [26].

**Table 1** Comparison of PCR methods used in TCR/BCR sequencing

| | Features | Advantages | Disadvantages | References |
|---|---|---|---|---|
| Multiplex PCR | Use multiple primers that are specific for different V/J genes | Permit amplification of variable region for TCR/BCR sequencing | Multiple primers influenced by small variations in annealing kinetics which results in variable gene bias<br>Produce redundant cDNA molecules lead to artificial antibody clones and diversity | [25] |
| Template-controlled multiplex PCR | Use a synthetic repertoire that includes a template for every V/J combination | Correct amplification bias | | [26] |
| 5'RACE PCR | Use reverse primers that bind downstream of the variable domain | Avoid amplification bias | Longer products demanding long-read capability of sequencing platform | [27, 28] |
| UMI-labeling PCR | Add a random library of sequences within the primer as a unique sequence tag | Correct sequencing error, avoiding artificial clones | | [29] |

*PCR* polymerase chain reaction, *TCR* T cell receptor, *BCR* B cell receptor, *5'RACE* rapid amplification of cDNA ends, *UMI* unique molecular identifiers

Another alternative PCR method is 5'RACE PCR, which provides a less biased PCR library using primers that bind downstream of the variable domain. The long PCR products resulting from this method is another challenge for sequencing platforms [27].

Sequencing techniques have been continuously evolving to be deeper and more precise; currently, there are three widely used state-of-the-art high-throughput sequencing platforms available. A detailed comparison of mechanisms, sequencing depth, and other critical features of each platform is provided by the following references [30–33]. In summary, insertions and deletions of nucleotides (due to imperfect interpretation of homopolymeric stretches) are considerable for the Roche 454 platform [34], while substitution errors are predominant in the Illumina platform [35, 36]. The overall error rate of Illumina platform is lowest, while that of Ion Torrent is the highest among the three platforms [37]. Error-correcting algorithms (k-mer spectrum, multiple sequence alignment, and suffix tree [31]) and bioinformatic tools are available for each of these different platforms [32].

PCR and sequencing errors inevitably result in artificial clones (especially antibody clones); these errors thus produce an overestimation of repertoire diversity. The common statistical strategy for both PCR and sequencing error removal is to eliminate low-abundance and low-quality sequences (i.e., those with a low Phred score), but this method leads to a significant loss of sequencing information. To rescue these sequences, low-quality CDR3 sequences can be mapped and merged to high-quality sequences with allowed mismatches at low-quality positions [33]. Recently reported unique molecular identifiers that label each starting molecule combined with MIGEC (molecular identifier groups-based error correction) correct these PCR and sequencing errors more efficiently than other quality- and frequency-based strategies [38].

Determination of the V-D-J gene segment from which the CDR3s are rearranged, as well as identification of point mutations, is often achieved using the ImMunoGeneTics database [39]. Many integrated bioinformatic tools for data processing have been recently developed [40–51]; these tools provide various statistical approaches for diversity estimation, repertoire comparison, clustering analysis, and somatic hyper-mutation analysis. In particular, Software for the Ontogenic aNalysis of Antibody Repertoires (SONAR) is capable of investigating specific lineages for ontogenic analysis of neutralizing antibody lineages; this method has been validated in several HIV antibody lineages [52]. Despite the availability and use of these versatile tools by many different investigators, no standardized strategy for bioinformatic analysis and visualization strategy is currently acknowledged.

# Applications

## *Assessing the Adaptive Immune Response After Infection or Vaccination*

Diversity is the most essential characteristic of TCR/BCR receptor immune repertoires, because when infection with a novel pathogen occurs, it is primarily the diversity of this T/B cell pool that determines whether a specific complementary paratope will occur [53]. Estimating the diversity and tracking the changes in clonal populations during the clinical course of infection provide important insights into the immune response and disease status. Several different methods may be used to describe the diversity of these lymphocyte repertoires at different levels; these include the number of somatic mutations, the CDR3 length, and VDJ recombination [54]. In addition, statistics such as the Simpson index [55, 56] and some nonparametric methods are commonly used. Decreases in the overall diversity of the immune repertoire have been observed after various antigen exposures, including HIV, influenza, and human herpes virus, which implies expansion of particular T/B cell clones [56–59]. Our group has compared changes in the diversity of the TCR beta chain and BCR heavy chain after H7N9 virus infection. Interestingly, these results demonstrated that the diversity of the BCR heavy chain began to increase 2 weeks after an H7N9 infection, while the T cell receptor beta chain repertoire continued to contract. In addition, a more diverse BCR repertoire and a less diverse TCR beta chain repertoire in convalescent phases correlated with improved prognoses, implying differences in the response process of humoral and cellular immunity [25].

A complete clinical infectious course is sometimes difficult to track, especially those caused by viruses, because of the incubation period as well as the typical delay in the etiological diagnosis. Vaccination of volunteers is an ideal substitute for investigating such immune repertoire responses due to the convenience of drawing blood samples at well-defined time points [60]. Studies using vaccines, such as those for influenza and TT, have revealed dynamic changes in the size and diversity of antibody repertoires before and after antigen stimulation [54, 61–63]. Comparison of postvaccination responses suggests divergent repertoire properties among individuals, with different age groups and even with successive immunization of the same individual with different influenza vaccines (TIV and LAIV) [54, 62, 64]. The maximum clonal response has been found to occur 7 days after vaccination, but the magnitude of response varies between individuals despite an identical immune challenge, which may be influenced by previous exposure, age, and other concurrent immune responses. After repeated challenging by the same antigen, with sequencing of samples taken from the same individual, the memory B cell clones are more impressive after the second stimulation. When using substantially different vaccine compositions in the immunization of the same individual, the resulting antibody repertoire analysis has identified the recall response of cross-reactive B cell clones, which is a novel strategy for antibody screening [61].

Antibody repertoire immune responses recorded by sequencing data have also been useful in testing the role of adjuvants in eliciting broad-spectrum antibodies. For example, Wiley and co-workers have evaluated the immune response of mice immunized with malaria vaccine by analyzing IgG immune repertoires. They found that the TLR agonist used as adjuvant increases the diversity of the IgG variable region, which is related to improved ability of the antibodies to recognize a broad spectrum of epitopes [65]. These studies exemplify a new level of the understanding of vaccine response and have pioneered the use of HTS in vaccine design.

## *Antigen-Specific Signature of Immune Repertoires for Diagnosis*

In infected patients, antigen-specific T/B cell immune repertoires are created in response to antigen exposure both in the circulation and in the peripheral tissues. Immune repertoire sequencing provides broad information that includes crucial antigen-specific clones; these clones have the potential to halt the spread of any pathogens [66]. T/B cell repertoire-based diagnostic marker discovery is a method to identify those antigen-specific T/B cell clones with stereotyped features from infected individuals. The advantage of these new kinds of biomarkers is that high-throughput sequencing data provides a large number of candidate sequences for biomarker investigation. Instead of using single biomarkers such as PSA or AFP in diagnosis, a combination panel of selected sequences may establish a pathogen-specific sequence library for diagnosis, which provides the potential for unprecedented sensitivity and specificity. Moreover, immune responses have demonstrated dramatic differences in CDR3 sequences responding to same pathogens across individuals and age groups. This intrinsic divergence between individuals is the major obstacle in finding “public” sequences as optimal biomarkers for specific infections.

These features can be assessed at different levels, such as gene rearrangement, identical or similar CDR3 sequence overlap, and certain CDR3 length. After influenza H1N1 vaccination, the dominant clonotype of Ig heavy chains has the same V-J gene rearrangement, CDR3 length, and somatic mutation position in CDR1 and CDR3 as seen with previously reported influenza antibodies [64]. However, in this reported study, the convergent dominant sequence was only found in one individual. Further researches in a broader population that includes non-dominant sequences are needed. A more successful example is reported in Ig repertoires related to dengue virus infection. Using cross validation and other approaches, stereotyped CDR3 sequences or CDR3 lengths that have high prevalence in the acute dengue samples have been found to be specific to acute dengue infection; this response is either absent or of low prevalence in the healthy and post-convalescent population [59].

Identification of pathogen-specific sequences also helps in differential diagnosis between infectious and noninfectious diseases. Comparing PBMC-derived T cell

clonotypes specific to a given virus with T cells from different origins (allograft-derived and urine-derived lymphocytes) provides a new methodology for differential diagnosis of two posttransplant complications – BKV-associated nephropathy and acute cellular rejection; this method provides a glimpse of possible applications of T cell sequencing for diagnostic purposes [67]. In addition, a recent study investigated sequencing data for CDR3 amino acid motifs that have been reported to be specific for a particular pathogen and has succeeded in identifying CDR3 sequences identical or similar to these motifs in postvaccination volunteers [68]. Of interest is the fact that the results of this study suggest that low-frequency sequences (rather than dominant sequences) possess the probability of becoming promising biomarkers.

## *Identifying Antibody Sequences from HTS Data*

Recombinant monoclonal antibodies have demonstrated great potential for the treatment of specific infections. In recent years, several strategies have been used to discover antibodies against specific antigens; these include phage display libraries, single B cell expression, and B cell immortalization [69]. Combined with subsequent bioinformatic tools and/or traditional screening tools, B cell repertoire data can provide an enormous variety of antibody sequences for screening, which has great potential for the development of protective monoclonal antibodies.

Predicting antigen specificity completely from analysis of BCR sequences has not been possible to date. However, a relatively direct method for identifying such sequences is based on the similarity of amino acid sequences to previously reported antibodies. Researchers have successfully found sequences of high identity with the broadly neutralizing antibodies and strain-specific antibodies from established antibody repertoires of patients with influenza infection or vaccination [64]. Some of these sequences have proven to have neutralizing activity, validating the potential for deep sequencing-based antibody identification. Moreover, another method using the frequency rank of heavy chain and light chain sequences to predict the function of antibody sequences has been reported successful in mouse models [70].

Another strategy for identifying neutralizing active clones is the phylogenetic analysis of antibody repertoires; this method has been validated in HIV infection [71, 72]. In this strategy, the sequences of one or more known antibodies are utilized as "seeds" to find all transcripts in the dataset that are from the same lineage ("seeded lineage assignment"). From this whole antibody repertoire, the heavy or light chain sequences derived from the germline IGHV or IGLV gene that is the same as template antibody are isolated and then compared with the germline gene for "divergence" and with the template antibody sequence for "identity." The sequences with high divergence and high identity were then selected for ontogenic analysis of neutralizing antibody lineages. Sequences located at the branches of known neutralizing antibody have been shown to be new antibody sequences of the same lineage.

Predicting T cell specificity based on the TCR heterodimer sequence is more difficult than predicting antibodies because of the highly variable nature of each of the components of the TCR-peptide-MHC complex [73]. Due to the challenges posed by the highly variable CDR3 loop of the TCR as well as the complexity of predicting protein-protein interactions [74, 75], experimental functional tests for mining antigen-specific T cells might be a more fruitful approach [76].

## *Guiding Vaccine Development*

Recent advances in HTS-based antibody sequencing may provide the largest benefit for the field of vaccine development, particularly for vaccine development for chronic HIV infection. Eliciting protective immune responses to HIV by immunization has confronted several major obstacles, including the extremely long time it required to generate effective broadly neutralizing antibodies, impairment of the host immune function in chronic infection, unusual features of HIV Env, and coevolution of the virus in response to the host antibody response [77, 78].

Deep sequencing analysis has identified rare variants of known HIV-neutralizing antibodies and also has elucidated the ontogeny of these neutralizing antibodies [71, 72, 79, 80]. These findings have cast a light on antibody-guided vaccine development. In the following studies, this HTS-based phylogenetic strategy greatly facilitated the investigation of coevolution of neutralizing antibodies and virus mutants [81]. Combined with long-term follow-up studies, these results illustrate how mutations in specific envelope sites allow the virus to escape certain neutralizing antibodies and how the virus, with the help of secondary neutralizing antibodies, may become sensitive to the neutralizing antibody [82–84]. These studies suggest a promising pathway to elicit broadly neutralizing antibodies by sequential immuni-

**Table 2** Applications of host immune repertoire analysis

| Applications | | References |
|---|---|---|
| Assessing the adaptive immune response | Tracking the changes in diversity and clonal populations | [25, 55–59] |
| | Identifying repertoire properties among individuals after vaccination | [54, 61–63] |
| | Testing adjuvants for vaccine | [65] |
| Antigen-specific signatures for diagnosis | Identifying stereotyped features of T/B cell repertoires that are specific to antigen | [59, 67, 68] |
| Identifying antibody sequences | Predicting antigen specificity through identity, frequency, or phylogenetic analysis | [70–72] |
| Guiding vaccine development | Elucidating the ontogeny of HIV-neutralizing antibodies | [71, 72, 79, 80] |
| | Investigating coevolution of neutralizing antibodies and virus mutants | [81, 83, 84] |
| | Providing candidates for future vaccine designs using structure of neutralizing antibody family | [86] |

zation with selected immunogens [81, 85]. Furthermore, structural investigation of the neutralizing antibody family may provide candidates for future vaccine designs [86] (Table 2).

## Conclusions

HTS has transformed our understanding of formation of the human immune repertoire during infection. The potential for further HTS investigations of the immune repertoire in clinical settings is enormous. Additional clinical investigations of the dynamic changes and sequence signatures of immune repertoires would greatly enhance our understanding of the immunopathogenesis of various infectious diseases. Questions remain about how the alterations of the immune repertoire response and various manifestations of CDR3 sequences are related to the severity and stages of certain infections as well as how to predict the abundance of protective immunoglobulins and/or T cells from a given sequence library. In terms of therapeutic discoveries, identification and production of functional antibodies and T cells should greatly assist and promote the development of passive immune therapies and vaccines. Advances in high-throughput sequencing of the immune repertoire during health and disease should provide an expanding and comprehensive view of the adaptive immune response in the very near future and will open the door to more rationale immunotherapy for infectious diseases.

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# Recombinant Protein-Based Diagnostics for Viral Hemorrhagic Fevers

**Masayuki Saijo**

## Introduction

The term "viral hemorrhagic fever" describes a severe multisystem syndrome in which multiple organ systems are infected/damaged; the vascular system is typically involved, and vascular damage results in hemorrhage (i.e., bleeding). The syndromes of viral hemorrhagic fever include well-known entities such as Ebola virus disease, Marburg virus disease, Crimean-Congo hemorrhagic fever, Lassa fever, as well as other less well-known entities. The RNA viruses causing viral hemorrhagic fevers are grouped in four distinct families: arenaviruses, filoviruses, bunyaviruses, and flaviviruses. These families and the viral hemorrhagic fever syndromes associated with each viral family are shown in Table 1 [1]. Moreover, there continue to be new viral hemorrhagic fever syndromes being discovered. For example, a tick-borne bunyavirus disease similar to Crimean-Congo hemorrhagic fever, severe fever with thrombocytopenia syndrome, recently has been found to be endemic to East Asia.

All of the RNA viruses causing viral hemorrhagic fevers should be manipulated in high-containment (biosafety level 4 (BSL-4)) laboratories, which are designed for working safely with dangerous and exotic agents that pose a high risk of laboratory infections and life-threatening disease. A number of viral hemorrhagic fevers (VHFs) are severe virus diseases with high case fatality rates; these include Ebola virus disease (EVD, formerly named Ebola hemorrhagic fever), Marburg virus disease (MVD, formerly named Marburg hemorrhagic fever), Crimean-Congo hemorrhagic fever (CCHF), and Lassa fever (LF). Ebolavirus and Marburg virus (EBOV and MARV, respectively) of the family *Filoviridae* cause hemorrhagic fever in humans and nonhuman primates, in which there are high mortality rates, sometimes reaching 50–90% of infected individuals [2–8]. CCHF virus (CCHFV) is a member of the family *Bunyaviridae*, genus *Nairovirus* that causes an acute viral hemorrhagic

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**Table 1** Viral hemorrhagic fevers in humans

| Family | Genus | Species | Virus member abbreviation |
|---|---|---|---|
| *Arenaviridae* | | | |
| | *Mammarenavirus (old world)* | *Lassa mammarenavirus* | LASV |
| | | *Lujo mammarenavirus* | LUJV |
| | *Mammarenavirus (new world)* | *Chapare mammarenavirus* | CHPV |
| | | *Junin mammarenavirus* | JUNV |
| | | *Machupo mammarenavirus* | MACV |
| | | *Sabia mammarenavirus* | SABV |
| *Bunyaviridae* | | | |
| | *Nairovirus* | *Crimean-Congo hemorrhagic fever virus* | CCHF |
| | *Phlebovirus* | *Rift Valley fever virus* | RVFV |
| | | *Severe fever with thrombocytopenia syndrome virus*[a] | SFTSV |
| | *Hantavirus* | *Dobrava-Belgrade virus* | DOBV |
| | | *Hantaan virus* | HTNV |
| | | *Puumala virus* | PUUV |
| | | *Saaremaa virus* | SAAV |
| | | *Seoul virus* | SEOV |
| | | *Sin Nombre virus* | SNV |
| | | *Tula virus* | TULV |
| *Filoviridae* | | | |
| | *Ebolavirus* | *Bundibugyo ebolavirus* | BDBV |
| | | *Sudan ebolavirus* | SUDV |
| | | *Tai Forest ebolavirus (formerly cote d'lvoire)* | TAFV |
| | | *Zaire ebolavirus* | EBOV |
| | *Marburgvirus* | *Marburg marburgvirus* | MARV |
| *Flaviviridae* | | | |
| | *Flavivirus* | *Dengue virus* | DENV1–4 |
| | | *Kyasanur Forest disease virus* | KFDV |
| | | *Omsk hemorrhagic fever virus* | PHFV |
| | | *Yellow fever virus* | YFV |

fever with a high mortality rate ranging from 10 to 40% [9]. LF is a viral hemorrhagic fever caused by Lassa virus (LASV), an Old World arenavirus. Many cases of LF occur in Western Africa in countries such as Guinea, Sierra Leone, and Nigeria [10–15].

The causative agents EBOV, MARV, CCHFV, and LASV for these four viral hemorrhagic fevers (VHFs) are internationally categorized as biosafety level 4 (BSL-4) pathogens. This designation means that manipulation of these infectious

viruses requires a BSL-4 laboratory. This designation also means that development of diagnostic systems for VHFs is difficult for facilities without BSL-4 laboratories. Moreover, there are no BSL-4 laboratories in most of the VHF-endemic countries.

As the magnitude of international trade and travel is continuously increasing, there is a significant risk that the HFVs from endemic areas could be introduced to virus-free countries. For example, a patient with MVD died in the Netherlands in 2008; this patient was infected with MARV during her stay in Uganda (ProMed mail of Archive number 20080711.2115). Other examples include patients with EVD reported in the USA in 2014; these cases were associated with the 2014–2016 EVD outbreak in Western Africa (Table 2). More than 20 cases of LF have been reported outside the endemic region in areas such as the USA, Canada, Europe, and Japan [16–21]. Human-to-human infection is also common among the caregivers for the patients in the LF-endemic regions. Furthermore, human-to-human infection also was reported in Germany; this is the first time that such transmission has been seen in an economically advanced country [22].

Therefore, the development of laboratory diagnostic systems for VHFs is an important topic even in countries without endemic VHFs. Manipulation of infectious hemorrhagic fever viruses such as EBOV, MARV, CCHFV, and LASV requires a BSL-4 laboratory. However, BSL-4 laboratories typically are available only in economically advanced countries, indicating that development of diagnostics for VHFs with using infectious HFVs is difficult for most VHF-endemic regions and countries.

To avoid the need for BSL-4 laboratories, diagnostic methods using recombinant viral antigens have been developed. These diagnostic methods offer significant advantages not only in diagnosis of but also in investigational studies on VHFs. In this chapter, these diagnostic systems using recombinant viral antigens for VHFs are reviewed.

## Pathogens Involved in Viral Hemorrhagic Fevers

### *General Characteristics of Hemorrhagic Fever Viruses*

All VHF viruses are zoonotic viruses. Most hemorrhagic fever virus syndromes in humans have a high morbidity and mortality; therefore, a pandemic outbreak due to VHF infections has never been reported. The life cycles and routes of infection for EVD, MVD, CCHF, and LF are shown in Fig. 1.

