http://dx.doi.org/10.4314/bajopas.v7i1.8

Bayero Journal of Pure and Applied Sciences, 7(1): 37 – 45
Received: April 2014
Accepted: June 2014
ISSN 2006 – 6996

APPLICATIONS OF MOLECULAR DIAGNOSTIC TECHNIQUES FOR INFECTIOUS DISEASES

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ABSTRACT
Diagnosis is concerned with identifying the cause of a disease or precise and consistent outcomes that are results of direct or indirect actions, reactions and interactions between the cause of a disease and the host. That outcome, if accurate, would help the clinician in disease management, or the epidemiologist in identifying trends of diseases or the administrator in policy and decision making. Traditionally, infectious disease diagnosis involves identifying the causative agents of infectious diseases through the direct examination, culture and often immunological tests on clinical specimens. The traditional diagnostic techniques have varied sensitivities and specificities which influence their choice and applicability in a particular setting for the diagnosis of infectious diseases. However, the limitations of many traditional techniques particularly low specificity and long turnaround time often necessitate initiation of treatment before results are made available. Molecular diagnostic techniques involve a variety of techniques that explore the use of nucleic acid molecules for the identification of a particular pathogenic organism. These techniques include nucleic acid-based typing system, nucleic acid analysis without amplification, polymerase chain reaction (PCR) and other nucleic acid amplification techniques. Applications of molecular detection methods for infectious diseases have resolved many of the problems of the traditional diagnostic techniques, due to their exquisite sensitivity and specificity that allow the accurate and timely detection of very small numbers of organisms. This paper examines the principles and applications of molecular biology techniques in the identification of the causative agents of infectious diseases either in a routine setting or as research tools.
Keywords: Infectious diseases, Molecular diagnosis, Polymerase Chain Reaction

INTRODUCTION

Infectious disease is a clinically manifested disease of man or animal resulting from the entry and development of an infectious agent or its products in a susceptible host (Benenson, 1995). Infectious agents could be Bacteria, Viruses, Fungi and Parasites. The infectious disease process comprises of complex interactions between six components collectively referred to 'infection chain' (Willey *et al*., 2008). Components of the infection chain are the infectious agent, its reservoir, porta of exit, transmission routes, portal of entry and the susceptible host (Engelgirk and Duben-Engelkirk, 2008).

Diagnosis of infectious diseases is traditionally based on demonstrating the presence of an infectious agent or its product in the host during the course of the disease, consistent with what a German physician, Robert Koch established in his famous "Koch's postulates" according to the relationship between *Bacillus anthracis* and anthrax disease (Millar *et al*., 2007).

The traditional conventional techniques used for the diagnosis of infectious diseases; microscopy, culture, serology and imaging are all consistent with the principles of Koch's postulates. Microbial phenotypic characteristics, such as protein, bacteriophage, and chromatographic profiles, as well as biotyping and susceptibility testing, phage and chromatographic analysis are also used in most routine laboratories for identification and differentiation of microorganisms (Tang *et al*., 1997). However, the last ten years of the twentieth century allowed for an exponential increase in the knowledge of techniques in molecular biology which allowed for significant developments in many areas of the life sciences, including infectious disease diagnosis.

This paper aims to examine the principles and applications of molecular biology techniques in the identification of the causative agents of diseases caused by bacteria, viruses, fungi and parasites in a routine practice or as research tools.

Traditional Diagnostic Techniques for Infectious Diseases:

Traditionally, identification of the causative agents of infectious diseases is made through the direct examination and culture of clinical specimens (Tang *et al*., 1997).

1. Microscopy

Microscopy, involves the use of various types of microscopes for observing magnified versions of microorganisms in clinical samples. Microscopes are optical instruments with specific resolving power (a limit to what can be seen through the instrument) or resolution. The naked eye has an estimated resolution of 2mm compared to that of compound microscope, scanning electron microscope

and transmission electron microscope with resolutions of 0.2µm, 20nm and 0.2nm respectively (Engelgirk and Duben-Engelkirk, 2008).

