

Chapter 19

Diagnosis and Monitoring of Infections

Abstract As molecular techniques for identifying and detecting microorganisms in the clinical microbiology laboratory have become routine, questions about the cost of these techniques and their contribution to patient care need to be addressed. Molecular diagnosis is most appropriate for infectious agents that are difficult to detect, identify, or test for susceptibility in a timely fashion with conventional methods. During the last 10 years, the detection of infectious disease agents has begun to include the use of nucleic acid-based technologies. Diagnosis of infection caused by parasitic organisms is the last field of clinical microbiology to incorporate these techniques and molecular techniques (e.g., PCR and hybridization assays) have recently been developed for the detection, species differentiation, and phylogenetic analysis.

Keywords PCR · Infections · Detections · Diagnosis · Sexually transmitted diseases · Vector borne diseases · Viral infections · Bacterial infections · Fungal infections · Molecular methods · Molecular epidemiology

19.1 Prologue

The tools of molecular biology have proven readily adaptable for use in the clinical diagnostic laboratory and promise to be extremely useful in diagnosis, therapy, epidemiologic investigations and infection control. Although technical issues such as ease of performance, reproducibility, sensitivity, and specificity of molecular tests are important, cost and potential contribution to patient care are also of concern. Molecular methods may be an improvement over conventional microbiologic testing in many ways. Currently, their most practical and useful application is in detecting and identifying infectious agents for which routine growth-based culture and microscopy methods may not be adequate.

Nucleic acid-based tests used in diagnosing infectious diseases use standard methods for isolating nucleic acids from organisms and clinical material. Because the target DNA or RNA may be present in very small amounts in clinical specimens, various signal amplification and target amplification techniques have been

used to detect infectious agents in clinical diagnostic laboratories (Vaneechoutte and Van Eldere 1997). Although mainly a research tool, nucleic acid sequence analysis coupled with target amplification is clinically useful and helps to detect and identify previously uncultivable organisms and characterize antimicrobial resistance gene mutations, thus aiding both diagnosis and treatment of infectious diseases. Automation and high-density oligonucleotide probe arrays (DNA chips) also hold great promise for characterizing microbial pathogens.

Although most clinicians and microbiologists enthusiastically welcome the new molecular tests for diagnosing infectious disease, the high cost of these tests is of concern. Despite the probability that improved patient outcome and reduced cost of antimicrobial agents and length of hospital stay will outweigh the increased laboratory costs incurred through the use of molecular testing, such savings are difficult to document.

19.2 Concept of Diagnosis and Monitoring of Infections

Over the past several years, the development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases. As technology advances, diagnostic tests continue to improve, and each year, we are presented with new alternatives to the standard procedures. Given the plethora of diagnostic alternatives, diagnostic tests must be evaluated to determine their place in the diagnostic armamentarium. Criteria include sensitivity and specificity, positive and negative predictive values, likelihood ratios for positive and negative tests, and receiver operating characteristic curves. The prevalence of disease influences the diagnostic utility of tests, particularly when diseases are rare or very frequent (Fardy, 2009).

Microbial phenotypic characteristics, such as protein, bacteriophage, and chromatographic profiles, as well as biotyping and susceptibility testing, are used in most routine laboratories for identification and differentiation. Nucleic acid techniques, such as plasmid profiling, various methods for generating restriction fragment length polymorphisms, and the polymerase chain reaction, are making increasing inroads into clinical laboratories (Wagar, 1996).

PCR-based systems to detect the etiologic agents of disease directly from clinical samples, without the need for culture, have been useful in rapid detection of unculturable or fastidious microorganisms (Schluger et al., 1994). Additionally, sequence analysis of amplified microbial DNA allows for identification and better characterization of the pathogen. Subspecies variation, identified by various techniques, has been shown to be important in the prognosis of certain diseases. Other important advances include the determination of viral load and the direct detection of genes or gene mutations responsible for drug resistance. Increased use of automation and user-friendly software makes these technologies more widely available. In all, the detection of infectious agents at the nucleic acid level represents a true synthesis of clinical chemistry and clinical microbiology techniques (Tang et al., 1997).

19.2.1 Detection and Identification of Pathogens Without Target Amplification

Commercial kits containing non-isotopically labeled nucleic acid probes are available for direct detection of pathogens in clinical material and identification of organisms after isolation in culture (Table 19.1). Use of solution-phase hybridization has allowed tests to be performed singly or in batches in a familiar microwell format.

Although direct detection of organisms in clinical specimens by nucleic acid probes is rapid and simple, it suffers from lack of sensitivity. Most direct probe detection assays require at least 10^4 copies of nucleic acid per microliter for reliable detection, a requirement rarely met in clinical samples without some form of amplification. Amplification of the detection signal after probe hybridization improves sensitivity to as low as 500 gene copies per microliter and provides quantitative capabilities. This approach has been used extensively for quantitative assays of viral load (HIV, hepatitis B virus [HBV] and hepatitis C virus [HCV]) (Table 19.1) but does not match the analytical sensitivity of target amplification-based methods, such as polymerase chain reaction, for detecting organisms.

Table 19.1 FDA-approved molecular diagnostic tests for infectious diseases

Test	Method	Company
<i>Chlamydia trachomatis</i> detection	PCR ^a ; LCR; TMA; Hybrid capture	Roche Abbott Gen-Probe Digene
<i>Neisseria gonorrhoeae</i> detection	LCR; Hybrid capture	Abbott Digene
<i>C. trachomatis/N. gonorrhoeae</i>	Screening/detection; Hybridization	Becton-Dickinson
<i>Mycobacterium tuberculosis</i> detection	PCR TMA	Roche; Gen-Probe HPV screening Hybrid capture Digene
CMV	Hybrid capture	NASBA Digene Organon Teknika
Grp A strep detection	Hybridization HIV quantitation PCR	Gen-Probe Roche
<i>Gardnerella, T. vaginalis,</i> and <i>Candida</i>	Hybridization	Becton-Dickinson
Culture confirmation for bacteria and fungi	Hybridization	Gen-Probe

^aPCR = polymerase chain reaction; LCR = ligase chain reaction; TMA = transcription-mediated amplification.

