MOLECULAR DIAGNOSIS OF MULTI DRUG RESISTANT TUBERCULOSIS
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Abstract
Drug resistant tuberculosis has been reported since the early days of introduction of anti-tuberculosis therapy but recently multi drug resistant tuberculosis (MDR-TB) and extensively drug resistant-tuberculosis (XDR-TB) has been an area of growing concern and is posing threat to the global efforts of tuberculosis control. Current WHO report states that the prevalence of primary and acquired rifampicin resistance in India is 2.8 % and 17.2 % respectively. Rifampicin resistance is of special concern because it is the most effective bactericidal drug against Mycobacterium tuberculosis. More than 95 % of all mutations are located in a 81 bp (1294—1375 bp) core region (Rifampicin Resistance Determining Region ,RRDR ) of the rpo B gene between codons 507—533 with the most common changes in codons Ser 531 Leu , His 526 Tyr, and Asp 516 Val. These changes occur in more than 70 % of rifampicin resistant. Thus rifampicin resistance acts as a surrogate marker of MDR-TB. The molecular methods to detect rifampicin resistance include methods such as Line Probe Assay (LiPA), DNA sequencing, Single-Strand Conformational Polymorphism (SSCP), Heteroduplex Analysis (HA), Molecular beacons (MB) and Polymerase chain reaction--Enzyme Linked Immunosorbert Assay (PCR-ELISA) .Among these, the Line Probe Assay is most advanced. The major advantage of PCR based molecular methods is the speed by which the result can be obtained.

Keywords: Rifampicin resistance, Multi drug resistant tuberculosis (MDR-TB), rpo B gene

1. Introduction
Drug resistant tuberculosis has been reported since the early days of introduction of anti-tuberculosis therapy but recently multi drug resistant tuberculosis (MDR-TB) has been an area of growing concern and is posing threat to the global efforts of tuberculosis control. Prevalence of MDR-TB in a community mirrors the functional state and efficacy of tuberculosis control programme and realistic attitude of the community towards implementation of such programmes1. MDR-TB is almost twice as common in tuberculosis patient co-infected with Human Immunodeficiency Virus (HIV) compared to tuberculosis patient without HIV2. The mean survival time for patients who are co-infected with HIV and MDR-TB is about 2 months from the time of diagnosis, with 1 year mortality rate of 60 % as compared to 30 % in non-HIV infected persons2. Of late, there has been a special concern for conversion of poorly managed MDR-TB to XDR-TB (Extensively drug resistant-tuberculosis) which is the untreatable form of tuberculosis 4. The combination of XDR-TB and HIV is virtually the death sentence for the patient. Thus, the alarming increases in MDR-TB, the emergence of XDR-TB and rapid mortality of MDR-TB and XDR-TB patients with HIV co-infection, have highlighted the urgency for rapid molecular diagnostic methods.

1.2 Definitions: Multi-drug resistant tuberculosis (MDR-TB) is defined as disease due to M.tuberculosis that is resistant to Isoniazid (H) and Rifampicin (R).
As per the latest definition, XDR-TB is a subset of MDR-TB with additional resistance to any fluoroquinolone (Ciprofloxacin, Ofloxacin etc) and one of the second line injectables namely Kanamycin, Capreomycin and Amikacin5.

2. Magnitude of problem:
There are an estimated 490,000 new MDR-TB cases every year and 30,000 new XDR-TB cases every year6. According to the 4th Global report, The WHO/ IUATLD Global project on Anti-tuberculosis Drug resistance Surveillance (2002—2007), the epidemiology of MDR-TB in India and Worldwide 7 is shown in tables I, II and III.

2.1 TB and HIV: There is a close association between TB and HIV, each potentiating the impact of the other. HIV infection is now considered as the most potent risk factor for
tuberculosis. It not only increases the risk of reactivating latent *M. tuberculosis* infections but also leads to the rapid progression to clinical TB soon after natural infection\(^7\). An HIV positive person is six times (50-60 \% lifetime risk) more likely to develop tuberculosis disease once infected with TB bacilli, as compared to an HIV negative person, who has a 10 \% lifetime risk\(^5\). TB is the leading cause of death among HIV infected people in Africa and a major cause of death elsewhere. It is the most common presenting illness among people living with HIV who also face threat of multi-drug resistant TB (MDR-TB) \(^6\).

### 2.2 MDR-TB and HIV:

Although HIV infection does not itself increase the chance of drug resistance occurring, both MDR-TB and XDR-TB are very serious threats to HIV positive people, whose weakened immune systems (depleted CD \(4 + \) T-lymphocytes) render them unlikely to fight off tuberculosis naturally. The mean survival time for patients who are co-infected with HIV and MDR-TB is about 2 months from the time of diagnosis, with 1 year mortality rate of 60 \% as compared to 30 \% in non-HIV infected persons\(^3\). Thus it is important that such cases are diagnosed at the earliest for proper management. Thus an early and rapid diagnosis is required for the management of tuberculosis, especially the MDR and XDR variety.

