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NUCLEIC ACID AMPLIFICATION AS USED IN THE DIAGNOSIS AND MANAGEMENT OF VIRAL DISEASES: A *REVIEW*

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ABSTRACT
Diagnostic virology is of great importance particularly in disease management and for epidemiological purposes, but is slow and results are obtained when the patient has recovered or succumbed to the infection. In addition, consideration of antiviral chemotherapy is in short supply even where preliminary investigation results are available. Some viruses are highly unculturable, fastidious or hazardous to the laboratory personnel and diagnosis depends on serological methods or culture in an expensive bio-safety level. Molecular diagnostic techniques do not depend on pathogen isolation or growth or on the detection of an immune response to the pathogen, rather uses genotypic characteristics to identify specific pathogen. Nucleic acid of a given pathogen is unique, and therefore nucleic acid analysis can be used for unequivocal identification via amplification to increase the amount of material available for analysis. Nucleic acid-based diagnostic methods are extremely sensitive, reliable and to some extent affordable, and are widely used for clinical microbiology to detect pathogen.
Keywords: Amplification, Diagnosis, Nucleic acid, Viral Diseases

INTRODUCTION

Microorganisms were first recognized as cause of diseases more than a century ago by the pioneered laboratory work of Koch and Pasteur. From then until the present day, microbiology laboratories have made significant contributions to the epidemiology of diseases. Despite remarkable scientific and technological advances, outbreaks of infectious diseases still occur and new infectious diseases continue to emerge. (Satcher, 1995). Many factors contribute to disease emergence (Lederberge *et al.*, 1992), and the epidemiology of many infectious diseases is becoming more complex. Fortunately, extremely sensitive and specific molecular techniques have recently been developed to facilitate epidemiologic studies. Nucleic acid amplification and analysis have emerged as particularly powerful analytic methodologies. These methodologies have prompted scientists to modify Koch's postulates as the standard of proof for a microbial aetiology of a given disease (Fredricks and Relman, 1996).

The development and application of molecular techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases. Amplification techniques are making increasing inroads into clinical laboratories; detect aetiologic agents of diseases directly from clinical specimens, without the need for culture, and for rapid detection of unculturable or fastidious microorganisms (Yi-wei tang *et al.*, 1997). Nucleic acid amplification provides the ability to selectively amplify specific targets present in low concentration to detectable levels; thus, amplification based methods offer superior performance, in terms of sensitivity, over the direct non amplified tests. Several different amplification-based strategies have been developed and are available commercially. The adaptation of amplification-based test methods to commercially available kits have served to optimize user acceptability, prevent contamination, standardize reagents and testing conditions, and make automation a possibility (Tang and Persing, 1999).

The advent of nucleic acid amplification and detection has resulted in a change from conventional laboratory methods that rely on phenotypic expression of antigens or biochemical products, to molecular methods for the rapid identification of a number of infectious agents. It has become increasingly incorporated into the clinical laboratory, particularly for the detection and characterization of virus infections and for the diagnosis of diseases (Speers, 2006).

During the course of *bacterial* evolution, genes that code for essential housekeeping molecules have remained highly conserved. For example, mutation of genes that code for proteins or RNAs whose function is constant and vital for the survival of *bacteria* occurs at a much slower rate than those for genes that are under strong selective pressure. Because medically relevant *eubacteria* have a common origin, molecular biologists can construct evolutionary (or phylogenetic) trees based on analysis of genes that code for these conserved macromolecules (Woese, 1987). In this fashion, previously characterized species of *bacteria* can be easily identified by genomic sequencing, and the phylogenetic position of new (and previously unsequenced) species can also be determined.

No equivalent, broadly conserved molecules exist among *viruses*, and so it is not possible to construct a single phylogenetic tree of all *viruses*. However, conserved and variable genes have been identified for

numerous groups of *viruses* and provide a molecular basis for determining relatedness within the respective groups. Genomic sequence analysis is of particular value in distinguishing closely related *viruses*.

AMPLIFICATION METHODS

Several strategies for the amplification of nucleic acids have been described, and include the following, Amplification of the nucleic acid target (e.g., polymerase chain reaction 'PCR', strand displacement amplification, and self-sustaining sequence replication), Amplification of nucleic acid probe (e.g., ligase chain reaction and Q-beta replicase) and Signal amplification (e.g., branched-probe DNA assay). PCR is the most widely used method.

