Overview of Molecular Diagnosis in Medical Microbiology

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Abstract

Clinical microbiology diagnosis became objectively possible at the end of 19th century with the seminar contributions of researchers such as Louis Pasteur, Robert Koch and other colleagues. The concept of diagnosis then was the ‘culture and isolate’ paradigm, which accelerated to a large extent the growth of bacteriology, as a number of bacteria were able to fulfill the Koch’s postulates, but delayed the growth of other fields of microbiology most especially virology because viruses were not culturable outside of host cells. This paradigm, together with the application of microscopy, serology and the use of animal models in research in the early 20th century, constituted the ‘first revolution’ in the field of microbiology that is referred to as the conventional microbiological diagnosis. Although this conventional diagnosis has remained valuable, assessments have shown that many of the conventional techniques are not demonstrably ‘fit for purpose’ in the 21st century. This has necessitated the consideration of complementary or alternative technologies, the molecular diagnosis, which has ushered in a ‘second revolution’ in microbiology that is as profound as the first in its impact on our understanding of the microbe-human interactions in health and disease. In this mini-review, an overview of the technologies underlying molecular diagnosis in microbiology is presented. The application of these molecular methods in clinical microbiology laboratory to ensure accurate, reliable and timely release of microbiological test results for better patients’ management and outcome is highlighted.

Keywords: clinical microbiology, conventional, molecular, diagnostics
INTRODUCTION
The pathogenic basis of infectious diseases can be historically linked to Robert Koch in the late 19th century who proved the germ theory of disease with his works and those of others on Bacillus anthracis, which gave birth to the popular Koch’s postulates of microbial pathogenicity [1]. The diagnostic concept of ‘culture and isolate’ paradigm in this era accelerated to a large extent the growth of bacteriology as a number of bacteria were able to fulfill the Koch’s postulates, but deter seriously the field of virology because viruses were not culturable outside of host cells [2, 3]. The introduction of serology and use of animal models in research in early 20th century however started the field of virology, and together with the ‘culture and isolate’ paradigm, constituted the ‘first revolution’ in the field of microbiology referred to as the ‘conventional microbiological diagnosis’ [4]. This revolution has impacted the field of microbiology up till the present time.

The conventional microbiology technology however has a lot of draw backs; (i) it requires significant laboratory investment in ‘big ticket’ items such as autoclaves, microscopes, incubators and associated equipment, which have become so much part of the furniture of hospital laboratories that the scale of these investments is often overlooked; (ii) the conventional techniques have to be performed by extensively trained, skilled operatives, not the least because the materials being handled are usually infectious; (iii) the techniques have proved difficult to automate microbiology methods, in contrast to other branches of clinical laboratory science; (iv) there are many variations in terms of media and growth conditions for different organisms; (v) many deficiencies of culture methods exist as not all organisms can be cultured, and those that can be cultured are often missed due to the lack of a suitable sample, which has led to a concentration of diagnostics on those organisms that can be cultured, with less emphasis on those that cannot, irrespective of the importance of the diseases that they cause; and (vi) characterization of bacterial isolates at the ‘subspecies’ or ‘strain’ level is frequently imprecise, or requires specialist reagents and/or techniques, or is not reproducible [4].

Reassessment of the conventional diagnosis showed that many of these techniques are not demonstrably ‘fit for purpose’ in the 21st century, necessitating the consideration of complementary or alternative diagnostic techniques, the molecular diagnosis [4]. The ‘second revolution’ in microbiology which is as profound as the first in its impact on our understanding of microbe-human interactions in health and disease is the “molecular technologies”. This revolution began in the last 20 years of the 20th century when Stanley Falkow applied the molecular Koch’s postulates to microbial pathogenicity [5]. The increasing application of DNA-based or ‘molecular biology’ approaches has gathered speed in the first two decades of the 21st century, as access to the data locked in microbial genomes provides a wealth of opportunities for the clinical microbiologists.

CATEGORIES OF MOLECULAR TECHNIQUES
Although, molecular diagnosis entails the analysis of biomolecules such as DNA, RNA, protein or lipid for organism identification, there are basically two categories of molecular techniques [6]; (i) protein-based techniques such as Western blot, multi-locus enzyme electrophoresis (MLEE), sodium dodecylsulphate-polyacrilamide gel electrophoresis (SDS-PAGE), matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and protein sequencing; and (ii) nucleic acid-based techniques such as plasmid analysis, hybridization, amplification and direct nucleotide sequencing techniques. However, the nucleic-acid techniques are nowadays usually the ones referred to as ‘molecular biology’ techniques [7]. A common step to almost all these ‘molecular biology’ techniques is nucleic acid extraction (NAE).

