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Introduction

Molecular biological methods for the detection and characterization of microorganisms have revolutionized diagnostic microbiology and are now part of routine specimen processing. Polymerase chain reaction (PCR) techniques have led the way into this new era by allowing rapid detection of microorganisms that were previously difficult or impossible to detect by traditional microbiological methods. In addition to detection of fastidious microorganisms, more rapid detection by molecular methods is now possible for pathogens of public health importance. Molecular methods have now progressed beyond identification to detect antimicrobial resistance genes and provide public health information such as strain characterization by genotyping. Treatment of certain microorganisms has been improved by viral resistance detection and viral load testing for the monitoring of responses to antiviral therapies(1).

nucleic acid amplification and detection methods developed in the past decade are useful for the diagnosis and management of a variety of infectious diseases. The most widely used of these methods is the polymerase chain reaction (PCR). PCR assays can detect rapidly and accurately the presence of fastidious and slow-growing microorganisms, such as *Chlamydia*, mycoplasmas, mycobacteria, herpesviruses and enteroviruses, directly from clinical specimens. Commercial PCR assays for the diagnosis of tuberculosis and genital *C. trachomatis* infection are now routinely used in many diagnostic laboratories(2).

Although the diagnosis of diseases is still primarily based on conventional methods (staining, culture, biochemical analysis), a series of advanced molecular diagnostic tools are increasingly introduced and incorporated in the workflow of clinical laboratories worldwide. These tools, such as polymerase chain reaction, northern blotting, restriction length polymorphism, flow cytometry, southern blotting, fluorescent in situ hybridization, agarose gel electrophoresis and pyrosequencing that are based on either the properties of nucleic acids (deoxyribonucleic acid and ribonucleic acid) or proteins of the target agents, have improved the efficacy, accuracy and speed of detection and identification of disease causing agents and characterization of the diversity of pathogens. They are mainly involved in medicine, ribonucleic acid analysis, criminal case determination, origin and evolution of species, deoxyribonucleic acid fingerprinting, forensic analysis, immunophenotyping, deoxyribonucleic acid studies, single nucleotide polymorphism, resequencing, tag sequencing and microbial identification. Therefore, advanced molecular diagnostic tools are new class of diagnostic tests that are used for identification of nucleic acids or proteins to test the status of infectious diseases and genetic disorders. But, they have some limitations like expensiveness, high technical need and sensitivity for even minor changes during procedures. So advanced molecular diagnostic tools have a cutting edge role in the diagnosis of animal and human diseases and they should be implemented in different diagnostic sectors(3).

Molecular microbiology techniques

Target nucleic acid amplification methods

Polymerase Chain Reaction

PCR is the most widely used nucleic acid target amplification technology. For PCR, a DNA sequence (template) is amplified in a buffered reaction solution containing oligonucleotide primers, thermostable DNA polymerase, deoxynucleotides triphosphates (dNTP), and magnesium or manganese ions. Other additives, used to enhance amplification, are common but not essential for the reaction to occur. The reaction solution is placed into a thermal cycler, which heats and cools the reaction components, exposing them to consecutive cycles of varying temperatures. In each temperature cycle, three steps occur: denaturation (heating to high temperatures to separate DNA into single strands), primer annealing (lowering the temperature to allow for primers, synthetic oligonucleotide strands designed with a sequence that is complementary to the ends of the original target gene sequence, to anneal to the single stranded DNA and create a partial double strand), and primer extension (addition of dNTPs to the ends of the bound primers by DNA polymerase, thereby creating a new synthetic piece of double-stranded DNA [the amplimer or amplicon], which is complementary to the original template strand). The annealing and extension steps can be combined into a single step for some reactions. With the exception of the first cycle, the number of amplicons will theoretically double with each cycle, resulting in an exponential increase in the quantity of amplicon, until the reaction reaches a plateau phase caused by depletion of reaction components. After a 36-cycle amplification, amplicon is present in the reaction tube at a theoretical concentration of approximately 6.9 X $1O^{1O}$ copies per each original template. The exact concentration of amplicon is lower when the reaction efficiency is less than 100%.(4)

Modifications of PCR

Several modifications of conventional PCR exist and require definition. For multiplex PCR, two or more primer sets are used in the same reaction mix. Multiplex PCR can be used to identify multiple microorganism or multiple nucleic acid targets within one microorganism, in a single reaction mix. For nested PCR reactions to occur, one set of primers is used initially. Then products of the first reaction are amplified with fresh reaction components (in most cases) and a second set of primers, internal to the first. Nested PCR can be likened to broth enrichment techniques used to allow certain microorganisms to grow prior to agar subculture and thereby increase the possibility of detection. Although nested PCR may sometimes be more sensitive than non-nested PCR, the nested method is prone to contamination by aerosolized amplified DNA. Because amplicon is physically transferred to a fresh reaction vessel and/or exposed to a

second set of primers and fresh reaction components prior to the second (nested) PCR step, amplicon contamination of subsequent reactions can easily occur. The product of the first reaction cannot be inactivated, because its activity as a template must remain intact for the nested protocol to function. Nested PCR is not recommended for use in clinical microbiology laboratories, unless the reaction is modified for performance in a single vessel, which remains closed throughout the amplification. A one-vessel nested approach can be accomplished by using physical separation of the two reactions in the same vessel or by using different temperatures for annealing the external and internal primer pairs. In situ PCR is a general term used to describe the amplification of DNA (or cDNA) inside an intact cell (e.g., in a paraffin-embedded tissue section), and provides a means of determining the disposition of a microbe within tissue. Reverse transcriptuse-PCR (RT-PCR) is used to detect RNA, either because an infectious agent's nucleic acid is naturally present as RNA and not DNA (e.g., HCV), or to detect transcribed microbial DNA as a marker of active infection. In RT-PCR, an enzyme with reverse transcriptase activity is used to create complementary DNA (cDNA) from an RNA target. The cDNA is then amplified by PCR. Various other modifications of PCR are used in clinical laboratories. Broad-range PCR involves the use of PCR primers targeted to highly conserved regions of DNA (e.g., 16s ribosomal DNA [rDNA]) so that, depending on the primer sets used, the target can be amplified from most bacteria. Broad-range PCR has been used in the research setting to identify unknown pathogens (e.g., Tropheryma whippelii, the Whipple's disease-associated bacteria) and has applications in the clinical microbiology laboratory. For example, the Applied Biosystems MicroSeqB 16s rDNA Bacterial Sequencing kit is used to amplify 16s rDNA of bacteria isolated by culture. Subsequent sequencing of the amplicon may be used for species-level identification and is described in subsequent sections. Direct sequencing of amplified 16s rDNA derived from patient specimens is also a possibility, although sensitivity and specificity issues need to be addressed prior to application of this approach outside the research setting. Quantitative PCR may be used to assess the amount of a microbe present in a patient specimen and can be performed in a number of ways. Semi-quantitative or limiting dilution PCR, the most basic of methods, involves titer determinations of the target template by end point specimen dilution prior to PCR. For more advanced, truly quantitative molecular assays, an internal or external quantitative standard (or calibrator) is incorporated. For quantitative competitive PCR, an internal quantitative standard (or calibrator) is included in the amplification reaction, along with the target sequences and competes with target sequences in the PCR reaction; therefore, standard curves of the competitor molecule correlate in inverse proportion target. An example is the AMPLICOR HIV-1 MONITORTM Test (Roche Diagnostics Corporation, Indianapolis), wherein the internal quantitative standard is a constructed segment of nucleic acid that uses the same primer binding sites as the target molecule, but has an internal sequence that differs from that of the target sequence and therefore allows differentiation from the target sequence. Current applications of quantitative PCR testing include measuring of viral load for pathogens such as HIV 1, CMV, HBV, HCV, and Epstein-Barr virus (EBV). Quantitative results may be important for guiding therapy.(4)