As these molecular methods are further defined and become more widely available in the next few years, physicians will need to understand their clinical applications and be aware of their potential advantages, limitations and clinical utility (Fredricks and Relman, 1999; Birkenmeyer and Mushahwar, 1991 and Tang *et al*., 1997).

Polymerase Chain Reaction (PCR)

Developed in 1983 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research laboratories for a variety of applications (Bartlett and Stirling, 2003; Saiki *et al*., 1985 and Saiki *et al*., 1988), including detection and diagnosis of infectious diseases. It is a technique that amplifies a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The PCR is commonly carried out in a reaction volume of 10–200µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction.

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles (Fig. 1), with each cycle commonly consisting of 2-3 discrete temperature steps. Most protocols call for cycles that have three temperature steps. The cycling is often preceded by a single temperature step (called hold) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers (Rychlik *et al*., 1990).

Initialization step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR (Sharkey *et al*., 1994). This is followed by denaturation step and is where the first regular cycling event takes place. It consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA. The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis leading to extension or elongation step. The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C (Chien *et al*., 1976 and Lawyer *et al*., 1993) and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment. Occasionally final elongation can be performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products. In multiplex PCR the assay is mordified to include several primer pairs specific to different DNA targets to allow amplification and detection of several pathogens at the same time (Marie *et al*., 2000).

Reverse transcription PCR is a modification of this method used when the initial template is RNA rather than DNA. In this case the enzyme reverse transcriptase first converts the RNA target into a complementary DNA copy (cDNA). This cDNA can then be amplified by standard PCR methods (Marie *et al*., 2000).

In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions. Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants (Joseph and David, 2001).

This usually involves spatial separation of PCR-setup areas from areas for analysis or purification of PCR products, use of disposable plastic ware, and thoroughly cleaning the work surface between reaction setups. Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products, and the usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA.

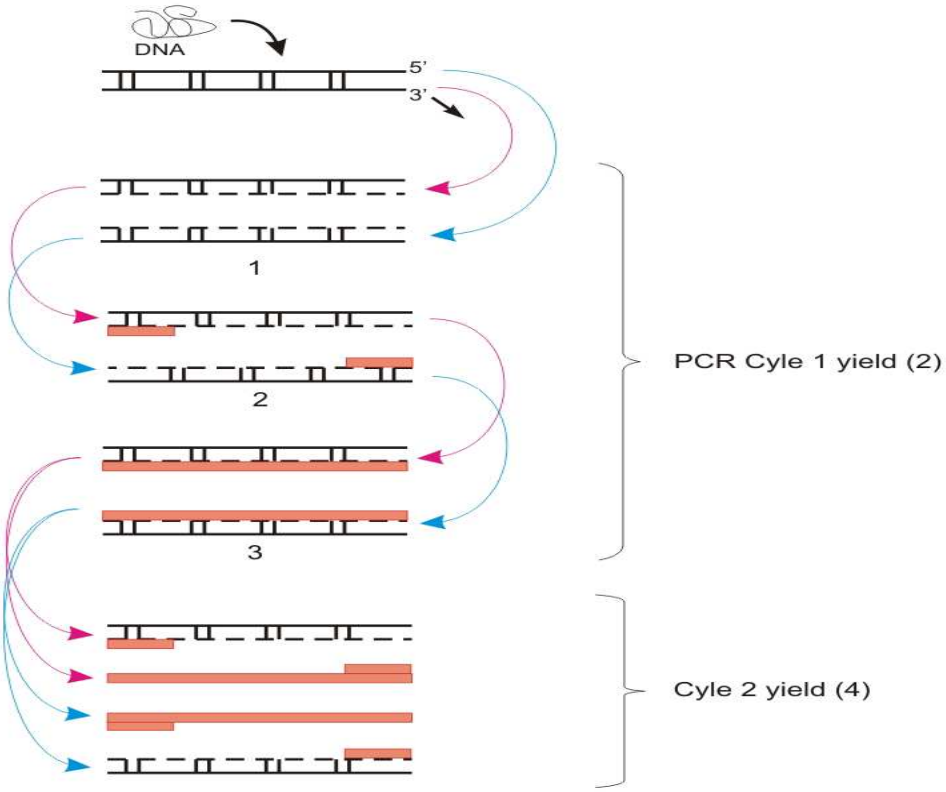

Figure 1: Schematic drawing of the PCR cycle. (1) Denaturing at 94–96 °C (2) Annealing at ~65 °C (3) Elongation at 72 °C (Sarkinfada, 2004)

