

# Molecular Techniques For Diagnosis Of Tuberculosis - A Review

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**Abstract:** Despite discovery of tubercle bacillus more than a hundred years ago and advances, tuberculosis still remain one of the major health problems facing mankind, particularly in developing countries. Traditional diagnostic methods are time consuming. Rapid, sensitive and accurate diagnostic techniques are needed for detection of *M. tuberculosis* (MTB) in specimens for successful diagnosis of tuberculosis. Accurate and rapid diagnosis of tuberculosis and initiating optimal treatment would not only enable a cure patient but also will curb the transmission of infection and disease to others in the community. In order to avoid delays in therapy and prevention of MultiDrug Resistant) Tuberculosis (MDR TB transmission, new genotypic methods based on hybridization technique and PCR have been proposed. Recently there is marked increase in development of molecular assays designed to detect MTB complex and resistance to rifampicin ( RMP) and ( isoniazid ) INH. These are based on Nucleic Acid Amplification Technology (NAAT), hybridization assays, microarrays, real-time PCR and sequencing based assays. Drug resistance detection methods detect most common resistance mutation genes associated with resistance. They are line probe assays, Expert RIF, microarrays and sequencing, line probe assay are approved by WHO for first line DST (drug susceptibility test). Line probe assay can be used for DST without need of culture.

**Key word:** Molecular Techniques, Diagnosis, Tuberculosis

## Introduction:

Tuberculosis caused by MTB remains global emergency and it continues to kill 1.7 million people globally each year.<sup>(1)</sup> In India 1.8 million tuberculosis cases occur annually ,they account for 1/5th world tuberculosis cases and 2/3rd of South East Asia region ,this makes India the highest burden country in world.<sup>(2)</sup>

Microscopy using Z-N stain and culture are traditional methods and are unable to diagnose tuberculosis in all suspected cases, due to the fact that they are time consuming, labor intensive and high concentration of bacteria must be present in samples to be detected.

Accurate, rapid diagnosis of tuberculosis and initiating optimal treatment would not only enable and cure these patients but also will prevent transmission of infection and disease in the community. Prevalence of tuberculosis is further complicated by MDR strains. Globally estimated 3.6% of new cases and 20.2% of previously treated cases have MDR-TB. Extensively drug resistant tuberculosis (XDR- TB) has been reported by 92 countries. On an average an estimated 9.6% of MDR cases have XDR TB.<sup>(2)</sup>

Most patients of tuberculosis are not being screened for drug resistance due to lack of laboratory resources and rapid tests .Over last few years recent advances in

molecular techniques have revolutionized the diagnostic microbiology. This article will review molecular methods used in modern clinical microbiology laboratory for diagnosis of tuberculosis.

## Molecular Methods:

Molecular methods used for diagnosis of tuberculosis can be divided into 3categories.

Detection of mycobacteria from clinical specimens - NAAT such as the target amplification techniques- Polymerase Chain reaction (PCR) , Transcription Mediated Amplification (TMA) , Strand Displacement Amplification (SDA ) , Nucleic Acid Sequence Based Amplification (NASBA) , Probe amplification technique - Ligase chain reaction (LCR) ,Signal amplification technique - branched DNA ( b DNA) are used for detecting MTB complex in clinical specimens.

Advantages of NAAT are, their sensitivity to detect 10 bacilli which is higher than microscopy (but slight lower than culture) , specificity 98-100% , rapid results in hours , Less training and infrastructure than that for conventional cultures and antibiotic susceptibility test (AST) and diagnosis of extra-pulmonary tuberculosis. Disadvantages are advanced laboratory infrastructure, high cost, complexity and most of them work better with

smear positives than smear negatives. Culture is still needed for species identification, confirmation and AST.<sup>(3)</sup>

PCR - PCR technique is used for detection of MTB complex from clinical specimens. It allows detection and amplification of sequences of DNA; in such a way that amount amplified can be visualized and identified. If appropriate sequence is selected 10-1000 organisms can be identified. Targets commonly used in PCR is IS 6110. This is specific for MTB complex and is present up to 20 times in genome thus offers multiple targets for amplification.<sup>(4)</sup>

#### A) In house PCR assay -

These PCR protocols amplify genus specific and MTB complex specific DNA regions. Insertion element 6110 and 16S r DNA are most common targets. Other regions used for amplification include the rpo B gene coding for  $\beta$  subunit of RNA polymerase gene coding for 32 kd protein, 38k D, the hsp 65 gene, the dna J gene, MPB 64 gene and 16s-23S r RNA internal transcriber spacer.