### 3. Molecular mechanism of multi-drug resistance:

The development of clinical drug resistance in tuberculosis is shown in figure I. Mutation in the genome of *Mycobacterium tuberculosis* that can confer resistance to antituberculosis drugs occurs spontaneously with an estimated frequency of \(3.5 \times 10^{-6}\) for isoniazid and \(3.1 \times 10^{-8}\) for rifampicin\(^8\). The clinical drug resistance is classified as acquired resistance when drug resistant mutants are selected as a result of ineffective treatment or as primary resistance when a patient is infected with a resistant strain. MDR-TB (resistance to isoniazid and rifampicin) will thus occur mainly in circumstances where sequential drug resistance follows sustained treatment failure.

#### 3.1 Rifampicin resistance and *rpo B* gene:

Rifampicin was introduced in 1971 as anti-tuberculosis drug. It is the most potent anti-tuberculosis agent and thus included in all ATT regimes. Rifampicin resistance is of special concern because it is the most effective bactericidal drug against *Mycobacterium tuberculosis*. It is a better sterilizing agent than isoniazid. It permeates all tissue membrane including the blood-brain and blood-placental barrier and is equally effective against intracellular as well as extracellular bacilli. Rifampicin is the only antitubercular drug that is effective against “persisters” or dormant bacilli which are found in solid caseous lesions, all other drugs being inactive\(^9,10\). In this context, it has a distinct advantage over isoniazid. This makes the detection of its resistance very important. The complete genome sequence of *Mycobacterium tuberculosis* consists of 4043 genes\(^11\). Out of these, mutation in the *rpo B* gene is responsible for resistance to rifampicin. The *rpo B* gene consists of 3267 bp of which the Rifampicin Resistance Determining Region (RRDR) consists of 81 bp as shown in figure II.

### 3.2 Molecular mechanism of action of rifampicin:

Rifampicin interferes with transcription by the DNA dependent RNA polymerase. RNA polymerase consists of four different subunits (\(\alpha \beta \beta\prime \sigma\)). The \(\beta\) subunit is the catalytic site. These four subunits (\(\alpha \beta \beta\prime \sigma\)) are encoded by *rpo A*, *rpo B*, *rpo C* and *rpo D* genes respectively. Rifampicin binds with the \(\beta\) subunit of RNA polymerase, hindering transcription and thereby killing the bacteria (bactericidal). So the mutation in the *rpo B* gene conferred conformational changes leading to defective binding of rifampicin to RNA polymerase making it ineffective as bactericidal agent.

### 3.3 Rifampicin resistance, a surrogate marker of MDR-TB:

Extensive studies on the *rpo B* gene in rifampicin resistant isolates of *mycobacterium tuberculosis* identified a variety of mutations and short deletions in the gene. A total of 69 single nucleotide changes; 3 insertions, 16 deletions and 38 multiple nucleotide changes have been reported\(^12,13,14,15,16\). More than 95 \% of all mutations are located in a 81 bp (1294—1375 bp) core region (Rifampicin Resistance Determining Region, RRDR) of the *rpo B* gene between codons 507—533 with the most common changes in codons Ser 531 Leu, His 526 Tyr, and Asp 516 Val. These changes occur in more than 70
% of rifampicin resistant isolates\textsuperscript{12,13,14,15,16}. Worldwide, the most frequent mutations of \textit{rpo B} gene are Ser 531 Leu followed by His 526 Tyr and His 526 Asp\textsuperscript{16} as shown in figure III.

Rifampicin resistance can be a surrogate marker for MDR-TB since more than 90 % of rifampicin resistant isolates are also isoniazid resistant\textsuperscript{13,17,18}.

3.4 Molecular methods to detect rifampicin resistance: MDR-TB is not a clinical diagnosis. It depends on the culture and drug sensitivity testing (DST) for confirmation of diagnosis. At present, culture and drug sensitivity testing is the ‘gold standard’ test for identification of rifampicin resistance. Culture can take up to 6 wks before a definitive result can be obtained and another 2-4 weeks are required for drug susceptibility testing\textsuperscript{14}.

Thus, the alarming increases in MDR-TB, the emergence of XDR-TB, rapid mortality of MDR-TB and XDR-TB patients with HIV co-infection, and long time taken for diagnosis by culture and DST, have highlighted the urgency for rapid molecular diagnostic methods.