Ligase Chain Reaction

Also called ligase amplification, first described in 1989 by Wu Wallace (1989), is a probe amplification technique rather than producing amplicon through polymerization of nucleotides. It uses both DNA polymerase and DNA ligase to drive the reaction. Each cycle results in a doubling of the target nucleic acid molecule (Wiedmann *et al.*, 1994). Oligonucleotide probes are annealed to template molecules in a head to tail fashion with the 3^{I} end of one probe abutting the 5^{I} end of the second. DNA ligase then joins the adjacent 3^{I} and 5^{I} ends to form a duplicate of one strand of the target. A 2^{nd} primer set; complementary to the 1^{st}, then uses this duplicated set (as well as the original target) as a template for ligation (Barany, 1991; Chernesky *et al.*, 1994 and Schachter *et al.*, 1994).

Branched Probe-DNA Amplification (bDNA)

This is a form of signal amplification, developed by Chiron Corp. Multiple probes as well as multiple reporter molecules are used to increase the signal in proportion to amount of target in the reaction (Sanchez-Pescador *et al.*, 1988 and Urdea *et al.*, 1991). It is used for specific nucleic acid chains, and is typically used to detect *Retroviruses* such as *HIV*. However, the test can be used to detect other types of RNA or DNA targets (Murphy *et al.*, 1999).

APPLICATION OF NUCLEIC ACID AMPLIFICATION TECHNOLOGY

Traditionally, the clinical medical microbiology laboratory has functioned to identify the aetiologic agents of infectious diseases through the direct examination and culture of clinical specimens; which is limited by the number of organisms present and the ability of the laboratorian to successfully recognise the pathogen. Also the ability of the pathogen to propagate and the choice of appropriate media. Some microorganisms are either unculturable at present, e.g., *viruses*, extremely fastidious or hazardous to laboratory personnel. In these instances the diagnosis depends on the serologic detection of a humoral response or culture in an expensive bio-safety facility. In community medical microbiology laboratories these facilities may not be available, or it may not be economically feasible to achieve targets, thus, cultures are often sent to referral laboratories.

The addition of nucleic acid amplification technology to the microbiology laboratories has resolved many of these problems. The exquisite sensitivity and specificity of these methods allow the accurate detection of very small number of microorganisms (Ichiyama *et al*., 1996 and Jackson *et al*., 1996 and Whelen *et al*., 1995). In selected situations, the limitation imposed by the inability of an organism to be cultured and the selection of appropriate media and culture conditions may be replaced by the use of nucleic acid amplification. Microbial DNA/RNA extracted from clinical specimens may be analysed for the presence of various organisms-specific nucleic acid sequences regardless of the physiological requirements or variability of the organisms (Fedorko *et al*., 1995; Sandhu *et al*., 1995; Schwab *et al*., 1996 and Tang *et al*., 1997).

Incorporation of nucleic acid amplification technology into the diagnostic microbiology laboratory

Nucleic acid amplification methods have the potential to significantly influence the diagnosis and management of a variety of infectious diseases. Conventional laboratory diagnostic methods require a minimum of 24hours, and in many cases significantly longer. Certain pathogenic organisms (*viruses*) are not easily detected by routine culture methods and require specialized procedures. Rapid non culture diagnostic tests relying on antigen detection by immunofluorescence or enzyme immunoassay, or using DNA probes, may have variable diagnostic sensitivities or specificities as compared with cultures. Molecular methods with amplification and detection of target nucleic acids have generally been found to have superior sensitivity and specificity and have the potential to provide results within hours of collecting specimen. Currently available commercial tests using PCR for the diagnosis of *viral* infections include those able to detect *HIV, Herpes simplex virus (HSV), Cytomegalovirus (CMV), Enterovirus, Hepatitis C virus (HCV), Hepatitis B virus (HBV), Rabies virus, Human T-lymphotropic viruses (HTLVs), Human Papillomavirus (HPV), and Hantivirus,* e.t.c.. Many of these assays are now routinely being used in clinical microbiology laboratories. Diagnostic test kits for many other *viral* infections are under development. Pilot studies have indicated the feasibility of designing broad range multiplex PCR assays with the capability of detecting a panel of microorganisms for clinical specimens, (Grondahl *et al*., 1999; Ley *et al*., 1998, Goldenberger *et al*., 1997 and Klausegger *et al*., 1999), while some are in the market, like the *HPV-Neisseria gnorrhoea* and *Chlamydia trachomatis*, capable of detecting ranges of high/low risk *HPV*s and the 2 other infections in a single test reaction.