The commercial probe systems that use solution-phase hybridization and chemiluminescence for direct detection of infectious agents in clinical material include the PACE2 products of Gen-Probe and the hybrid capture assay systems of Digene and Murex (Table 19.1). These systems are user friendly, have a long shelf life, and are adaptable to small or large numbers of specimens. The PACE2 products are designed for direct detection of both *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in a single specimen (one specimen, two separate probes). The hybrid capture systems detect human papilloma virus (HPV) in cervical scrapings (Ciotti et al., 2004), herpes simplex virus (HSV) in vesicle material, and cytomegalovirus (CMV) in blood and other fluids (Cinque et al., 1995). All these tests have demonstrated sensitivity exceeding that of culture or immunologic methods for detecting the respective pathogens but are less sensitive than PCR or other target amplification-based methods.

The signal amplification-based probe methods for detection and quantitation of viruses (HBV, HCV, HIV) are presented in an enzyme immunoassay-like format and include branched chain DNA probes (Chiron) and QB replicase (Gene-Trak) methods (Table 19.1). These methods are not as sensitive as target amplification-based methods for detection of viruses; however, the quantitative results have proven useful for determining viral load and prognosis and for monitoring response to therapy.

Probe hybridization is useful for identifying slow-growing organisms after isolation in culture using either liquid or solid media. Identification of mycobacteria and other slow-growing organisms such as the dimorphic fungi (*Histoplasma capsulatum*, *Coccidioides immitis*, and *Blastomyces dermatitidis*) has certainly been facilitated by commercially available probes. All commercial probes for identifying organisms are produced by Gen-Probe and use acridinium ester-labeled probes directed at species-specific rRNA sequences (Table 19.1). Gen-Probe products are available for the culture identification of *Mycobacterium tuberculosis*, *M. avium-intracellulare* complex, *M. gordonae*, *M. kansasii*, *Cryptococcus neoformans*, the dimorphic fungi, *N. gonorrhoeae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Haemophilus influenzae*, *Enterococcus* spp., *S. agalactiae*, and *Listeria monocytogenes*. The sensitivity and specificity of these probes are excellent, and they provide species identification within one working day. Because most of the bacteria listed, plus *C. neoformans*, can be easily and efficiently identified by conventional methods within 1–2 days, many of these probes have not been widely used. The mycobacterial probes, on the other hand, are accepted as mainstays for the identification of *M. tuberculosis* and related species.

19.2.2 Nucleic Acid Amplification

Nucleic acid amplification provides the ability to selectively amplify specific targets present in low concentrations to detectable levels; thus, amplification-based methods offer superior performance, in terms of sensitivity, over the direct (non-amplified) probe-based tests. PCR (Roche Molecular Systems, Branchburg, NJ) was the first such technique to be developed and because of its flexibility and ease of

performance remains the most widely used molecular diagnostic technique in both research and clinical laboratories. Several different amplification-based strategies have been developed and are available commercially (Table 19.1). Commercial amplification-based molecular diagnostic systems for infectious diseases have focused largely on systems for detecting *N. gonorrhoeae*, *C. trachomatis*, *M. tuberculosis*, and specific viral infections (HBV, HCV, HIV, CMV, and enterovirus) (Table 19.1).

Given the adaptability of PCR, numerous additional infectious pathogens have been detected by investigator-developed PCR assays (Table 19.2). In many instances, such tests provide important and clinically relevant information that would otherwise be unavailable since commercial interests have been slow to expand the line of products available to clinical laboratories. In addition to qualitative detection of viruses, quantitation of viral load in clinical specimens is now recognized to be of great importance for the diagnosis, prognosis, and therapeutic monitoring for HCV, HIV, HBV, and CMV. Both PCR and nucleic acid strand-based amplification systems are available for quantitation of one or more viruses.

The adaptation of amplification-based test methods to commercially available kits has served to optimize user acceptability, prevent contamination, standardize reagents and testing conditions, and make automation a possibility. It is not clear to what extent the levels of detection achievable by the different amplification strategies differ. None of the newer methods provides a level of sensitivity greater than that of PCR. In choosing a molecular diagnostic system, one should consider the range of tests available, suitability of the method to workflow, and

Table 19.2 Noncommercial nucleic acid-based tests for clinically important viral and bacterial pathogens

Organism	Specimen type	Clinical indication
Epstein-Barr virus (EBV)	Cerebrospinal fluid (CSF)	EBV lymphoproliferative disorder
Herpes simplex virus (HSV) types 1 and 2	CSF; Vitreous humor	Encephalitis
Varicella-zoster virus (VZV)	Various tissues	VZV reactivation
JC virus	CSF	Progressive multifocal leukoencephalopathy
Enterovirus	CSF	Aseptic meningitis
Parvovirus B19	Amniotic fluid; serum	Hydrops fetalis; Anemia
Adenovirus	Urine; tissues; blood;	Immunocompromised patients, transplant recipients
<i>Ehrlichia</i>	Blood; human	Granulocytic and monocytic ehrlichiosis
<i>Bordetella pertussis</i>	Nasopharyngeal aspirate	Whooping cough
<i>Legionella pneumophila</i>	Respiratory	Atypical pneumonia
<i>Chlamydia pneumoniae</i>	Respiratory	Atypical pneumonia
<i>Mycoplasma pneumoniae</i>	Respiratory	Atypical pneumonia
<i>Helicobacter pylori</i>	Gastric fluid; stool	Peptic ulcer disease

All tests use polymerase chain reaction. The list is not all-inclusive.

cost. Choosing one amplification-based method that provides testing capabilities for several pathogens is certainly practical.

Amplification-based methods are also valuable for identifying cultured and non-cultivable organisms. Amplification reactions may be designed to rapidly identify an acid-fast organism as *M. tuberculosis* or may amplify a genus-specific or “universal” target, which then is characterized by using restriction endonuclease digestion, hybridization with multiple probes, or sequence determination to provide species or even subspecies delineation. Although identification was initially applied to slow-growing mycobacteria, it has applications for other pathogens that are difficult or impossible to identify with conventional methods.

