

Molecular methods used in clinical laboratory: prospects and pitfalls

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Introduction

The emergence and re-emergence of several infectious diseases that can potentially impact public health worldwide has been a topic of interest in clinical research. There are many complex factors which influence the emergence of new infectious diseases. Some of these factors include: genetic variation in the host and pathogen, societal behavioral changes and population dynamics, as well as environmental pressures on both humans and animals. To complicate matters, the accessibility of international travel has also enabled infectious agents to be transmitted worldwide with a rapidity never seen before in human history. It is this complexity that makes rapid identification of new, as well as recognized, disease-causing pathogens, an urgent matter in the public health sector. In order to treat at-risk individuals, limit disease transmission, improve health surveillance and monitor pathogenic effects on the environment, medical-care providers and laboratorians must be prepared to rapidly recognize new and unusual events and uncommon microbial agents, as well as unexplained human and animal illnesses (Peruski *et al.*, 2003).

Molecular methods can complement conventional culture-, antigen- and antibody-based methods for the

Abstract

The role of molecular detection, identification and typing or fingerprinting of microorganisms has shifted gradually from the academic world to the routine diagnostic laboratory. Molecular methods have been used increasingly over the past decade to improve the sensitivity, specificity and turn-around time in the clinical laboratory. Molecular methods have also been used to identify new and nonculturable agents. Many high-throughput molecular tests are now available commercially, which impacts on the infrastructure in many of the diagnostic laboratories. In this paper, we take an overall look at the use of molecular methods (prospects vs. pitfalls) based on our clinical and public health experience, particularly as they related to *Borrelia burgdorferi*, a vector-borne pathogen, *Treponema pallidum*, a re-emerging sexually transmitted global pathogen, and West Nile virus, a newly recognized virus in North America.

detection, identification and epidemiologic analysis of infectious microorganisms (Zaidi *et al.*, 2003). Disadvantages of conventional methods include their inability to distinguish new pathogens derived from a common ancestor, time-inefficiency while providing only satisfactory results and poor sensitivity and specificity in test results. Molecular methods are now emerging as important tools in all laboratories for routine detection and fingerprinting, as well as aiding in public health surveillance, which could potentially allow the rapid implementation of infection-control and intervention practices.

Despite the advantages and popularity of molecular diagnostic and fingerprinting methods, significant challenges must be addressed before these methods can be adopted in the clinical diagnostic laboratory. Although there are a number of molecular methods available, most clinical diagnostics laboratory test menus are limited to PCR or related detection assays. Advanced test menus that will inevitably make their way into more diagnostics laboratories include real-time PCR (RT-PCR) and DNA sequencing. As molecular methods have their pitfalls as well, clinical microbiologists, infectious disease physicians and epidemiologists must learn to understand the use and the limitations of molecular methods.

Prospects

Rapid, highly sensitive, specific tests easily standardized

One of the most powerful molecular diagnostic methods used in clinical laboratories is gene amplification by PCR. PCR primers are designed to hybridize to specific RNA, DNA or cDNA sequences within a gene. This enables the causative agent to be accurately identified with a detection limit that varies from 1 to 100 copies of a target amplicon in clinical specimens, which is far superior to its equivalent immunological assay (Zaidi *et al.*, 2003). This accuracy can be further increased using RT-PCR for detection, as selective probes targeting specific genetic sequences or melting curve analysis can be used to identify the amplicon. An example of a PCR method, showing increased sensitivity over its conventional method, is noted in Table 1 for the detection of *T. pallidum*, the causative agent for syphilis. Samples were collected from patients seen in the Sexually Transmitted Disease Clinic at the British Columbia Centre for Disease Control (BCCDC), Vancouver, BC. Of the 106 genital ulcer disease (GUD) samples tested, 28 (26%) were PCR positive but dark field (DF) microscopy negative, while only 1 (0.94%) specimen was DF microscopy positive and PCR negative. These results were not surprising, as DF microscopy can be particularly challenging if the fluorescence is weak or if there are few spirochetes present in the sample. The opposite is true for PCR, which can detect low numbers of organisms and differentiate organisms accurately. This example illustrates the magnitude by which molecular methods may reduce the number of false-negative tests being reported (Morshed *et al.*, 2001).