Nested PCR

When there is an extreme need for sensitivity and specificity in PCR, the process of nested PCR can be carried out. Nested PCR involves the sequential use of two PCR primer sets. The first primer set is used to amplify a target sequence (which increases the sensitivity for the second primer set); the amplicon generated then serves as the template for a second amplification using primers internal to those of the first amplicon. This secondary amplification proceeds only if the intended target was initially amplified; if the primary amplification was nonspecific, the secondary amplification would not occur (increased specificity). A major drawback of nested PCR is that the reaction vessel needs to be opened in order to add the second primer set, increasing the potential for contamination of the work environment with amplified DNA(5)

Non-PCR Target Nucleic Acid Amplification Methods

Several non-PCR target amplification methods exist, including transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), and strand displacement amplification (SDA). NASBA and TMA are isothermal (i.e., require no changes in the temperature of incubation) amplification methods wherein RNA target is reverse transcribed into cDNA, and then RNA copies are synthesized using an RNA polymerase. Modifications of TMA also exist for its use with DNA targets. Gen-Probe Inc. (San Diego), currently offers a TMA test for detection of Mycobacteriurn tuberculosis, C. trachomatis, and N. gonorrhoeae. A TMA assay for qualitative detection of HCV is available from Bayer Corporation. SDA is an isothermal amplification technique that is based on the ability of a restriction enzyme to generate a single-stranded site-specific "nick in double-stranded DNA and the ability of a (5' to 3' exonuclease deficient) DNA polymerase to initiate replication at the nick and displace the downstream non-template strand." Primers used in SDA contain both a target-specific region and a recognition site for a restriction enzyme. The restriction enzyme site is hemimodified, so that the restriction enzyme does not produce a nick in one strand. Once the nick is created by the restriction enzyme, the 3' end formed at the site of the nick initiates DNA synthesis, thereby displacing the newly created single strand. Each displaced strand is then available to anneal with amplification primers. The process continues with repeated nicking, extension, and displacement of new DNA strands, resulting in exponential amplification of the original DNA target.(4)

Signal amplification methods

An alternative to enzymatic duplication of target nucleic acid is to increase the signal generated from the probe molecule hybridized to the target nucleic acid. This is referred to as signal amplification. Commonly used signal amplification technologies include branched DNA and hybrid capture assays.(4)

Probe amplification methods

Probe amplification technologies, wherein the end product of the reaction is an amplified version of the original components used to detect the target, include the Ligase chain reaction (LCR), Q-beta replicase amplification, Invader TM technology and Cycling Probe Technology. In LCR, DNA template is denatured and subsequently hybridized to probes that are located next to each other on the template. Successful ligation of the probes depends on the proximal positioning and perfect base pairing of the 3' end of one probe with the 5' end of the other to complete a complementary DNA strand. Once the probes have been ligated, the ligation product, which now mimics one strand of the original target sequence, can serve as a template for ligation of more probes. Accumulated ligation products are detected using labeled probes. LCR-based assays are available for detection of C. trachomatis and N. gonorrhoeae.(4)

Sequence-based analysis

DNA sequence analysis has several applications in the clinical microbiology laboratory. For example, the technology can be used to determine HCV, HIV-1, HBV, and HPV genotypes; to determine the DNA sequences of bacteria and fungi for microbial identification post-culture, and to identify mutations associated with resistance to antimicrobial agents. A variety of DNA sequencing methodologies are available. Most are based on a modification of Sanger dideoxynucleotide triphosphate (ddNTP) chain termination chemistry in which synthetic ddNTP molecules that lack 3' hydroxyl groups normally present in the natural molecules are added to a DNA synthesis reaction. Whenever a ddNTP molecule is incorporated into the growing DNA strand of the usual dNTPs, the synthesis reaction is terminated. For dye-terminator cycle sequencing, four different fluorescent dyes are incorporated into each of the four ddNTPs (ddATP, ddCTP, ddGTP, and ddTTP). The end result is a collection of DNA fragments of different lengths, labeled with different dyes depending on the terminal nucleotide. Reaction end products are separated by electrophoresis and excited and detected by a laser. The resulting digital data are processed into electronic DNA sequencing data. Sequence data are analyzed using special software and displayed either as a linear string of one-letter nucleotide codes or as a graphic presentation (electropherogram)(4)

Clinical application of molecular microbiology

1. Pathogen detection, surveillance and discrimination

Identification and characterization of micro-organisms (both in clinical specimens and cultures) that cause infections are crucial for successful treatment, recovery and safety of patients. However, not every bacterial species can be successfully cultured in the diagnostic laboratory, and the available molecular tests are unable to detect emerging genetic features in successfully evolving pathogens that spread in humans, animals and the environment. Unrecognized

pathogens can easily cause hospital outbreaks, putting patients at risk during their hospital admissions. Sequence analyses can be used to answer different diagnostic questions, such as the genetic relationship of either bacteria or viruses, the detection of mutations in viral or bacterial genomes leading to resistance against antivirals or antibiotics, identification of fungi through sequence analyses of the 18S ribosomal deoxyribonucleic acid (rDNA) of the internal transcribed spacer (ITS) region and identification of bacteria through sequence analyses of the 16S rDNA(6)