1. **In 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 microbiologist and the hospital epidemiologist are facilitated greatly by the availability of the newer molecular epidemiological typing techniques (Banerjee *et al*., 1991; Pfaller; Troy *et al*., 1997; Van belkum *et al*., 1997 and Wenzel, 1992). Application of DNA probe-based assays allows the diagnosis of nosocomial infections caused by *Respiratory syncytial virus* (Ahluwalia *et al*., 1987), *Varicella zoster virus* and *Herpes simplex virus* (Schmidt *et al*., 1980). The ability to rapidly and unambiguously characterise organisms suspected of causing a disease outbreak is critical to public health and hospital infection control endeavours. Recent contributions to clinical and hospital epidemiology have depended on PCR. Several putative outbreaks of infections have been investigated by molecular techniques; nucleic acid amplification assays for the detection of *viruses*, such as *Herpes simplex virus, Cytomegalovirus, Enteroviruses* and *HIV* have proved to be useful for screening, diagnosis and management.

The Canadian blood services have recently adopted a nucleic acid amplification method to screen donated blood for *Hepatitis C* and *HIV* because of the enhanced sensitivities of these assays. PCR detection of *Herpes simplex virus* in cerebrospinal fluid have become the method of choice for the diagnosis of *Herpes encephalitis*, with sensitivity and specificity of 95% and 94% respectively (Lakeman and Whitley, 1995), obviating the need for a brain biopsy (Fredricks and Relman, 1999 and Lakeman and Whitley, 1995). *Enteroviruses* are among the most common causes of aseptic meningitis. PCR for the diagnosis of *enteroviral meningitis* using CSF samples have been found to be more significantly sensitive than conventional *viral* isolation (14% of specimens positive versus 10% positive respectively). (Hadziyannis *et al*., 1999 and Van Vliet *et al*., 1998). Moreover, the PCR assay can be completed within 1day, whereas cultures for *enteroviruses* typically require up to 5days for isolation of the virus. A PCR assay for *CMV* is available for detection of the virus in plasma or CSF specimens and has been useful in monitoring *HIV* and bone marrow transplant patients with *CMV* infection. The performance of this test has been comparable to that of antigen assays, with reported sensitivities and specificities of 95% and 98-100% respectively (Long *et al*., 1998). In contrast, the sensitivity of culture detection of *CMV* was only 42% (Long *et al*., 1998). Significantly, a PCR analysis was recently successfully used to identify the *Hantivirus*, agent responsible for an outbreak of fatal infections in the US south-west (Arikawa *et al*., 1990 and Tang *et al*., 1990). *Human papillomavirus (HPV)* is a common cause of dysplasia, intraepithelial neoplasia and carcinoma in the female genital tract. Nucleic acid amplification techniques in cervical swabs or fresh cervical biopsy specimens to determine *HPV* infection and viral types have provided helpful information for clinical assessment and treatment of patients (Fuchs *et al*., 1988 and Reid *et al*., 1987). Occasionally, cases of human rabies occur in which the history of exposure cannot be determined. Molecular techniques now allow such a determination. Three cases of human rabies were investigated by Smith *et al.* (1991) among immigrants