A decrease in turn-around-times through molecular testing methodology is another advantage molecular testing has over conventional culture-based testing. Automation of nucleic acid extraction coupled with RT-PCR results in a fast and accurate platform by which the presence of DNA can be confirmed immediately (LightCycler and TaqMan). Also, it permits large-scale screening of organisms for pathogens in only a few hours, unlike gel-based methods, which require manual labor from trained technicians. Overall, molecular techniques allow increased reliability and efficiency through automation. The example shown in Fig.

Table 1. Comparison between conventional dark field microscopy and PCR for the detection of *T. pallidum* in genital ulcer disease specimens using the primers detected the *po1 A* region

	Syphilis dark field microscopy: positive	Syphilis dark field microscopy: negative
PCR Positive	26	28
PCR Negative	1	51

1 is a molecular method for the detection of West Nile virus (WNV) RNA in mosquitoes. This public health surveillance program involves large-scale insect sampling, and the need for efficiency as well as accuracy is high. The efficiency and accuracy of this method cannot be matched by any conventional method (Shi *et al.*, 2001). Results can be obtained in < 4 h through the use of automated genetic material extractor and a real-time one-step reverse transcription PCR system in a 96-well format (Lanciotti *et al.*, 2000). This figure also illustrates the use of an internal positive control (IPC) in monitoring the extraction as well as the RT-PCR procedure, which ensures the test to be more specific and accurate (Eisler *et al.*, 2004). Well-designed multiplex primers and probes with IPC ensure the quality of the test. In addition, the closed system reduces the chance of cross-contamination.

Tools for identifying novel, noncultivable or slow-growing pathogens

For specimens containing unidentified pathogens, screening for a large panel of possible causative agents with molecular tests, such as universal primers for PCR and DNA sequencing, would be a reasonable strategy. This method results in the scope of testing with nucleic acid amplification tests being narrowed, as some organisms may have similar genes and sequences. Application of 16S rRNA gene sequencing is widely used for the identification of clinical isolates with ambiguous biochemical profiles and for bacteria that are noncultivable (Rolph *et al.*, 2001). A large database Ribosomal Database Project (RDP) for most eubacteria is available for sequence alignment and identification.

Norovirus, previously known as Norwalk virus, cannot be cultured *in vitro* and could only be identified by electron microscopy (EM) before the development of a PCR method. This molecular test not only allows a laboratory unequipped with EM to perform the test, but also dramatically increases the detection sensitivity. A study carried out at BCCDC Laboratory Services on Norovirus showed that EM only detected 17% of all PCR positive samples (McNabb *et al.*, 2002). Syphilis is another example by which PCR has aided in diagnosis. *Treponema pallidum* cannot be cultured on cell-free laboratory media, therefore PCR is a good alternative in diagnosis of primary and secondary syphilis, and the amplicon from PCR could be further tested by DNA sequencing to provide confirmative results (Liu *et al.*, 2001).

For slow-growing bacteria, such as *Mycobacterium tuberculosis*, molecular amplification methods have not only been valuable for reducing turn-around time (TAT) in identification, but they also avoid the need to perform a live culture procedure (Rene *et al.*, 1998). There is always an inherent health risk to laboratory personnel when performing direct tests on unknown or highly infectious agents. Molecular