Diagnosis of infectious diseases by using PCR involves target amplification methods and often nucleic acid sequence analysis of the amplicon. Unlike phenotypic characteristics that may be variable, molecular amplification methods take advantage of the use of stable genotypic characteristics. Another major diagnostic advantage of PCR is that the detection of nucleic acid does not require the labor intensive cultivation of microbial biomass. The bacterial genome provides variable regions for species identification and virulence factors or toxins loci can be useful for defining the pathogenicity of an organism. In addition to standard PCR, complete genome sequences have greatly facilitated the rapid development of other molecular identification methods for pathogen detection including nucleic acid hybridization (probes), ligase chain reaction , strand displacement amplification (SDA), transcription-based amplification and loop mediated isothermal amplification (LAMP). The enormous amount of nucleic acid sequence data from human pathogens has enhanced the diagnostic capabilities of sequenced based identification and genotyping methods of both common and uncommon micro-organisms.(7)

Examples of uses for molecular methods other than microorganism identification in the clinical microbiology laboratory*

Test	Examples
Viral load monitoring	Cytomegalovirus, Epstein-Barr virus hepatitis B, hepatitis C, @HIV
Viral genotyping	@HIV, hepatitis B, hepatitis C, human papillomavirus
Bacterial resistance detection	#MRSA, ^VRE, ♦ESBL containing *E. coli, K. pneumoniae* *M. tuberculosis*
Bacterial genotyping	*M. tuberculosis, N. meningitidis*
Broad-range PCR	Infective endocarditis, bacterial meningitis

Figure 1: clinical application of molecular methods(1)

A. Real-time PCR

Despite the development of alternative amplification technologies, PCR remains the most widely used method in both research and diagnostic laboratories for pathogen detection. Conventional PCR assays designed for single target detection have been broadly utilized by most large diagnostic microbiology laboratories for the past 15 years. Targets for human pathogens are based on specific loci for taxonomic identification, important phenotypic traits (such as antimicrobial resistance determinants), or virulence factors.

The targets are detected in real time from the sealed PCR plate and there is no post-PCR processing required, therefore, the risk of false-positive results due to amplicon carryover is substantially decreased compared to conventional PCR. In addition, most real-time PCR formats offer the option of melting curve analysis so that the amplicon can be distinguished from nonspecific product or primer-dimers. The performance of real-time PCR assays therefore has increased sensitivity and reliability of the reported results compared to those obtained by conventional PCR. Real-time PCR assays can be designed to allow the detection and simultaneous quantification of the PCR products over a wide dynamic range of quantitative linearity, which is an important requirement of many diagnostic applications.Because real-time

PCR assays are amenable to automation, most of these detection assays are run and completed in the clinical lab oratory setting in a shorter time than conventional PCR, therefore providing a more rapid test cycle turnaround time. Real-time PCR has truly become ‘walk-away’ molecular testing in the clinical laboratory setting. Despite this benefit, cost is a limitation of implementation in smaller laboratories with limited resources because of instrument cost and the price of a single real-time PCR reaction including DNA extraction can be more than three times of the cost of conventional PCR.(7)

B. DNA target sequencing

Nucleic acid sequencing has emerged as important tool for pathogen identification and discovery. Identification of human pathogens by partial or whole gene sequencing had not been widely adopted by clinical microbiology laboratories even a decade ago due to the labor and expense of bringing this technology in house, the lack of standardized methods for conducting analyses, and interpreting the results. Prior to 2000, DNA target sequencing had been primarily used for identification of organisms that were difficult to identify by using conventional phenotypic methods or recover by cultivation. DNA target sequencing for the rapid identification of bacterial, viral and fungal pathogens has since been increasingly implemented as a routine procedure by many clinical laboratories. In the last decade, DNA sequencing technologies have improved throughput capability and cost. Important guidelines have been published that provides interpretive criteria for identification of a wide range of clinically important bacteria and fungi to the species and genus levels. Commercial software is available that provides a quality assured database of known bacterial and fungal sequences for human pathogens. Some diagnostic laboratories also perform sequencing analysis of viral cDNA generated by reverse transcription.(7)

C. Molecular typing

Molecular typing of bacterial isolates is required for infectious diseases epidemiological surveillance, to detect laboratory cross contamination, distinguish relapse from re-infection, and for surveillance during infectious outbreaks. Molecular typing is therefore crucial for source tracking and epidemiology. Van Belkum and colleagues published a comprehensive review and guidelines for the validation and application of molecular typing methods for use in bacterial epidemiology. Among non-sequence-based methods, pulsed-field gel electrophoresis(PFGE), which separates macro-restriction DNA fragments has been the ‘‘gold standard’’ technique used by clinical laboratories to examine the molecular relatedness of bacterial isolates as well as yeasts and fungi. Provided that the PFGE method and interpretation are carefully standardized for a given microorganism, this method has robust reliability and intra- and inter-laboratory reproducibility. Similar DNA fragment-based methods have also been developed and used for clinical analyses including; restriction endonuclease finger printing analysis (REA), plasmid

typing, ribotyping, PCR fingerprinting, restriction fragment length polymorphism, and amplified fragment length polymorphism (AFLP). Multi-locus variable number tandem repeat analysis (MLVA) is another PCR-based method, which is used to determine the number and length of variable number tandem repeats (VNTRs) present in a genome due to slipped mispairing. Several MLVA systems have been developed for interpretation of a variety of species but few multicentre studies have been done. Due to the dedicated expertise, labor and specialized equipment needed to perform PFGE and other fragment-based Due to the dedicated expertise, labor and specialized equipment needed to perform PFGE and other fragment-based methods, clinical laboratories are increasingly turning to more rapid, semi automated, PCR-based or sequence-based methods to perform either rapid initial or routine genotyping analyses. The Diversilab system performs semi-automated repetitive-PCR analysis and has good to excellent agreement compared to several other typing methods for distinguishing several different types of micro-organisms including methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant Enterococcus, and commonly encountered dermatophytes dematophytes, Candida and fungi. This method has also been used to genotype Acinetobacter baumanii outbreaks strains, and extended-spectrum B-lactamase producing Escherichia coli and Klebsiella pneumoniae isolates. Semi-automated rep PCR (Diversilab) however does not always provide the same discrimination power as PFGE or other typing methods. Recognizing this limitation of this semi-automated platform, Diversilab may nevertheless be a useful tool for the rapid identification of nosocomial outbreaks (depending on the type of bacteria involved) when used in parallel with confirmatory analysis with greater discriminatory power.(7)