2. Biochemical characterization

Cultural technique is the traditional method of determining the bacterial or fungal cause of patient's infectious disease by growing the pathogen in pure culture and gathering information about its phenotype for identification. Biochemical tests are employed for further identification of the pathogen through detecting the presence or absence of specific enzymes or determining an organism's ability to catabolise various substrates (Cheesbrough, 1991; Engelgirk and Duben-Engelkirk, 2008). Gas-Liquid Chromatography (GLC) is frequently used in reference laboratories as an adjunct to other identification methods for bacteria and yeasts based on the analysis of acid and product of metabolism and analysis of cellular fatty acids.

3. Immunodiagnostics

Immunodiagnostic procedures (IDPs) help in the diagnosis of infectious diseases based on the detection of either antigens or antibodies in clinical specimens. One advantage of IDPs is that the results are often available on the same day that the clinical specimen is collected from the patient. Detection of a particular pathogen's antigen in a clinical specimen is an indication that the pathogen is present in the patient, thus providing direct evidence that the patient is infected with that pathogen. On the other hand, detecting antibodies against a particular pathogen is an indirect evidence of infection with that pathogen. However, the presence of antibodies to a particular pathogen could be attributed to present infection, past infection or vaccination against the organism (Engelgirk and Duben-Engelkirk, 2008).

4. Phage typing

Phage Typing involves the use of bacteriophages (viruses that infect and lyse bacteria), to type bacterial strains of a given species. Bacteriophages are often specific for strains within species, such that when a bacterial isolate is exposed to a panel of bacteriophages, a profile is generated—a listing of which bacteriophages are capable of infecting and lysing the bacteria (Pitt and Gaston, 1995; Bannerman *et al.,* 1995). The more closely related the bacterial strains, the greater the similarity of the bacteriophage profiles. Bacteriophage profiles have been used successfully to type various organisms associated with epidemic outbreaks (Hickman-Brenner *et al.,* 1991).

The traditional diagnostic techniques have varied sensitivities and specificities which influence their choice and applicability in a particular setting for the diagnosis of infectious diseases. Sensitivity refers to the ability of a diagnostic procedure to give a positive test when the pathogen is present, while specificity refers to the ability of a procedure to correctly identify non-infected persons.

Limitations of Traditional Diagnostic Techniques for Infectious Diseases

The limitations of many traditional techniques particularly low specificity and long turnaround time often necessitate initiating treatment of infectious diseases before results of investigations are made available. Direct examination is limited by the number of organisms present and by the ability of the laboratorian to successfully recognize the pathogen. For instance, it requires approximately 3,500 acid fast bacilli per ml (3.3×10^3 cfu/ml) of a sputum specimen to detect the bacteria in a Zhiel-Neelsen stained smear. Similarly, isolation of a pathogen from a clinical specimen depends on the ability of the pathogen to grow on artificial culture media and the laboratory personnel's choice of appropriate media for the culture.

Limited volume of the sample submitted for analysis may often hinder the possibility to culture all the targeted pathogens. Some microorganisms are unculturable, extremely fastidious, or hazardous to laboratory personnel. In these instances, an expensive bio-safety level II-IV facility is needed for culturing or resorting to the serologic detection of a humoral response. In a resource constraint health care setting, bio-safety facilities may not be readily available, or it may not be economically feasible to maintain the special media required for culture of all of the rarely encountered pathogens (Tang *et al.*, 1997). 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.

Immunodiagnostic procedures are often hindered by relatively low specificity due to false positive results such that most immunodiagnostic tests are used only as complementary to other more specific techniques (Sarkinfada *et al.*, 2003). Phage 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 (Bannerman *et al.*, 1995).

The Need for the Molecular Diagnostic Techniques for Infectious Diseases:

Applications of molecular detection methods for infectious disease diagnosis have resolved many problems associated with the traditional techniques. 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 by molecular technique clearly illustrates this point (Whelen *et al.*, 1995; Jackson *et al.*, 1996). Molecular techniques allow for the rapid and accurate identification of the causative agent of infectious disease 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.

Molecular Diagnostic Techniques

The total complement of genes in a given cell is referred to as genome, comprising two type of nucleic acids, Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA).

