

Clinical relevance of molecular microbiology

Abstract

Molecular methods for the detection and characterization of microorganisms have transformed diagnostic microbiology. Detection of microorganisms was previously laborious and time taking by conventional microbiological methods but now more rapid detection by molecular methods is possible for pathogens of public health importance. Detection of antimicrobial resistance genes and their characterization by genotyping is also feasible. Detection of viral resistance gene and testing of viral load for the monitoring of antiviral therapies are possible because of molecular technique and automation of molecular microbiology. This review will focus on basic molecular techniques and the clinical utility of these molecular methods in the management of infectious diseases.

Keywords: Amplification techniques, Automation, Infectious diseases, Molecular methods

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Abbreviations: TMA, Transcription Mediated Amplification; NASBA, Nucleic Acid Sequence Based Amplification; SDA, Strand Displacement Amplification; LAMP, Loop Mediated Isothermal Amplification; PCR, Polymerase Chain Reaction; LCR, Ligase Chain Reaction; CPT, Cycling Probe Technology; RT-PCR, reverse transcriptase polymerase chain reaction

Introduction

Molecular microbiology is the fastest growing discipline which plays significant role for the detection and characterization of microorganism. Rapid detection of microorganism by using these molecular methods has revolutionized routine diagnostic microbiology. Microorganisms which are fastidious, slow growing, non-viable, and non-cultivable may not be detected by conventional culture technique but can be identified by using molecular technique. The introduction of these molecular techniques and their automation was introduced in the form of PCR technology. And these automation when applied to various stages of DNA or RNA extraction, amplification and product detection together with real-time PCR further increase the utility of molecular detection in the clinical microbiology laboratory by making it more efficient and cost-effective.¹ This paper will provide an overview of the some basic molecular technique and clinical applications of molecular methods for infectious diseases.

Categorizing molecular techniques

Hybridization methods

Used for identification of nucleic acid strand, less sensitive than amplification methods. Hybridization methods are based on the ability of two nucleic acid strand each derived from different source that have complementary base sequence to specifically bond with each other and form a double-stranded molecule (hybrid). So, one nucleic acid strand is from the known organism while the other is derived from the organism to be detected and percent similarity.

Steps involved in hybridization reactions are - Production and labeling of single stranded probes - Probes are labeled short nucleic acids with known nucleotide sequences designed to hybridize with the target nucleic acid. Labeling can be done using radioactive or non-radioactive labels. The common radioactive isotopes used for labeling include ³²P, ³⁵S, and ¹²⁵I. The non-radioactive labels include biotin, digoxigenin and acridinium ester and addition of substrate results in production of coloured product, signaling the

positive hybridization reaction.² Preparation of single stranded target nucleic acid, the source of target nucleic acid can be microorganism from the clinical specimen or from the culture. The nucleic acid is extracted chemically or enzymatically. The nucleic acid is treated to stabilize as well as preserve structural integrity and then denatured to derive single strands. Mixture and hybridization of target and probe nucleic acid - The annealing of the target nucleic acid with the corresponding specific nucleotide probe under optimum conditions of temperature and salt concentration is called hybridization. Detection of hybridization reaction - The various methods of detection of hybridization reaction are radiometric i.e. by autoradiography (radioactive isotope labeled probes), colorimetric (biotin or digoxigenin labeled probes), fluorometric (fluorescein tagged avidin or antibody) or chemiluminescence (acridinium ester labeled probe).³ Hybridization is classified on the basis of its application into two types;

Solution phase hybridization: The aqueous environment speeds up the rate of hybridization. The target DNA is denatured and the single stranded target nucleic acid is mixed with single stranded probes is added, unhybridized single stranded nucleic acids are removed using S1 nuclease digestion and the hybridized dsDNA is recovered using trichloroacetic acid precipitation. Another common method called hybridization protection assay uses acridinium ester labeled probe to hybridize with the target. Upon addition of H₂O₂ hydroxide, the duplex emits light. This assay can be performed in few hours, because it does not require removal of excess of unbound single stranded DNA.⁴

Solid phase hybridization: The hybridization reaction occurs on a solid support such as nitrocellulose or nylon membrane.

a. Dot hybridization: The target (nucleic acid) from sample is affixed to a membrane in the form of dot, then processed to release target DNA and denature it to a single strand. The membrane is immersed into a solution containing labeled probes and allowed to hybridize. Unbound probes are washed away and the hybridized duplexes are detected according to the nature of the reporter molecules.