19.2.3 Detecting Antimicrobial-Drug Resistance

Molecular methods can rapidly detect antimicrobial-drug resistance in clinical settings and have substantially contributed to our understanding of the spread and genetics of resistance. Conventional broth- and agar-based antimicrobial susceptibility testing methods provide a phenotypic profile of the response of a given microbe to an array of agents. Although useful for selecting potentially useful therapeutic agents, conventional methods are slow and fraught with problems. The most common failing is in the detection of methicillin resistance in staphylococci, which may be expressed in a very heterogeneous fashion, making phenotypic characterization of resistance difficult. Currently, molecular detection of the resistance gene, *mec A*, is the standard against which phenotypic methods for detection of methicillin resistance are judged.

Molecular methods may be used to detect specific antimicrobial-drug resistance genes (resistance genotyping) in many organisms (Table 19.3). Detection of specific point mutations associated with resistance to antiviral agents is also increasingly important. Screening for mutations in an amplified product may be facilitated by the use of high-density probe arrays (Gene chips).

Despite its many potential advantages, genotyping will not likely replace phenotypic methods for detecting antimicrobial-drug resistance in the clinical laboratory in the near future. Molecular methods for resistance detection (Table 19.3) may be applied directly to the clinical specimen, providing simultaneous detection and identification of the pathogen plus resistance characterization. Likewise, they are useful in detecting resistance in viruses, slow-growing or nonviable organisms, or organisms with resistance mechanisms that are not reliably detected by phenotypic methods. However, because of their high specificity, molecular methods will not detect newly emerging resistance mechanisms and are unlikely to be useful in detecting resistance genes in species where the gene has not been observed previously. Furthermore, the presence of a resistance gene does not mean that the gene will be expressed, and the absence of a known resistance gene does not exclude the possibility of resistance from another mechanism. Phenotypic antimicrobial susceptibility testing methods allow laboratories to test many organisms and detect newly emerging as well as established resistance patterns.

Table 19.3 Molecular methods for detecting antimicrobial resistance^a

Organism(s)	Antimicrobial agent(s)	Gene	Detection method
Staphylococci	Methicillin; Oxacillin	<i>mec A</i> ^b	Standard DNA probe; PCR; Branched chain DNA probe
Enterococci	Vancomycin	<i>van A, B, C, D</i> ^c	Standard DNA; probe PCR
Enterobacteriaceae <i>Haemophilus influenzae</i> <i>Neisseria gonorrhoeae</i>	Beta-lactams	<i>bla</i> _{TEM} <i>bla</i> _{SHV} ^d	Standard probe; PCR and RFLP; PCR; sequencing
Enterobacteriaceae and gram-positive cocci	Quinolones	Point mutations in <i>gyr A, gyr B, par C</i> and <i>par E</i>	PCR and sequencing
<i>Mycobacterium tuberculosis</i> ^e	Rifampin Isoniazid Streptomycin	Point mutations in <i>rpo B; kat G, inh A, and ahp C</i> ; in <i>emb B; rps L</i> and <i>rrs</i>	PCR and SSCP PCR and sequencing PCR and SSLP PCR and SSCP PCR and RFLP
Herpes viruses ^f	Acyclovir and related drugs	Mutations in the TK gene	PCR and sequencing
Iv ^g	Nucleoside reverse transcriptase inhibitors	Protease inhibitors Point mutations in RT gene Point mutations in PROT gene	PCR and sequencing PCR and LIPA PCR and sequencing

^aAdapted from Pfaller and Herwaldt (1997).

^b*mecA* encodes for the altered penicillin binding protein PBP2a'; phenotypic methods may require 48 h incubation or more to detect resistance and are less than 100% sensitive. Detection of *mecA* has potential for clinical application in specific circumstances.

^cVancomycin resistance in enterococci may be related to one of four distinct resistance genotypes of which *vanA* and *vanB* are most important. Genotypic detection of resistance is useful in validation of phenotypic methods.

^dThe genetic basis of resistance to beta-lactam antibiotics is extremely complex. The *bla*_{TEM} and *bla*_{SHV} genes are the two most common sets of plasmid encoded beta-lactamases. The presence of either a *bla*_{TEM} or *bla*_{SHV} gene implies ampicillin resistance. Variants of the *bla*_{TEM} and *bla*_{SHV} genes (extended spectrum beta-lactamases) may also encode for resistance to a range of third-generation cephalosporins and to monobactams.

^e*M. tuberculosis* is very slow growing. Four weeks or more may be required to obtain phenotypic susceptibility test results. Detection of resistance genes in *M. tuberculosis* has potential for clinical application in the short term.

^fThere are no phenotypic methods sufficiently practical for routine clinical detection of resistance to antiviral agents. Genotypic methods represent a practical method for routine detection of antiviral resistance.

^gAbbreviations not defined in text: RFLP, restriction fragment length polymorphism; SSCP, single-stranded conformational polymorphism; LIPA, line probe assay; TK, thymidine kinase; RT, reverse transcriptase; PROT, protease.

19.3 Molecular Diagnostics of Infections

During the past 10–15 years, we have seen expansive growth of the use of molecular technology in the clinical laboratory for diagnosing infectious diseases (Jeffrey et al., 2001). As a result, many laboratories are able to offer more sensitive testing, faster turnaround times, and ultimately improved patient care. The gold standard in bacteriology largely remains culture, primarily due to cost accounting and the potential complex nature of associated infections (i.e., urine, wound, and respiratory cultures).

However, in circumstances in which there may be minute quantities of a specific pathogen present, the patient may have received antibiotics prior to specimen collection or the etiologic agent may require unusual culture conditions, molecular detection offers a great advantage to culture techniques. In many virology laboratories, molecular detection has supplanted cell culture techniques for the identification of several viral pathogens and in many cases has become the new gold standard. Though molecular techniques can offer an abundance of added benefits when used to augment current gold standards such as culture and/or serology, the optimal use of molecular methodologies in microbiology resides with specimens in which a limited number of pathogenic organisms are sought and in cases where the enhanced sensitivity and faster turnaround time of molecular methods far outweighs the increased cost.

