Chapter 15
Molecular Microbiological Testing

Abstract The application of molecular testing methods in the clinical laboratory has dramatically improved our ability to diagnose infectious diseases. Nucleic acid techniques, such as plasmid profiling, various methods for generating restriction fragment length polymorphisms and the polymerase chain reaction (PCR) are making increasing inroads into clinical laboratories. However, the clinical usefulness of molecular testing will only be maximized to its fullest benefit by appropriate and careful studies correlating clinical findings with assay results.

Keywords Molecular diagnostics · Infectious disease · Diagnosis · PCR · Microbiological testing · Molecular tests · Biotyping · Antibiogram · Resistogram · Bacteriocin typing · Ribotyping · Molecular techniques

15.1 Prologue

Over the past century microbiologists have searched for more rapid and efficient means of microbial identification. Clinical microbiologists and microbiology laboratories are experiencing changes due to evolving views on "healthcare delivery" as an economic activity, due to changes in the medical environment and the demographics of the workforce, and technical evolution. The identification and differentiation of microorganisms has principally relied on microbial morphology and growth variables. Cost-effectiveness of laboratory procedures has been achieved through consolidation and integration of laboratories. Consolidation offers economy of scale and reduction in numbers of on-site staff, but also leads to separation of microbiologists from their clinical colleagues. Integration puts different laboratory disciplines under a single management, and leads to reorganisation of laboratories along common work-lines. Cost-savings combined with on-site availability of laboratories are achieved at the expense of a reduction in the influence of microbiologists in the daily running of the laboratory.

Medically, there is growing emphasis on evidence-based diagnostics. Because of time-delays inherent in culturing, microbiology has a limited impact on patient outcomes. Increased clinical relevance of microbiological testing through rapid testing is mandatory. There is an increasing shortage in Europe and the USA of trained microbiology laboratory technicians and microbiologists. This reinforces the trend

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towards more automation and integration. Technological advances, particularly in molecular diagnostics, offer the possibility of rapid reporting and improvement of the impact of clinical microbiology on patient management. Molecular tests, however, fit perfectly the concept of an integrated laboratory and may further loosen the link between microbiologist and microbiology tests. The challenge for clinical microbiology will be to use new techniques to improve its cost-effectiveness and impact on infectious disease management (Pfaller, 2001). The future organisation of microbiology laboratories must support this but is itself of secondary importance.

15.2 Concept

Molecular diagnostics of microbial pathogens is an integral part of modern medicine. The development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases. The traditional methods of microbial identification rely solely on the phenotypic characteristics of the organism. Bacterial fermentation, fungal conidiogenesis, parasitic morphology, and viral cytopathic effects are a few phenotypic characteristics commonly used. When methods for microbial genome analysis became available, a new frontier in microbial identification and characterization was opened.

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. At this point, in the twenty-first century, we are on the threshold of another era of discovery, that of molecular diagnostics. Advances in molecular biology over the past 10 years have opened new avenues for microbial identification and characterization (Mullis, 1990). The development and use of new molecular microbiological testing, coupled with an ever-improving understanding of how best to use these precious drugs in the treatment of infection, offers the greatest hope yet for physician prescribing that can retard, or perhaps even reduce, the development of drug resistance in many microbial species.

Nucleic acid techniques, such as plasmid profiling, various methods for generating restriction fragment length polymorphisms, and the polymerase chain reaction (PCR), are making increasing inroads into clinical laboratories. 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 uncultivable or fastidious microorganisms. These technologies, however, like the determinations of phenotypic variables, are limited by microbial recovery and growth. Rapid techniques of nucleic acid amplification and characterization have significantly broadened the microbiologists′ diagnostic arsenal. 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.

15.3 Advent of Improved Diagnostics

Diagnostic Microbiology probably started in the late seventeenth century when the Dutch scientist Antoni van Leeuwenhoek made microorganisms visible for the first time. Since then, 3 major revolutions have taken place, all of which had a major impact on the field of clinical microbiology. The first revolution took place at the end of the nineteenth century after the development of solid culture media by Robert Koch. Bacterial culture, nowadays, still remains the cornerstone of clinical microbiology, although technical refinements have made available automated identification and antimicrobial susceptibility testing in most modern laboratories. The second revolution was the development of antigen/antibody detection in the sixties and seventies of the twentieth century.