NUCLEIC ACID EXTRACTION
This is the first process in almost all molecular biology techniques [8]. There are different methods of nucleic acid extraction (NAE) but each method has four basic procedural steps; (i) cell disruption by physical and/or chemical agents; (ii) removal of membrane lipids, proteins, and other nucleic acids; (iii) nucleic acid purification/binding from bulk, and (iv) nucleic acid concentration [9]. NAE method can be categorized into two groups. The first group is the chemically-driven method which include Cesium Chloride (CsCl) gradient centrifugation with Ethidium Bromide (EtBr), Guanidinium-Thiocyanate-Phenol-Chloroform, Cetyltrimethylammonium Bromide (CTAB), Chelex (resin) extraction, Alkaline extraction by Bimboim and Doly (for plasmid DNA) which is now commercially available as Plasmid Maxi kit (by Qiagen), and Poly(A) + RNA oligo(dT)-cellulose chromatography. The second group is the solid-phase method which include the use of silica matrices now commercially available as QIAamp DNA mini kit (by Qiagen), Glass particles, Diatomaceous Earth, Magnetic beads, Anion exchange materials, and Cellulose Matrix [9].
PLASMID PROFILING

Plasmids are extrachromosomal circular double stranded DNA found in most bacteria. Each bacterium may contain one or several plasmids. Plasmid profile analysis involves study of size and number of plasmids. After the cells are lysed, the nucleic acids are subjected to electrophoresis (Fig 1). This gives the size and number of plasmids present in the cells [10].

Since some species may contain variable number of plasmids or even unrelated bacteria may harbour similar number of plasmids, plasmid profiling may not provide useful information. However, identification of plasmid DNA still remains the principal means of identifying some organism such as Chlamydia trachomatis from clinical infections by molecular methods [11].

HYBRIDIZATION TECHNIQUE

This is the earliest method of detection of DNA in samples or cultures by using labeled probes (oligonucleotide or long DNA probes) followed by detection with detector. The different formats of detection include; autoradiography with radioactively labeled probe, colorimetry with biotin or digoxygenin-labeled probe, fluorometry with fluorescen tagged avidin or antibody, and chemiluminescence with acridinium ester labeled probe [12].

The principle of hybridization is based on the ability of the double stranded DNA to separate to single strands at the melting temperature (usually 94°C) and for the single strand to anneal or hybridize at a cooling temperature (of about 55°C) with another strand that has sufficient DNA sequence homology (Fig 2). A known labeled DNA strand can therefore be used to identify an unknown DNA in a sample or culture.

![Fig 1: Plasmid analysis](image1)

![Fig 2: Principle of hybridization](image2)
There are three different formats of hybridization; (i) solid phase hybridization in which DNA is extracted, separated by electrophoresis and then transferred to a 'solid support' such as nitrocellulose membrane or bead followed by probing with a labeled probe and detection e.g. Southern blot technique for DNA and Northern blot technique for RNA; (ii) solution phase hybridization in which DNA hybridization reactions occur entirely in solution, and this ensures rapid detection and complete reaction than in solid phase reaction e.g. hybridization protection assay; and (iii) in situ hybridization in which labeled probe is used to detect nucleic acid directly in tissue e.g detection of virus RNA or DNA in a paraffin embedded tissue using florescence in situ hybridization (FISH).

AMPLIFICATION TECHNIQUES

In amplification technique, nucleic acid is first extracted from specimens or cultures and then amplified using DNA/RNA polymerase enzymes to million copies followed by detection using various detection techniques including the use of hybridization probes. Because high copies of DNA/RNA or signals are produced from the sample or culture, the sensitivity of amplification techniques is far higher than that of hybridization.

There are three main groups of amplification techniques but derivatives of any of these are widely applicable [6, 7, 12]. The three main groups are; target amplification, probe amplification and signal amplification.

(i) Target amplification

Target amplification involves amplification of extracted nucleic acid target from the sample or culture. This could be thermal amplification as seen in polymerase chain reaction (PCR) technique with use of thermocycler for DNA and RNA, or isothermal amplification which is especially suited for RNA without a need for thermocycler, and includes such methods as self-sustaining sequence replication (SSSR) or nucleic acid sequence-based amplification (NASBA), transcription mediated amplification (TMA), strand displacement amplification (SDA) and loop mediated isothermal amplification (LAMP).