There is heterogeneity in results in these PCR due to sample volume, DNA extraction protocols, presence of inhibitors, smear positivity - negativity and type of PCR as uniplex and multiplex PCR. Uniplex PCR targets single whereas multiplex targets more than one target in single reaction. Usual target in PCR is IS6110 but is absent in 10-40 % of Indian isolates so multiplex PCR are more sensitive and specific.<sup>(5)</sup>

Real time PCR has been used increasingly and they focus on detection of MTB complex. Risk of cross contamination is less with real time PCR.<sup>(6)</sup>

#### B) Commercial available assay –

AMPLICOR MTB - Roche AMPLICOR MTB PCR test rely on standard PCR technique. It is PCR that amplify 16 S r RNA gene. Amplification and detection steps are carried out automatically by the Cobas Amplicor instrument. Once sample extraction is done by heating, the tube is placed in thermal cycler integrated in Cobas instrument, without further handling the amplified product will be transferred to detection station where the chromogenic reaction is developed and read<sup>(7)</sup> This technique is approved for use in smear positive respiratory specimen in suspected patient by FDA. It displays sensitivity of 97% , 40-70 % and 27-90% in smear positive pulmonary, smear negative pulmonary and extra-pulmonary specimens respectively with specificity >95% in all specimens. Results were comparable to culture and are obtained in 6-7 hrs.<sup>(8,9)</sup>

Real time PCR - E.g COBAS TaqMan MTB PCR test (Roche Diag) Artus M.Tuberculosis PCR kits (Qiagen) - Hybridization of amplified nucleic acid with fluorescent labeled probes spanning DNA regions of interest and monitored inside thermal cycler. Fluorescent signal increases in direct proportion to amplified product in reaction tube. MTB light cycler (Roche applied Science, Pennsburg, Germany) can be used for test. With high volume of template DNA sensitivity is close to 78% and specificity 98-100 % in smear positive, smear negative pulmonary and even in extra-pulmonary cases. Advantages of this technique are that it is also rapid technique results available within 2 hrs after DNA extraction and low risk of contamination since both detection and reaction in single tube.<sup>(8)</sup>

TMA - The amplified MTB direct test AMTD test (gen probe Inch) (San Diego, California) AND E-MTD. It is isothermal Transcription Mediated Amplification of portion of 16S r RNA with detection method that utilizes DNA probe specific for MTB complex. A MTB complex specific region of 16S r RNA gene produces DS r DNA due to combined action of reverse transcriptase and ribonuclease. In turn RNA polymerase catalyses the synthesis of multiple stretches of ribosomal RNA from the ribosomal DNA synthesized before. A new cycle starts when new ribosomal RNA undergoes further transcription by reverse transcriptase. The detection of amplified product relies on hybridization with specific SS DNA labeled with chemiluminescent molecule.

Sensitivity of method is increased by the presence, in each bacterium of high number of 16S r RNA target molecules about 2000 compared to only one copy of 16S r DNA. Process is performed manually until final reading in illuminometer. Thermal cyclers not needed. Turnaround time is 2.5 hrs.<sup>(10)</sup>

Technique is approved for smear positive and for smear negative respiratory specimens of clinical suspect<sup>(11)</sup>

Displays sensitivity of 92-100% in smear positives and 40- 93% in smear negative, 93% in extra -pulmonary tuberculosis with specificity >95 % in TB suspects.<sup>(8)</sup>