Early antigen/antibody testing tended to lack some sensitivity and specificity, a problem which is nowadays largely resolved; however, most of these tests still show a window period before detectable antigen/antibody levels appear in the patient. The third and final revolution, the nucleic acid (NA)-based detection of microorganisms started some 25 year ago and is still ongoing. Molecular diagnostic testing has made its way from basic research to become a permanent asset for the clinical microbiology laboratory. In fact, in clinical virology PCR has nearly completely replaced traditional methods like immunofluorescence to detect active viral infection (Tiveljung-Lindell et al., 2009). However, in other fields in clinical microbiology, the use of molecular diagnostics has remained more limited to those microorganisms that are either uncultivable or hard to culture, or those where conventional diagnostic procedures lack sensitivity (Fig. 15.1).

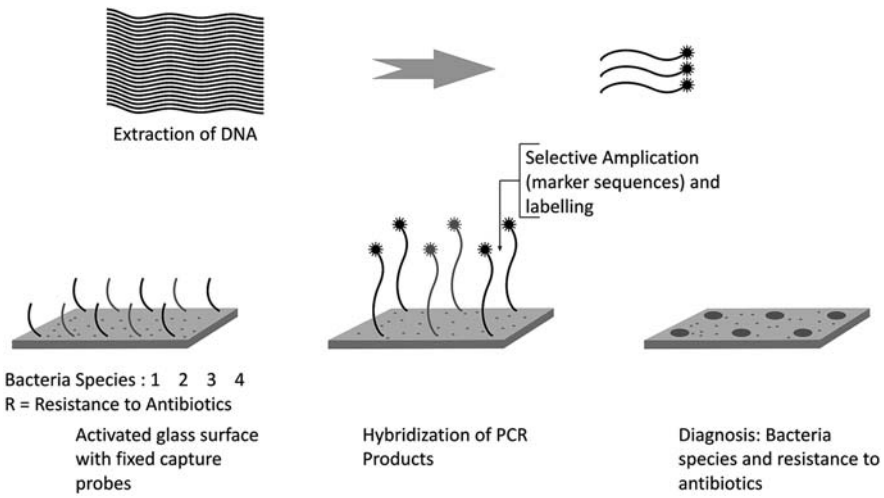

Fig. 15.1 Molecular diagnosis of bacteria using molecular diagnostic techniques

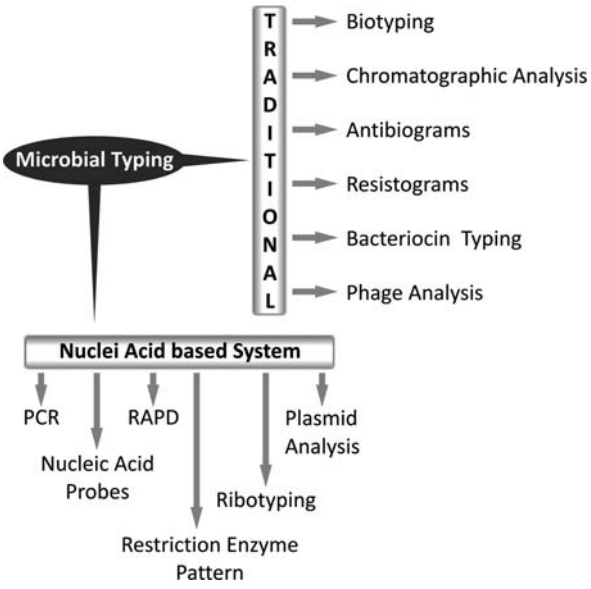

Fig. 15.2 Molecular diagnostic techniques for microorganisms

There are various diagnostic techniques for molecular screening of microorganisms (Fig. 15.2).

15.4 Traditional Microbial Typing

Molecular screening of particular at-risk populations for a group of possible pathogens is an exciting area of development in molecular microbiology. For example, numerous etiologic agents cause debilitating gastroenteritis in immunosuppressed patient populations, including mycobacteria (i.e., *M. avium* complex and *M. genevense*), parasites (i.e., *Cryptosporidum*, *Microsporidum*), viruses (i.e., rotovirus, Norwalk agent), and typical bacterial pathogens (*E. coli* variants, *Salmonella*, *Shigella*, and *Campylobacter*). Traditionally, different methods of detection are used for each group of intestinal pathogens. This requires special media, equipment, and expensive facilities for the culture of mycobacteria; expertise in the identification of parasites in ova and parasite stool preparations; virology facilities; and special media for the workup of bacterial enteric pathogens. Although these tests may be relatively inexpensive individually, an adequate workup for enteric pathogens can be quite costly. Some of the methods are discussed below:

15.4.1 Biotyping

Traditional microbial identification methods typically rely on phenotypes, such as morphologic features, growth variables, and biochemical utilization of organic substrates. The biological profile of an organism is termed a biogram. The

determination of relatedness of different organisms on the basis of their biograms is termed biotyping. In many instances, biotyping is used in conjunction with other methods to more accurately profile microorganisms (Tenover et al., 1994).

