Chapter 47
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 two decades 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 and user-friendly software 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. Indeed, every section of the clinical microbiology laboratory, including bacteriology, mycology, mycobacteriology, parasitology, and virology, have 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 has lagged somewhat behind. The purpose of this chapter is to review and discuss the interpretation and relevance of results produced by these advanced molecular techniques. Moreover, this chapter will address the "myths" of NAATs, as these myths can markedly influence the interpretation and relevance of these results.

C.W. Stratton (✉)
Departments of Pathology and Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, USA
e-mail: charles.stratton@Vanderbilt.Edu

Y.-W. Tang
Department of Laboratory Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 37212, USA
e-mail: tangy@MSKCC.org

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Myths of Nucleic Acid Amplification Tests

Myth 1: NAATs Are Extremely Sensitive

There are two aspects on sensitivity: analytical, which is determined by the limit of detection in a given specimen, and diagnostic/clinical, which is determined by the percentage of target in a patient (true positive) population. Most of NAATs possess excellent analytical sensitivity reaching ten copies of target genomes per reaction. This has made the NAATs essential for the detection of microbial pathogens from specimens such as cerebrospinal fluid (CSF) where there may be extremely low microbial loads [3–6]. However, in certain situations, the sensitivities of NAATs are inferior to conventional culture techniques in which a large volume of a specimen easily can be evaluated. Examples include blood cultures and sputum cultures for *Mycobacterium tuberculosis* [7–10]. Current NAATs are usually performed using very small volumes; until the processing of such specimens is greatly improved, the sensitivity of these NAATs will remain lower than conventional culture techniques.

Currently, most NAATs are designed in a monoplex format, i.e., one primer set for one specific microbial pathogen. Although the use of multiplex PCR amplification techniques is increasing in the clinical diagnostic field, these multiplex methods account for the minority of molecular testing. In conventional microbial culture techniques, a chocolate agar plate or a mixed cell line would allow the recovery of many different pathogens and/or multiple pathogens if they were present in a tested specimen. From this perspective, the diagnostic sensitivity of a monoplex NAAT may not be sufficiently high since it only detects the one pathogen that is being tested for rather than many different pathogens and/or multiple pathogens.

Myth 2: Real-Time PCR Is Extremely Sensitive as well as Objective

The term "real-time PCR" indicates a PCR procedure in which nucleic acid amplification and amplification product detection happen simultaneously. Real-time PCR methods often incorporate a FRET procedure, which allows the amplicon detection and identification to occur in real time in relation to the nucleic acid amplification. This avoids carryover amplicon contamination since the reaction is occurring in closed system. Moreover, real-time PCR allows accurate quantification of the nucleic acid. However, NAATs based on real-time PCR methods are not necessarily more sensitive than other NAATs [11]. In contrast, due to the nonspecific spontaneous FRET procedure, real-time PCR assays may be less sensitive due to the raised cutoff values. The arithmetic, spontaneous increase in fluorescent background emissions interferes with the exponential, specific energy emissions during the simultaneous detection procedure, producing potentially false-positive results. To overcome this nonspecific issue, the system has to either increase the cycle threshold (Ct) cutoff value or decrease the cycle numbers during the amplification, which results in decreased sensitivity.

Real-time PCR does provide a nice qualification procedure with a wide range covered; however, it is not ideal for qualitative assay 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 [4, 12]. In the later procedures, a separate detection and identification is used after amplification in which additional signal amplification (e.g., antigen–antibody linking) can be incorporated to further enhance the test sensitivity [12, 13].

Myth 3: NAATs Are Useful Tests for Assessing Therapeutic Efficacy

NAATs are often considered to be useful tests for assessing therapeutic efficacy. However, this is not true. NAATs detect microbial organism-specific nucleic acids; therefore, a positive NAAT result can occur with both alive and dead microorganisms, which is particularly true for those pathogens that have protective cell wall. 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 [14, 15]. Unlike the results of a function-based testing method, such as mycobacterial cultures, in the clinical setting, a positive PCR result after anti-tuberculosis therapy does not necessarily mean treatment failure. Therefore, DNA-targeted NAATs are usually not considered to be tests of cure. This is also true for sexually transmitted pathogens such as *Chlamydia trachomatis* and *Neisseria gonorrhea* [16].

Although DNA-targeted NAATs are not useful for therapeutic monitoring, mRNA-targeted NAATs may be used for assessment of antimicrobial treatment therapy. For example, 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 have demonstrated that such assays are useful in monitoring the efficacy of anti-TB therapy [14, 15].