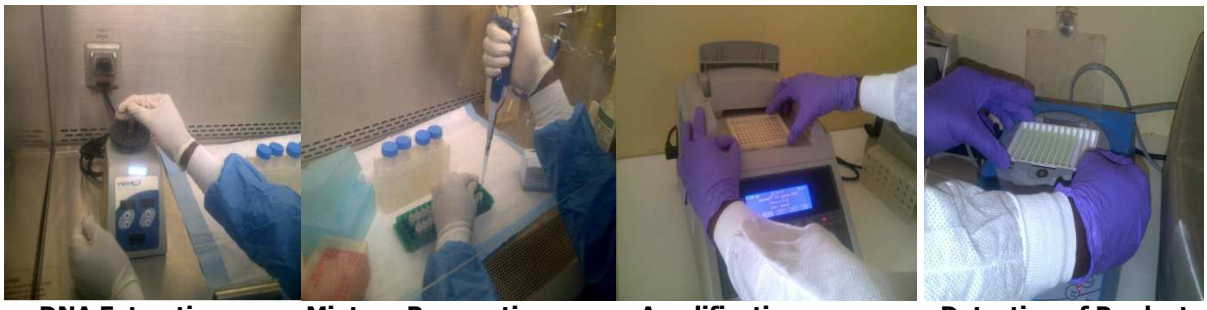
Genome carries all the genetic information that determines the characteristics of a particular organism that are in turn useful in identifying the organism or its genotype (genotype). The genetic information contained in the DNA is expressed through transcription and translation into protein, called its gene product (phenotype). The sequence of amino acids in polypeptide chain in turn determines the specific configuration into which the chain folds itself in forming the complete molecule of protein (primary, secondary and tertiary protein structure). Specific characteristics of a particular organism are determined by the nature and types of globular proteins that make up the enzymes system that mediate metabolic activities or the structural proteins that make up phenotypic identity.

Molecular diagnostic techniques involve a variety of techniques that explore the use of nucleic acid molecules for the identification of a particular pathogenic organism. These techniques include Nucleic acid-based typing system, Nucleic Acid analysis without amplification, Polymerase Chain Reaction (PCR) and other Nucleic Acid Amplification techniques.

PCR is a laboratory technique to obtain multiple copies of specific DNA fragments even from samples containing only minute quantities of DNA or RNA (WHO, 2011). The name, polymerase chain reaction, is derived from the deoxyribonucleic acid (DNA) polymerase enzyme used to amplify a piece of DNA by *in vitro* enzymatic replication. This process is known as a "chain reaction" due to the fact that the original DNA template is exponentially amplified in every cycle of replication. PCR has been extensively modified and is widely used in molecular biology, microbiology, genetics, diagnostics, and, clinical, forensic and environmental laboratories, besides several other applications.

General Principles of PCR

PCR is an enzyme-driven, primer-mediated, temperature-dependent process for replicating a specific DNA sequence *in vitro*. PCR technique involves extraction of target DNA from the clinical sample, amplification of the target DNA and detection systems (Figure 1). The principle of PCR is based on the repetitive cycling of three simple reactions, the conditions of which vary only in the temperature of incubation (Miller *et al.*, 2007). A design of oligonucleotide primers complementary to the ends of the sequences to be amplified is necessary. The primers are added to the reaction together with DNA polymerase and all is maintained in proper buffering conditions. Following heating to denature double stranded DNA and primer-dimers, cooling proceed to promote primer annealing and elongation. With the cycles the primers repeatedly bind to both the original templates of DNA and to complementary sites of newly synthesized DNA molecules. The final outcome of PCR reaction is the exponential increase in the total number of DNA molecules which is described by the mathematic formula of 2^n where n corresponds to the number of cycles (Zawacka-Pankau, 2011).



DNA Extraction **Mixture Preparation** **Amplification** **Detection of Products**

Figure 1: Some Important Steps in PCR Technique

The key enzyme of PCR is DNA polymerase. The golden and the most common standard enzyme is the thermostable polymerase isolated from bacterium *Thermus aquaticus* that occurs in geysers of over 110°C. The enzyme is heat-resistant and stable at temperatures up to 100°C. It binds to single stranded DNA and catalyzes the addition of free deoxyribonucleotides to 3′ end which promotes elongation of a new strand in 5′ to 3′ direction (Zawacka-Pankau, 2011). The PCR is commonly performed in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermocycler that heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction.