2. Pathogen discovery and characterization(rapid microbiological diagnosis)

Clinical microbiology laboratories use molecular methods to enhance the detection of pathogen(s) in critical clinical situations whereby phenotypic methods have failed to identify an etiological agent usually because the pathogen is fastidious or uncultivable and/or the patient has been given empiric antibiotic coverage prior to the collection and submission of clinical specimens. The organism may be cultivatable in the later situation but present in such low numbers that phenotypic methods are not sensitive enough for detection. Currently, the two most commonly used methods to detect such organisms include a microarray-based assay and a universal broad-range 16S PCR which utilizes species-specific hybridization probes, sequencing or electro-mass spectrometry for subsequent analysis of the product(s). Next-generation sequencing technologies may also be routinely employed in the future for pathogen detection and discovery in the clinical laboratory.(7)

A. Broad-range PCR

Although universal broad-range 16S rRNA gene PCR was originally developed for determination of microbial community diversity within complex bacterial populations in extreme environments, it has subsequently been used for pathogen detection where conventional bacteriological techniques fail to produce a diagnostic result. Broad-range PCR offers an important clinical benefit for analysis of critical culture-negative samples as it can detect bacterial nucleic acid present in a sample through targeting conserved bacterial sequences. Nucleic acid sequencing of amplicon generated by broad-range PCR allows identification of the organism(s) by comparison with known sequences deposited at Genbank or other databases. Previously uncharacterized microbes (novel pathogens) may also be detected because of the universal nature of primers/probes and the high sensitivity for detecting the presence of low copy number targets. Several investigators initially used amplification of conserved regions of bacterial ribosomal RNA and amplicon sequencing to identify human pathogens. Subsequently broad-range PCR has been extensively used non-selectively to detect bacterial DNA in clinical samples. Rather than perform multiple monoplex PCR assays on a given sample, broad-range assays are designed such that a single clinical sample can be simultaneously screened for multiple organisms (i.e., any bacterial species); however, amplicons may require cloning prior to sequencing in order to get an accurate result with traditional Sanger-based methods due to the potential mixed nature of amplified products.(7)

Interpretation of all broad-range PCR results should be correlated with the clinical picture due to the potential for DNA contamination from other sources. A ‘positive’ broad-range 16S PCR result should be correlated with the patient’s clinical history and examination findings along with the results of other pertinent diagnostic test information to determine the likelihood of a particular disease. For example, in the case of a cerebrospinal fluid (CSF) sample testing positive by 16S broad-range PCR for a bacterial meningitis pathogen, the patient’s clinical presentation and other CSF test results (i.e., CSF glucose, protein, cell count and culture) should be consistent with a diagnosis of acute meningitis. Further strength is also added to a 16S broad-range result of detection of a rare or unusual pathogen when the same micro-organism is detected in longitudinal samples, multiple samples of the same kind from several patients or several different specimen types. Interpretation of the presence of common specimen/culture contaminants by broad range 16S PCR may be particularly difficult when the isolate may also be a potential cause of infection (i.e., Streptococcus viridans sp. or coagulase negative staphylococci).(7)

B. Broad-range PCR with electrospray ionization mass spectrometry detection

A new commercial high-throughput bioidentification system (PLEX-ID, Abbott Molecular) has recently become available, that combines broad-range PCR using several gene targets with product detection and analysis by electrospray ionization mass spectrometry. It shows great promise for identifying a broad range of pathogens without prior knowledge of the pathogen’s nucleic acid sequence. PCR/ESI-MS can also provide results that include information about an organism’s genotype, resistance, or virulence profile. Although currently restricted to

epidemiological genomic studies and pathogen discovery, this technology may have a major diagnostic advantage in being able to provide detection, identification and characterization of a broad range of pathogens directly from clinical specimens. This instrument also has the potential to process up to 300 samples in a 24 h period.(7)

C. Microarrays

Microarrays are used as a high-throughput large-scale screening systems that allow for simultaneous identification and characterization of a very large number (up to several hundred thousand) of alleles of interest. Microarrays are therefore a very powerful tool with greater capacity (100–1000X) compared to other molecular methods (i.e., real-time PCR) that can only analyze a small number of targets. Microarray-based genotyping can potentially identify new genes by including sequences derived from related bacteria or identify polymorphisms that are detectable by oligonucleotide hybridization. DNA microarrays are also being used to diagnose infection by the identification of pathogens and simultaneous detection of drug-resistance and virulence-associated traits in these isolates.(7)

3. Microbial community analysis/including Genotyping

Characterizing the properties of pathogenic microbial communities is a new frontier for clinical microbiology because studies of the human microbiome demonstrate that there are far more unique species present at many body sites than are easily grown in the clinical laboratory. Several recent molecular studies of clinical samples from cystic fibrosis patients as well as complex abscesses have demonstrated the presence of a far more complex microbial flora than was previously found using phenotypic methods. Classic culture-based methods have most likely given clinical microbiologists a skewed view of the complex microbial community involved in some infections because only the cultivatable pathogens present in high numbers in a given clinical sample may be detected and reported. Escherichia coli is the prototypical gut micro-organism but this organism belongs to the c-proteobacteria phylum, which in its entirety only represents <1% of the total microbial population in the human intestine. E. coli has been thought of as a dominant resident in the human gut solely because it is readily cultivated in vitro.Because of the far greater discriminatory sensitivity of culture independent approaches, molecular methods are fundamental for characterizing complex human-associated microbial communities. Culture-independent molecular technologies have typically relied on either direct nucleic acid sequencing or electrophoresis in order to investigate community structure, community dynamics and community behavior. However, the revolution in high-throughput sequencing technologies in the last decade has allowed for unprecedented data output, orders of magnitude beyond traditional Sanger-based sequencing, at a fraction of the cost per nucleotide. Several high throughput sequence-independent community-profiling approaches are also available and include denaturing gradient gel electrophoresis, automated ribosomal intergenic spacer analysis and

terminal restriction fragment length polymorphism (T-RFLP) analysis. Here we will describe T-RFLP and deep 16S rRNA gene sequencing as tools for profiling microbial communities.(7)

A. Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is an approach that has been extensively used to profile fungal, archeal and eubacterial communities and has comprehensively been reviewed in the context of other fingerprinting techniques in the past. Traditionally, it has been used to profile environmental communities but it has also been exploited to investigate microbial communities associated with the oropharynx, gastrointestinal tract, lungs, vagina and other body sites. The technique utilizes one or both PCR primers labeled with a fluorescent dye (labeling both forward and reverse primers can improve resolving power) to amplify the universal 16S rRNA gene from a bacterial community of interest. The resulting amplicon is digested with one or multiple restriction enzymes (at four base pair recognition sequences), salts and primers are removed and the relative abundance of terminal restriction fragments (T-RFs) are resolved by polyacrylamide gel or capillary electrophoresis. The resulting data represents a semi-quantitative profile of the most dominant community members.(7)