Myth 4: NAAT Results Allow Direct and Objective Interpretation

Conventional microbiology methods are generally direct and objective in terms of interpretation of the results. A blood culture that is positive for *Staphylococcus aureus* strongly suggests that the patient has a staphylococcal bacteremia. A positive hepatitis A IgM antibody detected in serum implies a recent infection caused by this virus. However, positive NAAT results can be somewhat indirect and subjective

since the exact meaning of a positive NAAT result is only that the microbe-specific nucleic acid exists in the tested specimen. This can be caused by contamination, colonization and/or infection. Even when an infection is established, it can be either an acute or chronic infection.

Higher sensitivity may not always be clinically relevant. One good example is seen with the varicella-zoster virus (VZV). Previously, a positive VZV result, by either DFA or culture, would be interpreted as VZV being the causative pathogen and would be considered clinically relevant. PCR-based NAATs have increased VZV detection several hundred-fold such that a positive NAAT result for VZV from a skin or mucosal lesion specimen may be unclear as to the exact causative relationship of this virus to infection [17].

Clinical Relevance and Interpretation of Molecular Tests

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" [18]. 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 [19]. Since then, the techniques and pitfalls for blood cultures as well as the clinical implications of positive blood cultures have been well documented [20–23]. Not surprisingly, molecular and other non-culture-based methods for the rapid diagnosis of bloodstream infections have been widely evaluated [8, 10]. These studies along with earlier studies of blood cultures have illustrated some important points regarding the limitations of molecular assays for diagnosing bloodstream infections, which are described below:

Limitations of Molecular Assays for Diagnosing Bloodstream Infections

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 [10, 24]. For example, interpretation of DNAemia with coagulase-negative

staphylococci is problematic due to a false-positive rate that ranges from 60 to 80 % [22, 25]. In contrast, interpretation of DNAemia with *Ehrlichia* species is not a problem due to a true-positive rate of 100 % [26]. Interpretation of DNAemia has also been a problem in some studies where DNAemia is detected by PCR but not by blood cultures [24]. 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 [10, 27–32]. 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 [10, 24, 33].

Molecular Detection of Resistance Determinants

Another important issue for molecular diagnostic techniques is the need for molecular detection of resistance determinants [10, 24]. 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 [34]. 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 [34, 35]. The direct detection of resistance genes by molecular methods such as PCR to date has limitations due to the fact that relatively few resistance genes are firmly associated with phenotypic resistance [34–36]. 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 ascending, phenotypic methods for determining the level of susceptibility of bacterial isolates to antimicrobial agents are likely to remain clinically relevant for many years.

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 small [37–41]. 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 [40]. Clearly the volume of blood tested by molecular methods will also be important. Moreover, the Poisson distribution of these microorganisms is such that they are not evenly distributed [37, 42]. 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 [31]. The Poisson distribution may explain the moderate concordance between blood cultures and a molecular method reported in a study of post-surgical sepsis in adults [27].

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 and/or from indwelling vascular devices if the blood is obtained from such a device [23]. False-positive blood cultures have been recognized as a troublesome issue for decades, and such contamination will be no less important for molecular methods.

The Use of Molecular Assays for Diagnosing Tuberculosis

Tuberculosis remains one of the most important public health issues in the world. Tuberculosis results in approximately 1.7 million deaths each year, and the number of new cases worldwide is estimated at more than nine million; this is higher than at any other time in history [43]. Yet control of this treatable infection has been handicapped until recently by the lack of new diagnostic tests for the detection of *M. tuberculosis* and drug resistance [44]. 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 [44–46]. 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 described below.

The Limitations of Molecular Assays for Diagnosing Tuberculosis

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 [47], and Xpert MTB/RIF [47]. In comparison to mycobacterial culture, these molecular assays possess sensitivities approaching 90 %. 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.

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 [50–54]. 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 recent report from the Centers for Disease Control and Prevention [45]. 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 [55, 56]. In one of these reported cases [55], 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 *M. tuberculosis* Direct (MTD) test (BBL). 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 5×10 [5] organisms per ml, but were indeterminate at a concentration of 5×10 [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 [55].