The Steps of PCR Technique

The PCR typically consists of three basic steps:

(1) Denaturation: The first step of a PCR where the sample is heated to separate or denature the two strands of the DNA (>90^0C).

(2) **Annealing:** Following the denaturation step, the reaction temperature is lowered (usually 3-5^0C below the melting temperature of primer) to allow the oligonucleotide primers to bind to the single strands of the template DNA.

(3) Extension / Elongation: The final step of the PCR where the temperature is raised, typically to 72°C, allowing specific enzymes to synthesize a new DNA strand complementary to the DNA template.

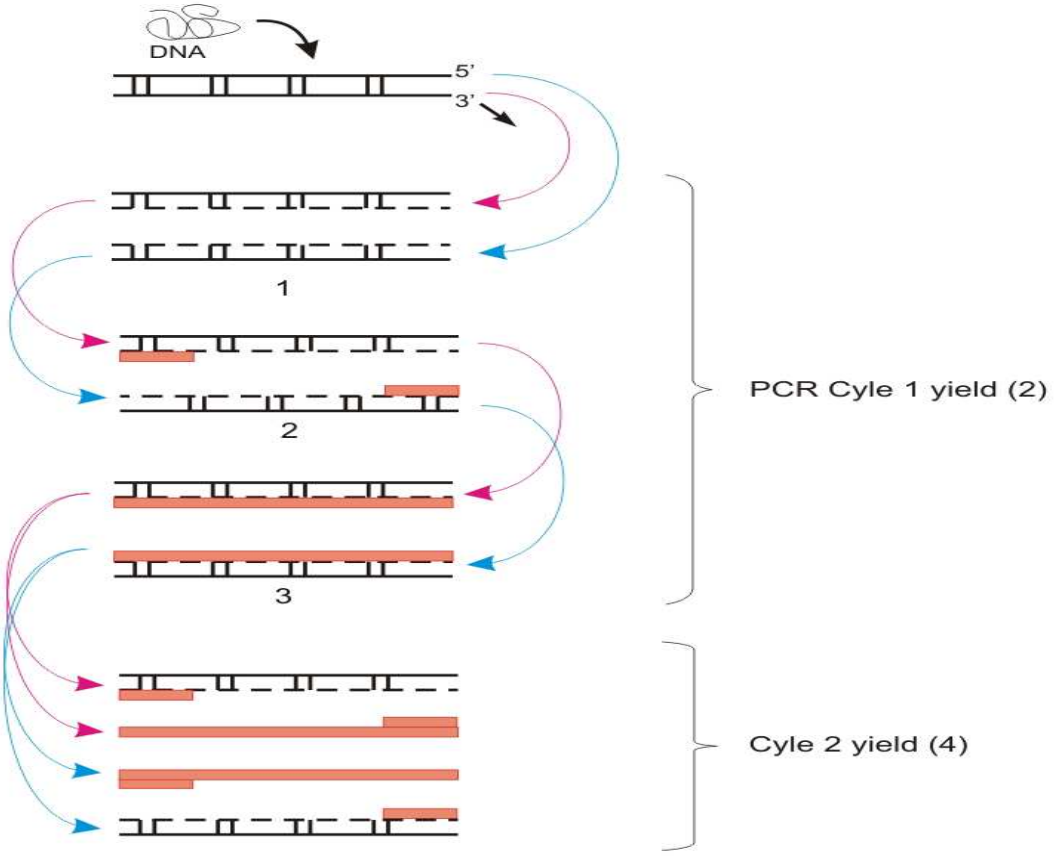


Figure 2: Schematic Diagram of Steps in PCR

One thermal cycle of these three steps theoretically doubles the amount of DNA present in the reaction. Typically about 25 to 45 cycles of PCR are performed depending upon the type of PCR used, the amount of initial template DNA and the number of amplicon copies desired for post-PCR processing (WHO, 2011).

Post-PCR Analysis

Post PCR detection system must accurately and reproducibly reflect the nature and quantity of the starting DNA template. Specialized methods used in post PCR analysis are usually tailored depending on specific applications. The simplest method uses agarose gel electrophoresis. After the electrophoresis, PCR products can be visualized by staining the gel with fluorescent dye such as ethidium bromide which binds to DNA and intercalates between the stacked bases (Figure 3). Confirmation of size of the DNA product is done by comparing the size with DNA ladder. The appearance of discrete band of the correct size may be indicative of a successful PCR amplification.