**Table 2** Outbreaks of EVD and MVD recorded

| Virus | Country | Year | Dead/patient | Description |
|---|---|---|---|---|
| MARV | Germany and Serbia/ Herzegovina | 1967 | 7/31 | Origin of MARV responsible for the outbreak was a monkey imported from Uganda |
| MARV | Zimbabwe and South Africa | 1975 | 1/3 | Index case was infected with MARV in Zimbabwe. Nosocomial infection occurred in a hospital in South Africa |
| SUDV | Sudan | 1976 | 151/284 | The first documented outbreak of VHF due to Sudan EVD |
| EBOV | DRC | 1976 | 280/318 | The first documented outbreak of VHF due to Zaire EVD |
| EBOV | DRC | 1977 | 1/1 | Sporadic outbreak |
| SUDV | Sudan | 1979 | 22/34 | |
| MARV | Kenya | 1980 | 1/2 | |
| MARV | Kenya | 1987 | 1/1 | |
| RESV | USA | 1989/1990 | 0/4 | Outbreak of EVD in nonhuman primates imported to the USA from the Philippines. Four persons were confirmed to be infected with Reston EBOV without any symptoms |
| RESV | Italy | 1992 | 0/0 | Outbreak of EVD in nonhuman primates imported to Italy from the Philippines |
| TAFV | Ivory Coast | 1994 | 0/1 | A veterinarian was infected with Ivory Coast EBO when she handled a dead chimpanzee |
| EBOV | DRC | 1995 | 244/315 | The epicenter of this outbreak is Kikwit, the DRC |
| EBOV | Gabon | 1996 | 21/31 | |
| EBOV | Gabon and South Africa | 1996 | 45/60 | Nosocomial infection occurred in a hospital, in which a nurse who took care of a doctor that was transferred from Gabon died |
| MARV | DRC[a] | 1998/1999 | 52/76 | Prospective study on MVD indicated that there were approximately 150 patients with MVD in this outbreak |
| SUDV | Uganda | 2000 | 149/394 | The epicenter of this EVD outbreak due to Sudan EBOV was Gulu District in Uganda |
| EBOV | Gabon and the DRC | 2001/2002 | 69/92 | The outbreak area was close to the border between the two countries |
| EBOV | The DRC | 2003 | 29/35 | Thirteen of the cases are laboratory confirmed and 130 are epidemiologically linked |
| SUDV | Sudan | 2004 | 7/17 | |
| MARV | Angola | 2004/2005 | 357/423 | Mortality rate exceeded 80%. Many children suffered from MVD in this outbreak |

| Virus | Country | Year | Dead/patient | Description |
|---|---|---|---|---|
| EBOV | The DRC | 2005 | 9/12 | One case was virologically confirmed (http://www.who.int/csr/don/2005_06_16/en/index.html) |
| EBOV | The DRC | 2007 | 187/264 | In this outbreak, only 26 cases were virologically confirmed to be the patients with EVD |
| BDBV | Uganda | 2007/2008 | 37/149 | The causative EBOV was confirmed to be genetically different from the three African EBOV subspecies |
| MARV | Netherland | 2008 | 1/1 | The patient was infected with MARV in Uganda |
| EBOV | The DRC | 2008 | 14/33 | |
| SUDV | Uganda | 2011 | 1/1 | |
| SUDV | Uganda | 2012 | 17/24 | |
| SUDV | Uganda | 2012 | 4/7 | |
| BDBV | The DRC | 2012 | 29/57 | |
| EBOV | 2014–2016 | Guinea | 2543/3811 | The causative Zaire EBOV was genetically different from the Zaire EBOV in Gabon and DRC. This outbreak had started in Guinea since December 2013 |
| EBOV | 2014–2016 | Liberia | 4809/10675 | |
| EBOV | 2014–2016 | Sierra Leone | 3956/14124 | |
| EBOV | 2014 | Nigeria | 8/20 | This outbreak occurred from the index case of EVD from Liberia |
| EBOV | 2014 | Mali | 6/8 | This outbreak occurred from the index case of EVD from Guinea |
| EBOV | 2014 | Senegal | 0/1 | |
| EBOV | 2014 | USA | 1/4 | Two patients were the cases of nosocomial infection |
| EBOV | 2014 | UK | 0/1 | |
| EBOV | 2014 | Spain | 0/1 | A case of nosocomial EVD |
| EBOV | 2014 | DRC | 49/66 | This outbreak was independent from the 2014–2016 western African outbreak |
| EBOV | 2015 | Italy | 0/1 | Imported case from western Africa |

[a]"DRC" indicates "Democratic Republic of the Congo"

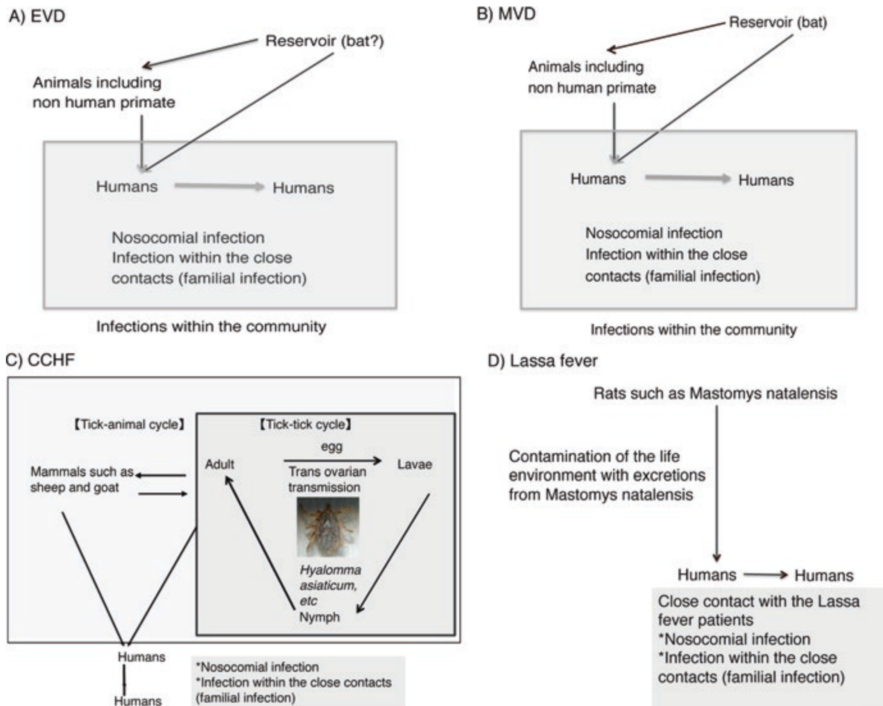


**Fig. 1** The life cycle of hemorrhagic fever viruses and mode and route of infection of humans with the viruses, which cause EVD (**a**), MVD (**b**), CCHF (**c**), and LF (**d**)

## *Ebola Virus and Marburg Virus Diseases*

EBOV and MARV are members of the family *Filoviridae*, with EBOV and MARV being classified in the genera *Ebolavirus* and *Marburgvirus*, respectively. The *Ebolavirus* genus comprises five viral subspecies, Zaire, Sudan, Tai Forest, Bundibugyo, and Reston ebolaviruses (EBOV, SUDV, TAFV, BDBV, and RESV, respectively). In contrast, the *Marburgvirus* genus comprises a single virus species, which is Lake Victoria MARV.

The natural host of EBOV most likely is species of fruit bats in Western and Central Africa [23, 24], although infectious EBOV has not yet been isolated from any mammals other than humans and nonhuman primates in Africa. In contrast, infectious MARV has been isolated from Egyptian fruit bats inhabiting a cave in Uganda, indicating that the natural reservoir of MARV is indeed fruit bats [25].

Although a primary case of EVD and/or MVD theoretically might result from exposure with either virus directly or indirectly from a reservoir such as the fruit bat, humans are usually infected with EBOV or MARV via close contact with contaminated blood, tissues, and/or other excretions from patients having viremic VHF infections caused by these two viruses.

After an incubation of 4–10 days, infected individuals abruptly develop flu-like symptoms consisting of fever, chills, malaise, and myalgia. Subsequently, these patients rapidly develop signs and symptoms that suggest systemic involvement; these include prostration, gastrointestinal symptoms/signs (anorexia, nausea, vomiting, abdominal pain, diarrhea), respiratory symptoms/signs (chest pain, shortness of breath, cough), vascular symptoms/signs (conjunctival injection, postural hypotension, edema), and neurological symptoms/signs (headache, confusion, coma) manifestations. Bleeding manifestations include petechiae, ecchymosis, uncontrolled oozing from venipuncture sites, gingival and oral mucosal hemorrhages, and bloody diarrhea. In later stages, the overall clinical status of VHF patients rapidly deteriorates due to multi-organ failure that usually includes disseminated intravascular coagulopathy; this rapid clinical decline usually results in death [2, 3, 26, 27].

## *Crimean-Congo Hemorrhagic Fever*

CCHFV is a member of the family *Bunyaviridae*, genus *Nairovirus*. Humans acquire infection primarily through tick (genus *Hyalomma*) bites or contact with fresh meat or blood from slaughtered viremic animals such as sheep, cattle, and goats (Fig. 1c).

The case fatality rate of CCHF is between 10 and 40%. The symptoms of CCHF patients vary in severity and may range from only fever or fever with flu-like symptoms to fever accompanied by hemorrhage and multiple organ failure that results in death. All CCHF patients appear to developed fever and joint pains. Orbital pain, backache, and headache also are common symptoms in these patients. In severe CCHF cases, hemorrhagic complications are seen (Fig. 2). In addition, oliguria is a common symptom and presumably is associated with renal failure caused by a direct effect of CCHFV vasculitis or by an indirect effect due to hypovolemic shock.

## *Lassa Fever*

LF is caused by LASV, an Old World arenavirus. Many cases of LF occur in Western Africa. Humans become infected through contact with infected excreta, tissue, or blood from the rodent, *Mastomys natalensis*, which is the reservoir host mammal for LASV. LASV can also be transmitted from human to human via mucosal/cutaneous contact or nosocomial contamination [11, 22].

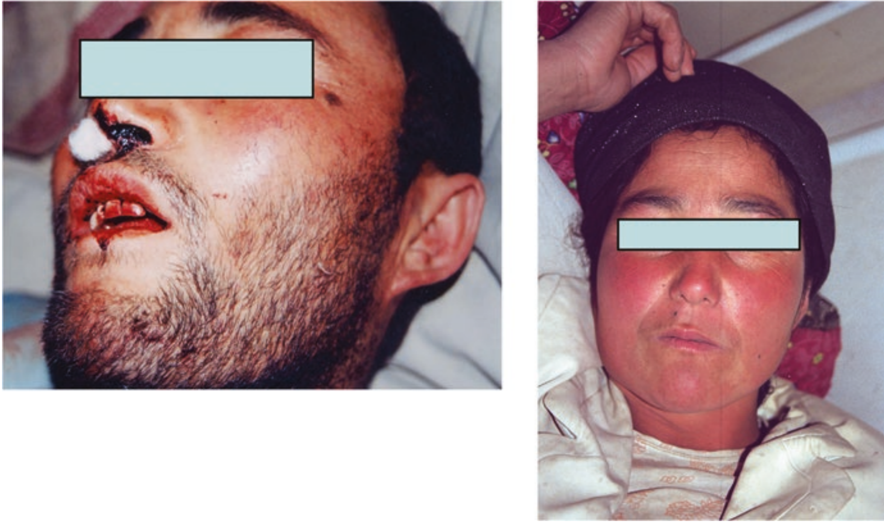

**Fig. 2** Clinical manifestations of the two patients with CCHF with hemorrhage (left) and flashed face (right). The patient shown in left panel was the 28-year-old shepherd with severe symptoms of hemorrhage from gingiva, nostrils, and rectum that was reported by our group [28]. The patient shown in the left panel was the patient reported previously [29]

## Epidemiology

### *The VHF–Endemic Areas*

The VHF-endemic areas are shown in Fig. 3. All the endemic areas of VHFs are resource-limited countries except for some CCHF areas such as Turkey, Greece, and Spain.

### *Ebola Virus Disease*

EBOV consists of five species, EBOV, SUDV, TAFV, BDBV, and RESV; these species were first isolated in the Democratic Republic of Congo (DRC, formerly Zaire), Sudan, Ivory Coast, Uganda, and the Philippines, respectively [8, 30]. Large-scale outbreaks of EVD have occurred in Western Africa from 2014 to 2016. In these outbreaks, 28,646 patients had suffered from EVD and/or were suspected of having EVD, and 11,323 of these patients have died (World Health Organization (WHO): http://apps.who.int/ebola/ebola-situation-reports). The first recognized outbreaks of EVD occurred in the DRC and Sudan in 1976 [31–34]. Since the initial discovery of EBOV and SUDV in 1976, a number of African countries have experienced outbreaks of EVD caused by one of the four known human-pathogenic EBOV species;

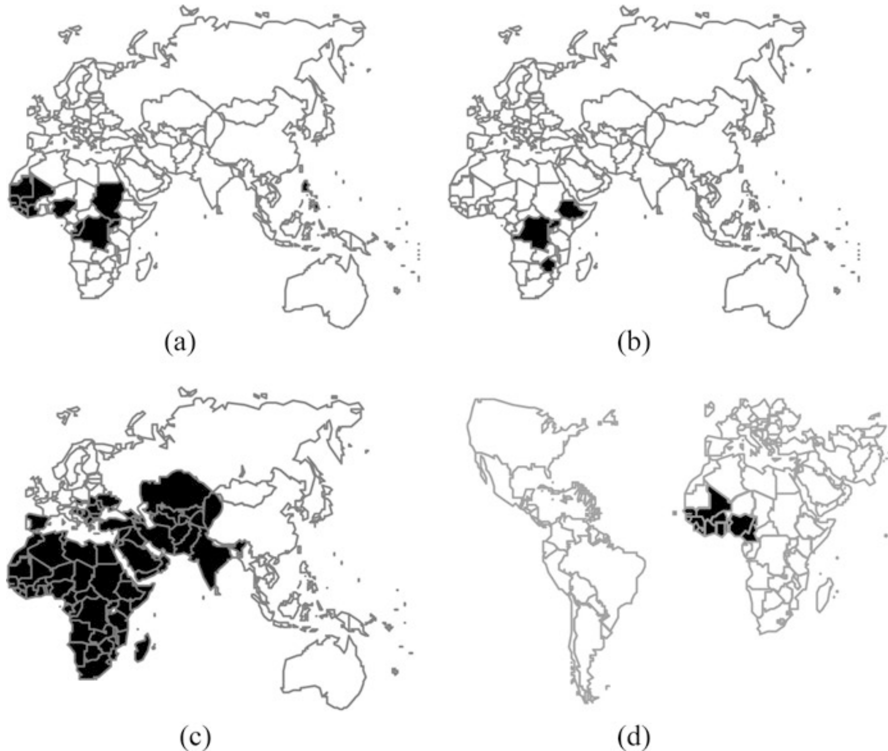


**Fig. 3** Endemic regions of EVD (**a**), MVD (**b**), CCHF (**c**), and LF (**d**). The areas shown in black indicate the endemic regions

EBOV, SUDV, TAFV, and BDBV [2, 7, 25, 35–44]. Outbreaks of EVD caused by the other EBOV species, RESV, occurred among cynomolgus macaques imported from the Philippines to the USA in 1989 [45]. RESV also had been introduced to the USA in 1989, 1990, and 1996, as well as to Italy in 1992, through importation of RESV-infected monkeys from the Philippines [45–48]. The outbreaks of EVD and MVD that have occurred are summarized in Table 2, based on the data issued from the WHO (http://www.who.int/mediacentre/factsheets/fs103/en/).

Several cases of nosocomial infections of EVD outside the endemic areas also have been reported. For example, a healthcare worker was infected with EBOV after taking care of a severely ill patient transported from Gabon [49]. The severely ill patient was a medical doctor, who was infected with EBOV from an EVD-patient in Gabon. Nosocomial transmission also occurred even in economically advanced countries such as Spain and the USA as a result of caregivers providing care for patients with EVD [50–52]. It is of note that the nosocomial transmission of EVD in Spain was the first report of human-to-human infection in economically advanced countries.

### *Marburg Virus Disease*

The first two documented outbreaks of MVD occurred first in Germany and then in Yugoslavia in 1967. Technicians and scientists suffered from MVD after they manipulated tissue materials collected from African green monkeys imported from Uganda. It has been suggested that the monkeys had already been infected with MARV when imported. Subsequent outbreaks have been seen. Three sporadic cases of MVD have been reported in Zimbabwe (1975) and Kenya (1980 and 1987) [7, 53–56]. From 1998 to 1999, there was a relatively large outbreak in the DRC [4, 5, 42]. The largest outbreak of MVD occurred in Uige Province, Angola, in 2004–2005, where 374 patients have been reported with a mortality rate of over 88% (http://www.who.int/csr/don/2005_08_24/en/index.html) [5]. In 2008, a fatal case of MVD in the Netherlands was reported (ProMed mail of archive number 20080711.2115). The patient contracted MVD in Uganda, developed symptoms after returning back to the Netherlands, and finally died. This case is the first case of imported MVD outside Africa.

### *Crimean–Congo Hemorrhagic Fever*

CCHF and human infections with CCHFV have been reported in Africa, Eastern Europe, the Middle East, and Central and Southern Asia [57]. Recently, CCHF patients have been reported in Spain, suggesting that CCHF is also endemic to Western Europe [58]. Cases of CCHF are thought to be significantly unreported, because the disease usually occurs in remote areas. Several imported cases of CCHF from Africa to Europe were reported: one was from Zimbabwe to the UK (ProMed-mail of archive number 19980109.0062) and the other was the case from Senegal to France (ProMed-mail of archive number 20041125.3152).

### *Lassa Fever*

LF is endemic to the Central and Western part of Africa. These endemic regions are closely related to the habitat of *Mastomys natalensis*. It is thought that LASV infects tens of thousands of humans annually and causes hundreds to thousands of deaths. Furthermore, it is noteworthy that the incidence of importation of LF cases to non-endemic countries from endemic regions is the highest among these four examples of VHFs.

## Diagnostic Methods

### *General Considerations for the Diagnosis of Viral Hemorrhagic Fevers*

In outbreaks of VHFs, infections are confirmed by various laboratory diagnostic methods. For most VHF outbreaks in Africa, regional and international organizations play an important role in diagnosing and managing these outbreaks.

Current diagnostic approaches include virus isolation, reverse transcription-polymerase chain reaction (RT-PCR) including real-time quantitative RT-PCR, antigen-capture enzyme-linked immunosorbent assay (ELISA), antigen-detection by immunostaining, and IgG- and IgM-ELISA using authentic virus antigens [8, 59–67]. Histological techniques including antigen-detection by immunohistochemical analyses are sensitive and useful methods, particularly for postmortem diagnosis [67]. Diagnosis by detection of viral antigens is most useful for patients in the early stage of illness, while serological diagnosis by detection of specific IgM and IgG antibodies is most useful for patients in later stages of their illness. Diagnostic methods for VHFs must be sensitive, specific, and reliable, because misdiagnosis may result in considerable turmoil in both endemic and non-endemic regions.

### *Antigen-Detection*

HFV infections often are rapidly fatal; thus, patients typically die before having an antibody response; this suggests that rapid diagnostic methods using antigen-detection are essential for the timely diagnosis of VHF infections. High titers of infectious HFVs usually are present in the blood and tissues of patients at early stages of VHF illnesses. Antigen-capture ELISA methods for detection of antigens from EBOV and SUDV [68, 69], MARV [70, 71], CCHFV [72], and LASV [73] have been developed by our group.

The target proteins are NPs of these HFVs. Monoclonal antibodies to the rNPs of selected HFVs have been produced and are used as capture antibodies. The polyclonal antibodies were raised in rabbit by immunizing these rabbits with rNPs being used as the immunizing antigen. The characteristics of each of the developed HFV-antigen-capture ELISAs are summarized in Table 3. Although monoclonal antibodies used in the antigen-capture ELISAs were produced by immunizing mice with rNPs, the NP-capture ELISAs detected not only the rNPs of these viruses but also the authentic virus NPs. Antigen-capture ELISAs have been developed for detecting the NPs of EBOV, SUDV, and RESV [68, 69] and also the NP of MARV alone [71]. The authentic MARV NP has successfully been detected. Furthermore, the antigen-capture ELISA has demonstrated an identical detection limit of MARV antigen to that of MARV genome amplification by the conventional RT-PCR [70].