15.4.2 Antibiograms, Resistograms, and Bacteriocin Typing

The susceptibility or resistance of an organism to a possibly toxic agent forms the basis of the following typing techniques. The antibiogram is the susceptibility profile of an organism to a variety of antimicrobial agents, whereas the resistogram is the susceptibility profile to dyes and heavy metals. Bacteriocin typing is the susceptibility of the isolate to various bacteriocins, i.e., toxins that are produced by a collected set of producer strains. By far, the antibiogram is the most commonly used susceptibility/resistance typing technique, most probably because the data required for antibiogram analysis are available routinely from the antimicrobial susceptibility testing laboratory (Dijkshoorn et al., 1996).

15.4.3 Protein Analysis

Commercially available antibodies are routinely used to specifically identify antigenic proteins from a wide variety of organisms. In some instances, the test may be used only to identify the genus and species of an organism. Monoclonal antibodies directed against the major subtypes of the influenza virus, as well as the various serotypes of *Salmonella*, are commonly used in speciation. Specific antigenic proteins may be detected by antibodies directed against these proteins in immunoblot methods (Mulligan et al., 1988).

15.4.4 Phage Analysis

Bacteriophages, viruses that infect and lyse bacteria, are often specific for strains within a species. The bacteriophage profile may be used to type bacterial strains within a given species. Bacteriophage profiles have been used successfully to type various organisms associated with epidemic outbreaks. However, this typing method is labor-intensive and requires the maintenance of bacteriophage panels for a wide variety of bacteria. Additionally, bacteriophage profiles may fail to identify isolates, are often difficult to interpret, and may give poor reproducibility (Hickman et al., 1991).

15.4.5 Chromatographic Analysis

Chromatographic analysis of short-chain fatty acid production is a routine method used to aid in the identification of anaerobic bacteria. Computer-aided gas–liquid chromatography is commercially available and is a means of microbial identification. This identification system utilizes the type and amount of cellular fatty acids

present in the lysate of an organism. Many species have unique cellular fatty acid chromatographic profiles (Stoakes et al., 1994).

15.5 Nucleic Acid-Based Typing Systems

Molecular techniques exist and are being developed that may be used to screen individuals within a particular patient population for the most probable etiologic agents of disease. Nucleic acids extracted from the stool of patients with gastroenteritis may be examined with organism- or group-specific nucleic acid primers and probes. In this manner, one single test may be used to single out the etiologic agent of disease among numerous possibilities.

15.5.1 Plasmid Analysis

Plasmid profile analysis was among the earliest nucleic acid-based techniques applied to the diagnosis of infectious diseases and has proven useful in numerous investigations (Wachsmuth, 1985). This method has also been widely utilized for tracking antimicrobial resistance during nosocomial outbreaks.

15.5.2 Restriction Enzyme Pattern

The relative simplicity of the RFLP profiles generated by PFGE facilitates application of the procedure in identification and epidemiological survey of bacterial pathogens. Fingerprinting, which combines PFGE with Southern transfer and hybridization, has been widely used in studying the tuberculosis nosocomial outbreak in human immunodeficiency virus (HIV)-positive populations (Kristjansson et al., 1994).

15.5.3 Ribotyping

Restriction patterns can be obtained by hybridizing Southern-transferred DNA fragments with labelled bacterial ribosomal operon(s), which encode for 16S and (or) 23S rRNA. This method, called ribotyping, has been shown to have both taxonomic and epidemiological value. Ribotyping assays have been used to differentiate bacterial strains in different serotypes and to determine the serotype(s) most frequently involved in outbreaks (Tenover et al., 1994).

15.5.4 Random Amplified Polymorphic DNA (RAPD)

RAPD has been used to differentiate strains of various species, various serotypes within species, and various subtypes within a serotype (MacGowan et al., 1993). It is, therefore, useful for determining whether two isolates of same species are epidemiologically related.

15.5.5 Nucleic Acid Probes

Nucleic acid probes allow the diagnosis of infections in which the organisms are not easily cultured or cannot be cultured at all. Detection of DNA with direct or culture-amplified gene probe technology has been applied to several organisms, including bacteria, viruses, mycobacteria, fungi, and even certain parasites. The technique has been also used to monitor growth as an indicator of drug resistance or to directly detect genes associated with antibiotic resistance. DNA probes have shortened the time required for probe assay.