who lived in areas of the US where animal *rabies* is either extremely rare or nonexistent. Isolates of rabies virus were obtained from 3 patients and compared with several methods with human and animal isolates that had been acquired from various geographic regions, including areas from which the 3 patients had emigrated. *Dog* isolates from the county or region of the 3 patients were included in the analyses. Complementary DNA copies of viral RNA were prepared and amplified by PCR; the molecular techniques used in this study allowed the 1st confirmation of this *viral* phenomenon. *Human T-lymphtropic viruses (1&2)* were also detected in peripheral blood samples to establish a clinical diagnosis using nucleic acid amplification technology. Available genomic sequencing data suggests that 2 types of *HTLV-1* isolates co-exist: so called cosmopolitan isolates, which have been acquired in Japan, the Americas, Africas and the Caribbean and have more than 97% nucleotide sequence similarity; and Australomelanesian variants (from new guinea, Australia Solomon islands) which by gene sequencing analysis are approximately 7% divergent from cosmopolitan strains.

2. In clinical diagnosis of viral infections:

The diagnosis of viral infections has been hampered for many years due to the cost, laboratory time and skilled personnel required for the cell culture systems used, together with the generally low sensitivity and slow growth of many viruses in artificial media. Serology is often unhelpful in the early stages of infection, specific antisera for the serology tests can be difficult to obtain, and the clinical detection of antibodies is relatively insensitive for a number of viruses. PCR technology has therefore improved the detection of a number of these viruses. *Herpes simplex virus (HSV)* encephalitis is a serious infection but diagnosis previously required brain biopsy in certain cases due to the low sensitivity of cerebrospinal fluid (CSF) culture and serology (Gilbert *et al*., 1999). PCR now allows the detection of HSV DNA from CSF with 95% sensitivity (Lakeman and Whitley, 1995) thus avoiding invasive brain biopsy. *Viral* meningitis, commonly caused by either *enteroviruses or HSV*, is more reliably detected by PCR when compared to culture (Van Vliet *et al*., 1998) and in a shorter time (one versus up to five days). HSV PCR can be multiplexed with other pathogens responsible for meningitis (Read *et al*., 1997). The detection of blood borne virus infection is also improved by both PCR and non-PCR molecular methods. Active *Hepatitis C virus (HCV)* infections are diagnosed by the presence of *HCV* RNA since the detection of antibody to *HCV* cannot distinguish between past and present infection. In terms of infectiousness only those with detectable *HCV* RNA have a significant risk of transmitting HCV by transfusion, organ transplantation, needle-stick injury or vertically to the child (Dore *et al*., 1997). Although infection with the *Human immunodeficiency virus (HIV)* is routinely diagnosed by serology, early HIV infection can be detected by *HIV* pro-viral DNA detection before HIV antibodies are confirmed by Western Blot serology (Dax, 2004). Vertical transmission of *HIV* infection is also detected in the infant using *HIV* pro-viral DNA detection (Luzuriaga and Sullivan, 1994). The Australian Red Cross Blood Service screens pooled samples from all donations for *HIV* and *HCV* using the Chiron Procleix *HIV-1/HCV* transcription mediated amplification assay, thus reducing the potentially infectious window period from 22 and 66 days to 9 and 7 days respectively (Seed *et al*., 2002).

Intrauterine infection of the foetus with *Cytomegalovirus (CMV)* (Palasanthiran *et al*., 2002), Rubella (Nourse, 2002), and *Varicella zoster virus* (Heuchan and Isaacs, 2002) can be detected by PCR testing of amniocentesis fluid. Genital ulceration due to *HSV*, usually due to *HSV type 2* infections, is now routinely detected by PCR in many clinical microbiology laboratories due to its increased sensitivity over viral culture. Molecular detection of respiratory viral pathogens from both upper respiratory specimens such as nasopharyngeal aspirates or throat swabs and lower respiratory specimens such as sputum or bronchoalveolar lavage fluid is cost-effective due to the prevention of hospitalisation, decreasing unnecessary testing and procedures, directing specific therapy, and reducing unnecessary antibiotic use (Henrickson, 2005). Large multiplex or tandem PCR assays testing for all the common respiratory viruses along with fastidious bacterial causes of pneumonia are now feasible providing a thorough yet cost-effective alternative to conventional detection methods. Uncommon yet significant respiratory viruses such as severe acute respiratory syndrome (SARS) *Coronavirus (SARS-CoV)* and *Influenza A/H5N1 (Avian influenza) virus* can also be incorporated into these assays thus acting as an in-built early detection system. During the SARS epidemic due to the *SARS-CoV*, PCR testing of respiratory specimens for other respiratory viruses was crucial to exclude a number of suspected cases which fulfilled the case definition for SARS. PCR detection was most helpful due to the ability to rapidly screen for many respiratory *viruses*. Subsequently a specific *SARS CoV* PCR has been developed for the early detection of *SARS-CoV* infection with a sensitivity of 50-87% early in the disease (Ng *et al*., 2003). Serology for SARS-CoV is up to 100% sensitive but of limited diagnostic value early in the disease when the risk of transmission is greatest (H_o *et al*., 2005). The recent *Avian influenza (H5N1)* outbreaks in South East Asia and beyond have also illustrated the need for rapid viral diagnosis.