**Table 3** Characteristics of the monoclonal antibodies and polyclonal antibodies used in the developed nucleocapsid protein (antigen)-capture ELISAs

| Target protein | Capture antibody | Recognition site of capture antibody | Detector antibody | Samples | Comments | Reference |
|---|---|---|---|---|---|---|
| EBOV NP | MAb, 3-3D, to Zaire EBOV | Carboxy-terminal region of Zaire EBOV NP | Rabbit serum raised to Zaire EBOV rNP | Blood, serum, tissue | The MAb, 3-3D, reacts with the NPs of Zaire, Sudan, Reston EBOVs and maybe react with NP of tai Forest EBOV | [68, 69] |
| MARV NP | MAb, 2A7, to MARV NP | Carboxy-terminal region of MARV NP (amino acid residues from positions 632 to 645) | Rabbit serum raised to MARV rNP | Blood, serum, tissue, other body fluids | The MAb, 2A7, reacts with MARV NP, but not with NPs of EBOV | [70, 71] |
| | MAb, 2H6, to MARV NP | Carboxy-terminal region of MARV NP (amino acid residues from positions 643 to 695) | Rabbit serum raised to MARV rNP | Blood, serum, tissue, other body fluids | The MAb, 2H6, reacts with MARV NP, but not with NPs of EBOV | [70, 71] |
| CCHFV NP | MAb, 1B7, to CCHFV NP | Central region within CCHFV NP (amino acid residues from positions 201 to 306) | Rabbit serum raised to CCHFV rNP | Blood, serum, tissue, other body fluids | Efficacy in diagnosis of CCHF was evaluated | [72] |
| LASV NP | MAb, 4A5, to LASV NP | Five-amino acid residues positioned from 439 to 443 | Rabbit serum raised to LASV rNP | Blood, serum, tissue, other body fluids | MAb, 4A5, reacts with NP of LASV, but not NP of LCMV[a] and JUNV[b] | [73] |

[a]LCMV indicates “lymphocytic choriomeningitis virus,” a member of Old World arenaviruses
[b]JUNV indicates “Junin virus,” a member of New World arenaviruses (Table 1)

The efficacy of the CCHFV-antigen-detection ELISA in diagnosis of CCHF has been evaluated [72]. Although the sensitivity of the nested RT-PCR for diagnosis of CCHF is higher than that of the antigen-capture ELISA, the CCHFV-antigen-capture ELISA has been confirmed to be effective, especially for the diagnosis of CCHF in patients before an antibody response. Antibodies to CCHFV present in serum may decrease the sensitivity in detection of antigens in the antigen-capture ELISA [72].

The LASV antigen-capture ELISA also has been developed. Unfortunately, the efficacy of the antigen-capture ELISA in the diagnosis of LF in patients has not yet been evaluated. However, this ELISA has been confirmed to have a similar sensitivity in detection of LASV antigen in serum samples collected from hamsters that have been experimentally infected with LASV [73].

## *Recombinant Protein–Based Serology: ELISA*

### Ebola Virus Disease

rNP of EBOV has been expressed using the baculovirus system in order to form a fusion protein with 6XHis-tag on the N-terminus [74]. The expressed rNP has been purified and used as antigens for IgG-ELISA.

### Marburg Virus Disease

It has been recognized that the carboxy-terminal half of the NP of EBOV and MARV possessed strong antigenicity [74]. Because the expression level of the full-length MARV rNP was not sufficient, the carboxy-terminal half of rNP of MARV and EBOV was expressed and used as antigens in the IgG-ELISA. These truncated rNP-based ELISAs were also confirmed to have high sensitivity and specificity in detection of antibodies [74].

### Crimean–Congo Hemorrhagic Fever

rNP of CCHFV has been developed using the baculovirus system in order to form fusion protein with 6XHis-tag on the N-terminus [75]. The expressed rNP has been purified and used as antigens for IgG-ELISA and IgM-capture ELISA [76]. It has been confirmed that the IgM-capture ELISA with an antigen, CCHFV rNP, is effective for the diagnosis of CCHF in the early phase of the disease [28]. The IgM-capture ELISA with rNPs of VHFs also might be effective in diagnosis of other VHFs such as CCHF, although further studies are needed.

### Lassa Fever

rNP of LASV also has been developed using the baculovirus system, purified, and used in IgG-ELISA [73]. Although the sensitivity of this assay system has not yet been evaluated using a relatively large number of serum samples of LF patients, the convalescent phase serum samples collected from the imported case to Japan have demonstrated a positive reaction in the rNP-based ELISA [18, 73].

**Table 4** Characteristics of recombinant antigen-based antibody-detection systems developed in the NIID

| Method | Origin of antigen | Antigen (amino acid position)[a] | Expression of recombinant protein | Sensitivity[b] | Specificity[c] | Reference |
|---|---|---|---|---|---|---|
| ELISA | EBOV | rNP | Recombinant baculovirus system | 13/14 | 50/51 | [74, 77] |
| | | Truncated rNP (361–739) | Transformation of *E. coli* with the expression vector | 13/14 | 50/51 | [74, 77] |
| | MARV[d] | Truncated rNP (341–695) | Transformation of *E. coli* with the expression vector | 3/3 | 62/62 | [74, 77] |
| | CCHFV Chinese strain 8402 | rNP | Recombinant baculovirus system | 13/14 | 107/109 | [75] |
| | LASV | rNP | Recombinant baculovirus system | 4/4 | 94/96 | [73] |
| IIFA | Zaire EBOV | rNP | Infection of HeLa cells with the recombinant baculovirus | 14/14 | 47/48 | [78] |
| | MARV | rNP | Transfection of HeLa cells with the expression vector | 1/1 | 96/96 | This article[e] |
| | CCHFV | rNP | Transfection of HeLa cells with the expression vector | 13/13 | 108/108 | [79] |
| | LASV | rNP | Transfection of HeLa cells with the expression vector | 4/4 | 96/96 | [73] |

[a]The amino acid position is counted from the translational initiation codon for each protein
[b]Sensitivity is defined as the number of samples showing a positive reaction in the developed antibody-detection system divided by the number of positive controls
[c]Specificity is defined as the number of samples showing negative reaction in the developed antibody-detection system divided by the number of negative controls
[d]"MARV" indicates "Lake Victoria MARV"
[e]"One serum collected from MVD patient showed a positive reaction, while 96 Japanese subjects showed negative reactions [70]". (Fig. 4)

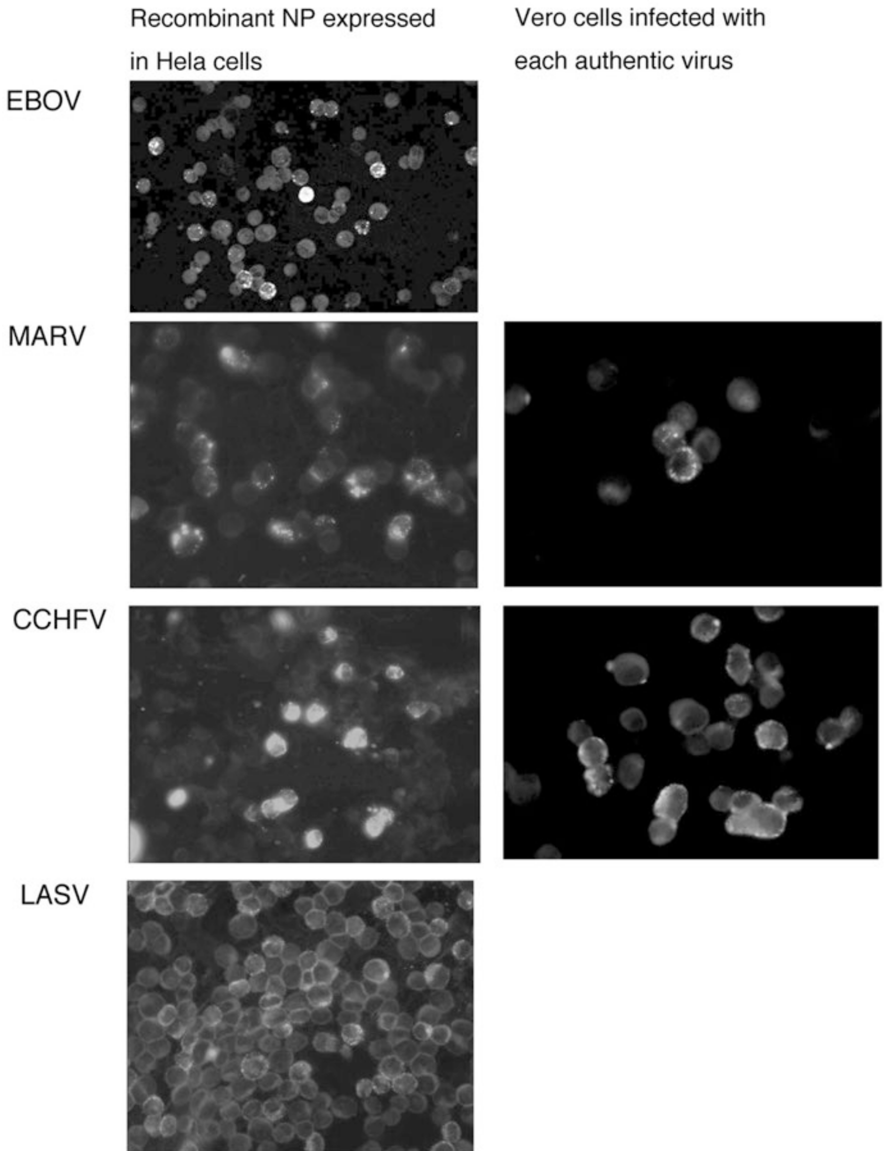


**Fig. 4** Immunostaining patterns of the recombinant NP of EBOV, MARV, CCHFV, and LASV expressed in HeLa cells expressed by the transfection with each expression vector (left panel). The immunostaining patterns of Vero cells infected with authentic MARV and CCHFV are also shown (right panel)

**Sensitivity and Specificity of these ELISA**

As shown in Table 4, all the IgG-ELISA with these rNP antigens have been determined to have high sensitivity and specificity.

## Recombinant Protein–Based Serology: Indirect Immunofluorescence Assay (IIFA)

Indirect immunofluorescent assay (IIFA) has been developed using the mammalian cells (HeLa cells), in which the rNP of ebolaviruses, MARV, CCHFV, or LASV were expressed. The rNPs of EBOV, MARV, CCHFV, and LASV were expressed in HeLa cells by transfection of the cells with an expression vector, pKS336 encoding the respective NP genes [73, 78, 79]. The rNPs of these HFVs were expressed with the form of granular patterns (Fig. 4). This HFV rNP-based IIFA was useful for detection of antibodies with high sensitivity and specificity (Table 4).

## Summary

The clinical characteristics, epidemiology, and diagnostic methods for selected VHFs have been reviewed. Because proper BSL-4 facilities for manipulation of infectious HFVs are limited, alternative methods for diagnosing these infections have been developed. However, these diagnostic methods should be established and available not only in the VHFs-endemic countries (regions) but also in the non-endemic areas. The recombinant protein-based diagnostic methods described for EVD, MVD, CCHF, and LF might offer advantages over traditional virus-based diagnostics in facilities without BSL-4 laboratories.

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# Standardization of Viral Load Determination for Monitoring CMV, EBV, and BK Viruses in Solid Organ Transplant Recipients

**Xiaoli Pang**

## Introduction

Molecular approaches such as nucleic acid amplification-based tests (NAAT) are increasingly gaining access to medical laboratories and diagnostic field for detection of pathogenic viruses, quantification of viral load (VL), and monitoring viral resistance and host response to therapeutic agents. NAAT is replacing conventional microbiological methods, such as pathogen propagation in culture, morphological identification of microbes, and color demonstration of antibody-antigen interaction, in an unprecedented pace as they often enable faster, more accurate, and higher sensitivity of quantitative measurements [1]. Currently, quantitative assay of nucleic acid testing (QNAT), like real-time quantitative polymerase chain reaction (qPCR) on VL determination, has become a mainstay in clinical management of cytomegalovirus (CMV), Epstein-Barr virus (EBV), and BK virus (BKV) diseases in solid organ transplant (SOT) and hematopoietic stem cell (HSC) recipients. However, a variety of different QNAT assays developed either commercially or by laboratory itself utilize different technique platforms, reaction chemistries, and calibration materials, leading to enormous variability in quantitative results in terms of numerical values. Given the heterogeneity of QNAT assays and lack of traceability to a standardized reference system, it is difficult to compare VL measurements between different laboratories [2–8]. This interlaboratory variability of measurement on CMV, EBV, and BKV VL observed makes it impractical to form a universal guideline on VL assessment, classify clinical stages of associated disorders, standardize intervention strategy, and evaluate treatment outcomes. Thus, it directs to impact on patient care. Establishment of the international reference standards for calibration

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of VL assays associated with three viruses is not only in need but also undoubtedly an important step in quality and application improvement of QNAT assays in solid organ transplant recipients. In an endeavor to achieve harmonization of QNAT assays on VL of those viruses and enable comparisons of biological measurements across laboratories worldwide, the first World Health Organization (WHO) International Standards (IS) for human CMV, EBV, and BKV were developed and approved by WHO's Expert Committee in collaboration with the National Institute for Biological Standards and Controls (NIBSC) (UK) in October 2010, October 2011, and June 2016, respectively [8–10]. This chapter focuses on the importance of establishing WHO IS reference controls; fundamental characterization of the interlaboratory variability of CMV, EBV, and BKV VL measurements in SOT recipients using QNAT assays; and remaining challenges in harmonization of CMV, EBV, and BKV VL assessment in SOT recipients with the latest findings of related studies.

## Calling for the International Standards for Viral Loading Determination

### *QNAT Assay for Monitoring CMV in SOT Recipients*

Regardless of major advances in its diagnosis and treatment, CMV, a member of the family *Herpesviridae*, remains the most important infectious agent causing significant morbidity and occasional mortality in SOT recipients. CMV may occur as a primary infection in CMV-seronegative organ transplant recipients, reactivation of latent endogenous virus, or reinfection with a different donor-transmitted strain among CMV-seropositive [11, 12]. CMV infection can be asymptomatic in most cases but could lead to pneumonia, encephalitis, retinitis, hepatitis, and gastroenteritis of CMV diseases in SOT recipients. Thus, measurement of CMV DNA in peripheral blood samples of SOT recipients has become standard clinical practice in many transplantation centers to guide the preemptive strategies of human CMV for disease prevention, to diagnose CMV disease, to monitor the response to antiviral therapy, to identify disease relapse, and to use it as a surrogate marker of anti-CMV drug resistance [13, 14]. It was also proposed that CMV levels in plasma could be used as safety markers in clinical trials of new immunosuppressive agents [15]. During the preemptive treatment, monitoring of CMV DNA levels and VL kinetics in blood over time using qPCR assay plays a critical role in governing the course of antiviral therapy. While cutoff values of CMV DNA level are currently defined by individual laboratories to specified patient groups for on/off antiviral therapy, a large variability of methodological sources are ascribed to the absence of a generally acceptable threshold of VL quantitation [1, 13]. Concurrently, most qPCR-based methods show a high degree of result variability particularly when the results are compared between institutions [2, 3, 16, 17]. The multicenter studies on comparisons of qPCR results of CMV quantitation demonstrated that unacceptable variations of plasma level of

CMV DNA were up to 4 log copies per millilitre (mL), which makes development of an international guideline for clinical management of CMV diseases and harmonization of interinstitutional results of CMV DNA difficult [3, 16, 17].

The qPCR is a dynamic process of nucleic acid quantification based on normalization of the time to signal strength against a calibration curve that is in turn generated by the use of calibration materials with "known" values. A slight deviation in any part of this complex procedure could theoretically affect result accuracy and precision. Although many reasons can potentially introduce variables for results such as differences in extraction methods, targeting gene fragments, and detection systems, the fact is that interlaboratory variability exceeds intra-laboratory variability of results remarkably [16]. The lack of universally accepted calibrators was considered to be a major issue in literature [18, 19]. The lack of available international quantitative standards for many of the commonly tested viral analytes has led to the use of a wide variety of materials, intuitively reducing the agreement of results when common samples have been tested by different centers. It has been strived to develop such an international reference material for CMV DNA result harmonization. Standardization of quantitative values of CMV DNA levels is important to ensure the portability of patient results among institutions, the data interpretation, and critical decision-making for clinical management of patients.

## *QNAT Assay for Monitoring EBV in SOT Recipients*

As a member of herpesvirus family, EBV possesses a high seroprevalence worldwide. Primary infection is generally asymptomatic but can lead to infectious mononucleosis in adolescents and young adults. Following primary infection the virus establishes lifelong persistence in B cells. The use of more potent and targeted immunosuppressive in SOT recipients with mismatched unrelated donors has an increased risk, resulting in emergence of EBV-associated posttransplant lymphoproliferative disease (PTLD) [20]. The observations in the mid-1990s that patients who developed PTLD often had high EBV VL in peripheral blood and that these high levels were found prior to the onset of clinical illness have led to the wide use of EBV VL assay in preemptive programs for disease prevention, diagnosis of symptomatic patients, and monitoring response to anti-EBV therapy [21]. EBV VL monitoring was also recommended as a means of therapeutic safety in clinical trial of new immunosuppressive agents [15]. Moreover, several centers have observed a reduction of PTLD incidence or mortality when the cohorts of patients who underwent routine EBV VL surveillance and preemptive interventions were compared to historical cohorts without surveillance and intervention [22, 23]. Currently QNAT has become the standard practice at most transplant centers to measure EBV VL in peripheral blood for the diagnosis and management of EBV-associated infections [24–27]. Although peripheral blood mononuclear cells, plasma, and whole blood have all been successfully used to detect EBV in the recipients, some studies suggest that whole blood is the most appropriate sample type as it contains both

cell-free and cell-associated EBV components [24–27]. However, QNAT assays based on qPCR platform present a relative high degree of result variability particularly between laboratories [4, 5, 16, 28]. The late studies on comparison of EBV DNA levels across multiple centers demonstrated that unacceptable variations were up to 4 log EBV DNA copies/L. As described in the CMV above, even though many reasons could potentially result in the variability, lack of universally accepted calibrators has been emphasized as a major factor which impacts harmonization of EVB VL quantitation [16]. The development of such international reference calibrator has been highly anticipated to improve result harmonization of EVB VL.

## *QNAT Assay for Monitoring BKV in SOT Recipients*

BK virus is a member of the *Polyomaviridae* family with double-stranded DNA. Primary infection is acquired in early childhood and is asymptomatic in a majority of cases. Consequently seropositivity across adulthood reaches as high as approximately 90% [29]. After a primary infection, the virus establishes its latency in kidneys and urinary tract with intermittent reactivation throughout life, whereas the virus is only detectable in <5% of healthy individuals [30, 31]. The clinical sequelae of BKV reactivation are confined to immunocompromised status. The BKV-associated disease most frequently seen is BKV-associated nephropathy (BKVN) after renal transplantation. BKV reactivation occurs up to 50% of kidney transplant recipients within the first year of transplantation. Among those 7–10% are advanced to BKVN, resulting in graft dysfunction or loss [32]. The underlying pathogenesis of BKVN is not well known [33]. Disruption of the balance between BKV replication and host immune defense system is generally thought as a key element of BKV-associated pathogenesis [34]. To date, due to lack of effectiveness of antiviral drugs [35], the mainstay therapeutic option for BKVN is a reduction of immunosuppressive, allowing reconstitution of recipient's immune defense system to clear the virus [36]. However, this approach could potentially increase a risk of graft rejection. According to Kidney Disease: Improving Global Outcomes (KDIGO) [37], the 2014 European guidelines [38], and the American Society of Transplantation (AST), all renal transplant recipients should be regularly screened for BKV replication in the plasma or urine to identify patients at increased risk of BKVN. A sustained BKV viremia level above the threshold of 4 $\log_{10}$ copies/ mL has been defined as "presumptive" BKVN [39]. High-level BKV viruria usually precedes viremia and potential nephropathy by 4–12 weeks. Thus, it is recommended to screen the BKV DNA VL in plasma monthly for the first 6 months of posttransplantation, followed by every 3-month screening until 2 years in order to guide therapeutic intervention for probable or proven BKVN.

The monitoring of BKV DNA VL using qPCR technology becomes a standard practice to predict and diagnose BKVN as well as manage BKV disease. So far there is the lack of well-designed multicenter comparison study using clinical specimens for evaluation of interlaboratory variability; current studies demon-

strated significant inter-assay variability in quantifying BKV DNA complicates the interpretation of these results and precludes establishing broadly applicable thresholds for clinical intervention [6, 7]. Quantitative results of BKV DNA reported by one assay may vastly be different from those reported by another, making inter-assay comparison of results impossible. Such a variability has been well known with negative impact on patient care, particularly when a discrepancy of BKV DNA VL was reported by two laboratories and clinical decision was to be made based on discordant quantitative results [16, 40–44]. Among those most cited factors attributing to assay variability of BKV DNA VL, the use of different standards for quantitation and primer/probe designs exempt from BKV genotypic variation were major considerations [40–42, 44]. An international reference calibrator of BKV DNA quantitation is in need for improving result harmonization and clinical management of BKVN.