NASBA - Genotype Mycobacteria Direct assay. It is based on Nucleic Acid Sequence Based Amplification (NSABA), an isothermal amplification technique applied to DNA strip technology. First step is Isolation of 23 S r RNA, second step is Amplification of RNA by NASBA and third step is reverse hybridization of amplified product on membrane strips using automated system. Assay is reliable, rapid with sensitivity 92% and specificity 100%, assay has ability for simultaneous detection of MTB

complex and 4 atypical mycobacteria - *M. kansasii*, *M. avium*, *M. intracellulare*, *M. malmoense* <sup>(12)</sup>

**Strand Displacement Amplification** - B D Probe Tec ET Direct TB system (B.D. Oxford) It uses DNA polymerase and isothermal Strand Displacement Amplification to produce multiple copies of IS 6110, an insertion sequence unique to MTB complex. It is a semi automated system. Some manipulation is required before introduction of sample in automatic instrument, each sample is first inactivated at 105 °C then sonicated to extract DNA, then transferred to a priming well at 72 °C and subsequently in amplification well at 54 °C in BD probe Tec instrument, the microplate containing sample and amplification reagents is incubated at 52.5 °C and fluorescence emitted is monitored. The method does not require thermal cycler, turnaround time is 3.5 to 4 hrs but is not approved by FDA. <sup>(13)</sup> Sensitivity 90-100% in smear positive, 33-100% in smear negative, 76% in extra-pulmonary specimens with specificity of >90% in all cases <sup>(8)</sup>

**LCR**-Abbott LCx *M. tuberculosis* assay (Abbott laboratories, Abbott Park, III)

It involves denaturation of DNA followed by annealing of 2 sets of adjacent primers to each strand of DNA. The primers themselves then ligate and the new ligation product created serves as template for next cycles ligation reaction. It is a commercial probe amplification system for detection of MTB complex. Sensitivity and specificity similar to both Gen Probe MTD and Roche Amplicor *M. tuberculosis* test. In contrast to these above two techniques steps of LCR are automated in LCx analyzer saving time. Assay is mainly used for respiratory specimens and gives results within 8hrs. <sup>(14)</sup>

**LAMP**- Loop-mediated isothermal amplification. It is a novel nucleic acid amplification method in which reagents react under isothermal conditions with high specificity, efficiency, and rapidity. LAMP is used for detection of MTB complex, *M. avium*, and *M. intracellulare* directly from sputum specimens as well as for detection of culture isolates grown in a liquid medium Mycobacterium growth indicator tube (MGIT) or on a solid medium. It uses the repetitive insertion sequence IS6110 as a target gene. Identical sensitivities were obtained for LAMP and nested PCR, but the LAMP assay was more rapid and cost-effective than the latter. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. Species-specific primers were designed. Simple procedure, starting with the mixing of all reagents in a single tube, followed by an isothermal reaction during which the reaction mixture is held at 63 °C for 60min

incubation time. Due to its easy operation without sophisticated equipment, it will be simple enough to use in small-scale hospitals, clinical laboratories in developing countries. <sup>(15)</sup>

**b DNA** - It is a signal amplification system where target NA sequence is not replicated through enzymatic amplification detection sensitivity is provided by amplification of the signal. Target used are IS 6110, 65kDa <sup>(16)</sup>

## 2) For Identification of species

### DNA probe

Probes are one of the most successful technologies for identification of mycobacteria. A Gen-probes-DNA probes are SS DNA oligonucleotides labeled with acridium ester that are complementary to target which is RNA, after sonication of sample probes are added to broken mycobacterial cells to form stable DNA:RNA complex. Following separation of labeled complex from unhybridized DNA, hybridization is detected by light emission in luminometer.