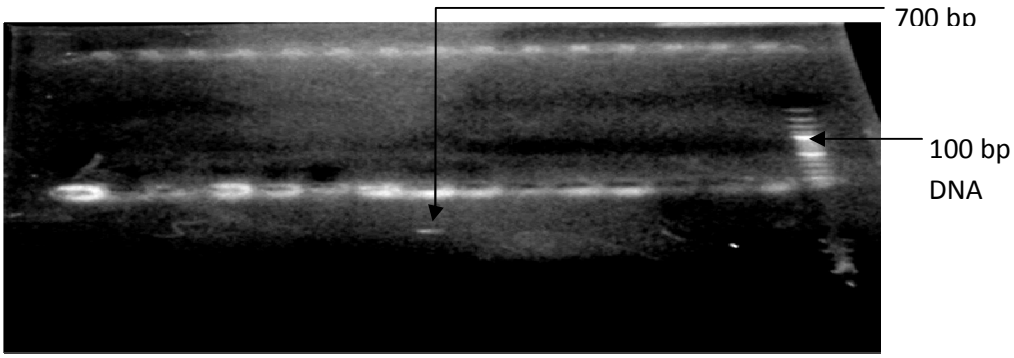


Figure 3: An agarose gel showing 700 bp of HPV type 16 (Source: Auwal *et al.,* 2012)

Other methods used for post PCR analysis include (1) Sequencing of the PCR product which is the gold standard but expensive and not widely available. (2) Restriction Fragment Length Polymorphism (RFLP). (3) Hybridization with a specific oligonucleotide probe - A wide variety of formats are available e.g. dot-blot, Southern blot, reverse hybridization, DNA enzyme immunoassay (WHO, 2011)

TYPES OF PCR

Specific PCR

Specific PCR is the simplest PCR approach which is designed for detecting specific target microbes. In specific PCR, primers are designed complimentary to a known DNA target and specific for the microbe being assayed. The primers should be so-designed so that they are strictly specific for the targeted microorganisms. As the result is specific for the detection of target microbes, this method can be used as a direct detection and identification method. This is the most widely used method in the diagnosis of infectious diseases. Many organisms, such as *Mycobacterium tuberculosis*, pneumococci, meningococci and *Burkholderia cenocepacia*, can be identified by specific PCR directly.

Reverse Transcriptase PCR (RT-PCR)

In Reverse Transcriptase or RT-PCR, a strand of RNA is initially reverse transcribed into its complementary DNA or cDNA using the reverse transcriptase enzyme. The resulting cDNA is further amplified by PCR. The reverse transcription step can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step PCR) depending on the properties of the reverse transcriptase enzyme used. The RT-PCR is used for detection of RNA viruses in clinical samples and in gene expression studies.

Multiplex PCR

Multiplex PCR refers to the simultaneous amplification of multiple selected target regions in a sample using different pairs of primers. In this version, multiple primer pairs are employed in the amplification mix so as to facilitate detection of multiple targets. Amplification products are finally differentiated by gel electrophoresis, sequence specific oligoprobes or in a real-time format, by melting curve analysis. Since multiplex PCR can be used to detect multiple genes of interest in one specimen, it can minimize the number of separate reactions and help conservation of time, reagents and samples that are of limited volume (Hayden *et al.*, 2008)