b. Sandwich hybridization: It utilizes two probes; an unlabeled probe that is attached to a solid support such as microtitre plate and serves to capture the target from the sample to be tested. The presence of this duplex is then detected using a labeled second probe that is specific for another portion of the target sequence.

c. Southern blot hybridization: DNA is extracted from the organisms and purified; it is cut into several fragments of various sizes using restriction enzymes. The resultant fragments are separated on gel by electrophoresis. The fragments move along the gel according to their molecular weight, where the smaller fragments migrate faster and farthest through the gel than the larger fragments. The target DNA is transferred on to nylon or nitrocellulose membrane. The membrane is dipped in a hybridization fluid containing labeled probe. Deionized formamide and detergents such as SDS are used to reduce non-specific binding of the probe. The hybridized duplexes are detected by radiometric, enzymatic or fluorometric methods depending on the nature of reporter molecule used.³

d. Northern blot hybridization: the target nucleic acid is single stranded RNA. In in-situ hybridization the microscopy glass slide with tissue specimen acts as the solid phase. From tissue sample, nucleic acid of the pathogen to be released and denatured to a single strand with the base sequence intact. Radio-labeled probes and fluorescent probes are commonly used.⁴ One can perform molecular hybridization while simultaneously viewing the histopathological structure of the specimen.

Clinical applications of hybridization techniques: Direct detection of pathogens e.g. in Pharyngitis (gp-A streptococci), genital tract infections (N. gonorrhoeae, C. Trachomatis) by Gen Probe Speciation of microorganisms Identification of culture isolates e.g. Identification of dimorphic fungi, mycobacteria etc by AccuProbe.⁵

Amplification Techniques

Target amplification: These systems amplify the target to large numbers. Some of these systems are:

- PCR-thermocycler is required. Isothermal amplification methods: there is no need for thermocycler - Commonly used methods are-
- NASBA (Nucleic acid sequence based amplification) / TMA (Transcription mediated amplification)
- SDA (Strand displacement amplification),
- LAMP (Loop mediated isothermal amplification).

Polymerase Chain Reaction: PCR can enzymatically amplify minute quantities of DNA or RNA to large number of copies in a short period hence provides ample target that can be readily detected. The basic steps in PCR are: Denaturation of DNA to single strands (94° C or higher for 15 seconds to 2 minutes) Annealing of primers to DNA (55° C, 1-2 mins) Extension by thermos table DNA polymerase (72° C typically 10-15 minutes). The same cycle is repeated for 30-35 times to produce approx. 109 copies / 2-3 hrs. All the steps of PCR are performed on the reaction mixture consisting of target DNA, primer pairs, thermostable DNA polymerase, deoxynucleotides (dATP, dTTP, dGTP and dCTP), buffer and Mg salt in the same test tube. Thermocyclers are used for the process. There are various methods to validate the PCR generated amplicons, as they serve to differentiate the target amplification from non-specific amplification.⁴ These methods include:

- Electrophoresis of the amplicons should be sufficient in most cases to demonstrate similarity between the expected and observed size of amplicons.
- Hybridization with probes for the known sequence within the amplified target sequence
- Nucleotide sequencing of amplicons.

Types of PCR

I. RT-PCR (Reverse transcriptase- PCR): Used to detect viral nucleic acid (of RNA viruses) in clinical specimen as well as to prepare cDNA library of mRNA. Using the enzyme reverse transcriptase, a complimentary copy of the RNA is made. A primer is annealed to the template RNA and with the help of reverse transcriptase, cDNA strand is synthesised by adding complementary base pairs. The template RNA is removed using the enzyme RNAse H leaving behind the newly generated single stranded cDNA, which can be amplified in PCR.

II. Nested PCR: Used for sensitivity and specificity.⁶ Nested PCR uses two sets of amplification primers. The first set of primers is used to amplify a target sequence and the second set of primers is used to amplify a region within the first target sequence.

III. Multiplex PCR: In multiplex PCR, two or more unique target sequences can be amplified simultaneously. Multiplex PCR can also be used to test for different organism on a single specimen. The primers are so designed that each amplification product is a unique size allowing detection and identification of specific organisms.

IV. Competitive quantitative PCR (QPCR): Competitive PCR is a quantitative PCR, which is used to quantify the amount of nucleic acid in the sample. It involves a competition between the target nucleic acid and the competitive DNA for amplification process. The competitive DNA requires the same primer pair and is added in known concentration. It differs from the target DNA in size or presence of unique restriction site which is required to accurately differentiate target DNA from competitor. Competitive PCR occurs due to the competition for the use of the primers. So, the amount of the target DNA can be estimated by comparing with the concentration of DNA competitor.