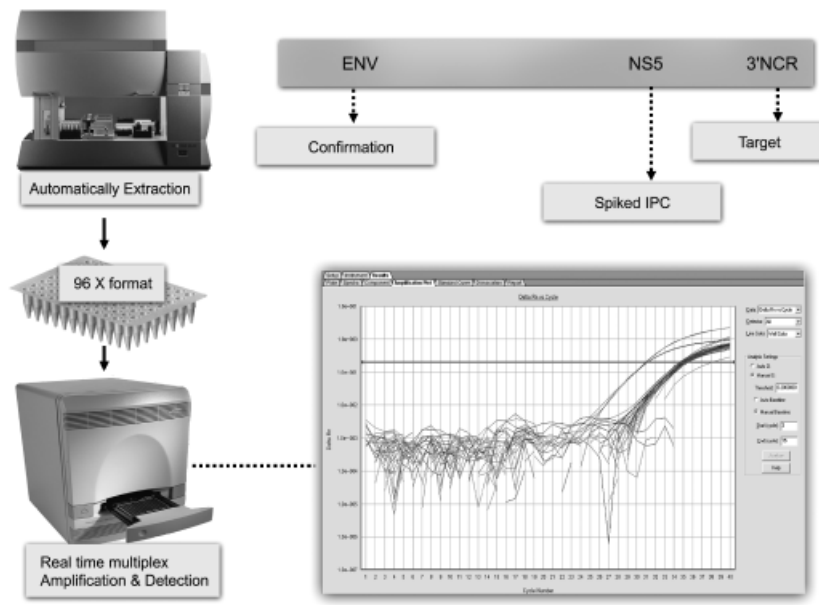


Fig. 1. Large-scale West Nile virus mosquito surveillance in an automated extraction and 96-well plate format real-time reverse-transcription PCR amplification and detection (unpublished data).

methods decrease this health risk for the laboratory workers significantly, because most specimens become noninfectious during the lysis and heating process of the nucleic acid extraction. This is advantageous, not only due to a decreased level of exposure for laboratorians during specimen manipulation, but also because there are a limited number of staff trained to work in biological containment facilities. Thus, more laboratories are enabled in the detection of potentially harmful novel pathogens.

Epidemiological tools

Sequence-based identification and strain typing, along with the development of probes for genetic markers, allows detailed strain fingerprinting. DNA fingerprint analysis can be used to study strain relatedness, as well as group heterogeneity for a particular organism. Thus, analysis can have a significant impact on patient management and disease control through early detection of disease clusters or outbreaks. This molecular typing has added a new dimension to studying the epidemiology of communicable diseases, by the recognition of unsuspected transmission and defining likely locations or modes of transmission, as well as quantification of the extent of transmission (Oiu *et al.*, 1992; Esteban *et al.*, 1996; Harpaz *et al.*, 1996; Krajdén *et al.*, 1998; Harrington & Bishai, 2004; Krajdén, 2005).

Figure 2 shows the information pathway and testing procedure used to track and detect *B. burgdorferi*, the causative agent for Lyme disease, from environmental specimens such as ticks and mice. In this schematic, both molecular and serological techniques are used. Cultures that are positive for spirochetes in BSK II media are confirmed to

be *B. burgdorferi* by PCR, immunofluorescence assays (IFA) and Western blots (WB), however PCR tests in this circumstance provide most of the confirmatory results (Morshed *et al.*, 2006). Figure 2a, illustrates the preliminary PCR performed to detect a gene found in most *Borrelia* species, *rrf-rrl*. If a positive result is obtained, confirmation is performed using the *ospA* gene as the target, which narrows the result down to the causative agent *B. burgdorferi*, as shown in Fig. 2b. The final test is to sequence positive *ospA* samples and identify the sequence using the National Center for Biotechnology Information (NCBI) website's basic local alignment search tool (BLAST). This identification can then be aligned with other *Borrelia* species sequences and placed in phylogenetic trees to determine their relatedness (Fig. 2c). Syphilis transmission may also be tracked using molecular techniques, such as PCR-restriction fragment length polymorphism (PCR-RFLP). Figure 3b shows the genotyping results of a PCR-RFLP in the *tpr* genes of *T. pallidum*. RFLP uses restriction enzymes to cut the amplicon into fragments that are separated on gel electrophoresis. This enables the distinct banding patterns for the isolate to be directly compared to those of the controls (Pillay *et al.*, 1998)

Monitoring treatment

The conventional management of infectious diseases may need revision as the near real-time detection of potential pathogens has allowed prompt prediction of drug resistance profiles, microbial virulence factors and host factors. This has enabled therapeutic and public health control measures to be implemented at a faster rate.