U-RFLP is inexpensive, high throughput, reproducible and can be a very effective tool for characterizing the dynamic changes that occur in human-associated microbial communities over time. Importantly, the technique is best suited for microbial communities with low to intermediate richness; in populations with greater than 50 distinct organisms the resolving power can be significantly reduced. Like all PCR-based assays, the technique suffers from amplification bias. Appropriate primer selection is essential; however, it must be noted that a catchall, perfect universal primer set does not exist for community profiling. Programs are available to optimize primer design and selection but can be limited by the incompleteness of current databases.(7)

B. Deep sequencing

Nearly 20 different next-generation sequencing instruments are currently on the market (and more are on the way), each with their own advantages and disadvantages. A detailed discussion regarding the technical aspects of all of these technologies is far beyond the scope of this article but excellent resources are available. Sequencing experiments can range from 14 days with the Genome Analyzer IIx (Illumina) instrument to a mere two hours with the new Ion Torrent instrument (Life Technologies). Single experiments can generate hundreds of Gigabases of data. Several of the platforms have been widely adopted and include the Roche 454 technology, the Solexa/Illumina platform and Life Technologies SOLiD platform. The incredible amount of sequencing throughput provided by these new instruments has or the first time, allowed for a deep characterization of complex microbial communities.(7)

Other clinical applications of deep sequencing

In addition to bacterial community analysis applications, high throughput sequencing has been successfully applied to sequencing en masse of total DNA extracted from a microbial community, a technique referred to as metagenomics. The most impressive example of the power of metagenomics to date is the sequencing of nearly 3 million protein coding genes in the fecal metagenome by deep Illumina-based sequencing. Viral patho gens can also be detected directly using an unbiased metagenomics approach to clinical specimens. During the 2009 H1N1 outbreak there were no laboratory tests initially available to identify the virus and it has been suggested that the virus may have been circulating for months prior to its identification. Metagenomics was capable of detecting the influenza virus at titers near the limit of detection of RT-PCR. This approach could play an important role in rapid detection of other novel pathogens during outbreaks of acute, unexplained illness. It has already been applied to the diagnosis of lymphocytic choriomeningitis virus infection in transplant recipients, in addition to defining the etiology of hemorrhagic fever outbreaks.(7)

4. Identification of Slow-Growing and Uncultivable Bacteria

16S rRNA gene sequencing and similar molecular identification methods have the additional advantage of shortening the time to identify slow-growing bacteria and being able to identify bacteria that are even not cultivable. It is well known that most Mycobacterium species, except the rapidly growing mycobacteria, usually take 6–8 weeks to grow in culture, and it often takes another few weeks to perform phenotypic tests using subcultures.(8)

5. Antimicrobial resistance testing

The increase in bacteria harboring antimicrobial resistance (AMR) is a global problem because there is a paucity of antibiotics available to treat multidrug-resistant bacterial infections in humans and animals. Detection of AMR present in bacteria that may pose a threat to veterinary and public health is routinely performed using standardized phenotypic methods.(9)

The determination of antimicrobial susceptibility of a clinical isolate is often crucial for the optimal antimicrobial therapy of infected patients. However, molecular assays for the detection of resistance have a number of limitations. New resistance mechanisms may be missed, and in some cases the number of different genes makes generating an assay too costly to compete with phenotypic assays. In addition, proper quality control for molecular assays poses a problem for many laboratories, and this results in questionable results at best. The development of new molecular techniques, e.g., PCR using molecular beacons and DNA chips, expands the possibilities for monitoring resistance. Although molecular techniques for the detection of antimicrobial resistance clearly are winning a place in routine diagnostics, phenotypic assays are still the method of choice for most resistance determinations.(10)

Resistance can be caused by a variety of mechanisms:(10)

(i) the presence of an enzyme that inactivates the antimicrobial agent;

(ii) the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent;

(iii) a mutation in the antimicrobial agent's target, which reduces the binding of the antimicrobial agent;

(iv) posttranscriptional or posttranslational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent;

(v) reduced uptake of the antimicrobial agent;

(vi) active efflux of the antimicrobial agent; and

(vii) overproduction of the target of the antimicrobial agent.

(viii) In addition, resistance may be caused by a previously unrecognized mechanism.

(ix) On the other hand, a gene which is not expressed in vitro may be expressed in vivo.

Molecular methods are often used in addition to phenotypic methods but are set to replace them in many laboratories due to the greater speed and accuracy they provide in detecting the underlying genetic mechanism(s) for AMR. Molecular characterization of the genetic mechanism(s) underlying a given phenotypic result, obtained by traditional antimicrobial sensitivity testing, is now an integral part of many clinical investigations in relation to bacterial infections, whether in humans or animals. In some cases, when phenotypic results are too time-consuming, non-conclusive, or unavailable, molecular analysis can be used to investigate the presence of a given gene or point mutation and thereby give direct support to ensure that an optimal treatment or control strategy is undertaken in a timely manner. In addition, molecular characterization is frequently used as an indirect method to aid in epidemiological investigations following an outbreak,when phenotypic data is not sufficiently detailed to control possible outbreaks involving resistant bacte ria. Finally, molecular characterization of antimicrobial resistance (AMR) determinants is also used for local, national, or even global surveillance of AMR.(9)

AMR Gene Detection

The use of PCR to detect the presence of AMR genes in a bacterial isolate or even in samples from different environments is commonplace. Due to the ease of designing conventional PCR primers, there are a plethora of PCRs that have been used to detect the presence of different AMR genes from bacteria, in both aerobes and anaerobes. A highly cited paper in this area is by

Schwartz, who discuss PCR used to monitor for the presence of vanA (encoding vancomycin resistance), mecA (encoding methicillin resistance), and ampC (encoding ampicillin resistance) in wastewater systems and water biofilms.(9)

E.g. Molecular Assays for the Detection of Drug Resistance in MTB

Previous studies demonstrated that in MTB strains, drug resistance is mainly acquired through spontaneous mutations, especially single nucleotide polymorphisms (SNPs), in the circular chromosome. For each anti-TB drug, mutations in one or several genes have been described, and each mutation relates to different levels of drug resistance. Mutation frequency also can be variable. For instance, 97% of the cases of resistance to RIF are linked to mutations in the *rpo*B gene. Mutations at codons 526–531 of *rpoB* show the highest frequency and confer high-level RIF resistance. Isoniazid (INH) resistance is acquired through mutations in the *kat*G, *inh*A and its promoter, *ahp*C, *ndh*, and *fur*A genes but mainly in *kat*G, *inh*A and its promoter. The most frequent *kat*G mutations (50–90%) are found at codon 315 and confer high-level resistance to INH.(11)