## Development of WHO International Standards for Viral Loading Determination of CMV, EBV, and BKV

### *CMV WHO International Standard for QNAT Assay*

A significant variability in the performance of QNAT assays suggests that a common assay calibrator may be the first critical step to improve the result harmonization. A collaborative study led by the NIBSC (UK) and the WHO Expert Committee on Biological Standardization (ECBS) was conducted to evaluate the suitability of candidate reference materials to harmonize measurements of CMV VL using a wide range of QNAT assays in 2009 [45]. Four candidate materials comprised of lyophilized Merlin virus, liquid Merlin virus, liquid AD169 virus, and purified HCMV Merlin DNA cloned into a bacterial artificial chromosome were shipped to 32 laboratories in 14 countries participating the study. Participating laboratories were selected based on their experience in CMV NAT assays and geographic distribution, including major clinical laboratories in medical centers and different manufacturers of in vitro diagnostic devices (IVDs) as well as reference, research, and quality assurance laboratories. For each assay run, a single estimate of $\log_{10}$ copies/mL was obtained for each sample, by taking the mean of the $\log_{10}$ estimates of copies/mL across replicates, after correcting for any dilution factor. A single estimate for the laboratory and assay method was then calculated as the mean of the $\log_{10}$ estimates of copies/mL across assay runs. Overall analysis was based on the $\log_{10}$ estimates of copies/mL or NAT detectable units/mL. Overall mean estimates were calculated as the means of all individual laboratories. Variation between laboratories (interlaboratory) was expressed as the SD of the $\log_{10}$ estimates and percentage geometric coefficient of variation (%GCV) of the actual estimates [8]. The results showed that the variability in the laboratory mean CMV concentrations derived from the virus samples was 2 $\log_{10}$ across the different assays. The variability for purified DNA sample

was relatively high (>3 $\log_{10}$). The agreement between laboratories was markedly improved when the potencies of the liquid virus samples were expressed relative to the lyophilized virus candidate. In contrast, the agreement between laboratories for purified DNA sample was not improved. Results indicated the suitability of the lyophilized Merlin virus preparation as the first WHO International Standard for HCMV QNAT assessment. It was characterized in October 2010 and approved by the WHO in Nov 2010. The standard has an assigned potency of $5 \times 10^6$ international units (IU) (NIBSC code 09/162) (Table 1). It is intended to be used to calibrate secondary references, used in HCMV QNAT assays with IU [8].

### *EBV WHO International Standard for QNAT Assay*

To resolve the same issue of variability for EBV QNAT assay, a proposal for the development of the first WHO IS for EBV was presented at the first Standardization of Genome Amplification Techniques (SoGAT) Clinical Diagnostics meeting held at NIBSC in June 2008 [46]. Several options for the selection of source materials and formulation of the candidate standard were discussed. The WHO ECBS approved the proposal and dedicated the NIBSC to proceed with the task [9].

Candidate materials were comprised of liquid and lyophilized cell-free live virus preparations of the prototype laboratory EBV strain B95-8 [46] as well as preparations of Namalwa [47] and Raji [48] cells containing integrated copies of an EBV viral genome. These strains represent well-characterized EBV genomes (type I viruses), which are frequently used in preparation of control materials for EBV NAT assays [9]. Namalwa and Raji cells contain 2 and 50–60 copies of the EBV genome, respectively, which are present as episomes. EBV B95-8 strain was isolated from a cell culture supernatant sample described previously by Lin Jung-Chung [49]. The preparations of an EBV B95-8 stock were described by Fryer et al. [9].

The B95-8 bulk preparations were formulated to contain approximately $1 \times 10^7$ EBV copies/mL in a final volume of 6.4 L Tris-albumin buffer. Approximately 250 mL of the liquid bulk was dispensed in 1 mL aliquots and stored at – 70 °C. The remaining bulk volume was immediately processed for lyophilization (NIBSC code 09/260). The Namalwa and Raji cells were propagated in RPMI-1640 medium and harvested at the log phase of growth. Three candidates, the B95–8 containing approximately $1 \times 10^7$ EBV copies/mL and Namalwa and Raji containing $1 \times 10^6$ cells/mL in PBS, were quantified using the EBV qPCR in order to determine the homogeneity of each candidate prior to dispatch for collaborative study [9]. The stability of lyophilized candidate 09/260 was assessed in an ongoing accelerated thermal degradation study at −70 °C, −20 °C, 4 °C, 20 °C, 37 °C, and 45 °C and showed very stable with tested temperatures. Candidate materials including lyophilized and liquid virus preparations of EBV B95-8 strain and preparations of Namalwa and Raji cells were shipped to 28 participating clinical laboratories, a small numbers of manufacturers of IVDs and reference and research laboratories

**Table 1** The 1st WHO International Standard for HCMV, EBV, and BKV for NAT

| | Material | Quantitation | Report unit | Stability | Shipping condition | NIBSC code | Release date | Ref |
|---|---|---|---|---|---|---|---|---|
| HCMV | lyophilized HCMV Merlin virus | $5 \times 10^6$ IU | International units (IU) | Stable up to 15 years at –20 °C | Ambient temperature or –20 °C | 09/162 | Oct 2010 | [8] |
| EBV | Lyophilized EBV B95-8 virus | $5 \times 10^6$ IU | International units (IU) | Stable up to 15 years at –20 °C | Ambient temperature or –20 °C | 09/260 | Oct 2011 | [9] |
| BKV | Lyophilized BKV virus (subtype1b-2) | 7.2 $\log_{10}$ IU | International units (IU) | Stable up to 15 years at –20 °C | Ambient temperature or –20 °C | 14/212 | Jun 2016 | [10] |

representing 16 countries. All participating laboratories were referred by a code number as samples 1–4 and allocated randomly. Participants were requested to test dilutions of each sample using their routine NAT assay for EBV on four separate occasions and to report the VL in copies/mL (positive/negative for qualitative assays) at each dilution of the sample. All results including details of methodology used were returned to NIBSC for analysis. The variability between the individual laboratory mean estimates for each candidate was 2.5 $\log_{10}$ copies/mL. The agreement between laboratories was improved when the potency of each candidate was expressed relative to the lyophilized B95-8 preparation. The results indicate the suitability of this candidate as the first WHO IS for EBV NAT. It was established on October 2011 by the WHO ECBS with an assigned potency of $5 \times 10^6$ international units (IU) showed in Table 1 (NIBSC code 09/260). It is intended to be used for calibration of secondary reference materials and in EBV NAT assays for improving the comparability of VL measurements in patients [9].

## *BKV WHO International Standard for QNAT Assay*

Evaluations on the proficiency panels of Quality Control for Molecular Diagnostics (QCMD) in 2007 and 2008 for both BKV and JCV highlighted large variability in QNAT assays, underscoring the need for greater standardization and the availability of international standards that could be used to calibrate the different working standards used by individual laboratories [10]. An international group convened in 2006 and 2008 to discuss the requirement for the international standardization of JCV NAT assays, alongside which BKV NAT assay standardization was also discussed. The proposed candidate BKV standards B (14/202) and D (14/212) comprised whole virus preparations of BKV type 1b-1 and 1b-2, respectively. Both standard formulations were cell-free, live virus preparations from productively infected cell culture. The candidate standards 14/202 and 14/212 have both been formulated in universal buffer comprising 10 mM Tris-HCl pH 7.4, 0.5% human serum albumin (HSA), and 0.1% D-(+)-trehalose dihydrate, to permit dilution into a sample matrix pertinent to the end user. The preparations were freeze-dried to ensure the stability of the product for a long term after an accelerated thermal degradation study had been performed at varied temperatures from −70 °C to 45 °C [10]. The candidate BKV reference materials in both the lyophilized and liquid state (Candidate 14/202, B and C; Candidate 14/212, D and E) were shipped to 36 participating laboratories, including research and clinical laboratories from 15 different countries, the manufacturers for BKV NAT IVD kits, and reference and EQA laboratories. All participating laboratories were randomly assigned a laboratory code by which to reference their data, thereby assuring laboratory anonymity.

Data were received from 33 laboratories with 35 quantitative datasets, and 3 qualitative datasets were analyzed. For the quantitative data, participants returned report of BKV results as copies/mL or $\log_{10}$ copies/mL. The viral copy values

obtained for both candidates showed good homogeneity across the vial contents. The mean copies/mL of 14/202 by in-house analysis (6.53 $\log_{10}$ copies/mL) was in agreement with both the quantitative and combined mean estimates of this reference (6.62 and 6.46 $\log_{10}$ copies/mL) obtained from the collaborative study. The mean copies/mL of 14/212 by in-house analysis (6.50 $\log_{10}$) was also in a reasonable range of agreement with the combined mean estimate of the reference (6.97 $\log_{10}$) obtained from the collaborative study. The lyophilized samples also showed better agreement with estimates compared with the liquid equivalents. Based upon the conclusion from the datasets received, it has been proposed that the candidate reference sample (14/212; 4092 vials) was established as the first WHO IS for BKV DNA NAT-based assays with an assigned potency of 7.0 $\log_{10}$ IU/mL per ampoule and released in May 2016 [10] (Table 1).

## Characterization, Improvement, and Challenges of the WHO International Standards

Evaluation on the variability of quantitative VL testing was conducted and reported since the first WHO IS for CMV, EBV, and BKV had been introduced as the secondary reference calibrators [50–55]. Overall result harmonization has been improved in varied degrees with different viruses. Semenova reported that the use of the WHO IS calibrators for EBV could improve the interlaboratory homogeneity of whole-blood EBV VL quantitation results. EBV whole-blood proficiency panel including 7 samples was tested by 12 participating laboratories using their own QNAT assays. The standard deviations (SD) were ranged from 0.41 to 0.55 when the results were expressed in $\log_{10}$ copies/mL and from 0.17 to 0.32 when results were given in $\log_{10}$ international units/mL, indicating a significant improvement of the variability of EBV VL in comparison with the previous study [4]. Lately the CAP proficiency testing surveys for QNAT assays on CMV, EBV, BKV, adenovirus (ADV), and human herpesvirus 6 (HHV6) from 554 laboratories were conducted to determine overall result variability of VL. The outcomes were stratified by assay manufacturer. Some improvements were seen when international units were adopted. This was particularly the case for EBV VL determination. However, the variability in VL results remains a challenge across all viruses tested. The implementation of WHO IS helped to reduce the variability more or less noticeably for certain viruses than others [55]. The multicenter comparative study showed that the variation of CMV VL results was acceptable but the EBV results were less accurate despite the use of WHO IS [50]. One of reasons was that some of the laboratories were using the panels comprised of "laboratory virus strains" rather than clinical specimens, which may not represent truly "variable values" in clinical testing. Regardless all efforts, the variability of VL measurements is present persistently. The reasons are largely unknown. One of the explanations is the WHO IS materials might not be commutable for different assays that are commonly used in diagnostic

laboratories [56, 57]. Recently, Preiksaitis et al. studied the impact of the first WHO IS for CMV DNA on the harmonization of results using clinical plasma samples [53]. Serial dilutions of the IS, a blinded panel of pooled CMV DNA-positives from 40 clinical plasma samples with known genotypes, and 10 negative plasma samples were tested across 6 laboratories using 10 qPCR assays calibrated to the WHO IS. The results showed that the variation of individual CMV DNA-positive samples was greater for clinical samples than the WHO IS dilutions. This implies that the variability of CMV DNA results derived from individual samples has been reduced by using the WHO IS, while the variability of CMV DNA results with clinical relevance persists, challenging meaningful inter-assay comparison of clinical results. It was also observed that the assays designed with amplicon sizes ≤86 bps yielded significantly high level of CMV VL compared to the assays with larger sizes of amplicons. This observation provides an evidence to support the hypothesis that CMV DNA in clinical plasma samples is likely fragmented [53]. In order to further confirm the hypothesis, three paired qPCR assays with variable amplicon sizes were designed for the measurement of CMV DNA fragmentation [58] in plasma samples obtained from 20 SOT recipients, CMV viral stock preparations (Towne, Merlin, AD169), and the first WHO IS. Interestingly, CMV DNA results were highly reproducible for three CMV viral stock preparations and the WHO IS. In contrast, CMV DNA results were very different for the plasma samples from 20 SOT recipients. CMV DNA measured by the assay with small amplicons was 2.6-fold, 3.4-fold, and 6.5-fold higher than that with long amplicons, indicating that CMV DNA from the patient plasma samples is highly fragmented and a portion of the CMV DNA in plasma is present in the form of extremely small fragments <138 bp [58]. This conclusion, however, should not be extrapolated to whole-blood sample type although plasma CMV DNA is a part of whole blood. Further studies on distribution of CMV DNA fragments with varied sizes in various blood compartments in different status of CMV diseases are warranted before and after antiviral therapy and in the presence of different immune response potencies. The findings from this study have significant implications for the interpretation of dynamic changes objectively in serial CMV VL in SOT recipients and guidance in design of qPCR assays for CMV DNA measurement.

There is limited study for characterization of variability and its impact of the first WHO IS for BK VL testing. Tan et al. reported comparison of one commercial assay (Altona) with an in-hour assay after calibration using the first WHO BKV IS to evaluate inter-variability of testing results from 161 clinical plasma samples. The results revealed similar regression lines, no proportional bias, and improvement in systematic bias, indicating that the use of a common calibrator improved the agreement between the two assays [54]. However, comparison between two assays only was not sufficient to provide solid conclusion. A study with multiple participating laboratories with different assays is needed.

## Future Considerations on Standardization of QNAT Assay for Viruses

### *Understanding the Nature-Occurring Form of NA* **In Vivo** *for Targeting Virus*

During the course of development and characterization of the WHO IS for CMV, EBV, and BKV was performed, we have learned that selected reference materials have direct influence on the results of calibration for QNAT assays. Cultured viruses, gene fragments, NA cloning of viruses, and clinical samples can all be used as reference materials, from which one of those materials will yield the best calibration result for specific QNAT assay for one or more targeting viruses. If the variability of QNAT assay results persists between the selected calibrator and clinical samples, further understanding of the nature-occurring form of NA in vivo for targeting viruses is very important as we have learned from the report of CMV DNA fragmentation in plasma samples of SOT recipients [58]. Study on what dominant fragment of the viral NA in vivo is, how the fragments of viral NA distribute in various blood components, and whether the levels of fragmented NA will be altered by clinical intervention will allow us to have insight about the origin of variability and to improve design of primes or probes for QNAT assays. As a result, harmonization of inter- or intra-laboratory variation of QNAT assay could be achieved for the targeting viruses using selected reference materials.

### *Proficiency Testing for CMV, EBV, and BKV NAT Assays*

The proficiency testing (PT) offers a unique opportunity to determine interlaboratory variation across a large number of individual diagnostic laboratories using shared reference specimens or standards if each lab has its own method to measure VL in shared specimens. However, current materials provided for proficiency testing for CMV, EBV, and BKV NAT assays are mostly comprised of nonclinical materials such as cultured virus strains, which may not represent the true "variable values" in clinical setting. As mentioned above, some of the studies have showed a persistent variability of CMV QNAT assays because of high fragmentation of CMV DNA in clinical plasma specimens [58]. It is highly recommended including adequate numbers of clinical samples in the PT panel to make meaningful comparisons of clinical results across laboratories and to promote interlaboratory communication regarding to VL levels and appropriate management of associated disorders.

## *Design of Primers and Probes*

The finding of the presence of fragmented CMV DNA in clinical plasma has again raised an essential question about design on primers/probes, amplicon sizes, and selecting genetic regions for targeting viruses. To realize the global harmonization among QNAT assay results for CMV, EBV, and BKV DNA, it is recommended that appropriate committees such as WHO ECBS, NIBSC, SoGAT, etc. should form a task force to work and set up a guideline on the principle of the assay design. The principle should include defined targeting regions of genes for each virus, adequate amplicon size matching with most of biological forms of clinical specimens, preferred sample types, and a set of primers/probes with corresponding reference standards and values.

## *Communication*

Numerous commercial QNAT assays are available for quantitative measurement of CMV, EBV, and BKV VL in different specimen types. It would be a benefit for individual laboratory to understand the assay's features clearly prior to make a choice of selecting an assay for targeting viruses. Most of user's manuals of commercial assays do not include sufficient information on gene targets, primer/probe sequences, and amplicon sizes. This makes troubleshooting difficult during application of the assay in clinical usage. It is encouraged that the industries in commercial assay development provide those essential information to users while the users should also feedback to their issues on performance of the assay in clinical diagnosis to the industries. The communication also means that the proficiency testing should be performed and results should be exchanged regularly among individual laboratories especially involved in diagnostics in the networking.

In summary, development and characterization of international reference standards for QNAT assay for monitoring CMV, EBV, and BKV viral load are truly frontier work to improve the commutability of inter-assay and interlaboratory results of NAT assays on the viruses. Significant improvement on harmonization of viral load determination for these viruses has been achieved using the first WHO IS across clinical laboratories for the past 5–7 years. Meanwhile, there are some challenges especially in the calibration of VL results from clinical samples using IS. New insights on large variability of QNAT assay on clinical samples have been accumulated. These works have provided important experiences and knowledge for standardization of VL determination for other human pathogenic viruses in near future.

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# Applications of Digital PCR in Clinical Microbiology

**Jessica N. Brazelton De Cárdenas and Randall T. Hayden**

## Introduction

Since its advent in the 1980s, PCR has gone through many iterations, and applications of PCR can be found in almost every life science laboratory. Originally used primarily for qualitative detection of microbiologic agents in clinical microbiology labs, many subsequent applications of PCR have focused on quantitative detection. Methodologies have varied over time and have included competitive endpoint PCR, real-time quantitative PCR (qPCR), and most recently digital PCR (dPCR). Like earlier endpoint and fluorescent probe-based detection methods, dPCR is appropriate for either DNA or RNA targets, with the addition of an RT step for analysis of the latter.

As noted elsewhere in this text, dPCR is based on the concept of limiting dilution [1–4]. PCR reactions are split into numerous partitions (tens to millions, depending on the system and method of separation), each of which becomes a microreaction. The positive or negative results of these microreactions are then counted directly at reaction endpoint, based on the presence or absence of PCR product, typically detected by the use of fluorochrome-labeled probes. Assuming sufficient sample dilution, Poisson statistics can be used to calculate the number of target molecules in the original sample. Simply counting the positive reactions would typically lead to an underestimation of the actual concentration in the sample due to some reactions containing greater than one target molecule [5, 6]. There are currently a few different dPCR platforms on the commercial market, and they differ primarily by partition method and number of partitions produced, among other characteristics. Each platform has its own strengths and weaknesses, and platform choice should be based on application, available reagents, and clinical

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**Table 1** Potential advantages of digital PCR over real-time PCR in clinical microbiology

| | |
|---|---|
| Not dependent on rate-based measurements or calibration curves | [8] |
| Less affected by amplification inhibitors and suboptimal amplification efficiency | [9–14, 85] |
| Less affected by mismatches in primer and probe sequences | [11, 20, 21, 78] |
| Absolute quantification of viral load, including monitoring viral load changes | [15–21, 34, 38] |

**Table 2** Potential applications of digital PCR in clinical microbiology

| | |
|---|---|
| Organism genome load determination – copies/unit volume ("DNAemia") | [16, 19, 22–24, 30–32, 74–76] |
| Genome cellular copy number determination | [28, 35–38, 46–48] |
| Quantitation of pathogens with high genetic diversity (HIV, BKV, etc.) | [11, 21, 30, 78–84] |
| Quantitation of pathogens in samples prone to amplification inhibition | [9, 10, 14, 85–87] |
| Standardization of viral load testing<br>Lot-to-lot testing of controls and calibrators<br>Reference standard for performance verification or assessment of commutability | [7, 50, 55, 66–70] |

setting, as with other instrument choices. There are few available publications providing guidelines for evaluation and reporting of quantitative digital PCR data [7]. For publication purposes, several items specific to digital PCR should be included, namely, mean copies per partition, total volume of the partitions measured, individual partition volume, partition number, template structural information, comprehensive details and appropriate use of controls, examples of positive and negative experimental results as supplemental data, and experimental variance or CI [7]. With these guidelines in place, publications using digital PCR may provide more reproducible data and reliable scientific reporting, irrespective of the platform used.

Despite differences among platforms, several potential advantages of using dPCR instead of qPCR have emerged in recent years (Table 1), leading many to explore applications for clinical diagnostic use. Digital PCR does not rely on rate-based measurements, nor on calibration curves, as with qPCR [8]. As dPCR is an endpoint method, it is often less affected by amplification inhibitors or by suboptimal amplification efficiency [9–14]. Additionally, dPCR can be used for monitoring viral load changes in response to antiviral therapy [15–21]. It has been surmised that many of the recognized weaknesses of qPCR, relating to poor uniformity of results, lack of standardization, and susceptibility to target diversity and matrix affects, could be addressed through the increased use of dPCR methods. Authors have increasingly demonstrated potential value in each of these areas. Potential applications of dPCR in the clinical microbiology laboratory are noted in Table 2 and explored below.