15.5.6 Polymerase Chain Reaction

Nucleic acid amplification technology is examined from the critical viewpoint of a clinical microbiologist working in a routine diagnostic bacteriology laboratory. Widely recognised limitations of amplification technology include those of false-positive and false-negative results, the difficulty of obtaining quantitative results, the problem of using this technology for susceptibility testing, and the difficulty of detecting routinely the wide range of possible pathogens contained in a clinical sample. On the positive side, amplification technology brings welcome new possibilities for rapid detection of specific pathogens in a sample, including viruses, slowly growing bacteria, fastidious or uncultivable bacteria, fungi and protozoa. Other possible applications include screening normally sterile clinical samples for non-specific bacterial contamination and the use of amplification-based DNA fingerprinting methods for identification and typing of microorganisms. Nevertheless, it is predicted that – in contrast to research and reference facilities – routine bacteriology laboratories will continue to rely on culture as the preferred "amplification method" for most diagnostic applications.

Nucleic acid amplification techniques increase sensitivity dramatically while still retaining a high specificity. Commercial systems for PCR detection of DNA targets of *Chlamydia trachomatis* and *Mycobacterium tuberculosis* are manufactured by Roche Molecular Systems (Loeffelholz et al., 1992). Numerous modifications of the standard PCR procedure have been developed since its inception. RT-PCR has played an important role in diagnosing RNA-containing virus infections, detecting viable *Mycobacteria* species, and monitoring the effectiveness of antimicrobial therapy. For diagnostic uses, multiplex PCR can be set up to detect internal controls or to detect multiple pathogens from a single specimen (Roberts and Storch, 1997). Another important technical modification is the development of broad-range PCR, in which conserved sequences within phylogenetically informative genetic targets are used to diagnose microbial infection. A broad-range PCR approach has identified several novel, fastidious, or uncultivated bacterial pathogens directly from infected human tissue or blood (Relman et al., 1996). In a recent report, Bannoehr et al. (2009) reported the first diagnostic test for the identification of *Staphylococcus pseudintermedius* involving a simple PCR-restriction fragment length polymorphism approach. The method allows discrimination of *S. pseudintermedius* from

the closely related members of the *Staphylococcus intermedius* group and other important staphylococcal pathogens of humans and dogs.

15.6 Current Application of Molecular Diagnostics

In recent years, the demand for quantification of nucleic acid targets has been growing (Crotty et al., 1994). By use of molecular methods, the microbial load of an infecting pathogen may be determined and its genotype may also be evaluated. Viral load data are used to monitor therapeutic responsiveness and may yield prognostic information regarding the progression of disease. Until recently, however, the task of quantitative nucleic acid amplification has been very difficult to accomplish. Because the amplification techniques yielded products in an exponential manner until a plateau was reached, any factor interfering with the exponential nature of the amplification process would therefore affect the result of the quantitative assay. In practice, many factors can affect the efficiency of the PCR reaction throughout the amplification procedures and result in the differences between theoretical and actual yields of the reaction. Now, however, kit-based technologies make it possible for many laboratories to carry out quantitative determinations.

15.6.1 Clinical Microbiology

Traditionally, the clinical medical microbiology laboratory has functioned to identify the etiologic agents of infectious diseases through the direct examination and culture of clinical specimens. Direct examination is limited by the number of organisms present and by the ability of the laboratorian to successfully recognize the pathogen. Similarly, the culture of the etiologic agent depends on the ability of the microbe to propagate on artificial media and the laboratorian's choice of appropriate media for the culture. When a sample of limited volume is submitted, it is often not possible to culture for all pathogens. In such instances, close clinical correlation is essential for the judicious use of the specimen available.

Some microorganisms are either unculturable at present, extremely fastidious, or hazardous to laboratory personnel. In these instances, the diagnosis often depends on the serologic detection of a humoral response or culture in an expensive biosafety level II–IV facility. In community medical microbiology laboratories, these facilities may not be available, or it may not be economically feasible to maintain the special media required for culture of all of the rarely encountered pathogens. Thus, cultures are often sent to referral laboratories. During transit, fragile microbes may lose viability or become overgrown by contaminating organisms or competing normal flora.

The addition of molecular detection methods to the microbiology laboratory has resolved many of these problems. The exquisite sensitivity and specificity of many molecular methods allow the accurate detection of very small numbers of organisms. The direct detection of *M. tuberculosis* nucleic acid from the sputa of smear-negative patients with tuberculosis clearly illustrates this point (Whelen et al., 1995). The

technology allows for the rapid and accurate identification of the etiologic agent in a time substantially shorter than traditional methods. This allows for earlier initiation of a focused antimicrobial regimen and decreases the likelihood of disease progression.