Molecular detection methods were developed following the 1997 Hong Kong outbreak (Yuen *et al*., 1998) and have the advantage of being rapid and able to be performed in many clinical microbiology laboratories. Specific serology needs live virus for the microneutralisation assay which is currently classed as a Biosafety Level 4 organism in Australia. Likewise direct immunofluorescence detection requires *Influenza type A/H5*-specific monoclonal antibodies (WHO, 2005). *Viruses* cause more infectious diarrhoea worldwide than *bacteria* and other pathogens. The diagnosis of viral diarrhoeal disease has improved with the development of PCR detection.

The method of choice for microbiological diagnosis of rotavirus from stool samples is PCR. *Norovirus*, a *calicivirus* formerly known as *Norwalk virus* and responsible for large outbreaks both in the community and health care facilities, can be diagnosed by electron microscopy, enzyme immunoassay and PCR but PCR is the most sensitive and rapid method. PCR is also the most sensitive method for the diagnosis of *Astroviruses* and enteric *Adenoviruses* (serotypes 40 and 41) (Clark, 2004).

3. In treatment monitoring:

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 (Katzenstein, 2003). Commercial tests are available and more recently ultrasensitive tests such as the Cobas Amplicor *HIV- 1* Monitor Ultrasensitive Test have been released, reducing the lower limit of *viral* detection to 50 copies/ml (Berger *et al*., 2005). *Viral* load testing is also used for the assessment and monitoring of responses to therapy in chronic *HCV* and *HBV* infection. *HCV* RNA *viral* loads are assessed in patients with genotype 1 *HCV* infections when monitoring for responses to combination interferon-alpha and ribavirin therapy. Patients who remain negative for *HCV* RNA 6 months after completing combination therapy for *HCV* infection almost always remain free of the virus in the longer term and have achieved a sustained virological response. If the *HCV* genotype 1 RNA is undetectable after 12 weeks of therapy there is a 75% chance of a sustained virological response. However, even if the HCV RNA remains detectable, a 33% chance of a sustained virological response remains if a 100-fold decrease in the viral load has occurred after 12 weeks of therapy (Fried *et al*., 2002). In *HBV* carriers with active liver disease *HBV* DNA loads are measured not only to assess patients regarding the need for either interferon-alpha or lamivudine (a DNA polymerase inhibitor) antiviral therapy but also to monitor their effectiveness. An increase in *HBV viral* load is also used as a marker of the emergence of lamivudine resistant *viral* mutants (Thomson and Main, 2004). *Cytomegalovirus* infection is a serious infection in bone marrow and solid organ transplant recipients together with *HIV*-infected patients but detection has been limited by the poor sensitivity of traditional culture methods (Long *et al*., 1998). *Viral* load testing by quantitative PCR is now the accepted standard for monitoring the emergence of *CMV* infection during immunosuppression and allows pre-emptive therapy prior to the emergence of clinical disease with high sensitivity when compared to culture (Emery *et al*., 2000).