The molecular methods to detect rifampicin resistance include methods such as A) Line Probe Assay (LiPA), B) DNA sequencing, C) Single-Strand Conformational Polymorphism (SSCP), D) Heteroduplex Analysis (HA), E) Molecular beacons (MB) and F) Polymerase chain reaction–Enzyme Linked Immunosorbent Assay (PCR-ELISA).

A. Line Probe Assay (LiPA): LiPA is the most advanced method for rapid detection of rifampicin resistance (alone or in combination with isoniazid)\textsuperscript{19}.

It involves following steps
i) DNA extraction from \textit{M. Tuberculosis} isolates or directly from clinical specimen
ii) PCR amplification of the RRDR of \textit{rpo B} gene using biotynylated primers
iii) Labelled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip as shown in figure IV.
iv) Captured labeled hybrids are detected by colorimetric development.

If mutation is present in one of the target regions, the amplicon will not hybridise with the relevant probe. Mutations are therefore detected by
i) Lack of binding to wild type probes (\textit{S_1\textsubscript{S_2\textsubscript{S_3\textsubscript{S_4\textsubscript{S_5}}}}})
ii) Binding to specific mutant probes (\textit{R_2\textsubscript{R_4\textsubscript{R_4b\textsubscript{R_5}}})} for the most commonly occurring mutations.

The post-hybridisation reaction leads to the development of colour bands on the strip at the site of probe binding and can be observed by naked eye.

Line probe assay performance have been adequately validated in direct testing of sputum smear-positive specimens and on isolates of \textit{M. Tuberculosis} complex grown from smear-negative and smear positive specimens. Direct use of LiPA on smear negative-clinical specimens is not recommended\textsuperscript{19}.

LiPA is a reliable, rapid and informative tool for the early detection and characterization of \textit{rpo B} gene mutation associated with rifampicin resistance in \textit{Mycobacterium tuberculosis}\textsuperscript{16}. But this method is too costly for use in routine laboratories, especially in developing country.

LiPA is recommended by WHO for rapid detection of rifampicin resistance since 27\textsuperscript{th} June 2008\textsuperscript{19}.

B. Sequencing: PCR amplification followed by DNA sequencing is the most commonly applied technique to characterize mutations in the \textit{rpo B} gene\textsuperscript{18}. The detection of rifampicin resistance by DNA sequencing is rapid, advantageous and provides a confirmatory result thereby aiding in initiation of immediate alternative drug therapy in patients who develop rifampicin resistance.

Rifampicin resistance as reported by various researchers is due to mutation in the Rifampicin Resistance Determining Region (RRDR) of \textit{rpo B} gene. They reported the most frequent mutations in RRDR of \textit{rpo B} gene are in codon 531 followed by codon 526 and codon 516\textsuperscript{16,18,21,22,23}. However, some of the previous researchers\textsuperscript{16} also reported codon 526 as the most common site of mutation leading to rifampicin resistance in MDR-TB cases.

The advantage of using DNA sequencing as a diagnostic modality for diagnosis of MDR-TB is that it can detect mutation outside RRDR which is responsible for 8-9 % cases of MDR-TB.

C. Single-Strand Conformational Polymorphism (SSCP): SSCP is a gel based method that can detect short stretches of DNA
approximately 175-250 bp in size. Small changes in a nucleotide sequence result in differences in secondary structures as well as measurable DNA mobility shifts that are detected on poly acryl amide gel electrophoresis. Till today various studies have applied PCR-SSCP to identify mutational changes associated with drug resistance in mycobacterium tuberculosis for frontline drugs like rifampicin and isoniazid. However, PCR-SSCP analysis is technically demanding and not sufficiently sensitive. Furthermore SSCP conditions must be carefully evaluated since not all mutations will be detected under the same conditions.

D. Heteroduplex analysis (HA): HA depends on the conformation of duplex DNA when analysed in native gels. Heteroduplexes are formed when amplicons from known wild type and unknown mutant sequences are heated and reannealed. The DNA strand will form a mismatched heteroduplex if there is a sequence difference between the strands of the wild type and tested DNA. These heteroduplexes have an altered electrophoretic mobility when compared to homoduplexes, since mismatches tend to retard the migration of DNA during electrophoresis. Recently, temperature mediated HA has been applied to the detection of mutations associated with mutation in rpo B gene. HA has certain disadvantages in that it is insensitive to G-C rich regions and is very time consuming.