V. Real time PCR: They have the ability to detect amplified target DNA by fluorescently labeled probes as the hybrids are formed (i.e. detection of amplicons in real time). By plotting the increase in fluorescence versus cycle number, the system produces amplification plots that provide quantitative picture of the PCR process. -Both amplification and product detection can be accomplished in one reaction vessel without ever opening hence cross contamination is lessened. -Accumulation of amplicons is monitored as it is generated, hence able to quantitative the amount of product and also determine the number of copies of target in the original specimen. Currently fluorescent probes are used for amplicons detection. SYBR Green I is non-specific and binds to dsDNA (both specific and non- specific). Fluorescent probes used are Taqman probes, Molecular beacons and FRET (Fluorescent resonant energy transfer) hybridization probes.⁷

VI. Helicase-dependent amplification: This technique is similar to traditional PCR, but uses a constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA Helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.

1. Transcription mediated amplification (TMA)/ Nucleic Acid Sequence Based amplification (NASBA): These are isothermal RNA amplification methods rather than DNA. The technique uses three enzymes in the reaction mixture; reverse transcriptase, RNAse H and DNA dependent RNA polymerase. In TMA the enzyme used for reverse transcription also has intrinsic RNAse H activity. So, in TMA only 2 enzymes used

while in NASBA, 3 enzymes used. In this technique, the cDNA is created using reverse transcriptase and this is used as a template for transcription. A cDNA copy of the target RNA is made using a specific primer that binds to the target RNA. Reverse transcriptase builds a complementary DNA, resulting in the formation of RNA-cDNA hybrid. RNase enzyme digests and removes the RNA strand from the hybrid leaving behind single strand of cDNA. The second primer binds to the cDNA strand and the reverse transcriptase enzyme extends it using cDNA as template, resulting in generation of dsDNA copy of the target RNA. Using RNA polymerase, several copies of antisense RNA are produced from this dsDNA molecule. These RNA molecules can act as template for the reverse transcriptase resulting in the production of dsDNA molecules and the same cycle is repeated again.⁷

2. **Strand displacement amplification (SDA):** Strand displacement amplification is an isothermal DNA amplification reaction based on a restriction endonuclease nicking its recognition site and a polymerase extending the nick at its 3' end, displacing the downstream strand. It requires restriction enzyme cleavage of the DNA sample prior to amplification in order to generate an amplifiable target fragment with defined 5'- and 3'-ends. Sample DNA is denatured at 95°C in the presence of excess of four specific primers that define target sequence.
3. **LAMP (Loop Mediated Isothermal Amplification):** This method relies on auto-cycling strand displacement DNA synthesis that is performed by a DNA polymerase and a set of two inner and two outer primers. In the initial steps, all four primers are used, but later during the cycling reaction only the inner primers are used for strand displacement DNA synthesis.⁴

Signal Amplification: They amplify the signal generated by the labeled probes.

- a. **Branched DNA (bDNA) probe system:** The key to assay is amplifier molecule with identical branches each of which can bind to three labeled probe i.e. capture probe, capture extendor (unlabelled) probe and labeled probe. The required nucleic acid target sequence is captured and hybridized with an unlabeled probe that has two hybridization sequences, one directed against the target sequence and the other capable of hybridizing with bDNA amplification multimer. This system is highly sensitive because the target nucleic acid has to bind both to the capture as well as target probes before the signals are amplified. Amplified signal is directly related to the number of targets present in the original sample.
- b. **Nucleic Acid Capture Assay (NACA):** Here the target molecule is directly captured onto the solid support and no branched oligodeoxyribonucleotides are used for detection and hence found more sensitive than bDNA assays.
- c. **Probe amplification technique:** Probe amplification methods amplify products containing only the probe sequence. It includes Ligase Chain Reaction, Cycling Probe technology etc.

Ligase Chain Reaction (LCR): LCR amplification is based on sequential rounds of template dependent ligation of two juxtaposed oligonucleotide probes. LCR allows the discrimination of DNA sequences differing in only a single base pair. Single stranded target DNA is incubated with oligonucleotide probes that bind to the target in an end-to-end fashion. A thermostable DNA Ligase then ligates (or joins) the two probes together. The resulting duplex is heated to separate the target DNA and the ligated probes. Both the separated

target sequence and the ligated probes now act as targets for the probes, which bind in an end-to-end fashion. These steps are repeated several times resulting in geometric probe amplification.⁸

Cycling Probe Technology (CPT): It employs a DNA-RNA-DNA fluorescent labelled probe at a constant temperature. The probe labeled with fluorescence one end and a Quencher the other, anneals to target DNA. An enzyme cuts the RNA region of the probe; the probe is no longer intact unquenching the signal, resulting in emission of fluorescence. Probe amplification is linear and not exponential. This application has been used to detect the *mecA* gene of MRSA.