Nested PCR

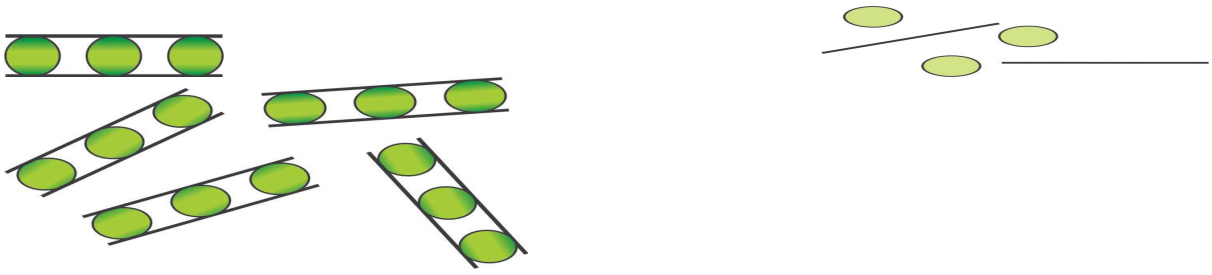
Nested PCR involves two successive PCRs, where the amplification product from the first PCR reaction is used as the template for the second PCR. Either one of the primers (semi-nested PCR) or both the primers (nested PCR) used in the second PCR may be different from the primers used in the first PCR. It has been employed to detect organisms present in low copy numbers in specimens, and has the benefits of enhanced sensitivity and specificity, the latter resulting also from a cleaner template provided by the first amplification (Terrango-Asensio and Avellon-Calvo, 2005).

***In-situ* PCR**

The PCR amplification reaction takes place within the cell which is often fixed on a slide. It can be employed for the detection of nucleic acid in small tissue samples. The PCR master mix is directly applied onto the sample on a slide, and then both are covered using a cover slip, and the latter is subjected to amplification in a thermocycler with a slide adaptor or *in-situ* adaptor (Pestaner *et al.*, 1994).

Real Time PCR

The Real Time PCR method is used for the detection and quantitation of an amplified PCR product as the reaction progresses in 'real time.' This new approach of PCR is based on the incorporation of a fluorescent dye where the increase in fluorescence signal, generated during the PCR, is in direct proportion to the amount of the PCR product. This modification avoids the requirement of a separate amplicon detection step, by employing fluorescent amplicon detection technology (using DNA-intercalating dyes such as SYBR Green or sequence-specific oligonucleotide chemistry such as TaqMan probes). Here, the fluorescent molecules added to the PCR mixture produce fluorescent signals which are detected simultaneously with the progress in amplification. The SYBR green fluoresces when it binds to double-stranded DNA (Figure 4A). When DNA is denatured SYBR Green is released causing a decrease in fluorescence (Figure 4B). As more PCR products are generated SYBR Green binds to more double-stranded DNA causing a net increase in fluorescence detected by the machine (Figure 4C).



A B C

Figure 4: SYBR Chemistry (Source: WHO, 2011)

Since SYBR green binds to any double-stranded DNA, after real time PCR amplification, the machine is programmed to perform a melting profile of the products to ascertain the specificity.

TaqMan reagent-based chemistry uses a fluorogenic probe for detection of specific PCR product as it accumulates during PCR cycles. When the probe is intact, reporter dye (R) emission is quenched due to its proximity to the Quencher (Q) (Figure 5).

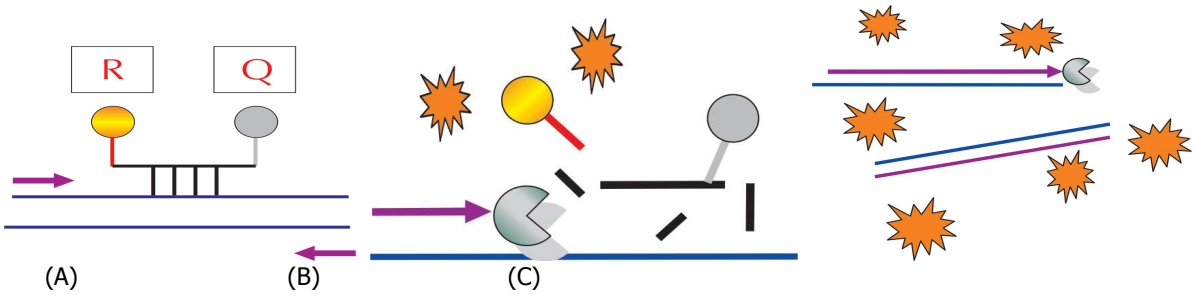


Figure 5: The Taqman Probe **(Source: WHO, 2011)**

All PCR products for a particular primer pair should have the same melting temperature (Tm) - unless there is contamination, mis-priming or primer-dimer artifact. In this melting curve (Figure 6), all samples are run with the same primer pair, but the sample that contained no DNA (the red line) shows a melting curve with a lower Tm compared to other samples; this is probably due to a primer-dimer artifact (WHO, 2011).

