Molecular Diagnosis of Multidrug-Resistant
Mycobacterium tuberculosis Isolates in and around Mumbai

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Mycobacterium tuberculosis (MTB), resistant to both isoniazid (INH) and rifampicin (RIF) and leading to multidrug-resistant tuberculosis (MDR-TB) can be successfully identified by an 8-week long phenotypic method. However, there is a need for faster diagnostic techniques. In the present study, we evaluated the use of PCR followed by sequencing as a rapid diagnostic tool and determined the incidence of rpoB and katG resistance-associated mutations in MTb isolates in and around Mumbai. Fifty-Five sputum samples from clinically suspected tuberculosis patients were decontaminated and screened for MTB using Ziehl Neelsen (ZN) staining and Lowenstein–Jensen (LJ) culturing. Extracted DNA from positive samples was subjected to conventional PCR for rpoB and katG genes. In addition, Drug Susceptibility Testing (DST) of isolates was performed by the standard proportional method. PCR products of selected samples of either type i.e., resistant and susceptible by both techniques were sent for sequencing. Fifteen samples (27.27%) were found to be resistant to both drugs phenotypically by DST and by PCR for rpoB and katG genes. Sequencing analysis revealed point mutations Ser 531 Leu for rpoB gene and Ser 315 Thr for katG gene. The results obtained by direct PCR followed by sequencing method were in accordance with phenotypic method (DST) with high sensitivity and specificity for both genes. The results suggest that the use of molecular techniques can be more suitable for rapid diagnosis of multi-drug resistance in clinical isolates of MDR-TB.

Key words: Multi-drug Resistant tuberculosis (MDR-TB), Polymerase Chain Reaction (PCR), Sequencing, rpoB & katG genes.

In 2012, with an estimated 8.6 million new cases and 1.3 million deaths every year, tuberculosis (TB) represents one of the most serious infectious diseases worldwide¹. The alarming emergence of multidrug-resistant TB (MDR-TB) cases, resistant to at least two drugs, including isoniazid (INH) and rifampin (RIF), and the recent emergence of extensively drug-resistant TB (XDR-TB), with additional resistance to a fluoroquinolone (FQ) and at least one of the three injectable second-line drugs, pose a significant threat to tuberculosis control²,³. Lack of adequate treatment, often due to irregular drug supply, inappropriate regimens, or poor patient compliance, is associated with the emergence of drug-resistant Mycobacterium tuberculosis⁴. In India, the incidence of all forms of TB was estimated to be 193/100,000 population in 2012⁴. In 2012, approximately 450,000 cases of MDR-TB were estimated throughout the world with 58 nations reporting at least one case of XDR-TB to the World Health Organization (WHO)⁵. Among them, India and China had the highest burden of MDR-TB, together accounting for almost half of the world’s total cases⁶. Previous studies have shown that 68% of the patients for whom drug susceptibility testing
(DST) was requested had multidrug-resistant tuberculosis. Therefore, there is an urgent need for rapid and accurate detection of MDR-TB in order to achieve immediate patient treatment and hence cure and from a public health perspective, to prevent transmission. TB detection using molecular techniques namely, polymerase chain reaction (PCR), PCR based restriction fragment length polymorphism (PCR-RFLP) and PCR based DNA sequencing are very rapid, more sensitive and reliable when compared to the conventional culture.

There are several in-house nested PCR (nPCR) standardized for the detection of M. tuberculosis from clinical specimens targeting MPB64, IS6110 and 16S rRNA genes. MPB64 is an immunogenic protein produced by certain mycobacterial strains while IS6110 is an insertion element present in single or multiple copies in M. tuberculosis complex isolates specifically. The collection of data from different countries has indicated that resistance to Rif in 90% of cases is due to mutations resulting in an amino acid substitution within the 81-bp core region of the RNA polymerase-subunit gene (rpoB), called the Rif resistance-determining region (RRDR). In contrast, INH resistance is mediated by mutations in several genes, most frequently within the katG gene, encoding a catalase-peroxidase which transforms INH into its active form and in the promoter region of inhA, encoding a putative enzyme involved in mycolic acid biosynthesis. An upregulation mutation in the inhA promoter region results in the overexpression of InhA and develops INH resistance via a titration mechanism. In the present study, we determined the incidence of resistance-associated mutations in two specific genes (rpoB and katG) of M. tuberculosis isolated in and around Mumbai and compared the results of sequencing with the results of phenotypic method (DST by proportional method).

**MATERIALS AND METHODS**

**Study Groups**

Fifty-five clinical samples from patients diagnosed with pulmonary tuberculosis were included in the study. Individuals more than 18 years of age, belonging to either sex and attending outdoor patient department or admitted to Government Sewree TB Hospital, Mumbai were selected for the study. Individuals less than 18 years of age and pregnant women were excluded from the study. A written informed consent was acquired from all individuals that were included in the study. Ethical approval for the study was taken previously from Institute Ethics Committee.

**Sample Collection and Processing**

A total of 55 sputum samples were collected from subjects during one year period from 2011 to 2012. The samples were transported to the laboratory and stored at 4°C within 4 h of collection and processed immediately for decontamination of sputum sample by NALC-NaOH Method.

**Phenotypic Method**

Detection of *Mycobacterium tuberculosis* was done by using Ziehl Neelsen (ZN) staining (Microscopy) and culturing on to Lowenstein–Jensen (LJ) medium. Drug Susceptibility Testing (DST) of isolates was performed by the standard proportional method on Lowenstein–Jensen (LJ) medium containing 0.2 mg Isoniazid per mL and 40.0 mg Rifampicin per mL, as described in RNTCP guidelines.