Sequencing and Enzymatic Digestion of Nucleic Acids

DNA sequencing means determining the order of nucleotides in a DNA molecule for which extraction of plasmid or chromosomal DNA is done. This technique can be used to study the structure of gene, detect mutations, and compare genetic relatedness and to design oligonucleotide primers. Different techniques are given below:

Restriction fragment length polymorphism (or variability): In nucleotide sequence is present in all organism including microbes. RFLP technique relies on the base pair changes in restriction sites, which arise due to mutations. Restriction enzymes (RE) cut DNA at specific 4-6 BP recognition sites and fragments separated according to molecular size by gel electrophoresis. Ethidium bromide staining is used to reveal the fragments under UV (260 nm) light. Differences resulting from base substitutions, additions, deletions or sequence rearrangements within RE recognition sequences can be detected with this technique e.g. it can be used for determination of strain variation in *M. tuberculosis*.

Pulsed Field Gel Electrophoresis (PFGE): In PFGE, electrical fields are applied alternatively from different angles which lead to separation of large DNA fragments; hence there is enhanced resolution of fragments that differ by few bases.

Other molecular methods

Line Probe Assay (LIPA): In LIPA, series of probes of interest are bound on nitrocellulose membrane/strip and amplicons is then applied to the strip which is just the reverse of southern blotting. Biotin labeled PCR product is then hybridized to the immobilized probes. After hybridization streptavidin labeled with alkaline phosphates is added and binds to the biotinylated hybrids. Clinical applications of LIPA HCV and HBV genotyping Mutation associated with HIV Mutation associated with mycobacteria Detect HPV subtypes medically important fungi.

Ribotyping: In ribotyping, restriction enzymes are used to cut the genes coding for rRNA into pieces, and then separated by gel electrophoresis. Universal probes that target specific conserved domains of ribosomal RNA coding sequences are used to detect the band patterns. Clinical application: Allow subtype differentiation of bacterial isolates beyond the species and subspecies levels.⁹

Spoligotyping: (spacer oligonucleotide typing) this technique is used as typing method for *M. tuberculosis*. Direct Repeats i.e. repeated sequences of base pairs are interspersed by non- repetitive DNA spacers and these spacers are usually unique in a genome. Hence spacers are amplified and detected. It can be done on nonviable strains, stored samples or directly on samples.⁶

DNA Microarray: It is a tool for analyzing gene expression. A DNA microarray (gene chip, DNA chip, or gene array) is a collection of microscopic DNA spots attached to a solid surface, such as glass, plastic or silicon chip (Probes). Each spot is designed to represent

one gene and these are hybridized with cDNA or cRNA by forming hydrogen bonds between complementary nucleotide base pairs under high stringency conditions. A high number of complementary base pairs in a nucleotide sequence mean tighter non-covalent bonding between the two strands. Later it captures a snapshot of which genes are expressed in a given cell at a given time and monitors the whole genome on a single chip. Hence microarrays use relative quantitation in which the intensity of a feature is compared to the intensity of the same feature under a different condition.⁷