**Line probes** - This technique includes PCR reverse hybridization with different specific DNA probes immobilized on a strip and colorimetric detection in automated instrument. Banding pattern is indicative of species. Two commercial line probes are The innoLipa Mycobacteria V2 and Genotype mycobacterium

**The innoLipa Mycobacteria V2** - It is based on amplification of mycobacterial spacer region 16S-23S rRNA for simultaneous identification. One strip identifies 17 most frequently isolated species including MTB complex *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. kansasii*, *M. xenopi*, *M. chelonae*, *M. gordonae*, *M. fortuitum* complex, *M. simiae*, *M. marinum*, *M. ulcerans*, *M. szulgai*. Sensitivity is 100% and specificity is 94% respectively <sup>(17)</sup>

**Genotype mycobacterium** - Procedure is multiplex PCR followed by reverse hybridization and line probe technology. Three types of kit are, the Genotype MTBC for distinguishing members of MTB complex, genotype mycobacterium common mycobacteria (CM) and Genotype Mycobacteria AS (Additional species) for nontuberculous mycobacteria (NTM). Genotype MTBC based on gyr B gene polymorphism, AS and CM use 23S rDNA as target. Amplicons generated in CM assay can be used for AS assay without doing 2nd PCR thus combined AS and CM can distinguish 30 species of NTM

Assays are rapid, easy to perform and easy to interpret and can be carried out without sophisticated instrument. Sensitivity and specificity compared to 16S rRNA gene

sequencing is 97.9 % and 92.4 % for CM and 99.3% and 99.4% for AS respectively.<sup>(18,19)</sup>

**PCR based sequencing** - PCR and Restriction fragment length polymorphism (RFLP) - Technique is used for identification of mycobacteria from solid as well as liquid culture. It amplifies gene coding for 65 -kDa heat shock protein and 16 S r RNA, followed by RFLP using restriction enzymes, fragments are then subjected to agarose gel electrophoresis and compared. Test requires a day and is cost effective and reliable.<sup>(20)</sup>

**Pyrosequencing** - It is a novel method of NA sequencing - by - synthesis that is based on detection of released pyrophosphate during DNA synthesis. Assay targets a portion of the 16S r RNA gene that contains a hypervariable region. Incorporation of the complementary nucleotides resulted in light generation peaks, forming a pyrogram, which was evaluated by the instrument software.<sup>(21)</sup>

**DNA Chips (Microarray)** - Chip is based on hybridization of fluorescently labeled PCR amplicons of unknown strain to DNA array containing nucleotide probe for 16S ribosomal RNA gene and rpo B gene. Pattern of hybridization and intensity is determined by scanning the chip using laser confocal fluorescence microscopy. It requires 2 hours for identification of mycobacterial species and RMP resistant allele.<sup>(22)</sup>

**3) Detection of drug resistance** - MDR TB are generally defined as resistant to at least isoniazid (INH) and rifampicin (RIF). The knowledge of susceptibility pattern of isolate is crucial for successful therapy.<sup>(23)</sup>

As the molecular basis of anti-tuberculous drug resistance is becoming clear, several molecular methods to detect mutation in MTB have been developed. Resistance to anti-tuberculous drug is primarily due to mutation in a series of genes. Most frequently found mutation for RIF resistant isolates are mutation in 81bp segment of rpo B gene that encode for  $\beta$  subunit of RNA polymerase. In 85% INH resistant strain mutation is in 2 genes kat G coding for catalase-peroxidase and inh A that takes part in fatty acid elongation. Mutation in emb B gene which plays role in synthesis of lipoarabinomannans and arabinogalactans are connected to ethambutol resistance. Pyrazinamide resistance in more than 70% is due to pnc A gene which code for pyrazinamidase that convert pyrazinamide to active form. Mutation in 16 S rRNA gene or the rps L gene that encodes for ribosomal protein 12S cause approximately 65 to 75 % resistance to streptomycin. Kanamycin resistance is due to mutation in rrs gene

encoding 16 S RNA. MDR strains arise by sequential accumulation of resistant mutation for individual drug. Molecular assays have ability to detect these mutations within few hrs<sup>(24,25)</sup>

**Line probe assay (LPA)** - These assays were endorsed by WHO in 2008 for molecular detection of drug resistance from smear positive patients at risk of MDR TB.<sup>(26)</sup> This method can be used for identification from culture or positive specimens of members of MTB complex as well as detects most common SNPs associated with resistance. Assays use probes specific only to MTB complex and additionally for detection of mutation responsible for drug resistance.