19.3.1 Sexually Transmitted Infections

Sexually transmitted infections (STIs) constitute an important world-wide public health problem (Msuya et al., 2009). The use of sensitive and specific laboratory methods for diagnosing this condition is crucial to reduce the transmission and sequelae of STI. *Neisseria gonorrhoeae* and *Chlamydia trachomatis* are the pathogens most frequently involved in urethral and cervical infection. Culture continues to be the gold standard for diagnosing gonorrhea. Nucleic acid amplification assays are considered the new gold standard for *C. trachomatis*, although culture is still the most specific technique. Genital ulcers due to *Treponema pallidum*, *Haemophilus ducreyi*, or herpes simplex virus have little clinical and bacteriological correlation; therefore, it is essential to establish the microbiological diagnosis. Lesions present in the primary or secondary period of syphilis can be diagnosed by dark field microscopy. Serologic diagnosis for the remaining periods is based on non-treponemal tests associated with confirmatory treponemal tests. Cell culture is considered the gold standard for herpes simplex virus although molecular methods also have a sensitivity and specificity near 100%. Currently, microbiologic diagnosis of *H. ducreyi* and venereal lymphogranuloma is achieved with the use of molecular methods on samples obtained from the ulceration or lymphadenopathy. The diagnosis of genital warts in immunocompetent patients is based on clinical findings in most cases because the lesions are sufficiently characteristic. Culture is considered the reference method in *Trichomonas vaginalis* infection (Vázquez et al., 2008).

Several new techniques for diagnosis of the sexually transmitted diseases have been developed during the past decade. The rapid, nonculture techniques for direct detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and herpes simplex virus are reviewed, and their advantages and disadvantages are compared with conventional diagnostic methods (Woods, 1995). Polymerase chain reaction tests were performed on specimens from consecutive male patients with genital ulcers to detect sexually transmitted pathogens. PCR was also performed for the detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Trichomonas vaginalis* on urethral specimens from consecutive subjects with dysuria or urethral discharge. Antibody tests for syphilis and Herpes simplex virus type-2 (HSV-2) and human immunodeficiency virus antibodies were performed. Men at risk of genital ulcers should be asked about relevant symptoms with and without prompting and examined clinically to maximize the likelihood of correct diagnosis and treatment. The finding of a high prevalence of HSV-2 and associated dysuria cautions against providing empirical treatment for gonorrhoea and chlamydia in ulcer patients with dysuria but without urethral discharge. Innovative strategies to limit the burden of HSV-2 infection in this population are required (O'Farrell et al., 2008).

Even then, microbiological screening is incorporated into STI control strategies; lack of access to appropriate services (especially in rural and remote areas), reluctance of at-risk populations to attend for treatment, fear of invasive genital examinations, and lower sensitivities of conventional diagnostic assays reduces the effectiveness of such programmes. Therefore, accurate, cost-effective, reliable diagnostic assays (preferably those which can be used in the field) are needed to impact on the incidence of the various STIs, as well as HIV. With the advent of molecular technologies, including target and signal amplification methods, diagnoses of STIs have been revolutionised and allow the use of non or minimally invasive sampling techniques, some of which are self-collected by the patient, e.g. first-void urine, cervico-vaginal lavage, low vaginal swabs, and tampons. Most studies evaluating such self-sampling with molecular diagnostic techniques have demonstrated an equivalent or superior detection of STIs as compared to conventional sampling and detection methods. These sampling methods can also be used to determine prevalence of STIs in various populations, but particularly those with difficult access to medical care (Garland and Tabrizi, 2004).

The presence of *Trichomonas vaginalis* (TV) is associated with an increased risk of coinfection with *Chlamydia trachomatis* (CT) and/or *Neisseria gonorrhoeae* (NG) in female patients presenting to the ED with symptoms consistent with a sexually transmitted disease (STD; White et al., 2005). Vaginal swabs are the specimens of choice when screening for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*: results from a multicenter evaluation of the APTIMA assays for both infections (Schachter et al., 2005). The Gen-Probe APTIMA Combo 2 (AC2) is a Food and Drug Administration-cleared nucleic acid amplification test (NAAT) for the detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* from urine and urogenital swab specimens. The Centers for Disease Control and Prevention have recommended confirmation of positive NAAT results in low-prevalence populations. APTIMA CT (ACT) and APTIMA GC (AGC) are two discrete NAATs for

C. trachomatis and *N. gonorrhoeae* detection that are suitable for confirming AC2-positive results because they target different nucleic acid sequences. Thus, ACT and AGC relative sensitivity compared to that of AC2 was 100%. All APTIMA assays detected more confirmed positive results than culture, DFA, and LCx. The performance of APTIMA assays was not altered by the use of various swab types and by long-term storage of specimens. All APTIMA assays are highly sensitive and rapid. ACT and AGC can be recommended for confirmation of positive results from other NAATs, such as AC2 and LCx (Boyardzhyan et al., 2004).

19.3.2 Vector Borne Disease

Lyme borreliosis caused by the spirochete *Borrelia burgdorferi* is now the most common vector borne disease in North America, Europe and Asia. It is a multi-systemic infection which may cause skin, neurological, cardiac or rheumatologic disorders. The use of serologic testing and its value in the diagnosis of Lyme disease remain confusing and controversial for physicians, especially concerning persons who are at low risk for the disease. Samples drawn from patients within four weeks of disease onset are tested by Western blot technique for both immunoglobulin M and immunoglobulin G antibodies; samples drawn more than four weeks after disease onset are tested for immunoglobulin G only (DePietropaolo et al., 2006).

Current serologic Lyme disease tests use whole borrelia cells as the source of antigen. These assays are difficult to standardize and to optimize for sensitivity and specificity.

The recently introduced highly sensitive PCR methodology can be used for amplification and subsequent identification of *B. burgdorferi* specific sequences. The diagnostic sensitivity of PCR was comparable to and even higher than in vitro culture. PCR was significantly more sensitive than a histological *B. burgdorferi* specific immunophosphatase-staining method. The sensitivity of PCR was 71%, which was superior to culture and serology. There is clear evidence for PCR being the most sensitive and specific test for detection of *B. burgdorferi* in skin biopsies from patients with both early and late dermatoborreliosis. PCR can be used as a diagnostic aid in these patients. However, in general the measurement of specific intrathecal antibody production in patients with neuroborreliosis was superior to PCR. Furthermore, strain differences were of importance for selection of suitable antigens for diagnostic assays and for vaccine development. Since then, *B. burgdorferi* isolates have been studied by phenotypic and genotypic traits and have been shown to be highly heterogeneous (Lebech, 2002).