DNA Line Probe Assays

Line probe assays (LPAs) are basically DNA–DNA hybridization assays that allow the simultaneous detection of different mutations by using multiple probes. After DNA extraction and target amplification, amplicons are hybridized to specific oligonucleotide probes that are complementary to the target sequences and are immobilized on the surface of a strip. After several post-hybridization washes to remove non-specific binding, the amplicon-probe hybrids are visualized by eye as colored bands on the strip. The turnaround time of the whole assay is 5–7 h.(11)

Real-Time PCR Assays

Real-time PCR is now broadly applied for the development of rapid diagnostic tests. Two main approaches are commonly used in real-time PCR: (i) the use of non-specific fluorescent dyes to detect any double-stranded DNA generated by PCR amplification, and (ii) the use of sequence-specific probes tagged with a fluorescent reporter for the specific detection of the hybridization between probes and amplicons[6]. Each probe has a specific melting temperature (Tm), and a Tm change reflects the presence of mutations in the target. This feature has been used to develop real-time PCR tests for drug resistance screening.(11)

Xpert MTB/RIF and Xpert MTB/RIF Ultra

Xpert MTB/RIF, a fast molecular-based test, is endorsed by WHO for the detection of the MTB complex and RIF resistance screening in suspected cases. This test was first recommended in 2010 for the diagnosis of pulmonary TB in adults from sputum specimens. Since 2013, it has been recommended also for the diagnosis of TB in children and of some specific extra-pulmonary forms. The Xpert MTB/RIF assay uses semi-quantitative nested real-time PCR to

amplify a fragment containing the 81 bp hotspot region of the *rpo*B gene (codons 507–533) that is then hybridized to five molecular beacon probes(11)

Advantages of molecular antimicrobial resistance testing methods(12)

For several reasons, genetic methods, compared to conventional susceptibility methods, have the potential to provide a more rapid and reliable assessment of antimicrobial resistance.

(i) Genetic susceptibility testing methods can be performed directly with clinical specimens obviating the need for isolation of the organism by culture.

(ii) These methods assess the genotype of the organism, whereas conventional susceptibility techniques asses the phenotype or expression of the genotype under artificial or laboratory conditions.

(iii) In some cases, genotypes may be discerned long before phenotypes can be determined due to the slow growth of the organism.

(iv) Some organisms cannot be cultured or are not easily cultured and so only genotypes can be determined in these cases.

(v) Genetic methods may lessen the biohazard risk which may occur with the propagation by culture of a microorganism, a requirement for conventional test methods.

Disadvantages of molecular antimicrobial resistance testing methods(12)

Several disadvantages exist for genetic methods compared to conventional phenotypic methods.

(i) They may lack sensitivity when only a few organisms are present in a sample. Refinements in techniques for concentrating nucleic acids in large volumes of clinical specimens hopefully will address this problem.

(ii) Different assays are required for each antimicrobial agent tested.

(iii) Resistance of a microorganism to a specific antimicrobial agent may occur via different mechanisms associated with different resistance genes or a large array of single or coincidental mutations.Pertinent to this and the prior point, is the concept that “with genetic testing methods, one only gets what one specifically is looking for.” This is in contrast to culture-based methods which are more comprehensive in assessing antimicrobial resistance. That is, by using the same culture-based assay, different forms of resistance can be detected. By virtue of the capacity to survey for different forms of resistance, culture-based methods also are useful for detecting emerging or new forms of antimicrobial resistance.

(iv) A genetic mechanism for resistance for some antimicrobics may not have been defined.

(v) Genetic methods may detect resistance genotypes that are expressed at levels that may not be clinically relevant. Examples of this include low-level vancomycin resistance encoded by *vanC* genes and poorly expressed extended-spectrum β-lactamase resistance.

(vi) False-positive results may occur due to contamination of the test sample with extraneous nucleic acid or residual nucleic acid from prior samples. This problem is of particular concern when nucleic acid amplification techniques such as PCR are used. However, the development of enzymatic and chemical sterilization methods for nucleic acid amplified by PCR has greatly enhanced the specificity for this technique.

(vii) Unlike for conventional culture-based susceptibility test methods, no standards exist for performing genetic testing methods.

(viii) Multicenter, controlled clinical trials to assess the accuracy, reproducibility, and clinical utility of these methods have only been conducted for a few of these tests.

6. Treatment Monitoring (check prognosis of disease)

Monitoring viral DNA or RNA loads has become the standard of care for several chronic viral infections. Measurement of viral load is performed either by competitive PCR systems, branched chain DNA signal amplification or more recently real-time PCR. HIV viral load testing is an integral component of the management of HIV infection. It is the major tool used to monitor the success of antiretroviral therapy and to detect the emergence of viral resistance, evidenced by a rise in the viral load despite ongoing therapy. HIV viral loads also predict progression of disease, and give prognostic information. Viral load testing is also used for the assessment and monitoring of responses to therapy in chronic HCV and hepatitis B virus (HBV) infection.(1)

7. Vaccine development

Vaccines were initially developed on an empirical basis, relying mostly on attenuation or inactivation of pathogens. Advances in immunology, molecular biology, biochemistry, genomics, and proteomics have added new perspectives to the vaccinology field. The use of recombinant proteins allows the targeting of immune responses focused against few protective antigens. There are a variety of expression systems with different advantages, allowing the production of large quantities of proteins depending on the required characteristics. Live recombinant bacteria or viral vectors effectively stimulate the immune system as in natural infections and have intrinsic adjuvant properties. DNA vaccines, which consist of non-replicating plasmids, can induce strong long-term cellular immune responses.e.g. Recombinant protein vaccines, Live recombinant vaccines using bacterial or viral vectors, DNA vaccines etc.(13)

8. Drug development

Genome sequence data can be used for the discovery of antibiotic targets for development of novel antibiotics. Resistance is more likely to happen when newly designed antibiotics are chemically similar to previous ones already rendered ineffective. Therefore ideally, new antibiotics should have novel mechanisms of action, which is the ultimate goal of the genome sequencing approach to discover novel antimicrobials.the essential attributes of good drug targets in the genomes of bacteria: Firstly, good drug targets must be essential for viability or required for disease; secondly, they must be unique to bacteria or at least significantly different from orthologous genes in humans; and thirdly, for broad spectrum antibiotics, the targets must be present in key pathogenic bacteria. Identification of such targets is mainly done by comparative genomic analysis using bioinformatics approaches such as sequence homology, structural homology, cluster analysis and motif analysis(14).