E. Molecular beacons: Molecular beacons are single-stranded oligonucleotide hybridization probes which can be used as amplicon detector probes in diagnostic assays. A beacon consists of a stem-loop structure in which the stem contains a fluorophore on one arm and a quencher on the other end of the arm as shown in figure V. The loop contains the probe which is complementary to the target DNA. If the molecular beacon is free in a solution it will not fluoresce, because the stem places the fluorophore so close to the non-fluorescent quencher that they transiently share electrons, eliminating the ability of the fluorophore to fluoresce. However, in the presence of complementary target DNA the probes undergo a conformational change that enables them to fluoresce brightly. Different colored fluorophores (different primers) can be used simultaneously to detect multiple targets (each target will give a different color) in the same reaction. Molecular beacons are very specific and can discriminate between single nucleotide substitutions. Thus they are ideally suited for genotyping and have been used in the detection of drug resistance in M. tuberculosis. But the limitations of molecular beacon technique are that limited genes and sites are targeted. By this method all mutations cannot be detected.

F. Polymerase chain reaction--Enzyme Linked Immunosorbent Assay (PCR-ELISA): After PCR amplification of rpo B gene, ELISA is done with the amplicons. The reverse primer is labelled with digoxigenin at the 5′ end so that after PCR the amplicons will contain digoxigenin at their 5′ end. The ELISA plates are streptavidine coated. In this ELISA wells, capture probes (5′ biotinylated), amplicons, enzyme linked antibody and chromogenic substrates are added. If there is change in nucleotide sequence in the rpo B gene then the capture probe will not hybridise with the amplicons and will not produce colour. Thus a positive result shows the absence of any mutation in that region, while a negative result indicate a lack of hybridization and presence of a mutation in the rpo B sequence corresponding to the probe. PCR-ELISA cannot identify the specific mutation causing rifampicin resistance but does indicate the region in which the mutation is located. Knowledge of the specific mutation conferring resistance is, however, not necessary for efficient patient management. The benefits of PCR-ELISA system lie in its speed and accuracy in identifying rifampicin resistant strains and it can be considered as a screening method for MDR-TB.

4. Applications
The major advantages of PCR based molecular methods are the speed by which the result can be obtained. By using the molecular methods the results can be obtained within few days in contrast to the culture and drug sensitivity testing (DST) takes about 6 weeks for diagnosing rifampicin resistance. It is emphasized that the molecular techniques are important for rapid diagnosis of rifampicin resistance and MDR-TB.

5. Summary
Recently MDR-TB has been a growing area of concern and XDR-TB is virtually the
untreatable form of tuberculosis. The mean survival time for patients who are co-infected with HIV and MDR-TB is about 2 months from the time of diagnosis, with 1 year mortality rate of 60% as compared to 30% in non-HIV infected persons. Thus, it is essential to detect MDR-TB at the earliest possible stage using the best possible diagnostic modalities.

Mutation in \( rpoB \) gene (3267 bp) is responsible for rifampicin resistance. More than 95% of all mutations are located in a 81 bp (1294—1375 bp) core region (Rifampicin Resistance Determining Region ,RRDR ) of the \( rpoB \) gene between codons 507—533 with the most common changes in codons Ser 531 Leu , His 526 Tyr, and Asp 516 Val. Rifampicin resistance serves as a surrogate marker for MDR-TB since more than 90% of rifampicin resistant isolates are also isoniazid resistant. The advantage of using PCR based molecular techniques lies in its speed and accuracy in identifying rifampicin resistance strain. The use of molecular techniques will reduce the time required to detect rifampicin resistance.

Although molecular methods are more rapid, and can be done directly from a clinical sample, there are important limitations when compared to conventional methods. These include a lack of sensitivity since not all molecular mechanisms leading to drug resistance are known, therefore not all resistant isolates will be detected. Some mutations are silent and do not confer resistance but they can be detected by these molecular techniques and can lead to wrong interpretation.

References
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Mycobacterium tuberculosis clinical isolates by DNA Sequencing; Indian J of Tubercul; 2005;52;132-136.


Table I. Estimates of MDR-TB among New cases (2002—2007)

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<th>No.of new TB cases</th>
<th>No.of MDR-TB cases</th>
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Table II Estimates of MDR-TB among Previously treated cases (2002—2007)

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Table III  Estimates of MDR-TB among all TB cases  (2002—2007)

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Fig. I. Acquired resistance develops due to natural selection

Fig II.  *rpo B* gene (3267 bp)

Fig III. RRDR (81 bp core region, codon 507-533) of *rpo B* gene

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<thead>
<tr>
<th>507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533</th>
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Fig IV. Line Probe Assay (LiPA)

- Marker line – for orientation
- Conjugate control line – internal control for the colour development system
- TB line-specific probe for *M. tuberculosis* complex.
- Wild type probes (S₁,S₂,S₃,S₄,S₅)
- mutant probes (R₁,R₂,R₃,R₄)

Fig V. Molecular Beacons (MB)