In selected situations, the limitations imposed by the ability of an organism to be cultured and the selection of appropriate media and culture conditions may be replaced by the use of molecular microbiology. Microbial DNA/RNA extracted from a clinical specimen may be analyzed for the presence of various organism-specific nucleic acid sequences regardless of the physiological requirements or viability of the organism (Monstein et al., 1996). For example, the inability to culture and analyze the principal etiologic agent of non-A, non-B hepatitis limited medical advances in this area. Using various molecular methods, however, investigators have been able to isolate hepatitis C virus (HCV) nucleic acid (Choo et al., 1989). Analysis and cloning of the HCV genome has provided the viral antigens necessary for the development of specific serologic tests. Currently, RT-PCR allows for the identification, quantification, and sequence analysis of the HCV genome in infected individuals (Hammerle et al., 1996).

Another unculturable microbe that has been specifically detected by PCR and probe analysis is *Tropheryma whippelii*, the causative agent of Whipple disease (Ramzan et al., 1997). Because of the inability of this organism to grow on conventional media and the lack of a serologic test, diagnosis of Whipple disease is usually based on clinical and specific biopsy findings. Patients with Whipple disease often have gastrointestinal manifestations and undergo endoscopy. Small bowel biopsies reveal foamy histiocytes filling the lamina propria. The definitive diagnosis is made with the identification of non-acid-fast, periodic acid-shift-positive, diastase-resistant bacillary forms within the histiocytes. Extraintestinal Whipple disease, principally seen as arthritis and central nervous system involvement, may be missed entirely unless the clinician and pathologist have a high index of suspicion. Even so, the diagnosis in such instances may prove difficult. Advances in the molecular detection of *T. whippelii* have resolved this dilemma. On the basis of bacterial 16S rRNA gene sequence analysis, an emerging pathogen, *Bordetella holmesii*, has been successfully identified in the immunocompromised hosts. Additionally, the DNA from a single clinical specimen, such as a knee fluid aspirate, may be tested for several etiologic agents in a differential diagnosis. In such instances, the specimen may also be analyzed for other fastidious and difficult-to-culture agents of infectious arthritis, such as *N. gonorrhea* or *Borrelia burgdorferi*.

As explained earlier, molecular methods may also be useful in instances of limited specimen volume (Buck, 1996). Even in low-volume specimens, enough DNA/RNA can often be extracted to allow performance of numerous molecular assays. However, though molecular methods are very sensitive, clinically relevant results are ultimately reliant on the submission of quality specimens.

Some organisms, although not difficult to culture, are encountered infrequently and require special media for isolation. In these instances, culturing may not be cost-effective for smaller laboratories because the reagents may expire before usage; these samples may also be sent to reference laboratories for culturing, for the sake

of economy. Again, fragile organisms may die in transit or become overgrown by contaminating bacteria, thereby making the subsequent culture useless. If molecular microbiology facilities are not available in community laboratories, nucleic acids extracted by the use of commercially available kits may be sent frozen to molecular reference facilities. Alternatively, if molecular facilities are available, PCR primers and probes for relatively rare microorganisms may be maintained frozen at –70°C for extended periods and used when needed. This may eliminate the need for special culture media and circumvent problems related to specimen transit. As molecular techniques become more widely available, the spectrum of rapid and cost-effective clinical microbiology testing available to smaller laboratories can be extended.

Molecular methods of detection may also play a role in laboratory safety. Organisms such as *Coxiella burnetti*, *M. tuberculosis*, *Coccidioides immitis*, and several viruses causing severe hemorrhagic fevers are laboratory hazards. These organisms are easily cultured, but may infect laboratory personnel and cause serious illness or death. The handling of these organisms requires specially trained personnel, special equipment, and expensive ventilated facilities. All of these increase laboratory costs. Molecular methods may be used to detect such organisms directly from clinical specimens, without exposing laboratory personnel to biologically amplified organisms. After the initial extraction procedure, only non-infectious materials are handled.

The molecular detection of microbes with a known susceptibility profile is an effective replacement of the traditional culture. An excellent example is the molecular detection of *Bordetella pertussis*. This organism is a relatively slow grower, requires specially supplemented and more costly media, and has a known susceptibility profile. The molecular detection of *Bordetella pertussis* can save time, lower laboratory costs with regard to special media, and allow for the more rapid initiation of effective therapy. If variable antimicrobial susceptibility profiles exist, culture for susceptibility testing is still necessary. Molecular methods for the detection of antimicrobial-resistant strains are in development and in the future may replace traditional susceptibility testing. Until then, molecular screening may be used to determine which patients should be cultured for subsequent susceptibility testing.