## Viral Quantitation

Quantitative testing of infectious pathogens can be used to diagnose disease, predict clinical outcome, determine response to therapy, or direct preemptive treatment. In many cases, treatment is dependent on accurate pathogen load determination. Quantitative testing for human cytomegalovirus (CMV) and other blood-borne viruses has become standard care for transplant and other immunocompromised patients. Several studies have now been published examining whether the promise of improved accuracy and precision is fulfilled when dPCR is applied to these analytes and others. The results show a variable picture in terms of potential clinical diagnostic utility. Several groups have shown increased precision of dPCR over qPCR for at least part of the analytical measurement range, with comparative sensitivity varying, in some cases showing qPCR to have an apparent advantage [16, 19, 22–24]. Beyond the question of whether this method offers improved performance characteristics over real-time techniques is the challenge of improving interlaboratory agreement. It has been demonstrated that results of dPCR may vary less than those of qPCR with the use of different reagents and platforms, particularly for DNA assays [16, 25], with the reverse transcription step introducing a higher risk of variability even in digital systems for RNA quantitation [8, 26]. However, other studies have not shown a consistent advantage. The use of dPCR for quantification and detection of hepatitis B virus in plasma led to under-quantification compared to qPCR assays, and qPCR assays remain more sensitive for this application [27]. Some have had better results with RNA targets, however. Compared to qPCR, dPCR had a lower coefficient of variation and was equally reliable for quantifying HTLV-1, making it an option for clinical testing in low-cellularity samples [28].

Early work comparing CMV quantitation between qPCR and dPCR indicated that the quantitative accuracy for dPCR was high and that linearity and quantitation correlated well with qPCR results [16]. Subsequent work showed dPCR to have increased precision at viral loads greater than $4\log_{10}$ copies/ml and equivalent sensitivity to qPCR assays, along with improved inter- and intra-assay precision, indicating potential value in more accurate monitoring of disease progression [19]. Later studies comparing CMV reagents, standards, and digital PCR platforms demonstrated a good correlation between various reagents and digital platforms, suggesting value in reducing interlaboratory variability [25, 29]. Digital methods have also been successfully used to monitor adenovirus (ADV), another important agent in the posttransplant setting. A study comparing multiplex dPCR to multiplex qPCR for CMV and ADV showed better performance of the multiplex dPCR assay when testing inhibition-prone samples, though the study did suffer from low numbers of positive samples [19]. While primarily a proof-of-concept work, dPCR assays for JC virus, BK virus, and EBV have been described in recent literature and could have an eventual purpose for posttransplant clinical monitoring [30–32]. Studies evaluating commutability of EBV standards for quantitative assays [32] and exploring the use of a multiplex dPCR assay for EBV quantitation in glioblastomas have also been published [33]. Viral load testing is an essential tool to assess

disease burden, rate of disease progression, and response to therapy for HIV, but is typically performed with qPCR. The use of dPCR instead of qPCR for an assay to quantify 2-LTR circles in cells of 300 infected patient samples resulted in a demonstrated increase in assay precision and accuracy improvement of dPCR over qPCR, suggesting it may be a viable option for regular HIV quantitation in a clinical setting [20]. A similar study also noted the promise of dPCR for viral load quantitation but described differences in HIV-1 DNA copies and episomal 2-LTR circles between the methods [34].

Because of the sample partitioning on which it is based, dPCR is especially well-suited for the detection of low numbers of target in a background of abundant endogenous nucleic acid. This, together with its high degree of precision, makes it useful for cellular copy number determination. This has been a primary use of dPCR in molecular oncology but is also potentially relevant for infectious diseases, particularly in measuring cellular reservoirs of HIV [28, 35–38]. Cell-associated HIV-1 RNA has been demonstrated to be a predictive measure of outcome to antiretroviral therapy and has been used as a biomarker to predict reactivation of latent HIV reservoirs [39]. Determination of cell-associated HIV-1 RNA by qPCR can be of limited accuracy near the lower limit quantitation [38–40]. Digital PCR assays may assist in quantitating low levels of viremia or latent HIV reservoirs in this subset of patients [38]. Similarly, resting memory CD4 + T cells can carry integrated viral genomes, such as HIV-1 (proviral DNA), and while they are not associated with the production of active virus, they can reactivate upon discontinuation of therapy [35, 37]. Digital PCR may provide a more precise and reproducible method for determining proviral load quantification compared to qPCR, especially in samples with low numbers of cells [28]. Because of the lower inter-assay variability of dPCR, it can be used to monitor proviral load infections over time and assess therapeutic effects [28]. There exists evidence for population-specific copy number variation of the CCL3L and CCL4L genes, which have been shown to have a protective anti-HIV-1 effect at higher copy number in some studies [36, 41, 42], though this was not repeatable by others [36, 43, 44], leading to controversy in the field [36, 45]. In part, these contradictory findings can be attributed to qPCR assay variability, something that may be alleviated by dPCR assays [36]. In this study, digital PCR was determined to be more accurate than qPCR for CCL4L copy number determination, especially at higher copy numbers [36]. Cellular copy number can also be used to assess chromosomal integration of viral genome. Chromosomal integration of human herpesvirus 6 (HHV-6) has been demonstrated in approximately 1% of the population [46], sometimes resulting in overdiagnosis and unnecessary treatment based on elevated peripheral blood DNAemia. Digital PCR has been used to rapidly and precisely identify patients with chromosomally integrated HHV-6, with one or two viral genomes per cell indicating integration [46]. Follow-up studies have used specimen pooling with dPCR to reduce the number of tests to screen large numbers of samples for chromosomally integrated HHV-6 [47]. Detection of active HHV-6 infection against a preceding background of chromosomal integration has been accomplished using a multiplexed dPCR assay [48].

## Standardization of Viral Load Testing

As noted above, the effort to reduce variability among laboratories and assays measuring organism load has been a challenging one. Digital PCR may reduce such variability as a primary diagnostic method but may also be used to help improve agreement of qPCR methods by serving as a much-needed reference standard against which calibrators and other control materials may be measured [29–31, 49, 50]. Several studies have already demonstrated poor interlaboratory agreement in quantitative assays measuring blood-borne viruses, including but not limited to CMV, EBV, and BK virus [49, 51–57]. As international consensus standards have been developed for these agents, we have seen some improvement in interlaboratory agreement; however, further progress is needed [16, 29–31, 58, 59]. In particular, WHO standards, for example, are consensus standards, developed using results from a group of validating laboratories, typically using various qPCR methods, without traceability to an absolute reference standard [60–65]. Integration of a reference standard method into this process could reduce variability among lots of standards, something that has been noted as a potential limitation of current materials [16, 29–31, 59]. Digital PCR could serve such a role and in fact has been used by some metrological groups for the production of quantitative standards [7, 50, 55, 66]. In addition, analysis of commercially produced quantitative secondary standards by dPCR has shown mixed results with variable degrees of bias compared to nominal values and to one another [16, 25, 29–31, 53, 58]. Digital PCR could function to improve uniformity of such materials and by individual laboratories for QC of tertiary standards, external controls, and other quality control materials which presently show variability among manufacturers or lots [16, 25, 29–31, 53, 58]. Digital PCR could also serve a valuable quality assurance role in determining reagent stability and consistency over time and based on different storage conditions. Finally, for analytes without international consensus standards or with limited availability of commercially produced materials for quality assurance, dPCR may enable end-user development and QA of assays with a high degree of precision and accuracy, a potentially much more challenging task when using only qPCR methodology [67–70].

## Quantitation of Nonviral Pathogens

While most potential clinical applications of dPCR that have been explored thus far relate primarily to viral pathogens, there are also many examples where it may prove valuable to nonviral pathogen testing. One such application is genotyping of bacterial pathogens concomitantly with quantification – as demonstrated for *Helicobacter pylori* [71]. A stool-based 16S dPCR assay for detection and quantitation of *H. pylori* and a dPCR cagA *H. pylori* genotyping assay were compared to serology and stool antigen tests [71]. The results of quantitative detection of *H. pylori* and allelic

typing of the cagA virulence gene were both associated with differences in gastric cancer risk, potentially aiding in early detection and intervention [71]. Different fluorescent hydrolysis probes were used to distinguish between genotypes of *cagA* using dPCR; *H. pylori* load in stool samples analyzed by dPCR showed good correlation with *cagA* serum antibody assays [71].

*Mycobacterium tuberculosis* is another important clinical pathogen to which dPCR has been applied. While *M. tuberculosis* is typically tested for respiratory tract specimens, it is often difficult to obtain those specimens from small children or asymptomatic patients. The ability to detect and quantify *M. tuberculosis* (Mtb) from the blood using dPCR may allow earlier detection and genotypic drug resistance monitoring [72]. In adult patients with symptomatic Mtb, the use of dPCR for therapeutic monitoring is also promising [12, 73].

Digital PCR methods have also been used to quantify parasite loads in infected patients. Digital and real-time PCR were compared for quantification of *Cryptosporidium* [74]. The precision of dPCR was better than qPCR except at lower DNA concentrations, and dPCR was less affected by the presence of inhibitors [74]. Cost analysis showed that dPCR was approximately twice as expensive as qPCR [74]. The density of malaria parasites in blood has also been quantified using dPCR and is useful in detecting subclinical infections [75]. A duplex dPCR assay was also developed and compared to qPCR assays for *Plasmodium* detection, quantification, and species differentiation [76]. The first set of primers was genus specific, while the second set differentiated the *Plasmodium* species [76]. The duplex dPCR assay had equal sensitivity to both the qPCR and dPCR singleplex species-specific detection assays and had higher sensitivity for the identification of minor *Plasmodium* species, especially in mixed infections [76]. The authors reported that qPCR had a lower limit of detection (LOD) of 22 parasites/mL when used together with a specimen concentration method, while the use of dPCR (also with specimen concentration) decreased the LOD to 11 parasites/mL [76]. Finally, dPCR has been used as a preliminary method for detection and species differentiation of *Babesia* [77], with an LOD of 10 gene copies/mL [77].

## Sequence Variation and Quasi-Populations

Many targets of human disease have high sequence diversity, sometimes a significant challenge for quantitative assay development. Primer or probe sequence mismatches can reduce amplification efficiency and lead to inaccuracies in quantification by qPCR [11, 21, 78]. BK viral load testing provides an illustrative case where considerable inter-assay variability exists among various tests [30, 78–80]. Recent studies have shown that only 68% of urine, whole blood, and plasma samples fell within an acceptable range of variation compared to expected values when tested using qPCR methods at different laboratories [80] and that different primer sets performed differently depending on the genotype [78]. Because of the marked differences in genotypes,

dPCR can be used to overcome some reduced amplification due to mismatches, though the quantification of a robust standard is ideal [30].

Human immunodeficiency virus (HIV) is another viral target known for high genetic diversity, often leading to challenges in accurate detection, population level surveillance, and development of treatment [81–83]. While it cannot completely overcome these challenges, the application of dPCR has demonstrated significantly increased precision over qPCR for measurement of HIV DNA, targeting both the pol and 2-LTR regions [20]. These authors also noted the relative insensitivity of dPCR to mismatches in primer and probe sequences, with an average reduction of under-quantitation of 57%, compared to qPCR [20]. This improvement of analytical precision for templates with primer and probe mismatches could have significant impact on antiretroviral therapy, which is often based on viral load and response to treatment.

In addition to being used to detect diverse genotypes, dPCR can also be used to detect single nucleotide polymorphisms in viruses that may lead to drug resistance. This application of dPCR has been thoroughly exploited in the field of oncology research but is a relatively new concept for clinical microbiology. With the worldwide threat of antimicrobial resistance, the development of new methods for early diagnosis and monitoring of drug resistance is increasingly important. This is well-illustrated by influenza, a virus well-known for a high mutational rate with rapid emergence of rapid mutation of antiviral resistance, often characterized by quasi-populations in a single sample [84]. Real-time PCR assays in one study showed a detection sensitivity of 5%, while dPCR methods could detect SNPs present in as little as 0.1% of a viral population [84]. Consistent with these findings, dPCR has shown a higher sensitivity for SNP detection compared to qPCR and greater sensitivity for mutational abundance in influenza resistance studies [22, 84].

## Amplification Inhibition

Over time, as nucleic acid extraction methods have improved, the challenge of PCR inhibition has diminished. However, it remains a challenge in clinical testing and can vary in degree among samples, patients, and matrices. As an endpoint method, dPCR might be expected to show less susceptibility to reaction inhibition, and several studies have confirmed this [9, 10, 85]. In one such study, samples were treated with substances known to cause inhibition of qPCR assays for CMV detection, including serial dilutions of SDS, EDTA, and heparin [9]. Digital PCR tolerated the addition of SDS and heparin better than qPCR; however, this tolerance was not noted for EDTA, indicating that while dPCR may be more resistant to PCR inhibition than real-time methods, the degree of resistance for both techniques is inhibitor dependent [9]. Similar findings were noted in a study comparing dPCR to qPCR and qLAMP for CMV detection and quantification [85]. Digital PCR was better able to tolerate addition of ethanol and plasma compared to qPCR but showed inhibition with the addition of 3.5 mM K2 EDTA [85]. The authors postulated that K2 EDTA

inhibited PCR by increasing molecular dropout, a phenomenon in which the template is present but the reaction does not occur or produce enough amplification to be detected by the instrument, and noted that other studies have also observed that inhibitors can exhibit variable effects on dPCR compared to other methods [85–87]. Another study, comparing dPCR to qPCR for detection of CMV and ADV in stool samples, showed dPCR had increased precision and equal sensitivity compared to qPCR in this typically inhibition-prone matrix, without the need for sample dilution [10]. Similarly, inhibition was seen using qPCR but not with dPCR for the quantitation of Shiga toxin-producing *E. coli* in fecal samples [14].

## Limitations and Perspectives

While promising in many respects, dPCR has many limitations which may hinder its adaptation for use in the clinical microbiology lab. Like many technologies, its initial development was aimed at research applications, many of which focused on its value for cellular copy number determination in molecular genetics work. As such, systems have not been designed with the robustness, throughput, or ease of use desirable for routine clinical testing. Many early systems required extensive specimen manipulation, not conducive either to efficient workflow or to an optimal level of contamination control. Most platforms have been limited to only two detection channels, and disposables are often expensive. Reagents may be platform specific, reducing flexibility for the use of available assays.

The low limit of detection afforded by dPCR can come with complications, primarily in determining thresholds for positivity and the possibility for false-positive results [24, 38, 88]. However, the propensity for false positives seems to be both assay and platform dependent [88]. Because some instruments have a fixed input volume, this may limit assay sensitivity compared to real-time methods where larger input sample volume can be utilized. Another primary consideration for implementation of dPCR over other assays is the expected dynamic range of target. As the upper limit of is directly dependent on partition number, most currently available dPCR systems only have a dynamic range of four orders of magnitude, compared to the dynamic range of qPCR, which is dependent primarily on the availability of calibration material in high concentrations. However, dPCR systems continue to be refined, and many of these limitations can be expected to be addressed. At least one system claims ten million partitions. With the difficulty of obtaining quantitative standards for some targets, this may provide a dynamic range exceeding current real-time methods.

The analysis of dPCR data can also affect its precision and accuracy. Despite many advantages, bias may still exist, as the technique relies on the assumption that the sample has full random distribution, that there is a clear differentiation between positive and negative partitions, that there is no variation in partition size, and that all template molecules in all partitions are amplified [88]. Some data analysis procedures have been developed to account for bias in dPCR experiments, though

further research is still needed before dPCR can supplant other molecular methods in the clinical microbiology lab.

Despite some limitations, dPCR is a promising methodology, with a wide range of potential applications in the clinical microbiology laboratory. Many current assays can be directly adapted to dPCR, affording potentially improved clinical assay performance and a powerful new tool for standardization and quality assurance. With increasing cost-effectiveness of dPCR systems, increased automation and user-friendliness, and increased throughput, dPCR may find further applications over time and an expanded role in clinical diagnostics.

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# Innovations in Antimicrobial Stewardship

**Abhijit M. Bal and Ian M. Gould**

## Introduction

Antimicrobial resistance is a growing problem that is fast approaching a point of no return. Despite the steady increase in resistance and a general consensus about the significance of this problem, antimicrobial overuse continues to go unchecked. An additional difficulty is the lack of new antimicrobial agents in the pipeline although some progress has been made to provide a stimulus to the industry in order to facilitate research and development in this field [1]. However, new agents are likely to be expensive at a time when healthcare budget is a crucial issue for most governments. In this context prudent use of antimicrobial agents under the umbrella of antimicrobial stewardship (AMS) attempts to minimize the damage. As a result, AMS is the buzz acronym in modern healthcare delivery. AMS received a boost when the challenge to find rapid and accurate methods of diagnosing bacterial infections was supported by the longitude prize.

## A Tradition in Need for Change

AMS has traditionally focussed on developing a cooperative network between the laboratory, the pharmacy, and the clinicians aided by the hospital antimicrobial management teams. Over the years, many hospitals have developed guidelines to streamline antimicrobial usage. An important principle of the guidelines is

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antibiotic de-escalation or rationalizing following the availability of laboratory results in relation to various infections. While sound in principle, the overall system is not efficient for various reasons. Only a minority of patients in the hospital have laboratory-proven infection, and so infections are over-suspected but underdiagnosed in terms of etiology even when the underlying pathology is not in doubt. The time taken to generate a definitive report in the traditional microbiology laboratory that relies on culture techniques often defeats the very purpose of AMS. In the absence of committed antimicrobial management teams on the ground, AMS does not figure high on the list of the clinical teams. The need for an overall AMS program may sometimes appear to conflict with that of the individual patient. It follows that design and implementation of a formal AMS program will benefit from technological improvements in the various pathways that contribute towards such a program. These are summarized in Box 1.

(a) Rapid diagnostic modalities and improvement in reporting.
(b) Novel educational tools.
(c) Broadening of the base of deliverers for the program.
(d) Introduction of programs specifically designed for various units.
(e) Improvement in communication between the various arms of the program.

In this chapter we highlight the newer technological interventions and innovations that have the potential to have a positive impact on AMS, thereby reducing the unnecessary use of antimicrobials in modern medicine.

## The Modernizing of the Laboratory

The laboratory occupies a nodal position in driving an AMS program. Morency-Potvin and colleagues have argued for a pivotal role of the laboratory in the "6 Ds of stewardship," namely, guiding the clinician with the help of rapid testing to establish etiology (diagnosis), prioritization of invasive cultures (debridement/drainage), providing guidance on the use of appropriate agents (drug), guidance on the appropriate dose and duration, and facilitating de-escalation based on authorized reports [2]. An important step in achieving the desired results of an AMS program is rapidity of testing. These could include rapid diagnostics to identify organisms with the help of matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and perform antimicrobial susceptibility, the use of non-culture-based methods such as molecular assays, and the use of biomarkers, e.g. procalcitonin.

Morgan et al. identified "diagnostic stewardship" as an important component of antimicrobial management that improves clinical behaviour [3]. The elements of diagnostic stewardship can be further classified into pre-analytic, analytic, and post-analytic streams. Limiting diagnostic tests to only the patients with symptoms is the first step for curbing unnecessary prescriptions. Emphasis on rapid diagnostics in the analytic phase would improve the turnaround times. As an adjunct, the use of biomarkers (e.g. procalcitonin) would help in understanding the significance of

culture results. Appropriate interpretation of test results (e.g. differentiating colonisation from infection) and suppression of broad-spectrum antibiotics fall within the remit of post-analytic stream.

Direct testing for bacterial detection and antimicrobial susceptibility has been shown to improve prescribing. Rivard and colleagues compared the antibiotic usage before ($n = 456$) and after ($n = 421$) the implementation of rapid microarray assay (Nanosphere Verigene ®)-based detection of Gram-negative bacteria from blood culture. Median time to switch to effective therapy was significantly reduced in the microarray group (8.8 h vs. 24.5 h, $p = 0.034$). The mortality rates between the two groups were similar, but the median length of stay in the hospital was reduced in the microarray group (7 days vs. 9 days, $p = 0.001$) [4]. The rapid microarray system takes advantage of the ability to detect a large number of DNA sequences in a single experiment [5]. While still based on the principles of nucleic acid hybridization, DNA microarrays are ideally suited for the detection of thousands of genes in comparison to real-time polymerase chain reaction (PCR) which detects fewer numbers of genes. PCR relies on amplification of a single strand of DNA which results in the generation of thousands of copies of the desired sequence following thermal cycling. Microarray on the other hand detects transcriptional activity by subjecting RNA to reverse transcriptase activity leading to the production of cDNA copies followed by binding of the fluorescent-labelled fragments to complementary oligonucleotides.