19.3.3 Viral Infections

Viral infections are common causes of respiratory tract disease in the outpatient setting. Some viruses, such as influenza, respiratory syncytial virus (RSV), cytomegalovirus (CMV), and varicella-zoster virus (VZV), are relatively common. Others, such as adenovirus, severe acute respiratory syndrome (SARS)-coronavirus,

Hantavirus, and the viral hemorrhagic fevers (VHFs), are rare but have an immense public health impact. In cases of acute LRI, rapid diagnosis of the pathogens may be very important for initiating antiviral therapy, stopping viruses affecting humans throughout the world. Current methods used in the diagnosis of infections with RSV A and B, influenza virus A and B, and HPIV-1, -2, and costs of treating children with LRIs exceed \$700 million in the United States. Respiratory syncytial virus (RSV) type A -3 include virus isolation, shell vial assay, antigen detection, and serology. Major limitations of these techniques include and type B, influenza A and B viruses, and human parainfluenza virus (HPIV) types 1, 2, and 3 cause about 80–90% of viral low sensitivity, low specificity, and prolonged time to completion.

Infants, the elderly, and individuals with compromised cardiac, pulmonary, or immune systems are at the greatest risk. The development of rapid molecular techniques such as gene amplification (e.g., PCR) has allowed for the detection of small risk of serious complications from these viruses. A multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex; Prodesse, Milwaukee) was developed and used to rapidly detect and quantitate RNA of respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3 in nasal wash specimens in a single test. Primers and probes originated from highly conserved regions of each viral genome (Fan et al., 1998). PCR will become the first-line diagnostic test for viral meningitis and encephalitis (Jeffery et al., 1997).

Molecular biology-based assays are invaluable tools for the management of chronic viral hepatitis. They can be used to test blood donations, diagnose active infection, help to establish the prognosis, guide treatment decisions, and assess the virological response to therapy (Pawlotsky, 2002).

Human papilloma virus are strictly epitheliotropic and host-specific. Currently, 57 different types based on deoxyribonucleic acid sequence rather than serology have been identified. Some papilloma virus types infect the skin, while others infect the oral or genital mucous membranes. Human Papilloma virus type 16 is often associated with invasive carcinomas of the uterine cervix and of their intra-epithelial precursors. If various techniques for the detection of human papilloma virus may be used (cytology, electron microscopy and immunocytochemistry), as will become apparent, nucleic acid hybridization is the most reliable method for viral diagnosis and genotyping (Morinet, 1991).

Zika virus (ZIKV) is an emerging mosquito-borne flavivirus circulating in Asia and Africa. Human infection induces an influenza-like syndrome that is associated with retro-orbital pain, oedema, lymphadenopathy, or diarrhea. Diagnosis of Zika fever requires virus isolation and serology, which are time consuming or cross-reactive. A one-step RT-PCR assay to detect ZIKV in human serum was successfully reported by Faye et al., (2008).

Detection of virus genomes and their mutants by molecular technology is essential in clinical practice for patients with hepatitis B virus (HBV) and hepatitis C virus (HCV) related liver diseases (Silva et al., 2006). A multiplex PCR (M-PCR) assay with colorimetric detection was devised for the simultaneous amplification of DNA targets from *Haemophilus ducreyi*, *Treponema pallidum*, and herpes simplex virus

(HSV) types 1 and 2. The M-PCR assay is more sensitive than standard diagnostic tests for the detection of HSV, *H. ducreyi*, and *T. pallidum* from genital ulcers (Orle et al., 1996).

Polymerase chain reaction is a highly sensitive technique for the detection of hepatitis B virus-DNA and hepatitis C virus-RNA in serum, liver tissue, and peripheral mononuclear blood cells. Polymerase chain reaction detection of hepatitis C virus-RNA in serum may be the only means of confirming acute hepatitis C infection and also of identifying viraemia in the chronic disease, particularly in anti-hepatitis C virus antibody-negative individuals. It can also be used for direct evaluation of mother to child hepatitis C virus transmission. As in hepatitis B, polymerase chain reaction can be used for monitoring reinfection with hepatitis C virus after liver transplant, and has proved invaluable in identification of different hepatitis C virus genotypes. The efficacy of antiviral treatment can also be monitored using polymerase chain reaction. Polymerase chain reaction has thus shown numerous advantages for disease detection and monitoring despite the limitations imposed, for example, by possible contamination problems and semiquantitative evaluations (Bréchet, 1993).

A nested polymerase chain reaction (PCR) was evaluated for the detection of cytomegalovirus (CMV) DNA in cerebrospinal fluid (CSF). CMV PCR on CSF is highly sensitive and specific. It should be considered a rapid and reliable diagnostic method for CMV infection of the central nervous system (Cinque, 1995).

Molecular detection of viruses has extended beyond the standard therapeutic monitoring of viral loads in specific patient populations. Cell culture techniques are insensitive due to the low viral burden typically found associated with encephalitis and perhaps also the presence of host neutralizing antibodies. CSF culture for HSV detects less than 2% of clinically determined adult HSV encephalitis cases and 40% of neonatal central nervous system (CNS) disease. In contrast, HSV NAA is positive in most adult cases resulting in sensitivity and specificity > 95% and is 75% sensitive and 100% specific for neonatal meningitis. The rapid diagnosis of HSV encephalitis can prevent a brain biopsy and rapidly determine the need for acyclovir therapy. Enterovirus is the most common cause of aseptic meningitis in the summer and fall months in temperate climates and accounts for 10–20% of encephalitis cases. Nucleic acid amplification has also been successfully applied to other etiologies of viral CNS disease, such as CMV and varicella-zoster virus (VZV), but these assays have not been implemented as broadly as those for HSV and EV, so are still transitioning to becoming the method of choice. It should be noted that not all encephalitis viruses are readily detected by NAA. For example, due to the short period of viremia in many carboviral infections (i.e., West Nile Virus), CSF NAA has low sensitivity, and the gold standard remains serology. CSF NAA false negative results can occur due to collection of CSF very early or very late in illness, rapid viral clearance in immunocompetent hosts, and NAA inhibitors. False positive CSF NAA results also occur primarily due to lack of data to suggest the detection of certain viral nucleic acids correlates with clinical CNS disease, but can also be caused by the presence of peripheral blood in the CSF. While CSF NAA is considered by many the diagnostic standard of care as discussed above, the lack of

standardized FDA-approved assays has made implementation of CSF NAA difficult in nonacademic settings. While most laboratories offering CSF NAA use qualitative methods, data indicate a role for quantitative CSF NAA in differentiating nonspecific presence of virus and virus-associated disease, to aid in prognosis for improved patient management, and in monitoring antiviral therapy.