At the beginning of this century we were just beginning to recognize the potential for use of molecular diagnostics in the rapid and accurate detection of infectious pathogens (Araj, 2000; Louie et al., 2000). There was a general acceptance that laboratories have started molecular testing in pathogen detection as well as resistance determination for individual patients. Perhaps the first molecular test that was successful in reducing antibiotic use was the application of PCR to the detection of enteroviral meningoencephalitis (Dumler and Valsamakis, 1999). In 2003, several reports appeared assessing the impact of early detection of infectious agents (Hallin et al., 2003), determination of specific antimicrobial agent resistance and the positive impact of rapid results on the prescribing of vancomycin. An important innovation by Lapierre et al. (2003) was the development and testing of a novel real-time PCR assay for direct detection of fluoroquinolone resistance in staphylococci, finding a 99.8% correlation between PCR results and MIC measurements, and thus demonstrating the potential to increase dramatically the speed of resistance

detection using this new technology. Stefanelli et al. (2003) in another excellent technological advance, were able to predict diminished penicillin susceptibility for all *Neisseria meningitidis* tested within a few hours using real-time PCR.

In a recent report (Ramond et al., 2009) for diagnosis of respiratory virus infections in young children, modern molecular diagnostics tools like quantitative real-time PCR assays, multiplex PCR, microarray hybridization in an integrated molecular diagnostic device, the Infiniti analyzer were successfully used for detection and analysis. Detection of adenoviruses of groups A, B, C, and E; coronaviruses HKU1, 229E, NL63, and OC43; enteroviruses A, B, C, and D; rhinoviruses of genotypes A and B; influenza viruses A and B; human metapneumoviruses (HMPV) A and B, human respiratory syncytial viruses (HRSV) A and B; and parainfluenza viruses of types 1, 2, and 3 allowed a thorough etiological assessment of respiratory viruses infecting children.

15.6.2 Clinical Epidemiology and Infection Control

The investigation and control of nosocomial infections is a complex issue that involves clinical, infection-control, and laboratory personnel. The efforts of both the microbiologist and the hospital epidemiologist are facilitated greatly by the availability of the newer molecular epidemiological typing techniques. Molecular diagnostic techniques have been successfully used in the investigation and control of classical and emerging nosocomial pathogens, such as the enterobacteriaceae, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, coagulase-negative staphylococci, enterococci, *Candida albicans*, *M. tuberculosis*, and *Chlamydia pneumoniae*. Application of DNA probe-based assays allows the diagnosis of other nosocomial infections caused by respiratory syncytial virus, varicella-zoster virus, herpes simplex virus, and legionella to be made in only a few hours. Several putative outbreaks of infections have been investigated by molecular techniques. Such examples include investigation of several temporally clustered cases of *Streptococcus pyogenes* invasive disease in Air Force recruits (Musser et al., 1994), a case cluster of lymphogranuloma venereum caused by *Chlamydia trachomatis* serovar L1 in homosexual men, and an outbreak of *E. coli* O157:H7 infection from contaminated deer.

Molecular techniques are being used increasingly in epidemiological and clinical investigations. Use of DNA hybridization assays in cervical swabs or fresh cervical biopsy specimens to determine HPV infection and viral types has provided helpful information for clinical assessment and treatment of patients (Morrison et al., 1991). By using PCR followed by automated direct sequencing, several studies have revealed that the most common genotypes of HCV. A new PCR-based HCV genotyping system has been recently developed to identify various HCV genotypes in clinical studies. Molecular techniques have been used to directly detect resistance genes or mutations that result in resistance in organisms. The *mecA* gene that codes for resistance to methicillin in *Staphylococci* has been detected by PCR and multiplex PCR assays (Ubukat et al., 1992). Defining the mutations responsible

for resistance to microbial agents has led to new methods for monitoring efficacy of antimicrobial therapy. Successful investigations have been carried out on both bacterial and viral resistance mechanisms.