The MALDI-TOF technology has revolutionized the clinical diagnostics in routine laboratories. The sample for analysis is mixed with an organic energy-absorbent compound termed matrix. As the matrix dries, it also causes crystallization of the sample. This mixture is subjected to ionization with a laser beam leading to the production of protonated particles which vary in their mass-to-charge ratio. These particles are analysed with the help of TOF analysers based on the fact that the time required for their flight towards a potential depends upon the mass-to-charge ratio. Kock and colleagues improvised on their routine MALDI-TOF-based diagnostics by attempting to identify the blood culture pathogen from early growth rather than waiting for the cultures to mature. The attempted early detection led to faster identification of species (188 min vs. 909 min with conventional diagnostic techniques). The authors were able to demonstrate an improvement in prescribing in 72% of patients [6].

Using GeneXpert methicillin-resistant *Staphylococcus aureus* (MRSA)/SA SSTI assay (Cepheid CA), Trevino et al. identified 5/6 (83.3% sensitive) positive and 89/94 (94.7% specific) negative MRSA specimens, with a high negative predictive value of 98.9%. The assay also correctly detected 3/3 (100% sensitive) positive and 90/97 (92.8% specific) negative methicillin-susceptible *S. aureus* (MSSA) specimens, again with a high negative predictive value (100%). In their study, presuming that the discontinuation of vancomycin and linezolid occurred a day after a negative PCR result, the utilization of these antibiotics was potentially reduced by 68.4% and 83%, respectively, when measured against the usage of these antibiotics in the respective cohorts [7]. The GeneXpert MRSA assay is based on real-time PCR which is based on the identification of staphylococcal protein A gene along with

detection of *mecA* gene and the junction between SCC*mec* and the remainder of the staphylococcal genome [8].

Rapid antimicrobial susceptibility testing with digital time-lapse microscope system (oCelloScope system) can also reduce the turnaround time. The system was found to have 96% overall agreement in the context of antimicrobial resistance profile for reference strains; clinical isolates, including multidrug-resistant isolates; and isolates from positive blood cultures. AST of clinical isolates ($n = 168$) demonstrated 3.6% minor, 0% major errors, and 1.2% very major errors of the oCelloScope system in comparison to conventional susceptibility testing. The net average time-to-result was 108 min which could lead to faster rationalizing of antibiotics [9]. The automated time-lapse microscopy is based on the principle of scanning simultaneous images at each time point resulting in measurement of real-time bacterial growth [10]. As applied to the specific situation of antimicrobial susceptibility, the digital time-lapse microscopy is able to detect the formation of early "microcolonies". For example, a sample from positive blood culture bottle is allowed to come in contact with freeze-dried antibiotics in a microwell, and after a short period of incubation, bacterial microcolonies are detectable by microscopic imaging [11]. The crucial determinant for success with this technology is providing the right environment to keep the cells alive by ensuring the supply of nutrients, maintaining adequate pH and temperature, and reducing phototoxicity and fluid loss by evaporation. Details of these aspects and also the requirement for the necessary software and hardware have been extensively discussed in the literature [12].

Rodel and colleagues applied the loop-mediated isothermal amplification (LAMP) assay eazyplex® MRSA assay that detects *S. aureus* and *S. epidermidis* along with the *mecA* and *mecC* genes in addition to two in-house assays for the detection of streptococci, enterococci, the *vanA* and *vanB* genes, *Pseudomonas* spp., *Enterobacteriaceae*, and the *bla* $_{\text{CTX-M}}$ gene family. The overall concordance between the rapid testing assays and the conventional methods was 87.5% with 100% concordance in relation to antibiotic susceptibility. Test results were obtained within 30 min which could have a significant impact on AMS. The major difference between LAMP assay and PCR is that LAMP assays are isothermal, i.e. the reaction proceeds at a single temperature (and hence thermal cyclers are not required). This is due to the fact that the assay uses multiple primers that bind to a number of regions on the target nucleic acid sequence. In addition, LAMP assays are rapid compared to PCR, tolerant to the matrix inhibitors, and yield a greater product volume [13].

In specific settings, e.g. paediatric units, Ray et al. prospectively investigated the utility of FilmArray Blood Culture Identification Panel (FA-BCIP) (which is a multiplex PCR-based system) that detects 24 pathogens within 60 min. Out of the 117 positive blood cultures that were subjected to FA-BCIP, 74 (63%) grew clinically significant organisms, while the remaining 43 (37%) were adjudged as contaminants. FA-BCIP results altered clinical management in 63 (54%) of the 117 bacteraemic episodes. Antimicrobials were commenced or altered in 23 (19%) episodes and de-escalated or discontinued in 29 (25%) episodes. Ten children were discharged

earlier than otherwise expected saving 14 bed-days [14]. FA systems are generally based on multiplex or nested PCR with targets for various bacterial and fungal pathogens. As evident, the technology involves amplification of target sequences unique to the pathogens it is designed to capture.

The overall usefulness of rapid testing is still undergoing evaluation. While these results are encouraging, it is necessary to exercise caution in terms of the usefulness of rapid testing unless supported by an AMS program. Cosgrove et al. demonstrated that the peptide nucleic acid fluorescence in situ hybridization (PNA FISH) assay that identifies organisms growing in blood cultures within 30–90 min from the time of Gram-positive stain was still not sufficient to influence therapy optimization, length of hospitalization, and hospital mortality. Thus one of the important roles of the stewardship team is to act as a link between the laboratory and the hospital wards so as to facilitate interpretation of the results and continuous education with the aim to improve prescribing behaviour.

## The Modern Tools and Gadgets

One of the drawbacks of AMS program is lack of participation of healthcare workers who are at the forefront of prescribing. Hospitals encourage the healthcare workers to access educational websites by hyperlinking both internal and external links. Dissemination of these links is often by way of emails. However, it has been shown that official emails are poorly accessed as compared to social media websites. In a study that promoted hand hygiene, Pan et al. found that their education video was accessed significantly more often on Facebook (38%) and YouTube (20%) compared to links provided on official hospital emails (12%) [15]. To take advantage of social media, Pisano et al. invited healthcare workers (residents in internal medicine) to participate in their AMS program. After an initial survey that evaluated their ability to engage with the program, participants received tweets and posts with educational content. A comparison of the pre- and post-intervention survey showed that the median antibiotic prescribing knowledge increased from a score of 12 (range 8–13) to 13 (range 11–15) ($P = 0.048$), and the residents felt more accustomed to accessing the stewardship website and relevant clinical pathways. This led the authors to infer that social media is a useful tool for AMS programs [16]. Social media is not without perils: Conway and Knighton's commentary on Pisano's investigation warns about the risks associated with overreaching the accepted boundaries in relation to social media [17]. In order to effectively sell AMS programs, the developers need to engage with the end-users and stakeholders at earlier stages of development and involve them during the process. In their position paper, Beerlage-de Jong and colleagues have provided an excellent summary of how to implement technology in the form of a toolkit comprising of information apps, decision-making pathways, apps that support administration of antibiotics, and apps for audits, alerts, and education [18].

## Decentralization of the Program

Another innovative mechanism to improve the effectiveness of the AMS program is to decentralize it providing ownership of the strategy to specific areas where its need is most felt. These include intensive care units, high dependency units, and outpatient therapy units but could also include paediatric and general adult wards. Transfer of responsibility to deliver the program may be associated with improved outcomes although there are limited comparative data to support this strategy, or whether in a close-knit environment, additional input in relation to antibiotic management improves the outcome. Dinh and colleagues compared the quality of antibiotic prescriptions in a 1-year period before the implementation of an AMS program (November 2012 to October 2013) with a 1-year period following its implementation (June 2015 to May 2016). A total of 34,671 and 35,925 consultations took place at their emergency department unit, of which 25,470 and 26,208 were outpatient consultations in the respective periods. Prescriptions for antimicrobial agents were generated in 769 (3.0%) and 580 (2.2%) consultations, respectively ($p < 0.0001$). There was significant improvement in adherence to guidelines with 484 (62.9%) non-compliances recorded before and 271 (46.7%) after the implementation of the program ($p < 0.0001$). There was reduction in unnecessary antimicrobial prescriptions (197 [25.6%] vs. 101 [17.4%], $p < 0.0005$), prescribing of antibiotics with inappropriate spectrum (108 [14.0%] vs. 54 [9.3%], $p = 0.008$), and length of treatment (87 [11.3%] vs. 53 [9.1%], $p > 0.05$) [19]. Not all AMS programs demonstrate beneficial outcome particularly in units which already practise responsible prescribing. Trupka et al. compared the routine antibiotic management (RAM) with an enhanced antimicrobial de-escalation program in a crossover trial. Of the 283 patients with suspected pneumonia on mechanical ventilation, 139 (49.1%) were allocated in the RAM group and 144 (50.9%) in the enhanced group. Clinical evaluation demonstrated an early treatment failure in 33 (23.7%) and 40 (27.8%) patients, respectively ($P = 0.438$). In the remaining patients, antimicrobial de-escalation occurred in 70 patients in each group (66% and 67.3%, respectively ($P = 0.845$)). There was no difference between groups in relation to the total antibiotic days (7 days in both groups), hospital mortality (25.2% vs. 35.4% [P = 0.061]), or duration of hospital stay (12 days vs. 11 days, $P = 0.918$) [20]. Thus the value of any incremental addition to an existing program needs to be balanced against the availability of resources.

## Broadening the User Base

The focus of AMS is the hospital clinician, but lately there has been an appreciation that the program needs to broaden its reach to include other healthcare workers. The new Medication Management standard published by the Joint Commission emphasizes interdisciplinary engagement by expanding the program to nursing and additional relevant staff. Monsees's review on this subject identified the need for education and training of nursing staff in relation to antimicrobial use [21]. In this

regard data from Scotland shed light on areas in need of improvement. Only 36% of nursing staff ($N = 900$) in the published data had sufficient knowledge of AMS, and only a fifth of respondents were familiar with this term [22]. Over the years, with increasing specialization of medicine, nurses feel less empowered in decision-making, while specialized clinicians have less understanding of AMS. In order to break this barrier, an inclusive strategy of AMS which goes beyond the physician might be the answer. Such a strategy will also help fill in the needs of AMS in hospitals which lack sufficient number of clinicians or where these roles are increasingly being taken by advanced practitioners. Olans et al. highlighted the input of nursing staff in the entire patient journey in the hospital including their crucial role in documenting drug allergies, and yet presently they have a limited role in AMS [23]. A further argument is the nursing role in reducing infections including central vascular catheter infection, catheter-associated urinary tract infections, and MRSA and *C. difficile* infection by improved infection control measures which in totality bring about quality improvement, an essential auditable component of modern medicine. It is important to emphasize that the role of nurses is not usually that of prescriber (although increasingly nurses are taking up such additional roles) but they can be effective facilitators of AMS. Gillespie and colleagues demonstrated that following an educational program, nurses felt empowered to question intravenous antibiotic prescriptions (with a rise from 14% to 42%) with an improved awareness of antibiotic resistance [24]. Similarly, pharmacist-driven AMS program have been shown to be successful in reducing the duration of hospitalization leading to a significant decline in the consumption of carbapenems, quinolones, and echinocandins thereby reducing the cost of therapy [25]. Additionally, Brink and colleagues have argued for the involvement of non-infection specialists in limited resource settings [26]. One more crucial aspect is the involvement of patients by way of information. There are obvious gaps in this area. Beardmore et al. noted the dichotomy between what the public is told in relation to completing the course of antibiotics and the complete lack of data in this regard [27].

## Specialized Programs

### *Antifungal Stewardship (AFS)*

AFS has generated a great deal of interest as a specialized program within the overall remit of AMS [28]. AFS has some unique characteristics that are different from antibiotic stewardship. These are:

1. Invasive fungal infections are usually monomycological and seldom mixed fungal infections unlike bacterial sepsis that can often be polymicrobial. This facilitates targeted therapy and rationalization of therapy.
2. Unlike antibiotics, development of resistance and its spread is not a major outcome of indiscriminate antifungal use when compared to bacterial infections. Fungi of medical interest do not possess plasmids. However, recent investigations have highlighted the emergence of resistant fungal infections.

3. Antifungal susceptibility is not routinely offered in centres other than reference laboratories. Rationalization of therapy is often based on predictable rather than actual susceptibility pattern.
4. There is more emphasis on cost savings in AFS as many agents are still under patent.
5. Guidelines for empirical, pre-emptive, and definitive therapy have been developed over the years with much more emphasis on diagnostic-driven treatment pathway.

AFS aims to limit prophylactic and empirical use of antifungal agents, reduce cost of therapy, and streamline therapy in order to improve management. The impetus for AFS is the recent availability of antifungal agents including newer triazoles (voriconazole, posaconazole, and isavuconazole) and echinocandins (anidulafungin, caspofungin, and micafungin) which allows early effective prophylaxis and therapy followed by de-escalation.

Invasive fungal infections are difficult to diagnose as their presentation is often non-specific. Moreover, invasive fungal infections occur mainly in patients who are severely immunocompromised. These patients may also have concurrent bacterial sepsis that clouds the symptomatology. Fungi, particularly moulds, are comparatively slow growing, and so there has been interest in testing for biomarkers. A combination of the serum galactomannan (GM) assay and PCR-based detection of *Aspergillus* DNA in blood was found to be useful in rapid diagnosis of invasive aspergillosis (IA) [29]. The *Aspergillus* lateral-flow device (LFD) is a highly specific point-of-care test for rapid diagnosis of aspergillosis [30]. The *Aspergillus* LFD is based on the principles of immunochromatography. The assay, which is highly specific, uses JF5, a monoclonal antibody which binds to an extracellular glycoprotein released by the fungus during the phase of active growth [31]. Thus, the assay differentiates between the active phase of multiplication and the sporulating phase. As the test is based on immunochromatography, it utilizes a secondary molecule such as goat anti-mouse fluorescent conjugate for the detection of JF5 monoclonal antibody obtained from mouse hybridoma cell line secreting the protein [30].

Maertens et al. have alluded to the newer biomarkers for the diagnosis of IA including the electronic nose (eNose) or the detection of bis(methylthio)gliotoxin (bmGT) [32]. The eNose is an exciting noninvasive diagnostic advancement which can be applied to several clinical situations. It has the ability to discriminate between mixtures of volatile organic compounds with the help of sensors. The technology uses a number of metal oxide sensors. The sampled air (e.g. from the breath) is directed towards these sensors which produce a characteristic response to the substance under investigation. The pattern of response of the sensors, which can be measured with a scatterplot, identifies the substance. The technology has also been validated for non-infectious conditions such as asthma and chronic obstructive pulmonary disease [33].

AFS has been successfully applied to patients with candidaemia. Following a protocol-guided switch from echinocandin to fluconazole, investigators were able to de-escalate treatment in 70% of patients at a median duration of 5 days leading to a cost saving in excess of £ 2000 per successful de-escalation [34]. Antibiotic

stewardship programs can by themselves influence AFS. Reduction in unnecessary use of antibiotics can lead to a reduction in the incidence of candidaemia, thereby limiting the use of antifungal agents for targeted treatment.

AFS program is not without its unique challenges. These include limited appreciation of the program, funding for personnel and laboratory equipment, lack of availability of rapid testing in hospitals, and resistance from prescribers. Because the opportunities for AFS are fewer, the sustenance of the program requires a higher degree of personal motivation. As resistance to antifungal agents is lower in scale compared to antibiotic resistance in bacteria, the main driver of the program may be perceived to be the cost of antifungal agents, and so with reducing cost of antifungal agents as a result of patent expiry, the need for the program may be less felt by hospital administration. It is important to emphasize that resistant species of fungi are getting increasingly common as evidenced by the recent outbreak of *Candida auris* in a major tertiary London hospital [35].

## *Antibiotic Heterogeneity*

Antibiotic heterogeneity is a specialized tool that has been studied extensively in mathematical models but only infrequently in clinical practice. Antibiotic cycling, also known as antibiotic rotation, involves withdrawal of certain antibiotics from clinical use and their substitution by agents with a different mechanism of action for a specified duration after which the antibiotic classes are rotated. Antibiotic mixing is heterogeneous at the level of individual patients rather than limited by specific time periods. In addition, supervised implementation of antibiotic heterogeneity has led to a novel concept known as periodic antibiotic monitoring and supervision (PAMS). While broadly resembling antibiotic cycling in its principle, PAMS is more in real-time because the ongoing pattern of resistance informs the use and restriction of antibiotics in the subsequent cycles. Takesue and colleagues have shown a reduction in the incidence of resistance in *Pseudomonas aeruginosa* following implementation of this strategy [36], but the benefits of PAMS have not been reproducible in other studies [37]. This is in line with more recent work demonstrating that heterogeneity is not a strategy with consistently favourable outcomes and can sometimes cause harm [38]. The Saturn project showed a lack of statistically significant difference in the prevalence of resistance during antibiotic cycling or mixing leading Beardmore and colleagues to conclude that personalized intervention programs that focus on individual patients perform better than cycling and mixing interventions [27].

## *Antibiotic Quotas*

The Cochrane review on AMS [39] and a recent systematic review and meta-analysis [40] confirm that many of the most successful hospital-based interventions to control antimicrobial resistance have involved targeted reduction in key classes of

antibiotics specifically associated with MRSA, *Clostridium difficile*, and multidrug-resistant Gram-negative bacilli. These classes include cephalosporins, quinolones, carbapenems, and macrolides. An important development in this area is non-linear time series analysis which allows definition of thresholds of antibiotic use. Over a defined time period, limitation of use of such classes, in hospitals or communities, to levels below a threshold avoids exacerbation of specific resistance problems. Two recent publications show the application of this concept to the successful control of MRSA and *C. difficile* [41, 42] and describe the quotas of key agents which may be applicable to other hospitals and communities, although such quotas are likely to vary depending on variables such as the local resistance issues and their dynamics, patient vulnerability, overcrowding, and infection control standards. Such data argue for as much diversity in antibiotic class prescribing as possible, limiting use of key classes of antibiotics, much as we ascribe carbon quotas to countries in the battle against global warming.

AMS has covered a lot of ground from the time that its need was first realized, but there are major challenges ahead. This chapter has focused on various innovations that have made an impact on performance aspects of AMS in hospitals only. The future of AMS lies in diversification that allows ownership of the program by frontline healthcare staff as a quality improvement strategy aided by technology that reduces the gap between prescribers and program managers. Community and non-medical use too need to be addressed as they comprise the vast majority of world-wide use, albeit perhaps not the major issues in resistance. Hospital stewardship has been most successful in reducing the use of key classes of antibiotics (squeezing the balloon), but as in community and non-human use, overall reduction in total use needs to be a priority.

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# Interpretation and Relevance of Advanced Technique Results

**Charles W. Stratton and Yi-Wei Tang**

## Introduction

Advanced techniques in the field of diagnostic microbiology have made amazing progress over the past 25 years due largely to a technological revolution in the molecular aspects of microbiology [1, 2]. In particular, rapid molecular methods for nucleic acid amplification and characterization combined with automation in the clinical microbiology laboratory as well as user-friendly software and robust laboratory informatics systems have significantly broadened the diagnostic capabilities of modern clinical microbiology laboratories. Molecular methods such as nucleic acid amplification tests (NAATs) rapidly are being developed and introduced in the clinical laboratory setting [3, 4]. Indeed, every section of the clinical microbiology laboratory, including bacteriology, mycology, mycobacteriology, parasitology, and virology, has benefited from these advanced techniques. Because of the rapid development and adaptation of these molecular techniques, the interpretation and relevance of the results produced by such molecular methods continues to lag behind. The purpose of this chapter is to review, update, and discuss the interpretation and relevance of results produced by these advanced molecular techniques.

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## The Use of Molecular Assays for Diagnosing Bloodstream Infections

Bloodstream infections have long been recognized as among the most severe manifestations of bacterial disease and were first described in 1940 by Keefer in his sentinel paper *The Clinical Significance of Bacteremia* [5]. The importance of the rapid diagnosis of bloodstream infections is not argued and serves to illustrate many of the issues involved in the interpretation and relevance of advanced techniques in diagnostic microbiology.

By 1940 when Keefer pointed out the clinical relevance of bacteremia, blood cultures were well established for the evaluation of febrile patients [6]. Since then, the techniques and pitfalls for blood cultures as well as the clinical implications of positive blood cultures have been well documented [7–10]. Not surprisingly, molecular and other non-culture-based methods for the rapid diagnosis of bloodstream infections continue to be widely evaluated [11–20]. These studies along with earlier blood culture studies have illustrated some important points regarding the limitations of molecular assays for diagnosing bloodstream infections, which are summarized in the following sections.