The Use of Molecular Assays for Diagnosing Respiratory Tract Infections

There is no doubt that respiratory tract infection 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 [57]. 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 [58]. Viral respiratory tract infections caused by pathogens such as the severe acute respiratory syndrome coronavirus (SARS-CoV) [59] and novel A/H1N1 influenza virus [60] can cause epidemic viral pneumonia in which some patients have respiratory failure with a significant risk of mortality [61]. Respiratory tract infections are also important in the ambulatory setting because of the documented overuse of antimicrobial agents in this patient population [62].

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 sputum. The diagnostic value of detecting *M. tuberculosis* in the sputum was recognized as early as 1884 [63], and evaluation of sputum became the cornerstone for the diagnosis of tuberculosis [43, 44]. Post-mortem studies in the late 1890s and early 1900s then established the role of other microorganisms such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *S. aureus*, and *Klebsiella pneumoniae* in non-tuberculous infections of the respiratory tract [64–66]. In 1902, the use of the Gram's stain was described [67]. The microscopic examination of sputum was followed by the introduction of sputum cultures for the diagnosis of bacterial pneumonia [68–70]. 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 [69] 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." 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." Luetscher opines in his paper [70] 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." These astute observations remain relevant 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 [71–76]. 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 [77, 78]. 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 [79], was not widely used for obtaining sputum for microscopy and culture until the 1970s when fiberoptic bronchoscopy became available [80]. Fiberoptic bronchoscopy also resulted in the use of bronchoalveolar lavage for diagnosing acute bacterial pneumonias [81]. Other methods adopted for obtaining uncontaminated sputum included transtracheal aspiration [72], percutaneous needle biopsy [76], and open-lung biopsy [71].

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 [82–88]. Indeed, the Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults 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 [89]. 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 rapidly emerged in the clinical microbiology laboratory as an important adjunct to traditional culture methods [90, 91]. It was quickly realized that molecular assays such as NAATs offered significant advantages over conventional methods for the detection of *Mycoplasma pneumoniae* [92, 93], *Legionella* species [94], and *Chlamydia* species [95]; 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 [96, 97]; 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 [98]. 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 [99]. Both user-developed and commercial molecular methods have quickly evolved and now allow rapid identification of multiple common viral pathogens causing respiratory tract infections [100–102]. 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 [57, 93, 103, 104]. Indeed, initial studies in which rapid molecular assays were combined with conventional diagnostic methods have demonstrated that this approach increased the etiological diagnosis of lower respiratory tract infections considerably [105, 106]. This was especially true for patients with adequate collection of sputum [105]. Of interest was the observation that NAATs increased both the diagnostic and treatment costs [106]. Finally, the diagnosis of hospital-acquired pneumonia is another potential area where the use of rapid molecular assays for respiratory pathogens may prove useful [58]. Currently clinical trials are needed to provide evidence for which molecular assays are best as well as how this molecular information should be applied in the clinical setting.

The Limitations of Molecular Assays for Diagnosing Respiratory Tract Infections

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 [58]. 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 are clearly going 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, 108]. 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 [109]. PhyloChip analysis demonstrated the presence of over 1,200 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 versus 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 [110]. Such neonatal airway colonization with Gram-negative bacilli is associated with a cytokine response as well as with severe bronchopulmonary dysplasia [111, 112]. The etiologic role of neonatal colonization in children with non-cystic fibrosis bronchiectasis is unclear at this time [113–115], but molecular methods may provide further insight into the pathogenesis of this disorder. Similarly, the etiologic role of bacterial colonization in the pathogenesis of chronic obstructive pulmonary disease [107–109, 116, 117] is currently being elucidated with the assistance of molecular methods.

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 [105] where mixed infections were frequently seen: these most commonly were *S. pneumoniae* together with a respiratory virus. These findings are not unexpected; a number of studies have reported an association between viral respiratory tract infections and invasive pneumococcal disease [118–120]. Molecular diagnostic methods employed in other studies of respiratory tract infections have confirmed the etiologic role viral respiratory tract infections and bacterial pneumonia [121–123].

Accuracy of Assay Development

An important issue for NAATs is whether the amplification products truly represent the target microorganism [104]. 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 [104, 124–127]. 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 *Bordetella 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 [128]. However, false-positive results have been reported due to the smaller copy numbers of IS481 existing in non-pertussis *Bordetella* species [129, 130]. 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 [131]. For afebrile patients who present with watery non-bloody diarrhea of less than 24 h duration, microbiologic investigation is usually unnecessary [131, 132]. In contrast, patients with a diarrheal illness lasting for more than one 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 [131–134]. 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 [134]. 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* [135]. 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 (TSCB) agar for *Vibrio* species [136]. 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 [137].