(ii) Probe amplification

This involves amplification of the probe that hybridizes to a target nucleic acid, and includes such methods as Q β replicase and Ligase Chain Reaction (LCR).

(iii) Signal amplification

This involves amplification of signal generated upon hybridization of a labeled probe to a target DNA e.g. branched DNA probe technology

Polymerase Chain Reaction

The technique of polymerase chain reaction (PCR) was described by Karry B. Mullis [13] who won the 1993 Nobel Prize for Chemistry because of this discovery. PCR mimics in vivo DNA synthesis by microorganism using DNA polymerase to amplify sequence of target nucleic acid but this reaction takes place in vitro in a tube [14]. The amplified fragments (amplicons) can then be separated by electrophoresis for identification or used as templates for sequencing, cloning (recombinant DNA technology), probes, or in forensics. There are two basic types of PCR procedures; conventional and ‘real time’ PCR.

i. Conventional PCR

Conventional PCR could be performed as simplex PCR, multiplex PCR, nested PCR, reverse transcriptase PCR (RT-PCR), competitive quantitative PCR etc [7, 12]. The key materials (PCR reaction mix) required to be in the PCR tube includes deoxynucleotides (dATP, dTTP, dGTP, dCTP), target DNA (extracted), primers (short DNA oligonucleotides in large number), Taq DNA polymerase (thermostable) from *Thermus aquaticus*, buffer, PCR water and others such as dimethyl sulphoxide (DMSO), glycerol etc. The key instrument is thermocycler (Fig 3).

Fig 3: Thermocycler

The PCR procedure occurs in a cycle of three reaction steps; denaturation usually at 94°C, annealing or hybridization with primers at 55-60°C and extension of the annealed primers at 72°C (Fig 4). This is usually performed for 30 to 35 cycles. The detection of PCR amplicon may be done first by separation on ordinary agarose gel electrophoresis (AGE) with DNA staining by Ethidium bromide or SYBR Green or LC Green dye (Fig 5 and 6). The amplicon may also first be digested by restriction enzyme (endonuclease) followed by separation using pulse field gel electrophoresis (PFGE) and restriction fragment...
length polymorphism (RFLP) analysis. Alternatively, the amplicon may be detected by a labeled DNA probes.

**ii. Real Time PCR**

In ‘real time’ PCR, amplification and detection of amplicons occur simultaneously in the same reaction vessel/tube and result obtained in ‘real time’ [12]. The PCR reaction incorporates fluorophores in the form of dyes or probes and uses a fluorescent detection system coupled to a thermal cycler (Fig 7), to allow each step of a PCR reaction to be monitored [15]. Real time PCR is extremely sensitive and allows qualitative detection as well as quantification of the amount of DNA templates in the starting reaction. The detection by fluorescent labeled probes can be by non-sequence specific dyes such as SYBR Green, Eva Green and LC Green, or by sequence-specific probes such as hydrolysis probes (TaqMan probes), hybridization probes, molecular beacons, or by scorpions [12, 15].
The three phases in ‘real time’ amplification are lag phase when amplification is yet to be detected (background noise), exponential phase when there is logarithmic increase in PCR products following a number of cycles (cycle threshold, CT) and plateau phase when exhaustion of reagents and inhibition occurs (Fig 8).

Absolute or relative quantifications of DNA in the samples can obtained by normalizing the CT relative to a reference (Standard Curve method) (Fig 9) or to another CT which is a reference target in the same experiment (Comparative CT method). Some of the benefits of ‘real time’ PCR over conventional PCR are rapid analysis (result in approximately 90 minutes or less), no need for post-amplification processing (no gels, auto-radiographs, etc), closed-tube helps control contamination, automated data collection and analysis, objective analysis as a result of controls and standards that can be built-in, high precision, sensitivity and reproducibility.

**DIRECT NUCLEOTIDE SEQUENCING**

The direct nucleotide sequencing is the most sequence specific molecular method that became available in the early 1970s by Maxam and Gilbert. Nucleotide sequencing can be for single nucleotide (gene), which may be single locus e.g. Staphylococcus protein A (Spa) or multiple loci e.g. multi-locus sequence typing [16, 17]. It could be large scale sequencing involving a whole libraries of DNA or cDNA e.g. a whole genome or a whole transcriptome of microorganism or human cell. The available sequencing technologies are Maxam Gilbert method, dideoxy nucleotide sequencing (Sanger sequencing) and the Next Generation sequencing.