This is PCR hybridization technique. Steps include extraction of DNA from mycobacterial isolates or from culture specimen, PCR of NA sequence, and hybridization of labeled PCR products with oligonucleotide probes immobilized on strip, colorimetric development that allow line to be seen.<sup>(2)</sup>

**First line probe assay** - INNO - LiPA Rif TB (innogenetics, Ghent, Belgium)

The kit has 10 oligonucleotide probes 1 specific for MTB complex, five wild type probes S1 to S5 and four probes for detection of most frequent mutations that cause resistance to RIF. More than 95 % strains have mutation within 81-bp hot spot region of rpo B gene. All probes are immobilized on nitrocellulose strip. A MTB isolate is considered susceptible to RIF if all wild type probe give positive signal and all probe for resistance are negative. Absence of 1 or more S probe is indicative of mutation that may be identified by one of the R probes assay show 100% correlation with results of conventional AST and specific of detection of MTB complex. Assay is recommended for use only on isolates where amount of DNA is large.<sup>(27)</sup>

**Second line probe assay** Genotype MTB DR (hain Lifesciences, Germany)

Originally developed as Genotype MTB DR assay, did not showed resistance detection to satisfactory degree, showed 90-95 % sensitivity in isolates with RIF resistance or low level INH resistance. They are then modified to Genotype MTB DR plus by including detection of more resistance mutations in rpo B for RIF resistance, inhA and kat G for INH resistance. This assay showed high sensitivity and specificity for RIF (more than 97% & and 99 % respectively) and 92 % for INH when compared to conventional AST. They are used on smear positive specimens or on isolates of tuberculosis.<sup>(28)</sup>

Third version GenoType MTB DR sl is designed to detect resistance to second line drugs fluoroquinolones , ethambutol, kanamycin, amikacin, capreomycin and can be used in combination with MTBDR plus test to detect XDR-TB.<sup>(29)</sup>

Major advantages of LPA is it can be performed on smear positive specimens. It gives susceptibility in approximately 5 hrs, without need of culture. Disadvantage of LPA are that they are labor intensive and require trained persons, well equipped laboratory<sup>(1,30)</sup>

**Xpert MTB / RIF-** One of the most promising new diagnostic technologies developed in recent years is the Gene Xpert ( Cepheid , Sunnyvale ,California USA ).It is designed for detecting MTB complex and direct genotypic DST from from unprocessed sputum or from sediment from concentrated specimen. It detects RIF resistance targeting RIF resistance determining region(RRDR) of rpo B gene .GeneXpert machine is used. It is fully automated, closed system that performs sample preparation and real-time PCR producing results in less than 2hrs .Sample reagent poured into sample tube,incubated for 15 min ,pipetted in Xpert cartridge and inserted in into geneXpert machine for processing .Sensitivity of test is 98.2 % and 72.5 % on smear positive and smear negative tuberculosis patients respectively and specificity is 99.2% .<sup>(31)</sup>

**Micro Array -** DNA microarray can be used to detect mutations associated with drug resistance in mycobacteria .It can simultaneously detect different drug resistant mutations of MTB.

DNA chips also have been designed to determine specific mutations associated with resistance to INH and RIF ( rpo B and Kat G And inh A ) It is oligonucleotide microchip coupled to PCR for detection of resistant mutations. Results are comparable to DNA sequencing and conventional AST .<sup>(3,32)</sup>