4. In viral genotyping and resistance testing:

HIV genotyping for the detection of drug resistance is the standard of care to guide antiretroviral therapy and complements *viral* load assessment. Several databases are available such as the Stanford reverse transcriptase and protease database (http://hivdb.stanford.edu) where sequences can be checked for resistance mutations. Genotyping is also critical to the management of chronic viral hepatitis. There are six *HCV* genotypes geographically distributed throughout the world. The genotype is the single strongest determinant for success with combination therapy and all patients wishing to undergo therapy firstly undergo *HCV* genotyping. Those with chronic genotype 2 or 3 *HCV* infections receive 6 months of therapy with a 76% chance of success compared to a 56% chance of success for those with genotype 1 *HCV* infection receiving 12 months of therapy (Fried *et al*., 2002). Active chronic infections with HBV treated with lamivudine require surveillance for the emergence of lamivudine resistant viral mutants. During lamivudine monotherapy point mutations at the active site of the polymerase gene (YMDD variants) occur with a frequency of 14-32% after one year in phase III studies, and in 42% and 52% of Asian patients after two and three years of therapy respectively (Dixon and Boehme, 2000). The emergence of lamivudine resistance is detected by a rise in *HBV viral* load and confirmed by sequencing of the active site of the DNA polymerase gene (Allen *et al*., 2006). The presence of *HBV* pre-core mutants may cause active liver disease despite the absence of HBeAg, the common marker for active hepatitis in hepatitis B infection. This may be due to either a premature stop codon point mutation in the precore gene (G1896A) or a mutation in the basal core promoter region down-regulating HBeAg production, both of which can only be reliably detected genotypically (Papatheodoridis and Hadziyannis, 2001). *Human papillomavirus (HPV)* is now accepted as the cause of almost all cervical cancers and *HPV* genotypes are now classified as either low or high-risk for the causation of these cancers. Screening for pre-neoplastic cytological changes has traditionally been performed by the Pap screening, but the detection of high-risk *HPV* infection is a useful adjunct. Since *HPV* cannot be routinely cultured in vitro, testing for the 15 high-risk genotypes of *HPV* requires molecular methods. Detection can be achieved by signal amplification, such as the Digene Hybrid Capture 2 assay which used to be the only diagnostic in vitro test approved by the Federal Drug Administration (FDA).This assay contains specific RNA probes directed toward the high-risk genotype DNA sequences which are detected by an antibody directed against the DNA-RNA hybrids formed (Hubbard, 2003). Detection of the high-risk genotypes can also be achieved by target amplification such as multiplex PCR, Uniplex PCR. Detection of these genotypes by molecular analysis can help in the assessment of equivocal Pap smears to define those women at risk of developing cervical cancer (Brestovac *et al*., 2005). Alternatively a normal Pap smear with a negative genetic test for the high-risk genotypes may indicate a longer period of time before re-testing.

With the advent of a genotype 16 *HPV* vaccine the role of this testing is likely to assume more importance.

Limitations of nucleic acid amplification technology in diagnostic microbiology laboratory

Despite the obvious advantages of these newer procedures, there may be some potential limitations. The accuracy -reproducibility of PCR assays (as the most widely used technique) depend on the technical expertise and experience of the operator. Specificity of the test may be affected by specimen contamination during processing, if non-specific primers are selected or if the PCR conditions are not optimal, allowing non-specific products to amplify. Contamination or amplification product carryover of even minute amounts of nucleic acid may result in the generation of billions of DNA copies that may lead to a false positive result. False negative results may occur because of the presence of substances in the specimen that inhibit nucleic acid extraction or amplification. The assay may also lack sensitivity if there is low inoculation of the micro-organism present in the clinical sample.

Interpretation of nucleic acid amplification test result is not always clear cut. For e.g., assays may detect the residual DNA of a pathogenic organism even after successful treatment (Dagan *et al.*, 1998), and it is not clear whether this represents the presence of a small number of viable organisms or amplified DNA for non-viable organism. In addition, the meaning of a positive PCR result has not been validated for all infections. For example, it is uncertain whether positive PCR test results for *CMV* from a patient's serum represent active disease or latent infection. These observations suggest that there is a need for interpretative guidelines based on a correlation of nucleic acid amplification test results with clinical outcome.

Finally, it must be acknowledged that performance of nucleic acid amplification techniques is generally more expensive than conventional diagnostic methods (Fredricks and Relman, 1996).

CONCLUSION

Molecular technology involving nucleic acid amplification and detection is a promising tool for the rapid, accurate diagnosis of a variety of *viral* infections, and for confirmation or detection or both of antiviral sensitivity and resistance. Molecular methods have therefore gone beyond simple detection of viral infections to become an integral component of the management of blood borne virus and other *viral* infections.

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