19.3.4 Bacterial Infections

The quest for the search of rapid, cheap, easy to perform and highly sensitive and specific tests has resulted into the introduction and application of various new tests for the diagnosis of bacterial meningitis. Most of these tests are based on immunological principles, viz. counter-immunoelectrophoresis, latex agglutination test, co-agglutination, radio-immunoassay, haemagglutination inhibition as well as study of immune profile of cerebrospinal fluid. Apart from these certain non-immunological tests viz. Limulus amoebocyte lysate test, gas liquid chromatography, nitroblue tetrazolium dye reduction test have also been evaluated to make the laboratory diagnosis of this important clinical entity rapid and more reliable (Ichhpujani and Bhatia, 1984)

The tuberculin skin test for immunologic diagnosis of *Mycobacterium tuberculosis* infection has many limitations, including being confounded by Bacillus Calmette-Guérin (BCG) vaccination or exposure to nontuberculous mycobacteria. *M. tuberculosis*-specific antigens that are absent from BCG and most nontuberculous mycobacteria have been identified (Tiwari et al., 2007). Mori et al., (2004) examined the use of two of these antigens, CFP-10 and ESAT-6, in a whole blood IFN-gamma assay as a diagnostic test for tuberculosis in BCG-vaccinated individuals.

An enzyme-linked immunoassay for diagnosis of tuberculosis was devised by using a shotgun immunoexpression library in the *_gt11* vector. DNA from a virulent *M. tuberculosis* strain was sheared and expressed to generate shotgun polypeptides.

Promising mycobacterial DNA cassettes were subcloned and expressed into the glutathione *S*-transferase (GST) fusion vector pGEX-5X-1 with a strong *tac* promoter and were expressed in *Escherichia coli* BL21. These recombinant proteins when used in ELISA differentiated BCG-vaccinated healthy subjects and patients with active tuberculosis and proved to be effective in detecting pulmonary as well as extrapulmonary tuberculosis, with an overall sensitivity of 84.33% and an overall specificity of 93.62% (Bisen et al., 2003). A simple, rapid and cost-effective glycolipid antibody based liposome agglutination assay viz., TB Screen Test, for diagnosis of patients with pulmonary and extrapulmonary tuberculosis with 94% sensitivity and 98.3% specificity was developed (Tiwari et al., 2005). The assay clearly differentiated healthy controls and *M. bovis* BCG-vaccinated subjects from those with active tuberculosis. A TB/M card test based on liposomal agglutination test was developed by Tiwari et al. (2007) for detection of glycolipid antigens of *M. tuberculosis*. The test was shown to be effective in detecting glycolipid antigens of *M. tuberculosis* in clinical samples from patients with active TB with as

low as 1 ng/ml analytical sensitivity, 97.4% clinical sensitivity and 96.9% specificity. The test was proved to be highly economical, rapid, simple and instrument free. The test seems fairly useful for mass testing of a variety of biological specimens (cerebrospinal, pleural and synovial fluids, serum, tissue biopsy extract) from patients with tuberculous meningitis, pulmonary TB and other extra-pulmonary TB in endemic countries.

The sensitivity of NAA detection of MTB in smear-positive respiratory specimens is 96.9%, and the specificity is 100%, whereas the sensitivity and specificity in smear-negative specimens is 72.0 and 99.3%, respectively. It should be noted that NAA of MTB does not replace the need for routine mycobacterial culture and susceptibility testing. In addition to the direct detection of MTB, techniques such as probe-based technology and sequence analysis can be applied to cultured isolates to decrease the time to identification over routine biochemical analysis. Rapid identification of MTB impacts not only patient care, but also infection control. Due to the increasing frequency of isolation of mycobacterial species associated with immunocompromised hosts and the increased incidence of multi-drug resistant MTB, it has become imperative to offer accurate yet rapid diagnostic tools for the detection and identification of mycobacteria (Tiwari et al., 2007). A debate exists regarding the gold standard for the laboratory diagnosis of *Bordetella pertussis*. Historically, culture plates collected at the patient's bedside (i.e., cough plates) have been considered the reference method. Although culture is very specific, its sensitivity suffers partially due to the organism's fastidious nature, but primarily because the highest sensitivity for culture occurs before patients are symptomatic. NAA remains positive for longer after therapy than culture, and NAA is also positive for a longer period after onset of symptoms. Therefore, NAA is useful for patients presenting later in their illness. NAA testing allows for same-day results and since erythromycin-resistant *B. pertussis* is still rare, a cultured isolate is rarely needed for antimicrobial susceptibility testing. Multiple studies have demonstrated significant increased detection of *B. pertussis* when comparing NAA to culture: reported PCR-positive, culture-negative samples range from 13 to 88%. However, due to potential false positive and false negative results with *B. pertussis* NAA procedures, it is strongly recommended that results be considered in the context of patient clinical presentation, and clinically inconsistent results should be confirmed by a second method.

NAA is also being used in bacteriology to detect antimicrobial resistance. Since antimicrobial resistance can be multi-factorial, this practice is limited to organisms in which the results can be interpreted with confidence in regard to the genotypic relationship to clinical treatment and/or infection control precautions. Such examples are direct detection of vancomycin-resistant *Enterococcus* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) from rectal and nares surveillance cultures, respectively. Screening patients for VRE and MRSA carriage is a key strategy for preventing the spread of these organisms in health care settings. NAA technology reportedly increases VRE detection by up to 120%. In addition, enterococci that confer low-level intrinsic resistance, and thus not considered "true" VRE, are accurately ruled out preventing unnecessary contact precautions

and contributing to hospital savings. NAA detection of MRSA has been shown to be equal in sensitivity to culture-based methods, but has the advantage of offering a faster turnaround time, thus impacting hospital cost savings. However, it should be noted that direct specimen testing for MRSA comes with limitations, often including a lower positive predictive value than conventional methods. More recently, new strains of MRSA have appeared that are associated with skin and soft tissue infections in outpatients and are called community-associated MRSA (CA-MRSA). The increasing incidence of CA-MRSA is causing overall rates of MRSA to rise. Therefore, it has become even more important to quickly and accurately identify resistant isolates.