### *Interpretation of DNAemia*

The detection of circulating microbial DNA (i.e., DNAemia) is, per se, a new diagnostic parameter that may or may not represent the presence of viable microorganisms in blood [12, 21]. For example, interpretation of DNAemia with coagulase-negative staphylococci is problematic due to a false-positive rate that ranges from 60% to 80% [10, 22]. In contrast, interpretation of DNAemia with *Ehrlichia* species is not a problem due to a true-positive rate of 100% [23]. Interpretation of DNAemia has also been a problem in some studies where DNAemia is detected by PCR but not by blood cultures [21]. A number of these “false-positive” PCR results have been considered clinically significant, based on either retrospective chart review or subsequent isolation of the pathogen from other relevant clinical specimens [12, 24–29]. Clearly, the continued clinical investigation of microbial DNAemia during sepsis and other critical illnesses is needed and will provide a better understanding of the biology of the microbial circulating DNA that underpins such molecular diagnostic techniques [12, 21, 30].

### *Molecular Detection of Resistance Determinants*

Another important issue for molecular diagnostic techniques is the need for molecular detection of resistance determinants [12, 21]. Antimicrobial susceptibility testing is recognized as important for confirming susceptibility to chosen empirical

antimicrobial agents as well as for detecting resistance in individual microbial isolates [31]. Current methods for antimicrobial susceptibility testing continue to be based for the most part on the detection of microbial growth or lack of growth in the presence of the antimicrobial agent being tested [32, 33]. The direct detection of resistance genes by molecular methods such as PCR or molecular probes to date has limitations due to the fact that relatively few resistance genes are firmly associated with phenotypic resistance [31–33]. For example, resistance genes associated with phenotypic resistance that can be found in Gram-positive cocci include *mecA*, *vanA*, and *vanB*. In contrast, the lack of consensus sequences among acetyltransferases and adenyltransferase genes from Gram-negative bacilli makes the molecular detection of aminoglycoside resistance difficult. Although molecular methods for antimicrobial susceptibility testing are improving, phenotypic methods for determining the level of susceptibility of bacterial isolates to antimicrobial agents continue to remain clinically relevant.

## *Volume of Blood Tested*

The volume of blood cultured is known to be an important variable in blood cultures because the number of microorganisms in blood may be small [34–36]. Typically in adults, there are fewer than 10 CFU/ml, and there may be less than 1 CFU/ml. In septic neonates, there is a sizeable subset with less than 4 CFU/ml [36]. Clearly the volume of blood tested by molecular methods will also be important [11, 16]. Moreover, the Poisson distribution of these microorganisms is such that they are not evenly distributed [37, 38]. This increases the likelihood that sampling a small volume of blood will miss a microorganism that is causing sepsis. Volume-related issues may explain the lower sensitivity seen with a molecular method (66.7%) than seen with conventional blood cultures in a study of neonatal sepsis [28]. The Poisson distribution may explain the moderate concordance between blood cultures and a molecular method reported in a study of postsurgical sepsis in adults [24].

## *Contamination of Blood Samples*

The sample of blood collected to assess bacteremia and/or fungemia, whether this analysis is done by blood culture or by a molecular method, can be contaminated with microorganisms from the skin during venipuncture, from transient bacteremias, and/or from indwelling vascular devices if the blood is obtained from such a device [8]. False-positive blood cultures due to contamination have been recognized as a troublesome issue for decades, and such contamination will be no less important for molecular methods [11, 13].

## The Use of Molecular Assays for Diagnosing Tuberculosis

Tuberculosis continues to be one of the most important public health issues in the world [39–41]. Tuberculosis results in approximately 1.7 million deaths each year, and the number of new cases worldwide is estimated at more than ten million; this is higher than at any other time in history [40]. Yet control of this treatable infection has been handicapped until recently by the lack of new diagnostic tests for the detection of *Mycobacterium tuberculosis* as well as by drug resistance [40, 42]. The development of molecular assays for the detection of *M. tuberculosis* as well as simultaneous detection of resistance to isoniazid and/or rifampin promises to greatly assist TB control efforts although there are important limitations of these molecular methods that must be understood when interpreting the results and considering the relevance of such molecular techniques [42–44]. Indeed, none of these molecular methods eliminates the need for mycobacterial cultures, and all require a laboratory infrastructure that can accommodate molecular testing. Specific limitations of these molecular methods in both interpretation and relevance will be summarized in the following sections.

### *Limited Sensitivities*

There currently are a number of different molecular assays for detecting the presence of *M. tuberculosis* in sputum. These include PCR, transcription-mediated amplification, loop-mediated isothermal amplification [45], simultaneous amplification testing [45], and Xpert MTB/RIF [45–47]. In comparison to mycobacterial culture, these molecular assays possess sensitivities approaching or exceeding 90% [45]. In general, these molecular methods work better with smear-positive than with smear-negative sputum specimens; none are more sensitive than mycobacterial cultures. The sensitivity for patients with smear-negative sputum can be increased by the use of bronchial aspirates [48] or bronchial lavage fluid [49] but is still not as sensitive as mycobacterial cultures.

### *Assessment of Therapeutic Efficacy*

NAATs detect microbial organism-specific nucleic acids; therefore, a positive NAAT result can result from both live and dead microorganisms, which is particularly true for mycobacteria that have thick, waxed cell walls. The best example of this is the detection of *M. tuberculosis* DNA in sputum where the dead microbial pathogen DNA can remain un-degraded due to the fatty acid-rich cell walls [50, 51]. Unlike the results of a function-based testing method, such as mycobacterial cultures, in the clinical setting, a positive PCR result after antituberculosis therapy does not necessarily mean treatment failure. The application of mRNA-targeted NAATs

has been demonstrated for monitoring of tuberculosis therapy. Anti-TB therapy regimen selection is largely empiric. Treatment may not be modified until weeks or months later as results of antimicrobial susceptibility tests become available. Because the half-life of bacterial mRNA is extremely short compared to rRNA or genomic DNA, molecular assays that target mycobacterial mRNA better reflect mycobacterial viability. The ability of mRNA-based assays to distinguish viable from nonviable organisms has demonstrated that such assays are useful in monitoring the efficacy of anti-TB therapy [50, 51].

## *Molecular Detection of Resistance Determinants*

There currently are a number of different molecular assays for detecting gene mutations associated with resistance to a particular antituberculosis drug [46, 52, 53]. There are always gaps between basic research and clinical application as some of the drug resistance mechanisms remain unknown, while new resistance-related mutations are emerging. In addition, all molecular assays basically include a DNA amplification step and are categorized by the manner in which the amplified DNA is detected except for sequencing, which has some distinct advantages over the other methods. None of these methods, including sequencing, are able to detect all resistant strains although sequencing comes the closest to doing so. The major limitation of these molecular methods, except sequencing, is that they detect only known mutations in a defined site or region, as their design is dependent upon known mutations. The advantage of sequencing for molecular detection of mutations of drug resistance can be seen by a report from the Centers for Disease Control and Prevention [43]. This study used DNA sequencing to detect resistance to the first-line antituberculosis drugs isoniazid, rifampin, pyrazinamide, and ethambutol and to the second-line drugs amikacin, capreomycin, kanamycin, ciprofloxacin, and ofloxacin. The molecular data were compared to phenotypic data. Sensitivity and specificity values for the first-line and second-line drug loci were, in general, excellent and supported the use of DNA sequencing to detect drug resistance in the *M. tuberculosis* complex.

## *Misidentification*

Although uncommon, misidentification has been reported with molecular assays for tuberculosis [54, 55]. In one of these reported cases [54], a patient presented with inguinal lymphadenopathy as well as erythema nodosum-like lesions on his legs and forearms. A biopsy of an enlarged inguinal lymph node demonstrated caseating granulomata and numerous acid-fast bacilli on Ziehl-Neelsen staining; a portion of this node was sent for mycobacterial culture and molecular analysis. In addition, a skin biopsy of a forearm nodule was done; this revealed acid-fast bacilli that were

morphologically typical of *Mycobacterium leprae*. A diagnosis of leprosy was made based on the clinical presentation and the skin biopsy results. However, the lymph node sent for mycobacterial culture and molecular analysis was positive by the Gen-Probe Amplified *Mycobacterium Tuberculosis* Direct (MTD) test. Although leprosy was still considered to be a correct diagnosis due to the clinical presentation and the skin biopsy findings, the possibility of this patient also having tuberculosis could not be ruled out until the culture results were known. Therefore, the patient was treated for both leprosy and tuberculosis until cultures at 7 weeks as well as additional PCR testing of lymph node material for *M. tuberculosis* were reported to be negative. A root cause analysis was done in order to investigate this misidentification. *M. leprae* culture material was obtained from the National Hansen's Disease Programs at Louisiana State University; these mycobacterial organisms were tested with the Gen-Probe MTD test and were positive at a concentration of $5 \times 10^5$ organisms per ml but were indeterminate at a concentration of $5 \times 10^4$ organisms per ml. The investigators concluded that a high concentration of *M. leprae* in a clinical specimen could lead to a false-positive result with the Gen-Probe MTD test [54]. *M. leprae* has also been misidentified as *M. intracellulare* by the COBAS AMPLICOR *M. intracellulare* test [55].

## The Use of Molecular Assays for Diagnosing Respiratory Tract Infections

There is no doubt that respiratory tract infections other than those caused by *M. tuberculosis* also are of considerable clinical importance. Lower respiratory tract infections continue to be a leading cause of death due to infectious diseases in the United States as well as worldwide [56]. Hospital-acquired pneumonia is considered to be one of the most difficult treatment challenges in infectious diseases in part because results of culture and antimicrobial susceptibility testing can take 48 h or longer [57]. Viral respiratory tract infections caused by pathogens such as the severe acute respiratory syndrome coronavirus (SARS-CoV) and novel A/H1N1 and A/H7N9 influenza virus can cause epidemic viral pneumonia in which some patients have respiratory failure with a significant risk of mortality [58]. Respiratory tract infections are also important in the ambulatory setting because of the documented overuse of antimicrobial agents in this patient population [59].

Despite the obvious clinical importance of respiratory tract infections, the diagnosis of lower respiratory tract infections has always been problematic due, in large part, to issues related to the optimal collection and evaluation of respiratory specimens. Post-mortem studies in the late 1890s and early 1900s then established the role of other microorganisms such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* in nontuberculous infections of the respiratory tract [60–62]. In 1902, the use of the Gram's stain was described [63]. The microscopic examination of sputum was followed by the introduction of sputum cultures for the diagnosis of bacterial pneumonia [64–66].

Of note in these early reports describing sputum cultures was the recognition that collection of the sputum was important. For example, Hastings and Niles in a 1911 publication point out that, "Exudates formed in portions of the respiratory tract that are normally sterile may be collected and treated in a way that will prevent contamination [65]." These investigators further define a "clean sputum, i.e., one containing only two or three types of bacteria and free from buccal squamous cells, and a dirty sputum, i.e., one containing a varied bacterial and fungoid flora and buccal squamous cells, are readily recognized on microscopic examination." They also state that, "A dirty sputum is not suitable for bacterial examination and should be discarded for a second or third clean specimen from the same patient." Leutscher opines in his paper that, "The patient should be instructed to expectorate into the bottle or dish only what he is certain comes from his 'boots,' and also be made to understand that very little is wanted, but that that little must be choice [66]." These astute observations remain relevant more than a century later.

Clearly, the pitfalls of collecting expectorated sputum specimens suitable for microscopic examination and cultures were recognized early in the twentieth century. In the 1960s, these pitfalls were again being articulated and addressed [67–72]. In particular, contamination by microorganisms present in the upper respiratory tract (i.e., nasal-oral-pharyngeal regions) was considered to be a major issue with expectorated sputum [73, 74]. Because of these pitfalls, a number of alternative methods have been used to obtain better sputum specimens. Bronchoscopy, although introduced early in the twentieth century and used on occasion for aspirating pus from larger airways [75], was not widely used for obtaining sputum for microscopy and culture until the 1970s when fiber-optic bronchoscopy became available [76]. Fiber-optic bronchoscopy also resulted in the use of bronchoalveolar lavage for diagnosing acute bacteria pneumonias [77]. Other methods adopted for obtaining uncontaminated sputum included transtracheal aspiration [68], percutaneous needle biopsy [69], and open-lung biopsy [67].

Despite these continued attempts to obtain appropriate sputum specimens that are more clinically relevant, the usefulness of sputum cultures has continued to be questioned in numerous reports [78–84]. Indeed, the Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults continue to recommend that pretreatment Gram stain and culture should be performed only if a good quality sputum sample can be obtained and quality performance measures for collection, transport, and processing of this sputum sample can be assured [85, 86]. It must be remembered that sputum collection is the "weakest link" in the "chain" of evidence that provides the etiologic diagnosis of pneumonia.

Assuming that sputum collection is done correctly, the next issue is making sure that any microbial pathogen present in the sputum can be identified. It is not surprising that molecular assays for the detection and characterization of microorganisms have rapidly emerged in the clinical microbiology laboratory as an important adjunct to traditional culture methods [87–89]. It was quickly realized that molecular assays such as NAATs offered significant advantages over conventional methods for the detection of *Mycoplasma pneumoniae* [90, 91], *Legionella* species [92, 93], and

*Chlamydia* species [94]; moreover, these three respiratory pathogens did not require concomitant susceptibility testing results from clinical isolates. Similarly, the advantage of NAATs for the laboratory diagnosis of pertussis was recognized very early [95, 96]; PCR testing is now considered by the CDC to be an important tool for diagnosis of pertussis especially in the setting of the current resurgence of pertussis disease as it can provide timely results with improved sensitivity over culture [97].

The inherent problems associated with the detection and identification of respiratory viruses by culture and/or serologic methods also resulted in the early application of molecular assays for rapid detection and characterization of respiratory viruses [98]. Both user-developed and commercial molecular methods have quickly evolved and now allow rapid identification of multiple common viral pathogens causing respiratory tract infections [99–101]. In addition to identification of viral respiratory pathogens, it was appreciated that rapid molecular assays would also offer significant advantages for diagnosing recognized bacterial pulmonary pathogens causing community-acquired pneumonia [56, 91, 102]. Indeed, initial studies in which rapid molecular assays were combined with conventional diagnostic methods have demonstrated that this approach considerably increased the etiological diagnosis of lower respiratory tract infections [103, 104]. This was especially true for patients with adequate collection of sputum [103]. Of interest was the observation that NAATs increased both the diagnostic and treatment costs [104]. Finally, the diagnosis of hospital-acquired pneumonia is another potential area where the use of rapid molecular assays for respiratory pathogens may prove useful [66]. Clinical trials are beginning to provide evidence that molecular assays for pneumonia as well as bloodstream infections and sterile site infections are useful for providing rapid diagnosis of infections in the critically ill [105, 106].

### *Sputum/Specimen Collection*

Clearly the same limitations of conventional sputum culture methods for diagnosing respiratory tract infections are also limitations for molecular methods. In particular, the collection of sputum continues to be the most important aspect for the diagnosis of lower respiratory tract infections even when molecular assays are used [57]. These new molecular methods will not guarantee that the microbiology laboratory will receive the optimal sputum sample to analyze.

### *Complexity of Pulmonary Microbiome*

Another important aspect of molecular assays for the diagnosis of respiratory infections is that these methods have begun to reveal the complexities of the pulmonary microbiome. Indeed, recent applications of molecular assays have revealed a more diverse microbiota than previously recognized in the airways of patients with

chronic pulmonary disease [107–109]. For example, comprehensive profiling of the airway bacterial communities was accomplished using a culture-independent microarray, the 16S rRNA PhyloChip, of a cohort of COPD patients requiring ventilatory support and antimicrobial therapy for exacerbation-related respiratory failure [110]. PhyloChip analysis demonstrated the presence of over 1200 bacterial taxa representing 140 distinct families, including many that were not previously detected in airway diseases. A core community of 75 bacterial taxa was noted in all patients; many of these microorganisms were known pathogens in airway diseases.

### *Colonization Versus Infection*

Given the fact that the pulmonary microbiome is more complex that previously appreciated, the obvious question then becomes which microorganisms are colonizing and which are causing infection. One might also ask if there is any real difference between colonization and infection in the airways. Molecular identification of bacteria in the lower airways of preterm infants has revealed that early bacterial colonization of the airways with diverse species occurs within the first 3 days of life of intubated preterm infants [111]. Such neonatal airway colonization with Gram-negative bacilli is associated with a cytokine response as well as with severe bronchopulmonary dysplasia [112, 113]. The etiologic role of neonatal colonization in children with non-cystic fibrosis bronchiectasis is unclear at this time [114, 115], but molecular methods are providing further insight into the pathogenesis of bronchopulmonary dysplasia in these infants [116]. Similarly, the etiologic role of bacterial colonization in the pathogenesis of chronic obstructive pulmonary disease is currently being elucidated with the assistance of molecular methods [107–110, 117, 118].

### *Simultaneous Detection of Multiple Pathogens*

The extreme sensitivity of molecular methods such as NAATs may result in simultaneous detection of multiple pathogens from sputum specimens. Detection of multiple pathogens in sputum by molecular methods has already been reported in community-acquired pneumonia where mixed infections were frequently seen: these most commonly were *Streptococcus pneumoniae* together with a respiratory virus [104]. These findings are not unexpected; a number of studies have reported an association between viral respiratory tract infections and invasive pneumococcal disease [119, 120]. Molecular diagnostic methods employed in other studies of respiratory tract infections have confirmed the etiologic role of viral respiratory tract infections and bacterial pneumonia [121–125]. For respiratory samples, cycle-threshold-value-based semiquantitative interpretation of qPCR results has been suggested. Etiological relevance is assumed if cycle-threshold values are low, suggesting high pathogen loads [126].

### *Accuracy of Assay Development*

An important issue for NAATs is whether the amplification products truly represent the target microorganism [103]. Molecular methods that employ DNA sequencing are often considered completely accurate with 100% sensitivity and specificity. This, unfortunately, is not the case. There are a variety of technical factors such as the influence of contaminating DNA from other sources on the sequencing template, the selection of the primers used for the amplification, the quality of the base-calling software, and the method used for compiling the "consensus sequence" from multiple forward and reverse reactions [102, 127, 128]. Inappropriately chosen gene targets and regions will result in false positives and negatives. The insertion sequence element IS*481*, found in several hundred copies in the *B. pertussis* genome, is frequently used as a target for *B. pertussis* detection and has a much greater analytical sensitivity than assays with single-copy target sequences, such as that of the pertussis toxin promoter [129, 130]. However, false-positive results have been reported due to the smaller copy numbers of IS481 existing in non-pertussis *Bordetella* species [131, 132]. The accuracy of assay development is often not appreciated by the non-molecular microbiologist or the clinician.

## The Use of Molecular Assays for Diagnosing Enteric Infections

Most acute diarrheal illnesses are self-limited or viral [133]. For afebrile patients who present with watery non-bloody diarrhea of less than 24 h duration, microbiologic investigation is usually unnecessary [133, 134]. In contrast, patients with a diarrheal illness lasting for more than 1 day, especially when the illness is accompanied by fever, bloody stools, recent antimicrobial use, hospitalization, or systemic illness, should have a microbiologic evaluation of their diarrheal stool [133–136]. The microbiologic stool evaluation for such enteric infections has for many decades relied upon the analysis of bacterial cultures and/or microscopy to detect ova and parasites [136, 137]. For nosocomial diarrhea or patients with a history of recent use of antimicrobial agents prior to the onset of diarrhea, the microbiologic stool evaluation should focus on the diagnosis of toxigenic *Clostridium difficile* [138]. For persistent diarrhea in patients with a history of international travel, the microbiologic stool evaluation may require special selective and differential agar such as thiosulfate citrate bile salts sucrose (TCBS) agar for *Vibrio* species [139]. Finally, the noroviruses are the most common cause of non-bacterial enteritis worldwide: the laboratory diagnosis of noroviruses depends on the detection of virus particles by EM, detection of viral antigens by EIA, or detection of viral RNA by real-time PCR [140, 141].

Given the complexity of conventional methods for the microbiologic evaluation of a stool specimen from a patient with a diarrheal illness, it is not surprising that determining the microbiologic etiology of an enteric infection had been an elusive goal prior to the advent of molecular methods [142, 143]. Enteric infections due to

the broad range of potential pathogens such as viruses, bacteria, protozoa, and helminths are well suited for multiplex molecular assays. Indeed, multiplex molecular assays for most of these enteric pathogens have been described [143, 144]. Gastrointestinal pathogen panel tests generally correctly identified pathogens identified by conventional testing; however, these tests also generate considerable additional positive results of uncertain clinical importance [145]. Two commercial syndromic multiplex tests including Luminex xTAG gastrointestinal pathogen panel and BioFire FilmArray gastrointestinal test have received FDA clearance for in vitro diagnostic use in the United States [146–150]. Although multiplex PCR tests have shown superior sensitivity to conventional methods for detection of most pathogens, it will be important for both clinicians and microbiologists to appreciate the limitations of these molecular assays.