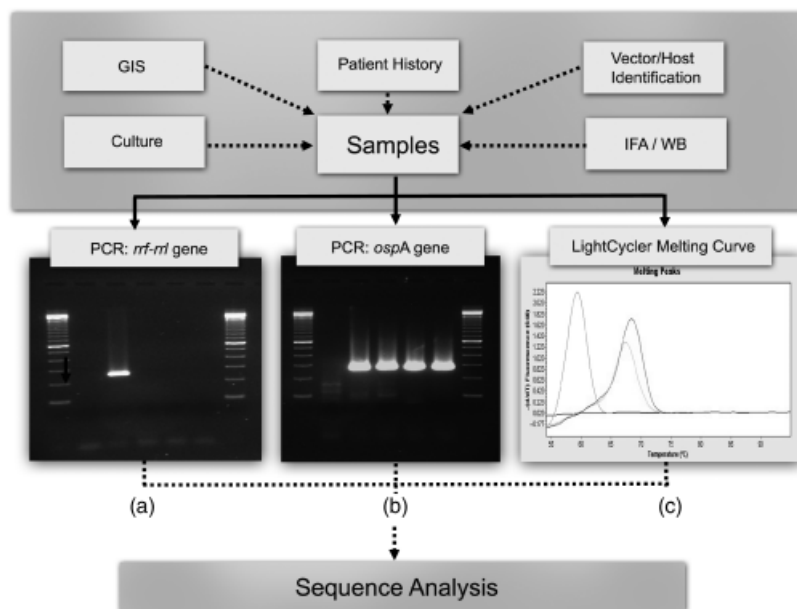


Fig. 2. Lyme disease testing: (a) PCR detecting *Borrelia* species, (b) PCR specified for *B. burgdorferi*, (c) sequence cluster analysis. GIS, Geographical Information System (unpublished data).

Multiple isolates of an infectious agent, obtained from the same individual, may or may not represent the same strain. Multiple strains may indicate a reinfection with a new strain, failure of therapy or the presence of coincidental contamination. The definition of each situation may be difficult by traditional culture or biochemical methods. PCR and DNA sequencing to differentiate and define these strains may be useful in answering the clinical or public health questions in these situations.

Molecular assays can also be used to monitor the efficacy of therapy. The viral load of the immunodeficiency virus (for example, HIV) is an important indicator for physicians using a treatment plan for HIV-infected individuals (Nkoghe *et al.*, 2004). Genotyping assays have also been used for disease management. For example, patients with Hepatitis C virus (HCV) genotypes other than type 1 respond better to interferon therapy (Lee *et al.*, 2002a,b). The optimal duration of treatment varies depending on the HCV genotype identified in the diagnostic laboratory. This molecular approach may also be useful in deciding whether monotherapy or a combination therapy should be used (Richter, 2002).

Molecular testing may also be widely used to detect microbial drug resistance. The detection of resistance genes in *M. tuberculosis*, for example, has potential for clinical application in the near future. This approach has also been used in managing the treatment of *Treponema* infections (Lukehart *et al.*, 2004). Figure 3c illustrates the detection of azithromycin resistant *T. pallidum* using PCR-RFLP analysis, by detecting a known mutation in the 23S rRNA gene sequence.

Pitfalls

Sampling, shipping and storage

It is important to characterize the specimen, as this will determine the best sample type and when optimal collection should occur. Specimen collection time, handling conditions, storage temperature and transport to the laboratory are important parameters. For example, the viral titer of the West Nile virus is 10-fold lower per 24 h period at 28 °C compare to 4 °C (Mayo & Beckwith, 2002). Therefore, it is very important to ensure specimens are collected properly and kept at the proper temperature after collection. The storage of nucleic acids after extraction is also important. Of note, the optimal storage temperatures for DNA and RNA are not the same. DNA must be stored at 4 °C or at -20 °C. However, RNA ideally should be stored at -80 °C, but often -20 °C is acceptable for short-term storage (Halfon *et al.*, 1996).