19.3.5 Fungal Infections

The incidence of invasive fungal infections has increased considerably in recent years. Mycetomas are chronic, granulomatous, subcutaneous infections caused by either actinomycetes bacteria or eumycetes fungi. The disease is endemic in the tropics and is characterised by a slow progression with risks of bone and visceral involvement. There have been notable advances in improved molecular techniques for species identification. Carbapenems, oxazolidinones and triazoles have emerged as promising therapeutic options, but access to drug therapies in developing countries remains limited by the poor availability and high costs (Ameen and Arenas, 2008). The molecular assay using PCR-ELISA help in the diagnosis of invasive fungal infections at the early stage of infection, before clinical manifestations (Badiee et al., 2009).

19.3.6 Practical Applications of Molecular Methods in the Clinical Microbiology Laboratory

Commercial kits for the molecular detection and identification of infectious pathogens have provided a degree of standardization and ease of use that has facilitated the introduction of molecular diagnostics into the clinical microbiology laboratory (Table 19.1). The use of nucleic acid probes for identifying cultured organisms and for direct detection of organisms in clinical material was the first exposure that most laboratories had to commercially available molecular tests. Although these probe tests are still widely used, amplification-based methods are increasingly employed for diagnosis, identification and quantitation of pathogens, and characterization of antimicrobial-drug resistance genes. Commercial amplification kits are available for some pathogens (Table 19.1), but some clinically important pathogens require investigator-designed or “home-brew” methods (Table 19.2). In addition, molecular strain typing, or genotyping, has proven useful in guiding therapeutic decisions for certain viral pathogens and for epidemiologic investigation and infection control.

19.3.6.1 Molecular Epidemiology

Laboratory characterization of microbial pathogens as biologically or genetically related is frequently useful in investigations. Several different epidemiologic typing methods have been applied in studies of microbial pathogens (Table 19.4). The phenotypic methods have occasionally been useful in describing the epidemiology of infectious diseases; however, they are too variable, slow, and labor-intensive to be of much use in most epidemiologic investigations. Newer DNA-based typing methods have eliminated most of these limitations and are now the preferred techniques for epidemiologic typing. The most widely used molecular typing methods include plasmid profiling, restriction endonuclease analysis of plasmid and genomic DNA, Southern hybridization analysis using specific DNA probes, and chromosomal DNA profiling using either pulsed-field gel electrophoresis (PFGE) or PCR-based methods. All these methods use electric fields to separate DNA

Table 19.4 Genotypic methods for epidemiologic typing of microorganisms^a

Method	Examples	Comments
Plasmid analysis	Staphylococci enterobacteriaceae	Plasmids may be digested with restriction endonuclease, only useful when organisms carry plasmids
Restriction endonuclease analysis of chromosomal DNA with electrophoresis	Enterococci; <i>Staphylococcus aureus</i> <i>Clostridium difficile</i> ; <i>Candida</i> spp	Large number of bands difficult to interpret not amenable to computer analysis
PFGE	Enterobacteriaceae; Staphylococci Enterococci; <i>Candida</i> spp.	Fewer bands; Amenable to computer analysis very broad application
Genome restriction fragment length polymorphism analysis; ribotyping, insertion sequence probe fingerprinting	Enterobacteriaceae; Staphylococci <i>Pseudomonas aeruginosa</i> <i>Mycobacterium tuberculosis</i> <i>Candida</i> spp.	Fewer bands; computer analysis Sequence-based profiles Automated
PCR-based methods: repetitive elements PCR spacer typing, selective amplification of genome restriction fragments, multilocus allelic sequence-based typing	Enterobacteriaceae <i>Acinetobacter</i> spp. Staphylococci <i>M. tuberculosis</i> HCV	Crude extracts and small amounts of DNA may suffice
Library probe genotypic hybridization schemes: multilocus probe dot-blot patterns, high-density oligonucleotide patterns	<i>Burkholderia cepacia</i> <i>S. aureus</i> <i>M. tuberculosis</i> High-density	Unambiguous yes-no result Less discrimination than other methods Couple with DNA chip technology

^aThe table contains examples of available methods and applications and is not intended to be all-inclusive; Adapted from Pfaller.

fragments, whole chromosomes, or plasmids into unique patterns or fingerprints that are visualized by staining with ethidium bromide or by nucleic acid probe hybridization. Molecular typing is performed to determine whether different isolates give the same or different results for one or more tests. Epidemiologically related isolates share the same DNA profile or fingerprint, whereas sporadic or epidemiologically unrelated isolates have distinctly different patterns. If isolates from different patients share the same fingerprint, they probably originated from the same clone and were transmitted from patient to patient by a common source or mechanism.

Molecular typing methods have allowed investigators to study the relationship between colonizing and infecting isolates in individual patients, distinguish contaminating from infecting strains, document nosocomial transmission in hospitalized patients, evaluate reinfection versus relapse in patients being treated for an infection, and follow the spread of antimicrobial-drug resistant strains within and between hospitals over time. Most available DNA-based typing methods may be used in studying nosocomial infections when applied in the context of a careful epidemiologic investigation. In contrast, even the most powerful and sophisticated typing method, if used indiscriminately in the absence of sound epidemiologic data, may provide conflicting and confusing information.

19.3.6.2 Financial Considerations

Molecular testing for infectious diseases includes testing for the host's predisposition to disease, screening for infected or colonized persons, diagnosis of clinically important infections, and monitoring the course of infection or the spread of a specific pathogen in a given population. It is often assumed that in addition to improved patient care, major financial benefits may accrue from molecular testing because the tests reduce the use of less sensitive and specific tests, unnecessary diagnostic procedures and therapies, and nosocomial infections. However, the inherent costs of molecular testing methods, coupled with variable and inadequate reimbursement by third-party payers and managed-care organizations, have limited the introduction of these tests into the clinical diagnostic laboratory.