The TB-Biochip oligonucleotide microarray system is designed to detect and identify thirty mutations within the RRDR. These mutations are found in >90% of RIF resistant strains .Each element (an acrylamide gel pad) of the microarray contains an immobilized oligonucleotide whose sequence matches that of either a wild-type or mutated segment of the RRDR. Hybridization of the microarray with fluorescently labeled target DNA produces a spatial pattern of fluorescence intensities corresponding to the efficiencies of hybridization of the labeled target DNA to the various oligonucleotide probes. In the TB-Biochip system, the fluorescence intensities are recorded using a charge-coupled device camera, and the

relative intensities of fluorescence for the elements representing wild-type sequences and mutant sequences for each codon are compared using imaging software and automated computer-assisted interpretation of hybridization results. The isolate is designated RIF susceptible if the fluorescence of each of the wild-type elements is greater than the fluorescence of any of the corresponding mutant elements The isolate is designated as RIF resistant if the fluorescence of any one of the mutant elements is greater than the fluorescence of its corresponding wild-type element. TB biochip system displayed sensitivity of 80% and specificity of 100% relative to conventional DST results for RIF resistance.<sup>(33)</sup> Microarrays can be used to detect second line drug resistance in MTB.<sup>(34)</sup>

**PCR DNA sequencing -** This is a automated DNA sequencing of PCR product to detect drug resistance mutations. The main advantage of sequence based approach is that the data is virtually unambiguous because resistance mutation is either present or absent .Initially the region frequently associated with resistance mutation is amplified and then amplicons are sequenced in order to determine presence or absence of specific mutations .This method needs expensive equipment and expertise.<sup>(6)</sup>

**PCR - Single strand conformation polymorphism (SSCP)-** It is based on conformational distortion that a nucleotide substitution can cause in single strand DNA fragment ,this change leads to an electrophoretic mobility different to that of the wild type single strand fragment, the procedure involves amplification of DNA fragment including the region of interest by PCR, denaturation and running this fragment in polyacrylamide gel together with denatured wild type reference sample. Mobility shifts in clinical sample indicate mutation. It has 100% specificity for RIF and INH resistance and sensitivity 96% and 87% for RIF and INH respectively using 4 genetic regions rpo B, kat G, inhA and ahpC.

Nested PCR linked SSCP is also used on sputum sample to detect M. tuberculosis and to determine RIF susceptibility. Results are concordant with conventional AST and DNA sequencing of culture isolates .Assay does not identify precise mutation so is less precise than DNA sequencing .Use is limited because of its labor extensiveness and high technical skill .<sup>(35)</sup>

**Pyrosequencing -** RIF resistance can be detected rapidly by pyrosequencing. Target was an 180 bp region of rpo B gene amplified by PCR and subjected to pyrosequencing analysis using 4 different sequencing primers in 4 overlapping reactions. It offers high accuracy and results

are comparable with LPA and phenotypic BACTEC 460 method.<sup>(36)</sup>

**Realtime PCR** - It is used for detection of mutations responsible for INH and RIF resistance. Method exhibit 85% and 98 % sensitivity and 100% specificity for detection of mutation responsible for INH and RIF resistance respectively.<sup>(37)</sup>

**Conclusion** - Molecular techniques have good sensitivity and specificity; give rapid results in hours, so they help in rapid confirmation in smear positive samples. They require less training and infrastructure than for conventional cultures and AST. Their use is limited because of advanced laboratory infrastructure, high cost, complexity and most of them work better with smear positives than smear negatives. Culture is still needed for species identification, confirmation and AST. Several molecular assays are commercially available, such as Amplicor TB assay, COBAS taqman MTB PCR test, Artus M. tuberculosis PCR kits, amplified MTB direct test. Current technology for drug resistance detection varies greatly in terms of time, cost and complexity. Line probe assay are recommend by WHO as the current gold standard for first line DST but not for second line DST. Common mutation associated with drug resistance have been described and molecular techniques targets these resistance associated mutations to identify drug resistant tuberculosis. Most important drug in treatment is rifampicin. RMP resistance is particularly suitable for molecular DST because 95 % RIF resistance is due to mutations present in 81 bp region of the rpo B gene known as RRDR.

Resistance to INH and second line drug is more complex requires detection of mutation in multiple genes for good correlation to phenotypic tests. In future improved multiplex PCR can make molecular DST methods more powerful.

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