15.7 Promise of Molecular Testing

New diagnostics will also need to sense those settings where infection is actually occurring. To avoid unnecessary prescribing of antimicrobial agents, rapid tests will be particularly useful when they can indicate what type of antibacterial, antiviral or symptomatic therapy (in the absence of infection) is most beneficial. The newest and most exciting possibility for specific outpatient diagnosis is that based upon amplification technology such as PCR. Rapidity of testing and cost are always key issues. With the advent of real-time PCR we finally have as a reality access to a reliable diagnostic test with same-day results. The cost of this testing also now approximates to that of conventional culture-based processing. Thus, we are on the threshold of microbiology diagnostics that, when carried to its expected conclusion, can provide sufficient information to permit specific application or avoidance of antimicrobial chemotherapy.

The newer applications of molecular diagnostics known as gene “chip” and “microarray” and “nanoparticle” technology offer the potential to solve many remaining impediments to rapid detection of important infectious agents in health care. Since these technologies do not require organism viability, and thus avoid any adverse effect of longer specimen transport, they can be successfully applied to both the in- and outpatient settings. Also, the resulting test rapidity theoretically will provide relevant information within a few hours, which would limit any necessary empirical treatment to one or two doses. Several companies currently possess the technical expertise and laboratory research infrastructure to bring a useful diagnostic testing approach to the clinical trial stage very shortly. One example is the new technology company Nanosphere, Inc. (Northbrook, IL, USA), which is developing gold nanoparticle technology to detect molecular DNA, RNA and protein biomarker targets using automated instrumentation, without the need for prior amplification (Ehghanian et al., 1997).This testing could detect likely pathogens responsible for important clinical scenarios, such as respiratory disease symptom complexes, implicating the key bacterial, viral or atypical microbial pathogens responsible. Simplified automation opens the potential for testing to be done near the patient at a peripheral site. If molecular testing methods were widely applied, it could be done at a very manageable cost and would have a significant impact on lowering unnecessary antibiotic prescribing.

Thus, the application of molecular testing methods in the clinical laboratory has drastically improved our ability to diagnose infectious diseases. However, the clinical usefulness of molecular testing will only be maximized to its fullest benefit by appropriate studies correlating clinical findings with assay results. As methods become more refined, automated, and standardized, the use of amplification methods to detect infectious agents will become more valuable. Because amplification

methods and the interpretation of their results are continuously evolving and becoming more refined, it is imperative for clinical microbiologists as well as clinicians to remain current and knowledgeable in all aspects, including the chemistry and microbiology of the infectious organism as well as in general medicine. Questions regarding basic laboratory issues including the clinical need, the cost benefit for testing the patent, and other issues as well as direct and indirect costs must be addressed and answered. Of great import, the specific clinical niche for the amplification test (i.e., the clinical question that a PCR result will answer) must also be delineated. And finally, whether an amplification assay is and remains the best approach must be addressed during as well as after assay development. On the short term, there has been an increase in molecular diagnostic testing within the clinical microbiology laboratory. The role of culture will therefore move from a primary detection tool to an epidemiological tool. On the long run, developments will be technology driven, eventually leading to "real-time" point of care diagnostics.

However, some additional hurdles remain other than the technological issues. For example, in the United States the estimated cost of regulatory requirements for approval and ongoing modification of multitargeted molecular tests, added to the expense of first developing them (in excess of $100 million) are considered prohibitive by the industry. This is due primarily to the fact that regulations have not changed to encompass test platforms having the capacity to detect a variety of genetic targets from multiple microbial species, with the aim of providing a unified diagnosis based on interpretation of a complex pattern using multiple results. Because of what is at stake – managing antimicrobial agent resistance – it clearly appears in the best interest of all to develop a modernized set of regulations that facilitate development and application of this testing.

15.8 Assay Validation-Analytic Sensitivity and Specificity

Once optimized and its specificity confirmed yet prior to its validation in a clinical setting, an amplification assay must be evaluated for its analytic sensitivity and specificity using a background milieu of clinical material. However, it is essential that key decisions be made prior to further assay development and evaluation employing clinical material. First, the type of specimen (or specimens) acceptable for assaying by PCR must be determined as well as the physical criteria for the specimen's (or specimens') suitability for analysis, including optimum source and volume, appropriate collection method, transport and storage conditions, and specimen longevity. The choice of specimen(s) plays a key role in the performance and interpretation of test results because if any of the criteria are not fulfilled, the sensitivity and specificity of the assay will vary accordingly (Noordhoek et al., 1996). This becomes of paramount importance in quantitative amplification assays used to determine viral clearance such as for HCV (Van Vliet et al., 2001). In general, plasma or serum is preferred over whole blood or leukocytes when the target organism is predominantly extracellular (e.g., HCV RNA, human immunodeficiency virus-1 RNA).