Not all molecular diagnostic tests are extremely expensive. Direct costs vary widely, depending on the test's complexity and sophistication. Inexpensive molecular tests are generally kit based and use methods that require little instrumentation or technologist experience. DNA probe methods that detect *C. trachomatis* or *N. gonorrhoeae* are examples of low-cost molecular tests. The more complex molecular tests, such as resistance genotyping, often have high labor costs because they require experienced, well-trained technologists. Although the more sophisticated tests may require expensive equipment (e.g., DNA sequencer) and reagents, advances in automation and the production of less-expensive reagents promise to decrease these costs as well as technician time. Major obstacles to establishing a molecular diagnostics laboratory that are often not considered until late in the process are required licenses, existing and pending patents, test selection, and billing and reimbursement.

Molecular screening programs for infectious diseases are developed to detect symptomatic and asymptomatic disease in individuals and groups. Persons at high risk, such as immunocompromised patients or those attending family planning or obstetrical clinics, are screened for CMV and *Chlamydia*, respectively. Likewise, all blood donors are screened for bloodborne pathogens. The financial outcome of such testing is unknown. The cost must be balanced against the benefits of earlier diagnosis and treatment and societal issues such as disease epidemiology and population management.

One of the most highly touted benefits of molecular testing for infectious diseases is the promise of earlier detection of certain pathogens. The rapid detection of *M. tuberculosis* directly in clinical specimens by PCR or other amplification-based methods is quite likely to be cost-effective in the management of tuberculosis. Other examples of infectious disease that are amenable to molecular diagnosis and for which management can be improved by this technology include HSV encephalitis, *Helicobacter pylori* infection, and neuroborreliosis caused by *Borrelia burgdorferi*. For HSV encephalitis, detection of HSV in cerebrospinal fluid (CSF) can direct specific therapy and eliminate other tests including brain biopsy. Likewise, detection of *H. pylori* in gastric fluid can direct therapy and obviate the need for endoscopy and biopsy. PCR detection of *B. burgdorferi* in CSF is helpful in differentiating neuroborreliosis from other chronic neurologic conditions and chronic fatigue syndrome.

As discussed earlier, molecular tests may be used to predict disease response to specific antimicrobial therapy. Detection of specific resistance genes (*mec A*, *van A*) or point mutations resulting in resistance has proven efficacious in managing disease. Molecular-based viral load testing has become standard practice for patients with chronic hepatitis and AIDS. Viral load testing and genotyping of HCV are useful in determining the use of expensive therapy such as interferon and can be used to justify decisions on extent and duration of therapy. With AIDS, viral load determinations plus resistance genotyping have been used to select among the various protease inhibitor drugs available for treatment, improving patient response and decreasing incidence of opportunistic infections.

Pharmacogenomics is the use of molecular-based tests to predict the response to specific therapies and to monitor the response of the disease to the agents administered. The best examples of pharmacogenomics in infectious diseases are the use of viral load and resistance genotyping to select and monitor antiviral therapy of AIDS and chronic hepatitis. This application improves disease outcome; shortens length of hospital stay; reduces adverse events and toxicity; and facilitates cost-effective therapy by avoiding unnecessary expensive drugs, optimizing doses and timing, and eliminating ineffective drugs.

Molecular strain typing of microorganisms is now well recognized as an essential component of a comprehensive infection control program that also involves the infection control department, the infectious disease division, and pharmacy. Molecular techniques for establishing presence or absence of clonality are effective in tracking the spread of nosocomial infections and streamlining the activities of the infection control program. A comprehensive infection control program uses

active surveillance by both infection control practitioners and the clinical microbiology laboratory to identify clusters of infections with a common microbial phenotype (same species and antimicrobial susceptibility profile). The isolates are then characterized in the laboratory by using one of a number of molecular typing methods to confirm or refute clonality.

19.3.6.3 Challenges and Opportunities

The field of molecular infectious disease testing has grown so rapidly that the diagnostic industry has not kept up. To fill this void, independent investigators have turned to the development of user-defined, or “homebrew,” molecular detection methods in the clinical laboratory. The implementation of user-defined NAA testing has revolutionized clinical molecular infectious disease testing. In addition, commercially-available non-FDA-approved NAA assays are increasingly becoming available as analyte specific reagents (ASRs). Though all reagents necessary for the amplification reaction can be purchased commercially, assay development and verification studies must be performed by individual laboratories. In many cases, there are no comparative studies between user-defined NAA procedures, including ASRs, limiting the comparative value of assays between institutions (particularly in viral load monitoring) and restricting the application of such procedures to more experienced laboratories. It is not without considerable cost that a molecular infectious disease diagnostic lab is developed. It represents an institutional commitment because the costs may only be offset when analysis of hospital-wide cost savings is employed (i.e. shorter hospital stays, decreased use of unnecessary antibiotics). The costs incurred not only stem from instrumentation purchases, but also from the dedicated, expert staff required for such testing. Since many academic medical centers have resorted to implementing user-defined assays, verification and validation studies are substantial and require extensive resources, including time, staff, and expertise. These studies are crucial to defining the performance of the assay and determining appropriate clinical utilization. The FDA, diagnostic companies, and major molecular infectious disease laboratories need to work together to resolve the poor standardization that exists between laboratories using user-defined assays or ASRs.

19.4 Conclusion

The applications of molecular technology in clinical microbiology are endless, but challenges also abound. We are still learning what many NAA results mean in terms of infectious etiology. With the use of molecular technology to detect potential etiologic agents of disease, we need to remember Koch’s postulates. Is the mere presence of an organism’s nucleic acid convincing evidence of disease causation? Undoubtedly, additional clinical scientific evidence is needed to make such a claim, and such evidence or lack thereof should be considered when interpreting molecular infectious disease results. Though there is still much to be learned regarding

the appropriate application and interpretation of molecular infectious disease testing, there are numerous exciting opportunities on the horizon. User-defined assays and ASRs have allowed experienced laboratories to offer critical diagnostic services that have yet to become available with FDA clearance. As investigators refine molecular applications for infectious disease testing, diagnostic companies market such applications, quality control and government organizations standardize results, and as costs associated with implementation decrease and reimbursement increases, molecular infectious disease testing will not only be available in academic medical centers and reference laboratories, but will also transition to community hospitals, thus more globally impacting patient care.

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