False-positive and false-negative results

Molecular diagnostic tests, as any other laboratory assays, may produce false-positive and false-negative results. Stringent quality controls to detect these false results must be used in the diagnostic laboratory. Even a very small amount of amplified genetic product may contaminate laboratory reagents and equipment (Zaidi *et al.*, 2003), and cause significant quality assurance repercussions to a laboratory at any point during a testing procedure (Nowak *et al.*, 1996; Neumann *et al.*, 1998; Zanghellini *et al.*, 1999; Niesters *et al.*, 2000). Good aseptic techniques, correct handling of

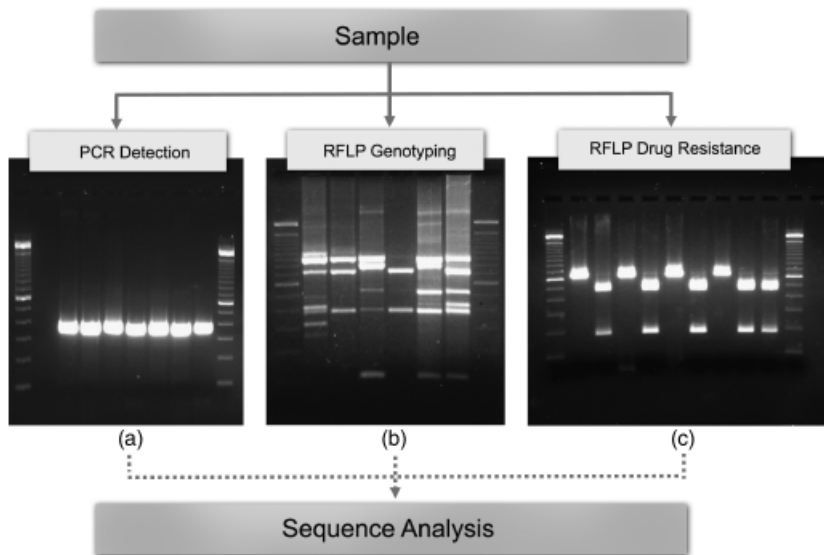


Fig. 3. The use of PCR for diagnosis, genotyping and azithromycin-resistance detection in *T. pallidum*. (a) PCR based on the *poIA* gene. (b) PCR-RFLP of the *tpr* genes. (c) PCRRFLP to detect mutation in the 23S rRNA gene (unpublished data).

infectious materials and exacting clean-up of work areas are all necessary in preventing contamination. Automated equipment may further reduce the potential for contamination (Krajden, 2005). Some laboratorians also choose to incorporate uracil in amplification products then predigest reaction mixes with uracil-N-glycosylase; this helps decrease the likelihood of contamination (Neumaier *et al.*, 1998).

Nonspecific genetic amplification may arise from other sources besides direct contamination. They may come from the environment (for example, normal flora from a stool sample) or the host gene (patient blood and tissue). Some of these amplifications can have a similar sized amplicon on gel electrophoresis, or may have the same melting temperature in a melting curve analysis. Unless a confirmatory assay is performed, this could result in a false positive. Good experimental design, such as selecting primers with higher annealing temperatures and BLAST searching in Genbank to determine specificity of primers, helps decrease the possibility of nonspecific amplification. Other approaches to addressing this problem include the use of a 'hot start' or 'touchdown' technique, probe hybridization, RFLP or nested PCR.

Figure 4 illustrates how nonspecific binding or cross-contamination may lead to a false-positive result. The gel photograph indicates that the sample may have the infectious agent in question, as shown by the weak band (circled) which corresponds to a band the same size as the positive control. The amplicon was sequenced and subjected to a BLAST search to determine its best match in the NCBI database. The final result was a match to a human DNA gene. Therefore, molecular method standard operating procedures must be set up to include stringent quality assurance procedures and confirmatory tests.