Once the acceptable specimen type (or types) has been delineated, the method for extraction must be selected. There are numerous methods in the literature for extracting either DNA or RNA from clinical specimens; however, they are beyond the scope of this discussion. A host of commercially available kits are available from which to choose as well as a number of automated methods for specimen preparation. These commercially available kits and automated systems are advantageous over manual methods of extraction in that they are more rapid and cost-effective and provide more consistent results. Nevertheless, the most optimal method for extracting nucleic acid must also be determined for each specimen type. The volume of specimen to be used for extraction must be taken under consideration; sample volumes vary considerably depending on the amplification method, but most assays use between 100 and 250 [μ]l. Lastly, the method for detection of the target must be selected. Again, numerous options are available, such as detection by agarose gel electrophoresis or hybridization capture assays employing the capture of a biotinylated amplicon by a target-specific probe and detection by streptavadin-horseradish peroxidase etc.

In the mid-1980s, the polymerase chain reaction (PCR) methodology for the amplification of minute amounts of target DNA was successfully developed and then introduced into clinical use; such technology has led to a revolution in diagnostic testing. Nucleic acid amplifications have become crucial in the diagnosis of selected infectious disease agents. Initially, molecular diagnostic tests were performed only in highly specialized or research laboratories; however, these assays are now more widely performed in all sections of the clinical laboratory. This has been largely a result of commercial assays, such as those advocated by the Food and Drug Administration, having been introduced into the market. Clearly, as the technology advances, additional commercial, Food and Drug Administration-approved methods will become available and enable even the smallest laboratory to employ amplification technologies for the detection of microorganisms. Nevertheless, in the meantime, there is a significant demand for amplification assays to detect the presence of a variety of microorganisms in clinical specimens for which there are no commercially available kits. Thus, clinical laboratories, particularly those associated with academic medical centers, must frequently develop their own "in-house" assays to accommodate the demand for the laboratory diagnosis of infectious diseases by amplification methods.

Despite the significant advantages and strengths that amplification methods offer in terms of the rapid and sensitive detection of infectious agents as well as the quantification of pathogens such as human immunodeficiency virus-1 to monitor therapy or disease outcome, there are limitations and caveats to these assays that must be understood. For example, studies have demonstrated that there is significant variation in the ability of "home-brew" or in-house assays among clinical laboratories to reliably detect infectious agents. Moreover, as studies are published that correlate clinical findings with results of amplification tests, it has become exceedingly evident that although molecular diagnostic assays enhance diagnostic capabilities, their results must be clearly interpreted within the clinical context and performance of the laboratory assay. In other words, a thorough understanding of the parameters of the molecular assay, including the respective procedural

limitations and the target organism's microbiology and pathogenesis, is critical for the proper interpretation of results. Clearly, ongoing clinical research to correlate amplification results with clinical findings as well as strict adherence to guidelines for method validation for in-house PCR assays are prerequisite.

In general, PCR and other amplification tests are perceived as expensive. Not only are there costs for reagents and equipment, but there are also training costs associated with teaching microbiologists to perform these molecular diagnostic assays. Through rapid diagnosis, more expensive and/or invasive procedures may be prevented, hospital stays shortened, or the unnecessary administration of antibiotics prevented. Presently, in light of the expense of these assays coupled with an ever-increasing demand for testing as the list of clinical syndromes increases for some infectious agents, the appropriate use of these assays is imperative. Studies centering on the use of molecular assays in terms of cost and other benefits are now being published. More of these types of studies need to be performed so that the appropriate use of molecular assays is accomplished.

15.9 Conclusion

New pathogens will continue to emerge, and as soon as they are recognized will need to be incorporated into existing diagnostic test menus. Mixed viral and bacterial infections can also be daunting tasks for diagnosis and therapy, but if the molecular tests developed not only delineate all that is present (signals) but also differentiate what is responsible for illness in the host (sensors), then we will be at a true crossroads in the treatment of infectious diseases. Dunne et al. (2002) described a future scenario where this testing develops as we envisage here, and postulated that by the year 2025 sophisticated samplers will painlessly obtain the necessary material, followed by automated analysers to process simultaneously DNA, RNA, protein, glycopeptides and exopolysaccharides to detect any of a possible 168 pathogenic microbes as well as toxins and resistance genotypes, all completed and yielding a diagnosis within 15 min. We believe this future is attainable even earlier, and that bringing these advances to fruition is critical for mastering antimicrobial agent prescribing in order to manage their use in a rational way and finally reverse the increasing resistance that threatens these precious agents we have as necessary aids to all our health. The technology and vision are here, we just need the will to do it.

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