Applications of molecular techniques in microbiology

1. Molecular Assays For The Detection Of *C. Trachomatis* And *N. Gonorrhoeae*: Two assay formats, nucleic acid hybridization and nucleic acid amplification, have been developed for the detection of these organisms. Compared to culture, the first-generation hybridization assay (Pace-2, Gen-Probe) demonstrated improved sensitivity for detection of *C. trachomatis* and *N. gonorrhoeae*. Second-generation assays with even greater sensitivity have been developed. These tests include one hybridization signal amplification assay (Digene Hybrid Capture II CT/GC) and a number of NAATs.
2. Detection of Human Papillomavirus (Diagnosis of non-cultural agents: e.g. Human papilloma virus, Hepatitis B virus etc.)
3. Direct probe hybridization initial testing for HPV was performed using the Southern blot transfer technology and radio isotopic labeled probe. Another approach to using direct probes was in combination with a second blotting technique known as a dot or slot blot. This technique does not require enzyme digestion or electrophoresis and can evaluate numerous specimens by applying the nucleic acids directly to a membrane with subsequent hybridization with a probe of interest. Finally, probes have also been applied directly to tissue sections or cells on a slide as an *in situ* hybridization (ISH) assay.⁷ The Ventana Inform HPV assay is a chromomeric ISH test that can differentiate between episomal or cytoplasm viral replication and viral integration. Polymerase chain reaction Assays utilizing target amplification, on the other hand, are based on the *in vitro* amplification of a target nucleic acid sequence. Many PCR assays for HPV detection have used type-specific or consensus primers. The type-specific assays typically identify sequence variations in the E6 or E7 genes, whereas the consensus primers target conserved regions in the L1 capsid gene. There are many advantages to the PCR technology for such screening applications, including automation capabilities, turnaround time, multiplexing, sensitivity/specificity, multiple specimen types, and small-specimen volume.
4. Molecular Diagnostics For HIV
5. The Amplicor HIV Monitor™ Assay (Roche Diagnostics, Indianapolis, IN) is a RT-PCR-based viral-load assay. The plasma RNA is extracted with a lyses reagent containing guanidine thiocyanate and quantitation standard (QS) RNA. The QS that is added into each sample permits quantification of HIV-1 RNA by comparison of resulting optical densities following amplification and detection. A 142-bp sequence in the gag gene of HIV and the QS (primers SK145 and SKCC1B) are amplified by RT-PCR in a single reaction. The primers are biotinylated at the 5' ends and the HIV-1 and QS amplicons are detected in separate wells of a microwell plate (MWP) coated with HIV-specific and QS-specific oligonucleotide capture probes, respectively. The bound, biotinylated amplicons are quantified with an avidin-horseradish peroxidase (HRP) conjugate and a colorimetric reaction for HRP. The HIV-1 RNA copy number is then calculated from the known input copy number of the QS RNA. TAQMAN The next generation of PCR-based techniques for monitoring HIV-1 will utilize real-time RT- PCR (Taqman) technologies. Similar to the molecular beacons, TaqMan assays allow for amplification and detection to be performed in a single closed tube, virtually eliminating amplification product carryover contamination. The principle of TaqMan real-time PCR is based on the cleavage of an internal probe by the 5'-3' exonuclease activity of Taq polymerase during amplification.
6. Detection of HCV
7. HCV RNA qualitative assays determine only the presence or absence of HCV RNA. Two primary methods are used in such assays: reverse transcriptase polymerase chain reaction (RT-PCR) and transcription- mediated amplification (TMA). Quantitative HCV RNA the methods used for qualitative HCV RNA determination can be adapted to quantitative measurement by using a known amount of a synthetic standard and comparing the amounts of HCV amplified to the amount of standard amplified using a calibration curve. For hepatitis C genotype several techniques have been developed to determine the particular genotype and subtype of HCV causing infection in an individual. As discussed earlier, such techniques typically target the 5'-untranslated and/or core regions of the HCV genome, the most highly conserved regions. Because most amplification methods for HCV RNA also target the 5'-untranslated region, qualitative PCR methods can be used to provide amplified RNA for use in determining the genotype.
8. Detection of Cytomegalovirus
9. Polymerase chain reaction assays and the hybrid capture assay are designed to detect CMV DNA; they can be either qualitative or quantitative assays. On the other hand, the NASBA assay detects pp67 mRNA, which is transcribed late in the viral replicative cycle. Because the NASBA assay is isothermal, it can detect pp67 RNA without interference of CMV DNA. The NASBA assay is qualitative. The in-laboratory-developed PCR assays utilize both standard and real-time PCR methods and their performance varies as a result of differences in specimen type, nucleic acid extraction methods, target gene, primer sequence, quantitation standard, and detection method.
10. Fastidious, slow-growing agents: e.g. *Mycobacterium tuberculosis*, *Legionella* etc.
11. Highly infectious agents that are dangerous to culture: e.g. *Francisella*, *Brucella*, *Coccidioides immitis* etc. *In situ* detection of infectious agents: e.g. *H. pylori*, *Toxoplasma gondii* etc.
12. Antiviral/ antibacterial drug susceptibility testing: e.g. HIV to assess drug resistance, Determination of resistant genes like Mec A gene, Van genes For the purpose of molecular epidemiology: e.g. identify point sources for hospital and community-based outbreaks and to predict virulence for confirmation of culture.

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None.

Conflicts of interest

None.

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