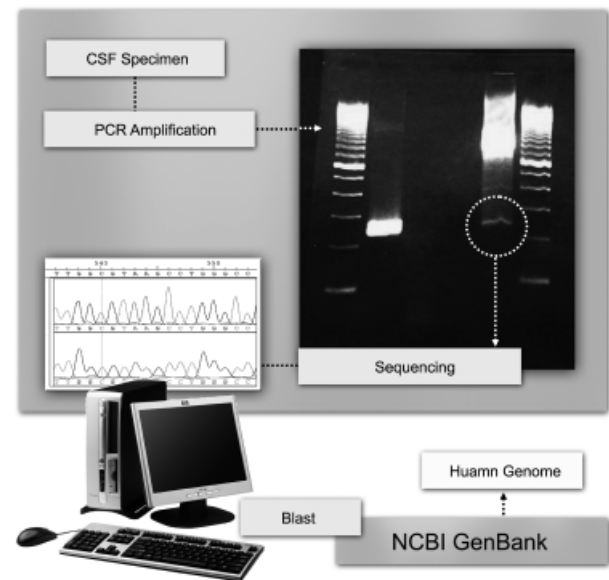


Fig. 4. Amplicon from patient CSF in a *Borrelia* PCR assay has the size same as the positive control on gel electrophoresis. It is confirmed to be a nonspecific amplification from human DNA after a DNA sequencing comparison.

False-negative test results may be difficult to detect because they can be the result of insertions or deletions in the genome of an organism (Papin *et al.*, 2004) mixed infections, incorrect timing for sample collection or incorrect handling of specimens, or inhibition of material within the sample. Insertion or deletion within the genome of an organism may only be confirmed by sequencing methods. These mutations would go unnoticed in a basic PCR reaction, which may simply show a

different-sized amplicon compare to the positive control. Further analyses must be performed to confirm the PCR results. A sample containing a mixed infection may produce multiple bands in the PCR reaction or only one band reflecting the dominant infection. The amplification of different organisms may be the result of the primers used in the PCR reaction not being specific enough to the organism in question.

As for any laboratory diagnostic test, the timing of the specimen collection must be considered. Some organisms have a long incubation period within the host; therefore it may take several weeks to months before the etiological agent is detectable. In such a case, if samples are collected too early from an individual, diagnostic testing will not be useful.

Inhibitory substances in the specimen may also pose challenges. Substances such as heparinized blood, triglycerides, haemoglobin, some therapeutic drugs or extraction reagents, may cause false-negative results; no PCR product is detected (Holodniy *et al.*, 1991; Elbeik *et al.*, 2004) even though the infectious agent is actually present. In some cases, this can be corrected by diluting the sample. The dilution of sample will cause the inhibitory substance to be diluted and allow amplification to proceed. The best way to detect inhibitors is to use internal positive controls with samples tested. This control would be subjected to the same extraction, amplification and detection procedure as the sample to ensure that laboratory procedures are carried out correctly, and it assists in the determination of the inhibition.

Difficulties in validating orphan testing

Orphan tests are tests carried out for pathogens, but are not performed by many laboratories. In general, they have not been extensively validated. Some examples include tests for Lyme disease, Bartonella, Q-fever and Hanta virus. Because few diagnostic laboratories test for these pathogens, it may be very difficult to obtain positive controls and specimens sufficient to validate these assays. Therefore, in some instances, there is a need to use artificial or manufactured controls. An example of this is the use of Armored RNA for WNV testing (Eisler *et al.*, 2004). The alternative for the lack of positive controls is the sharing of confirmed positive specimens between laboratories. With either circumstance, validation with these materials still poses problems, as many are not quantitative (the exact concentrations of the controlled substance is unknown).

Proficiency testing and quality management

Molecular diagnostic processes must be designed to ensure procedural elements are followed; tests must be validated against accepted, standard methods to ensure that results are equivalent. Signal-to-noise ratios need to be closely monitored and limits of detection analyzed carefully. Labora-

tories must participate in proficiency testing programs (External Quality Assurance) for the evaluation of test performance and interpretive accuracy (Krajden *et al.*, 1999; Lefrere *et al.*, 2004).