### *Lack of a Gold Standard for Diarrheal Etiology*

The absence of a gold standard for the microbiologic cause of symptomatic enteric infections means that the clinical significance of a detected pathogen may not always be clear [142]. Although conventional wisdom suggests that there should be one main pathogen causing a symptomatic enteric infection in a patient, the detection of multiple pathogens in some patients will challenge this thinking [142]. The question of stool colonization by a potential pathogen versus a pathogen that is truly causing gastroenteritis can be difficult [151]. This is apt to be particularly true for parasitic enteric infections. Moreover, the detection of RNA or DNA in a stool specimen does not necessarily mean a viable pathogen or that the pathogen is truly causing gastroenteritis.

### *Complexity of the Human Gut Microbiome*

Molecular assays including high-throughput sequencing techniques have begun to identify the vast communities of bacteria that inhabit the skin and gut in humans [152]. Despite these methods, the human gut remains relatively unexplored [152–154]. This complexity will continue to be a factor in the use of NAATs for diagnosing enteric infections if for no other reason than the influence of contaminating DNA from these gut microbes on the sequencing template.

### *Issues with Nucleic Acid Extraction*

The molecular diagnosis of an enteric infection will usually begin with extraction of nucleic acid from the specimen. Because this specimen is generally a diarrheal stool sample, the extraction step becomes a critical step in this molecular diagnostic

process. This is because stool is a complex mixture with multiple and diverse nucleic acids and amplification inhibitors. Investigators have noted that detection of a given target will reduced several logs when the target is placed in a stool mixture [142, 155]. This may result in enteric pathogens present in low numbers being missed. This is the reason that some investigators have used mass spectrometry as an identification method following isolation of potential enteric bacterial pathogens from stool [155, 156]. In addition, extraction of DNA from ova and parasites may be more difficult that extracting DNA from bacteria [157]. Concentration of ova and parasites that may be present in low numbers may be required [158], as it is and has been for microscopic evaluation for parasites [159].

## *Requirement for Multiplex PCR*

Over 50 pathogens currently are recognized a potential causes of enteric infections [142–144]. This means that a multiplex PCR such as the Luminex bead method or the FilmArray real-time PCR assay must be used [146, 147, 149, 150]. Even a multiplex approach will likely require the use of a diagnostic algorithm or the use of several multiplex assays. The use of multiplex assays will create several technical problems that include difficulty with discrimination of multiple targets in a single reaction and reduced sensitivity. Multiplex assays also will cause some problems with interpretation due to detection of multiple pathogens. For example, one study that reexamined stool samples using PCR found that the detection rate increased for both viral and bacterial pathogens, but the detection rate for multiple pathogens also increased [142–144]. Similar to respiratory specimens, etiological relevance is assumed if cycle-threshold values of qPCR results are low which correlate to high pathogen loads [126].

## *Requirement for Quantitative PCR*

Molecular assays due to their high sensitivity may detect low levels of enteric pathogens with unclear clinical significance. For example, *Giardia* species are known to occur in stool at high rates in persons without diarrhea [160]. A recent study revealed that high level of norovirus fecal load was correlated with norovirus genogroup II infections and associated with development of severe clinical symptom at the time of diagnosis [161]. Therefore, the use of quantitative PCR methods may be needed in order to provide information that will be useful for interpreting the clinical significance; the assumption being that a higher burden is more likely to be associated with disease [142, 143]. Ultimately, this relationship of higher burden and symptoms of disease will need to be verified for many enteric pathogens for which this relationship has not yet been determined.

### *Detection of Resistant Determinants*

Antimicrobial resistance is increasing for many bacterial pathogens and is likely to happen with enteric pathogens such as *Shigella*, *Salmonella*, and *Campylobacter* [162–164]. Detection of resistance determinants may be necessary in the future and is likely to be difficult from stool samples due to the diversity of microorganisms present in stool [142, 163].

## The Use of Molecular Assays for Diagnosing Central Nervous System Infections

Central nervous system (CNS) infections can be life threatening if not diagnosed and treated early. The initial clinical presentation of most CNS infections is non-specific, which makes the determination of an etiologic diagnosis challenging. The laboratory evaluation of suspected meningitis/encephalitis (ME) is often complicated because the differential diagnosis is broad, and the clinical signs and symptoms do not suggest a specific microorganism. Clinicians often approach the laboratory evaluation of a CNS infection based on host factors, duration of symptoms, and potential environmental exposure; but cerebrospinal fluid (CSF) indices in combination with a broad range of microbiologic tests are usually required to identify a potential pathogen or to rule out infection [165, 166].

The conventional approach to CNS infection most frequently used in clinical microbiology laboratories includes direct microscopic examination, culture techniques, and antigen/antibody detection assays. These methods, although frequently utilized, have several important limitations. These limitations will be reviewed using the example of enteroviruses, which is among the most common causes of meningitis [167]. Direct microscopic examination of cerebrospinal fluid (CSF) is not useful for enterovirus. The sensitivity of enterovirus culture is only 65–75% and requires 4–8 days [168]. Moreover, some enteroviral serotypes such as *Coxsackievirus A* strains grow poorly or are non-cultivable [169]. Enteroviruses lack a common antigen among various serotypes, which makes detection of an antigen or antibody impossible. Similar issues are seen with the diagnosis of HSV infections of the CNS by conventional methods—the sensitivity of CSF cultures is extremely poor. Although the presence of HSV IgG antibodies in CSF can be used to diagnose CNS infections, such antibody production is delayed until day 10 or 12 of the infection making this method less useful for early diagnosis [170].

Nucleic acid in vitro amplification-based molecular methods are increasingly being applied for routine microbial detection. These methods are proving to be a significant improvement over conventional techniques and have the advantages of both rapid turnaround and higher sensitivity and specificity [165, 166]. For example, one study reported that 16S ribosomal ribonucleic acid (rRNA) PCR-based assays

were able to accurately detect the causative organism in 65% of banked CSF samples in comparison to 35% with the use of microscopy and culture [171]. In another report, the diagnostic yield from molecular methods was improved and was able to optimize antimicrobial therapy for patients with infectious meningitis when conventional methods provided a negative result [172]. Currently, molecular methods performed on CSF samples are considered to be the "platinum" standard, in contrast of the culture gold standard, for the diagnosis of CNS infections caused by viruses which are difficult to detect and identify [173–175]. US Food and Drug Administration (FDA)-approved PCR assays have been available for enteroviruses (Xpert EV; Cepheid, Sunnyvale, CA) and herpes simplex viruses (Simplexa HSV 1&2 Direct; Focus Diagnostics, Cypress, CA) for many years now with excellent results [166].

The relative simplicity and high-throughput detection of multiplex molecular assays make these an attractive option for screening and detection of a panel of microbial targets [176]. Several multiplex PCR assays targeting the most common causes of meningitis have been used to identify bacterial pathogens in CSF [165, 166]. The FilmArray meningitis/encephalitis (ME) panel (BioFire Diagnostics LLC, Salt Lake City, UT) is currently the only FDA-approved multiplex assay for CNS infections and detects six bacterial (*E. coli* K1, *H. influenzae*, *L. monocytogenes*, *N. meningitidis*, *S. agalactiae*, and *S. pneumoniae*), seven viral (cytomegalovirus, enterovirus, HSV-1, HSV-2, human herpesvirus 6 (HHV-6), human parechovirus, and VZV), and one fungal (*C. neoformans/gattii*) target in CSF [177, 178]. The integrated FilmArray system has a turnaround time of about an hour, with only 2 min of hands-on time [178–180]. The clinical usefulness of this device has been described in several recent reports [180–183].

Next generation sequencing-based (NGS) approach offers great potential for use in CNS infections. Both CSF specimens and brain biopsies can be used to further explore the use of NGS technology for pathogen detection and discovery [165, 166]. As demonstrated in a highly challenging clinical situation, metagenomics was successfully used to make a timely diagnosis of neuroleptospirosis in a 14-year-old boy with severe combined immunodeficiency who presented with recurrent bouts of fever, headache, and coma [184]. Similarly, high-throughput RNA sequencing performed on brain biopsy from an 18-month-old boy with encephalopathy identified a new astrovirus as the pathogen [185]. Although molecular methods will undoubtedly be widely used for diagnosing and monitoring CNS infections, it will be important for both clinicians and microbiologists to appreciate the limitations of molecular assays such as the multiplex PCR amplification-based syndromic panels. Results generated by these methods need to be carefully interpreted in light of the patient's clinical findings.

## *Limited Sensitivities and Subjective Cutoffs*

Real-time PCR-based monoplex assays do provide an excellent qualification procedure with a wide range of concentrations covered; however, such assays are not ideal for qualitative measurements as there are no objective criteria for determining

the cutoff point. This can be problematic when the microbial load in the tested specimen is extremely low. For example, HSV and *Chlamydia pneumoniae* detection in CSF specimens by real-time PCR is not as objective and sensitive in comparison to end detection PCR procedures [186, 187]. A negative FilmArray ME panel result does not exclude infection due to organisms that are not included in the panel, and false-negative results for targeted pathogens that are present in low quantities are still possible. Empirical antibiotics and/or acyclovir should still be administered when the clinical suspicion for bacterial infection or HSV encephalitis is high despite a negative meningitis/encephalitis panel.

## *False Positives*

Although multiplex PCR-based assays such as the FilmArray ME panel offer promising syndromic platforms for rapid diagnosis of CNS infections, many false-positive or unconfirmed ME panel results have been noted [178, 188]. The comprehensive list of targets included in the ME panel ensures that an actionable diagnosis is not likely to be missed, but the false-positive results are problematic. *Streptococcus pneumoniae* was the most frequent false-positive detection made by the ME panel [178]; clinically irrelevant positive detections of *H. influenzae* also have been revealed in our institution (Tang, unpublished data). False-positive CSF test results can potentially result in significant harm if they lead to unnecessary, potentially toxic antimicrobial therapy or unwarranted invasive procedures. Several of the false-positive ME panel results in this published study theoretically could have had untoward sequelae if therapy or invasive procedures have been implemented [178]. These observations highlight the importance of laboratory operating procedures designed to minimize carryover contamination, even when using a “closed” system such as the FilmArray. Operators should wear a mask when loading the FilmArray pouches and/or ideally use a biological safety cabinet or dead air box. Each laboratory must establish expected positivity rates for the individual targets contained in the ME panel in order to monitor for contamination [177]. Finally, interpreting the clinical significance of reactivated or latent *Herpesviridae* can be difficult. Providers must consider these results carefully in the clinical context.

## *Misidentified Pathogens*

An example of syndromic nucleic acid amplification test panels is the FilmArray system, which simultaneously detects a broad range of pathogens and has improved the diagnosis of many infectious diseases by reducing turnaround times and simplifying laboratory workflow. The rapid results obtained are useful for guiding antimicrobial therapy and improving infection prevention practices. However, when the

system detects multiple microorganisms, it may be difficult to determine which microorganism caused the clinical infection. This can be particularly important if the real pathogen is missed (most likely due to the low test sensitivity) while a different microorganism is detected. For example, Gomez et al. have described a case of tuberculous meningitis misdiagnosed as herpes simplex virus-1 encephalitis using the FilmArray ME panel [188].

### *Cost-Effectiveness*

As syndromic diagnostic panels ("one stone for two birds") become popular and widely used in clinical practice, laboratorians will be faced with guiding the rational use of these expensive technologies in the current absence of studies evaluating cost-effectiveness. FDA approval does not necessarily mean that the ME panel is going to be the right approach for all patients with CNS infections. Several factors should be considered before implementing the ME panel [177]. First, which patients should be tested? The pathogens targeted by the ME panel are most appropriate for immunocompromised patients with CNS infections, a setting where members of the *Herpesviridae* as well as *Cryptococcus* species are commonly seen and cause significant disease. In pediatric and adult patients with acute meningitis and a high clinical suspicion for a bacterial infection, the ME panel could reduce the time to diagnosis and may be particularly useful in situations where patients have received empiric antimicrobial therapy prior to a diagnostic lumbar puncture. Short of these selected situations, targeted molecular testing with prioritization by most likely pathogens should be the first consideration. Laboratory screening criteria that are based in part on CSF nucleated cell counts might be a way to minimize unnecessary testing for immunocompetent adults [177, 189–191]. It can be argued that performing targeted molecular testing based on clinical suspicion is likely to be more cost-effective for immunocompetent patients, especially when such testing can be done in-house. In summary, the ME panel appears to be an additional test that will not necessarily replace current approaches [177].

## The Use of Molecular Assays for Diagnosing Tissue Infections

The use of molecular assays for diagnosing tissue infections is another area that is rapidly evolving. For example, molecular assays have proven quite successful in the diagnosis of infectious endocarditis [192–202]. Indeed, a number of fastidious microorganisms causing endocarditis have been identified using molecular assays; these include *Tropheryma whippelii* [192], *Bartonella quintana* [194, 199, 200], *Bartonella henselae* [199, 200], and *Coxiella burnetii* [200]. This success has resulted in molecular assays being included in the best practices and guidelines for identification of

difficult-to-culture pathogens in infective endocarditis [202, 203]. Molecular assays of tissue have been useful for diagnosing necrotizing fasciitis caused by group A streptococci when cultures were negative or not available [204, 205].

Finally, molecular assays for fungal pathogens also have been widely studied and have the potential to be useful in the diagnosis of fungal tissue infections [206]. Fungal pathogens identified from tissue by molecular assays include *Paracoccidioides brasiliensis* [207], *Histoplasma capsulatum* [208], *Coccidioides immitis* [209], *Blastomyces dermatitidis* [209, 210], *Aspergillus fumigatus* [211, 212], *Absidia corymbifera* [211], and *Rhisopus arrhizus* [211, 212]. NAATs have been used to detect a variety of DNA and RNA viral pathogens in formalin-fixed, paraffin-embedded (FFPE) tissue specimens [213–216]. The use of molecular assays for diagnosing tissue infections will only increase over time [216]; therefore, the limitations of these molecular assays should be appreciated.

## *Fresh/Frozen Tissue Versus Formalin-Fixed, Paraffin-Embedded Tissue*

Fresh/frozen tissue is best for molecular testing and should be available if molecular testing is considered at the time of biopsy [216–218]. In contrast, FFPE tissue is usually available as often is the only tissue available when molecular testing is considered as an afterthought [206]. Accordingly, one of the most important limitations in the use of molecular assays for diagnosing tissue infections is considering these assays at the time of biopsy so that fresh tissue can be used or frozen for use later. The difference in sensitivity for PCR testing can be seen by a study in which fresh nonembedded tissues were found to have sensitivities for PCR detection of fungi of 97% versus only 68% for FFPE tissue [217]. The reason for this decreased sensitivity is that nucleic acids obtained from FFPE tissue are frequently damaged (i.e., cross-linked) and may contain PCR inhibitors [219, 220]. If FFPE tissue must be used, a housekeeping human gene must be amplified as a control [206, 213, 220].

## *Wide Diversity of Potential Microbial Pathogens*

The wide diversity of potential microbial pathogens that could potentially be detected in tissue is readily apparent. These pathogens could be viral, bacterial, fungal, or parasitic. This diversity will greatly influence the DNA targets and the PCR primers used as well as whether monoplex or multiplex PCR methods will be used. For example, species-specific identification of a wide range of clinically relevant fungal pathogens using Luminex technology required up to three different probes for each fungal pathogen using the internal transcribed spacer (ITS2) region, which is highly variable among genomes of individual fungal species [221].

### Choice of DNA Target, PCR Primers, and Amplification Method

The choice of the DNA target is important [222]. In general, molecular assays that target multi-copy genes provide the greatest sensitivity. Amplification methods should provide objective endpoint assessments for the PDR test used. PCR primers are important. For example, there is insufficient variation in the internal transcribed spacer (ITS1) region to differentiate certain species of fungal pathogens [223]; therefore, analysis of other regions such as ITS2 should be considered. False-positive results have been described with certain primer for *H. capsulatum* [208]. False-negative results have been found for *C. immitis* from FFPE tissue (73% sensitivity) versus fresh tissue (93% sensitivity) suggesting a primer problem, degradation, or inhibitors [209, 224]. Finally, it is estimated that approximately 10–20% of the sequences in GenBank are misidentified [225]. Currently there are relatively few commercial kits available for molecular testing using tissue specimens. If laboratory-developed PCR assays for tissues are used, they must be evaluated, verified, and validated by the laboratory before the results can be used for clinical diagnosis and patient care [223, 226].

### Issues with Nucleic Acid Extraction

DNA extraction from FFPE tissues is difficult and requires special protocols [225]. The amount of DNA extracted is usually quite small; reported methods show an amplification success rate between 60% and 80%. Commercial DNA extraction kits have been evaluated [225]; one method (TaKaRa) was noted to extract DNA for 69 of the 74 FFPE tissue samples from which a housekeeping gene could be amplified. Moreover, this method was cost-effective and had a non-laborious protocol. Successful extraction of RNA from FFPE specimens depends on the prompt original tissue processing and a well-developed extraction protocol [215, 220, 227].

### Low Number of Pathogens and/or Random Distribution in Tissue

When the number of pathogens is scant in tissues, the amount of DNA obtained may be insufficient to perform a PCR assay. Moreover, these pathogens are often randomly distributed in the tissue [37]. When FFPE tissue is used, a punch biopsy can be used to take a sample from an area noted to have inflammation and/or microorganisms by a stained slide from the same tissue block. The stained slide can be marked and then used to direct the location for the punch biopsy sample from the tissue block. The use of fresh or frozen tissue is more problematic as the selection of tissue will be random and may not contain microorganisms.

### *Simultaneous Detection of Multiple Pathogens*

As would be expected, molecular assays already have been noted to detect mixed infections. This may present difficulty in interpretation of the results. In particular, microbial diversity in endocarditis has been noted with cultivation-independent molecular techniques [226]. Multiple pathogens detected by molecular assays have also been reported in fungal infections [206, 227].

## Concluding Remarks

Outcomes from infectious diseases often depend on early and appropriate antimicrobial therapy [228]. Appropriate antimicrobial therapy is directly related to the length of time required for identification of the microbial pathogen [105, 106, 229]. Until recently, clinical microbiology laboratories have been handicapped by conventional, slow multistep culture-based techniques that require prolonged incubation times for many pathogens and are not able to isolate others. Clinicians unable by clinical judgment or diagnostic results to quickly and accurately identify a pathogen causing infection must adopt a conservative approach involving empiric therapy with broad-spectrum antimicrobial agents [230]. Fortunately, this cumbersome approach has rapidly changed over the last two decades because of the increasing utilization of molecular diagnostic techniques [105, 106, 231]. Indeed, molecular assays such as NAATs have initiated a revolution in the field of diagnostic microbiology due to their high sensitivity, specificity, rapid test turnaround time, as well as potential high throughput and automation. In particular, emerging commercial molecular tests for the diagnosis of bloodstream infections promise to further improve patient outcomes in septic patients [19]. The adaption of molecular syndromic methods for microbiology diagnostics to the point-of-care laboratory also promises to improve patient outcomes [232]. Molecular assays have been heralded as the “diagnostic tool for the millennium” [1, 3, 4]. However, molecular assays also bring some uncertainty such as that caused by false-positive results due to contamination from endogenous or exogenous sources of DNA [4, 8, 18, 20, 233]. For example, one study using a universal 16S rRNA PCR assays detected eubacterial DNA in blood samples from healthy subjects [234]. NAATs also may give false-negative results due to two principle reasons: (1) the relatively small sample required for PCR reactions and (2) technical problems associated with PCR processing [235]. Moreover, the results of molecular assays may be difficult to interpret and apply in the clinical setting. As NAATs are increasingly used in routine clinical microbiology laboratories, interpretation is expected to be more difficult as new tests are developed and more complicated multiplex assays emerge. For example, clinical relevance of positive NAATs in paraffin block specimens and multiple microbial organisms found in any specimen will need careful interpretation. As the usefulness of these molecular assays is determined by usage over time,

communication between the clinician and the microbiology laboratory is always suggested and will be increasingly important whenever an interpretation is needed. Finally, both the clinical microbiologist and the clinician must acquire a working knowledge of the principles, diagnostic value, and limitations of these molecular assays [1, 236, 237].

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