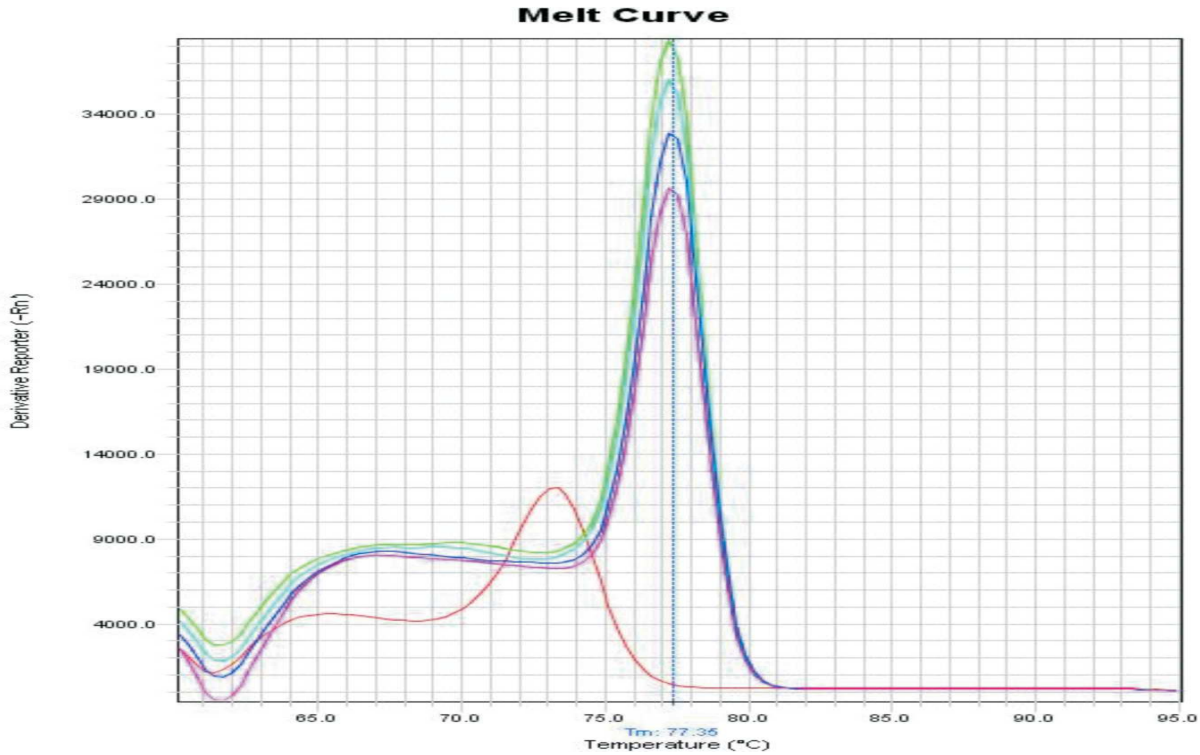


Figure 6: Melt Curve (Source: WHO, 2011)

Use of a closed system, reduced turnaround time, dynamic range of target detection, and feasibility for quantitation are a few of the advantages of this method. However, the need for coupling with melt curve analysis to increase its specificity is part of its disadvantages.

Applications of Molecular Techniques for Specific Infectious Diseases

With the increasing number of genomes of infectious pathogens being sequenced, catalogues of genes can be exploited to serve as amplification targets fundamental to the design of clinically useful diagnostic tests. As a result, over the past 2 decades the number of PCR assays developed commercially and in hospital-based laboratories ("inhouse") has continued to expand (Yang and Rothman, 2004). Molecular identification should be considered in four scenarios, namely (a) for the identification of an organism already isolated in pure culture, (b) for the rapid identification of an organism in a diagnostic setting from clinical specimens (c) for the identification of an organism from non-culturable specimens, e.g. culture negative endocarditis or (d) in clinical epidemiology and infection control (Tang *et al.*, 1997; Millar *et al.*, 2007). A summary of the applications of molecular techniques and the specific organisms targeted are presented in Table 1.