Proficiency testing for conventional methods usually includes test samples developed in another laboratory which are spiked with known amounts of the infectious agent in question. The laboratory being tested is required to detect, quantitate and report the agent. This is a challenge for molecular methods, because there is often not a single way to detect for a certain organism. Many molecular laboratories develop their own methods for testing a particular organism, or further adapt published methods to increase the sensitivity or specificity of a test. This leads to inter-laboratory variations in molecular methods, and thus makes it hard to validate by comparing to standard methods.

Rigorous quality control (QC) is required before many molecular methods are deemed acceptable for use in detecting infectious agents. Quality assurance (QA) in quality management systems (QMS) is also an important aspect of overall accuracy of clinical results. Test validation from the collection of samples through to the reporting of results must be carried out to ensure laboratories are providing accurate diagnoses.

The stability and integrity of any specimen must be maintained during handling and transportation, as factors such as storage times, transport times and anticoagulants will affect integrity (Holodniy *et al.*, 1995; Ginocchio *et al.*, 1997; Grant *et al.*, 2000; Lee *et al.*, 2002a, b). Not only is sample integrity an important quality issue, but the conditions of the extraction and the ongoing integrity of other test reagents are as well. Reagents such as premanufactured PCR master mixes, and primers and probes, need to be monitored in an ongoing, rigorous manner to ensure their composition and concentrations are maintained. Lastly, equipments used during the extraction, detection and analysis of specimens need to be tested as part of the QA system. For example, pipettes must be constantly calibrated to ensure accurate pipette volumes. Thermocycler temperatures must be checked regularly to ensure cycling conditions are exact.

The QA system in any laboratory will depend on whether the test has had regulatory approval, whether it is used for research or investigational studies, or whether it is an adapted procedure from a manufacturer's test. How often a test is being performed will also determine the QA required (Krajden, 2005).

Information technology

Understanding the character and coverage of the database used is important. An incomplete or 'polluted' database can result in problems particularly related to BLAST or typing by cluster analysis. The NCBI database is a good source for

genetic information on many different organisms; however, in many cases it is necessary to screen unvalidated or unrelated sequences. On the other hand, the amount of sequence data available is directly related to the level of sensitivity of the assay in identifying unknown agents. Sometimes these data are required in order to develop a molecular diagnostic test. Without other laboratories or research groups willing to cooperate, obtaining accurate information may be an informatics challenge.

Many molecular assays and their results are not integrated into the Laboratory Information Systems (LIS) at this time. They still depend on manual analysis, which is not standardized and sometimes more subjective. The interpretation of the results could vary from laboratory to laboratory. Even if some of them are integrated in the LIS, the choice of algorithms and analytical methods, including parameters and cut-off points, is still a challenge, particularly in dealing with border-line specimens. Many developments, however, are anticipated in this field as the benefits to patients, as well as to expand opportunities for microbial genome analysis, are clear.

Costs

One of the drawbacks of the use of molecular methods is the increased costs as compared to the traditional methods. The set-up costs may be significant, as specially designed rooms and designated equipment are generally required to reduce contamination. The instrumentation required, i.e. thermocyclers, sequencers or real-time machines, are also expensive. The costs also vary depending on the type of molecular method used. Once the initial set-up has been completed, funding to keep the assays running optimally with enhanced QA are required. Basic primers are inexpensive, but probes and specially designed primers required to increase sensitivity and specificity add extra cost. Therefore, the overall costs depend on the type of assays being performed, but in general this issue is a challenge. There are ways in which costs can be reduced (e.g. the pooling of samples) for screening samples, but strategies to address the funding issue are needed.

Conclusions

Although there are a large number of challenges to using molecular methods, it may be possible to overcome these issues. Use of these methods to provide accurate and timely information to health professionals is clearly advantageous. The use of molecular methods is expected to continually increase, particularly in diagnostic microbiology and public health laboratories geometrically. In the next decade, this will be a growth industry. The use of molecular methods, alone or in conjunction with conventional methods, has great potential to improve our success in the ongoing war against the spread and emergence of infectious diseases worldwide.

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