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 remains an elusive goal [138]. It is no wonder that molecular methods have been applied to the diagnosis of acute infectious diarrhea [138, 139]. 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, monoplex and multiplex molecular assays for many of these enteric pathogens have already been described [140–146]. Therefore, it will be important for both clinicians and microbiologists to appreciate the limitations of these molecular assays.

The Limitations of Molecular Assays for Diagnosing Enteric Infections

Lack of a Gold Standard for the Microbiologic Cause of Enteric Infections

The absence of a gold standard for the microbiologic cause of enteric infections means that the clinical significance of a detected pathogen may not always be clear [138]. Although conventional wisdom suggests that there should be one main pathogen causing an enteric infection in a patient, the detection of multiple pathogens in some patients will challenge this thinking [138]. 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 or an infectious pathogen.

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 [147]. Despite these methods, the human gut remains relatively unexplored [147, 148]. This complexity is likely 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 be reduced several logs when the target is placed in a stool mixture [138]. This may result in enteric pathogens present in low numbers being missed. This is the reason that some investigators have used molecular methods following isolation of potential enteric bacterial pathogens from stool [149]. In addition, extraction of DNA from ova and parasites may be more difficult than extracting DNA from bacteria [140]. Concentration of ova and parasites that may be present in low numbers may be required, as it is for microscopic evaluation.

Requirement for Multiplex PCR

Over 50 pathogens currently are recognized a potential causes of enteric infections [138]. This means that a multiplex PCR such as the Luminex bead method must be used. 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 [150].

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 [151]. 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 [138]. 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.

Molecular 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*. 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 [138].

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 [151–160]. Indeed, a number of fastidious microorganisms causing endocarditis have been identified using molecular assays; these include *Tropheryma whippelii* [151], *Bartonella quintana* [153, 158, 160], *Bartonella henselae* [158], and *Coxiella burnetii* [158]. 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 [161]. Molecular assays of tissue have been useful for diagnosing necrotizing fasciitis caused by group A streptococci when cultures were negative or not available [162, 163].

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 [164–174]. Fungal pathogens identified from tissue by molecular assays include *Paracoccidioides*

brasiliensis [165], *Histoplasma capsulatum* [164], *Coccidioides immitis* [174], *Blastomyces dermatitidis* [174], *Aspergillus fumigatus* [166], *Absidia corymbifera* [166], *Rhisopus arrhizus* [166], and *Candida* species [171]. NNATs have been used to detect a variety of DNA and RNA viral pathogens in formalin-fixed, paraffin-embedded (FFPE) tissue specimens [175–180]. The use of molecular assays for diagnosing tissue infections will only increase over time; therefore, the limitations of these molecular assays should be appreciated.

Limitations in the Use of Molecular Assays for Diagnosing Tissue Infections

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 [181, 182]. In contrast, FFPE tissue often is the only tissue available when molecular testing is considered as an afterthought. 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 [181]. 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 [183, 184]. If FFPE tissue must be used, a housekeeping human gene must be amplified as a control [179, 184].

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 [185].

Choice of DNA Target, PCR Primers, and Amplification Method

The choice of the DNA target is important [167]. In general, molecular assays that target multicopy 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 [186]; therefore, analysis of other regions such as ITS2 should be considered. False-positive results have been described with certain primer for *H. capsulatum* [164]. 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 [164]. Finally, it is estimated that approximately 10–20 % of the sequences in GenBank are misidentified [187]. 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 [186, 188].

Issues with Nucleic Acid Extraction

DNA extraction from FFPE tissues is difficult and requires special protocols [187]. 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 [187]; 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 [175, 179, 180, 184].

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, 42]. 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. 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 [188]. Multiple pathogens detected by molecular assays have also been reported in fungal infections [166].

Concluding Remarks

Outcomes from infectious diseases are directly related to the length of time required for identification of the microbial pathogen. 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. Fortunately, this cumbersome approach is rapidly changing because of the introduction of molecular diagnostic techniques. 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. Molecular assays have been heralded as the "diagnostic tool for the millennium" [189, 190]. However, molecular assays also bring some uncertainty such as that caused by false-positive results due to background contamination from exogenous sources of DNA [190, 191]. For example, one study using a universal 16S rRNA PCR assays detected eubacterial DNA in blood samples from healthy subjects [192]. 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 [193]. 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 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 [194, 195].

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