**i. Maxam Gilbert sequencing technique**

The “DNA sequencing by chemical degradation” and “wondering-spot analysis” methods described by Walter Gilbert and Allan Maxam at Harvard in 1973 based on DNA degradation and fragmentation is about the first sequencing technique described [18]. This method is now obsolete.

**ii. Sanger sequencing**

The Sanger sequencing was described by Frederick Sanger in 1977 at the Medical Research Council, Cambridge, United Kingdom [19], building on his earlier accomplishment of completing the entire amino acid sequence of insulin hormone, and the works of Padmanabhan and Wu on DNA sequence using synthetic location-specific primers [20]. Sanger’s “DNA sequencing by chain terminating inhibitors” is based on DNA amplification/synthesis using a mixture of deoxynucleotides and labeled dideoxynucleotides (chain termination nucleotides) at a ratio of usually 100 to 1, which allows separation of sequences by
electrophoresis (Fig 10). The Sanger method has been modified for throughput analysis (Fig 11).

**iii. Next generation sequencing**

The next generation sequencing (NGS) technologies use different approaches such as pyrosequencing, reversible terminator sequencing, sequencing by ligation, support oligonucleotide ligation detection (SOLiD), and real time sequencing, to achieve sequencing of large genome size of up to 50Gb per run, in a relatively short time (in days). The NGS technology was first made available in 2004 with 454 FLX (Roche), Illumina/Solexa in 2006, Applied Biosystems SOLiD in 2007, Helicos Heliscope and Pacific Biosciences SMRT in 2010, and Life Technologies Ion Torrent in 2011 [21, 22].

**PRACTICAL APPLICATIONS OF MOLECULAR TECHNOLOGIES IN CLINICAL MICROBIOLOGY**

There are several practical applications of these technologies in microbiology laboratory for accurate, reliable and timely diagnosis of clinical infections, as well as in research in bacteriology, virology and parasitology. Some of the applications are enumerated below.

**i. Diagnostic detection of microorganisms**

Application of molecular techniques in clinical microbiology laboratory enables; (i) detection of organisms that cannot be cultured or difficult to grow such as *Mycobacterium leprae*, *Treponema pallidum*, *Chlamydia*, *Rickettsia*, *Mycoplasma* and viruses; (ii) rapid detection of organisms that grow slowly e.g. *Mycobacterium tuberculosis*; (iii) detection of organisms that have become non-viable especially in partially treated infections; (iv) detection of pathogenic microorganisms in mix infections and differentiating pathogenic from non-pathogenic, toxigenic from non-toxigenic, and wild-type from vaccine-derived strains; (v) detection of previously unknown (novel) organisms e.g. Whipple Associated Bacillus (*Tropheryma whippelii*); (vi) detection of antimicrobial resistance in microorganisms; (vii) detection of organisms for which reliable diagnostic methods are not available; (viii). diagnosis of congenital infections, (ix) detection of microbial virulence factors, (x) detection of contaminating viruses in tissue culture, and (xi) detection of exact localization of virus infection or tumors in tissue by in-situ hybridization.

**ii. Monitoring disease progression**

Molecular technique give quantitative estimate of infectious agent burden that is important in monitoring disease progression e.g. viral load in HIV monitoring [23].

**iii. Epidemiological typing in outbreak investigation**

Molecular techniques are very useful in characterization of microorganisms beyond identification and therefore highly suitable for investigation of strain relatedness for epidemiological typing during epidemics or pandemics [24].

**iv. Microbiology research**

Molecular technologies provide microbiologists’ ample opportunities for research with access to data locked in microbial genomes that can lead to technology ‘leapfrogging’ in understanding previously refractory microorganisms and their roles in disease causation [25].

**v. Other applications**

Molecular techniques have useful applications in cases such as HLA typing, cancer diagnosis, anthropology and disputed paternity. Molecular techniques have useful applications in cases such as HLA typing [26], cancer diagnosis and monitoring [27], anthropology [28] and disputed paternity [29].

**CONCLUSION**

The arrays of molecular techniques available for diagnosis in clinical microbiology laboratory and for research in the 21st century are enormous. The optimization of these techniques, including making them available at the point-of-care, has the potential to revolutionize clinical microbiology practice with generation of accurate and reliable laboratory test results in a timely manner leading to re-
dution in turn-around-time, thereby contributing to favorable outcome of patients with infectious diseases.

**Declaration of Conflict of Interest**
The Authors declare that there is no conflict of interest.

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