Inhibition of nucleic acid synthesis: common drugs in this group include fluoroquinolones and rifamycins. Fluoroquinolones act by inhibiting DNA gyrase, an enzyme which introduces negative supercoils in the bacterial DNA prior to initiation of DNA replication. Fluoroquinolones also inhibit Topoisomerase IV, which is responsible for removing the separating daughter chromosomes at the end of a round of replication. Rifampin inhibit bacterial RNA polymerase, which occurs as a result of the antibiotic binding in the polymerase subunit deep within the DNA/RNA channel, causing direct blocking of the growing or elongating RNA.(14)

9. Differentiate live vs dead cells

The distinction between viable and dead cells is a major issue in many aspects of biological research. The current technologies for determining viable versus dead cells cannot readily be used for quantitative differentiation of specific cells in mixed populations. This is a serious limitation. We have solved this problem by developing a new concept with the viable/dead stain ethidium monoazide (EMA) in combination with real-time PCR (EMA-PCR). A dynamic range of approximately 4 log10 was obtained for the EMA-PCR viable/dead assay. Viable/dead differentiation is obtained by covalent binding of EMA to DNA in dead cells by photoactivation. EMA penetrates only dead cells with compromised membrane/cell wall systems. DNA covalently bound to EMA cannot be PCR amplified. Thus, only DNA from viable cells can be detected. We evaluated EMA-PCR with the major food-borne bacterium *Campylobacter jejuni* as an example. Traditional diagnosis of this bacterium is very difficult due to its specific growth requirements and because it may enter a state where it is viable but not cultivable. The conditions analyzed included detection in mixed and natural samples, survival in food, and survival after disinfection or antibiotic treatment. We obtained reliable viable/dead quantifications for all conditions tested. Comparison with standard fluorescence-based viable/dead techniques showed that the EMA-PCR has a broader dynamic range and enables quantification in mixed and complex samples. In conclusion, EMA-PCR offers a novel real-time

PCR method for quantitative distinction between viable and dead cells with potentially very wide application(15)

Viability PCR (v-PCR) as a method to selectively detect intact live cells has gained considerable interest over the last years with an increasing number of applications. The principle is based on treatment of microbiological samples with a viability dye prior to extraction of genomic DNA and its amplification. The dye is selectively taken up by membrane-compromised dead cells resulting in the degradation of their DNA upon light exposure and therefore inhibition of amplification. Although the treatment greatly helps to generate more meaningful data, one of the main drawbacks of the technique is currently that the exclusion of dead cell signals can be incomplete leading to false-positive signals. The resulting overestimation of live cell population is especially problematic for the detection of pathogens(16)

Propidium monoazide (PMA) can also be applied in combination with quantitative PCR (qPCR) for selective detection of intact cells. Co-incubation of PMA with deoxycholate can only be applied to gram-negative species. PMA incubation temperature has a strong effect on dye uptake by damaged cells. When working with low dye concentrations, PMA-qPCR efficiency can easily be improved by longer incubation times. Elevated dye incubation temperature and extended incubation time greatly improve exclusion of dead cell signals(16)

In order to address the limitation of conventional PCR, alternative PCR-based strategies have been developed. This article reviews two complementary strategies. One strategy, termed viability PCR, or vPCR, correlates viability with cell envelope impermeability. In viability PCR, microbes in samples are incubated with a membrane-impermeative reagent such as propidium monoazide (PMA). Upon photoactivation, PMA binds tightly to exposed DNA and interferes with PCR amplification. Nonviable cells with damaged membranes, and free nucleic acids, are not protected from the reagent, and their amplification is inhibited after the reagent-DNA complex is photoactivated. In contrast, viable cells with intact cell membranes exclude PMA, enabling strong quantitative PCR (qPCR) signals in the presence of the reagent. The operating principle is similar to microscopy-based live/dead staining, in which a membrane-impermeative DNA stain (typically, propidium iodide [PI]) is excluded from intact cells but penetrates and stains the DNA of membrane-compromised cells. In live/dead staining, inactivated cells are quantified relative to total cell counts by fluorescence microscopy or flow cytometry rather than by PCR(17)

The second strategy, termed “molecular viability testing” (MVT), correlates viability with the ability to rapidly synthesize a macromolecule (a species-specific rRNA precursor, or pre-rRNA) in response to a brief nutritional stimulus. Pre-rRNA synthesis upon nutritional stimulation is detected by reverse transcriptase-qPCR (RT-qPCR) measurement of species-specific pre-rRNA molecules. Pre-rRNAs in inactivated cells, and free nucleic acids, do not increase in numbers upon nutritional stimulation and therefore are excluded. the two methods have complementary

applications which are best understood in the context of the physiology of microbial viability and inactivation(17)

Flow cytometry: Propidium iodide (PI) is a small fluorescent molecule that binds to DNA but cannot passively traverse into cells that possess an intact plasma membrane. PI uptake versus exclusion can be used to discriminate dead cells, in which plasma membranes become permeable regardless of the mechanism of death, from live cells with intact membranes. PI is excited by wavelengths between 400 and 600 nm and emits light between 600 and 700 nm, and is therefore compatible with lasers and photodetectors commonly available in flow cytometers. This protocol for PI staining can be used to quantitate cell death in most modern research facilities and universities..(18)

10. Differentiation of toxigenic from non-toxigenic strains

Various molecular methods based upon oligonucleotide probes have been developed to detect and discriminate among harmful algal taxa. Among these molecular methods, fluorescence *in situ* hybridization (FISH), targeting ribosomal RNA (rRNA), has been often used for identification of harmful species in field samples.The FISH method is quite straightforward and target cells are directly visible by fluorescence microscopy. However, weak probe penetration, loss of cells during preparatory steps, and autofluorescence of the targeted cells, which can mask the fluorescence signal, have been reported(19).

11. Detection of mutations

Detection of rare mutations and site-specific modifications/damage in DNA is now plausible because of advances in high-throughput sequencing technologies, but sequencing error rates have remained a major impediment and set undesirably high noise thresholds for detection. Numerous modified library construction methods have recently been developed to generate redundant sequences from each original DNA fragment and thereby suppress error rates. Further elaborations such as circle sequencing, duplex sequencing, and locus-specific targeting have made improvements to yield and/or accuracy and broadened the range of applications(20)

Mutation detection using PCR: mutation is visualized by the loss of corresponding bands on the gel by comparing with reference bands.