Table 1: Applications of Molecular Techniques for Specific Infectious Diseases

Organism	Sample	Techniques	Infections
CMV	CSF, Blood	Qualitative PCR Real-time PCR	CNs Infection Congenital infection
Influenza and *parainfluenza viruses*	Respiratory samples	RT- PCR Real-time PCR Multiplex PCR	Flu Bronchiolitis Croup
HIV	Plasma	Real-time quantitative PCR (Viral load Detection	HIV/AIDS
Hepatitis	Serum or plasma Liver tissue	Real-time PCR Hybridization	Hepatitis (chronic)
Middle-East Respiratory Syndrome *Coronavirus (MERS-CoV)*	Respiratory samples	RT-PCR	MERS-CoV pneumonia
Group-B Streptococcus (GBS)	Vaginal Swab	Real-time PCR	Vaginitis
Neisseria Meningitidis	CSF	16s rDNA PCR	Meningitis
Helicobacter species	Biopsy of bone lesion	16s rDNA PCR	Osteomyelitis
Plasmodium falciparum	Blood	Nested PCR	Malaria
Methicillin-resistant *Staphylococcus aureus (MRSA)*	Blood	Multiplex PCR	Health Care Associated Infections
Multi-Drug Resistant *M. tuberculosis*	Sputum	Real-Time PCR(Gene Xpert)	Tuberculosis MDR-TB

Applications in Clinical Epidemiology and Infection Control

Study on the investigation and control of Health Care Associated Infections (HAIs) 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 HAIs pathogens. Some previous studies on the application of molecular techniques in clinical epidemiology are presented in Table 4.

Table 2: Application of Molecular Techniques in Clinical Epidemiology

Molecular Epidemiological Investigations	Techniques	Reference
MDR M. tuberculosis nosocomial outbreak among HIV-positive groups (Miami & New York)	PCR-RFLP Southern transfer Hybridization	Beck-Sague *et al*, 1992; Dooley *et al.*, 1992; Pearson *et al*, 1992
Clustered S. pyogenes invasive disease in Air Force recruits	Real-time PCR	Musser *et al.*, 1994
Cluster of LGV caused by C. trachomatis serovar L1 in homosexual men	PCR	Bauwens *et al.*, 1995
Outbreak of *E. coli* O157:H7 infection from contaminated deer jerkey	PCR	Keene *et al.*, 1997
Hantavirus mediated outbreak of fatal infections in the US southwest	Nested RT-PCR	Tang *et al.*, 1997

Limitations of Molecular Techniques in Diagnostic Microbiology Laboratory

Despite the obvious advantages of molecular diagnostic techniques, there are some potential limitations. The accuracy of PCR assays depends on the technical expertise and experience of the operator. Specificity of the test may also 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 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 performing molecular techniques is generally more expensive than traditional diagnostic methods (Fredricks and Relman, 1999).

Key Issues on Implementation of Molecular Diagnostic Techniques

Introducing new techniques is appropriate only when appropriate traditional methods provide poor results or are not cost effective. New techniques often require specialized equipment usually with costly maintenance contracts, and may be associated with limited education and training of laboratory staff in modern technologies. Medical laboratory scientific officers, clinical scientists and medical microbiologists must understand the principles of molecular based technologies to ensure proper handling of the specimens and appropriate interpretation and significance of results, hence specific training must be given priority.

Implementation of molecular diagnostic techniques for infectious diseases requires policy formulation that would ensure:

- Provision of equipment, reliable supplies and appropriate training for laboratory staff suitable for the level of care.
- Ensure the rational use of the Molecular Diagnostic Tests (MDT) relevant to the level of facility and the use of standard testing methodologies
- Appropriate deployment of MDT for each health care level only based on the health care package, public health, clinical importance, cost, suitability to the work environment, level of expertise of the service provider and the satisfaction of the user.

CONCLUSION

Molecular diagnostics are of wide applications and rapid development in the diagnosis of infectious diseases. Applications and scaling up of the molecular diagnostic procedures for infectious diseases require situation analysis and proper planning within the context of the health care system in which it is to be implemented.

Acknowledgements:

Special gratitude to Charles RABY of the World Health Organization's Regional office for South-East Asia for conveying the WHO's approval for me to use some of the illustrations (Figures 4, 5 and 6) from the document SEA-HLM-419.

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