Future directions of molecular microbiology techniques

Although larger clinical microbiology laboratories are increasingly developing their own molecular assays and procedures in house, several barriers exist in this process including rigorous validation for the population being tested, quality control, ensuring a high degree of

automation, access to dedicated technologists who are trained and experienced in molecular techniques, access to capital to purchase the necessary equipment, and the expense of operating a molecular microbiology laboratory alongside the phenotypic culture-dependent testing. However, implementation of culture-independent testing methods in clinical microbiology will increase over the next decade not only due to the continued development of multiplexing methods (real-time PCR, liquid suspension bead-based arrays) but also due to improvements in future generations of sequencing platforms that increase capacity and read length while reducing cost. High throughput sequencing technologies appear to be following Moore's law and increasingly the potential applications to clinical microbiology will become more apparent. For example, in addition to the bacterial community analysis, deep sequencing has been applied to viral communities in the human body. Deep sequencing of intra-patient HIV viral genomes has revealed an intriguing perspective on viral resistance and ecology. One can envision the next generation of clinical microbiologists having the ability to perform complex analyses as diagnostic and epidemiological tools such as routine genome wide SNPs, high throughput community analysis and streamlined metagenomic testing for novel pathogen discovery within a reasonable clinical turnaround time. However, although the clinical microbiology diagnostic techniques 'tool-box' has the potential to significantly increase in the future, it is very unlikely that culture-independent approaches will entirely replace cultivation of microbes because of the importance of strain stocks and phenotypic information is not easily inferred from genotypic data. As medical management of infectious diseases evolves so too will the need for alternate perspectives on complex chronic infections. In future, molecular approaches may also be routinely used to simultaneously investigate host susceptibility for particular types of infection, as well as determine pathogen induced phenotypic changes in host gene expression during microbial infections that prognosticate clinical outcome.(7)

Limitations of molecular microbiology techniques

Despite significant advantages of molecular diagnostics it cannot yet replace conventional methods for a range of infectious diseases since many common tests performed in the clinical microbiology laboratory are rapid and inexpensive. Advances in conventional technologies have resulted in many rapid antigen tests requiring only minutes for results and the modern automated culture systems allow relatively rapid identification and susceptibility testing. Unlike bacterial culture, which can detect a large number of cultivable bacteria without initially knowing the specific organism responsible, all PCR tests except broad-range PCR can only detect the organism whose DNA is complementary to the primers used.(1)

A. False Positive and False Negative Results

Another problem restricting the application of molecular techniques to routine diagnosis is that of false positive and false negative results. To avoid false positive results due to laboratory

contamination relatively large laboratory areas are required for physical separation of reagent preparation, specimen preparation and product detection areas together with a high level of staff training and skill. Poor primer design can also lead to erroneously positive results. Primers may be poorly designed such that incidental amplification of microorganisms other than those sought occurs. Also primers are designed based on the known sequences available through international databases but organisms or sequences yet to be discovered can subsequently reduce the specificity of the PCR.

False negative results may also be a problem. Some organisms such as mycobacteria are difficult to extract DNA from, reducing the sensitivity of the PCR.63 Substances in some clinical specimens such as sputum and faeces can degrade the DNA and RNA and other specimens may contain substances such as polysaccharides, haem and therapeutic drugs that inhibit the PCR enzymes.1 It is therefore important to include inhibitor checks for each specimen to ensure a negative PCR reaction is not actually an inhibited reaction. This can be done by incorporation of an internal amplification control to check for both inhibitors and successful DNA extraction.(1)

B. Lack of Uniformity in Molecular Testing

Molecular diagnosis is also complicated by the vast array of in-house PCR tests used in different laboratories. Commercial tests are available for a number of common and important infectious diseases such as HIV, hepatitis C and B, *C. trachomatis*, and *N. gonorrhoeae* but many infectious diseases are unlikely to have a commercial PCR test developed due to their rarity. Differences in primer selection (different genes or different sequences within genes), amplification format such as single round, nested or real-time PCR or one of the other nucleic amplification methods, and product detection methods such as ethidium bromide gel electrophoresis, DNA probes or sequencing make comparisons for sensitivity and specificity difficult.(1)

C. Differentiation between Infection and Disease

Since the presence of nucleic acid does not necessarily mean the presence of viable organisms a problem with interpretation of PCR results can emerge that does not occur with culture. For some infections such as invasive meningococcal disease the presence of meningococcal DNA from a sterile site has a very high positive predictive value. However, the detection of *P. jiroveci* in suspected PCP may have only a 50% positive predictive value in immunosuppressed patients since it may colonise as well as cause disease. Likewise herpes viruses such as Epstein-Barr virus (EBV), CMV and HSV are intermittently shed following primary infection without causing disease. Less sensitive detection methods such as culture have a higher specificity but quantitative PCR may be helpful in this regard since higher viral loads are usually more specific for disease. Quantification in viral infections such as HIV, CMV, EBV, HBV and HCV is well

established for assessing disease severity or monitoring response to treatment. In CMV disease viral load testing can monitor either increases of viral loads to threshold levels or rates of viral load increase to improve the positive predictive value for clinical CMV disease. Another approach is to detect RNA species that are usually degraded within minutes of cell death to indicate pathogen viability and replication.(1)

Conclusion

Molecular testing methods have the potential to replace many conventional microbiology laboratory assays. Recent refinements in technology have resulted in more user-friendly testing platforms. These platforms are automated and have lowered risks for contamination, decreased costs, and are faster than older platforms. The success of these technologies depends on their successful application to patient care. Quality issues include appropriate specimens for analysis, performance characteristics of different analytical methods, optimal specimen processing, the effects of PCR inhibitors, and false-positive results caused by contaminating nucleic acids. Quality control guidelines for molecular microbiologic diagnostic assays are in their infancy and require further development.

Additionally, the problem of "too much" sensitivity (brought on by the extreme sensitivity of these techniques coupled with the potential presence of small numbers of pathogenic organisms in asymptomatic individuals) should be considered. Potential problems when monitoring therapy (because molecular detection techniques do not generally have the ability to determine whether an organism is dead or alive) can also occur. Cost-effective test use, pathogen- or disease-targeted algorithms, and standardized methods will be necessary for the true value of these technologies to be realized. This is especially important, because, unlike traditional culture methods, most molecular microbiology methods are pathogen-specific. Clinicians familiar with the reasons why "pan-culture" (i.e., requesting all culture possibilities at once) is inadvisable should not use the same irrational approach when requesting molecular tests. The clinical usefulness of molecular testing will be maximized as targeted algorithms are developed and an understanding of molecular test ordering patterns is realized. Laboratory technicians and physicians must continue to apply and combine theories of traditional microbiology, clinical chemistry, and general medicine to the understanding and application of molecular diagnostics.(4)

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THE END!!!

THANK YOU!!!