**Extraction of DNA from MTB**

DNA from MTB processed sputum sample was extracted by Qiagen QIAmp DNA mini kit (Qiagen, Inc., Valencia, CA) method as per manufacturer’s instructions. Briefly, 200.0 µL of sputum sample warmed to room temperature (15-20°C) and 180.0 µL of lysis solution i.e ATL was added in 1.5 mL microcentrifuge tube. After briefly vortexing, it was incubated in dry bath (Benchmark Instruments) at 37°C for 3 hours. After addition of proteinase K and AL solution, the mixture was further incubated at 56°C for two hours in dry bath. A volume of 200.0 µL of 96% ethanol was added, mixed and the mixture was transferred on to a spin column. Following intermittent centrifugation and washing steps, 100.0 µL of elution solution i.e ATL, was added in 1.5 mL microcentrifuge tube. After briefly vortexing, it was incubated in dry bath (Benchmark Instruments) at 37°C for 3 hours. After addition of proteinase K and AL solution, the mixture was further incubated at 56°C for two hours in dry bath. An amount of 200.0 µL of 96% ethanol was added, mixed and the mixture was transferred on to a spin column. Following intermittent centrifugation and washing steps, 100.0 µL of elution buffer pre-warmed at 70°C was added to elute purified DNA. DNA yield from all the samples of extraction was determined on a NanoDrop Spectrophotometer (Thermo Scientific) by measuring the absorption at 260 nm, and purity was calculated based on 260/280 ratio.

**Detection of MTB by Conventional Nested PCR Using Suitable Primers**

Nested Polymerase Chain Reaction was used to amplify the IS6110 sequence of
**Mycobacterium tuberculosis** using primers as mentioned in Table 1. The outer and inner rounds of PCR were carried out using a PCR Master Mix (Fermentas Inc., Maryland, USA) as per manufacturer’s instructions with primer concentration being 0.4 µM and 0.7 µM respectively in a 25.0 µL reaction. The cycling conditions used were: 94°C/20s, 60°C/20s, 72°C/30s for 35 cycles to give a product size of 556 bp and 285 bp for outer and inner rounds respectively in a M/s. Takara PCR Thermal Cycler Dice (TP600 System) Instrument16. Amplified products were detected on 1.5% agarose gel infused with SYBR®Safe dye for visualization.

**Detection of Rifampicin & Isoniazid Resistance Gene by Conventional PCR Using Suitable Primers**

The conventional PCR was used to amplify *rpoB* gene sequence and *katG* gene sequence of *Mycobacterium tuberculosis* using primer as mentioned in Table 2. PCR primers were designed to amplify the regions between nucleotides 2335 and 2492 of the *rpoB* gene (codon 531 TCG?TTG and 526 CAC?GAC) and nucleotides 2759 and 2967 (codon 315AGC?AAC) of the *katG* gene11,17. PCR was carried out using a PCR Master Mix (Fermentas Inc., Maryland, USA) as per manufacturer’s instructions with primer concentration being 0.5 µM for both the genes and concentration of MgCl2 being 5.0 mM in a 25.0 µL reaction. The cycling conditions used were 95°C/45 s followed by 38 cycles of 95°C/15 s, 53°C (annealing temperature for the *rpoB* gene) and 60°C (annealing temperature for the *katG* gene) for 15 s, and 72°C/30 s to give a product size of 158 bp for the *rpoB* gene and 209 bp for the *katG* gene in a M/s. Takara PCR Thermal Cycler Dice (TP600 System) Instrument. Amplified products were detected on 1.5% agarose gel infused with SYBR®Safe dye for visualization. PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA Catlog No: 28106) as per manufacturers’ Instructions.

**DNA sequencing**

Direct sequencing of PCR products of selected samples and standard strains was outsourced to Ocimum Biosciences Ltd. The sequencing was performed with a 96-capillary 3730xl DNA Analyzer.

**RESULTS**

A total of 55 sputum samples from patients suspected of tuberculosis were subjected to preliminary screening by simple microscopic detection using the Acid Fast Bacilli (AFB) -ZN staining technique and culturing on LJ medium, of which 52 (94.55%) samples were found to be smear positive & 3 (5.45%) samples were found to be smear negative. However, 50 (90.91%) samples

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**Table 1. Amplification primer sequences of IS6110 gene for detection of M. tuberculosis by Conventional Nested PCR**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Position of IS6110</th>
<th>Sequences (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB1-F</td>
<td>555 - 572</td>
<td>CTCAAGGAGCACATCAGC</td>
</tr>
<tr>
<td>TB2-R</td>
<td>1111 - 1084</td>
<td>TCATAGGAGCTTCCGACC</td>
</tr>
<tr>
<td>Inner</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB3-F</td>
<td>590 - 609</td>
<td>CTCAGGTGTATACGTTGCC</td>
</tr>
<tr>
<td>TB4-R</td>
<td>874 - 855</td>
<td>CTCAGGTGTATACGTTGCC</td>
</tr>
</tbody>
</table>

**Table 2. Amplification primer sequences for Detection of Rifampicin (rpoB) and Isoniazid (katG) of M. tuberculosis resistance gene by Conventional PCR**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences (5’→3’)</th>
<th>Product size(bp)</th>
<th>Annealing temperature(°C)</th>
<th>Melting temperature(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB-FTR8</td>
<td>GTGCACGTCGCGGACCTCCA</td>
<td>158</td>
<td>55</td>
<td>68.4</td>
</tr>
<tr>
<td>rpoB-RTR9</td>
<td>TCGCCGGCATCAAGGAGT</td>
<td>158</td>
<td>55</td>
<td>61.0</td>
</tr>
<tr>
<td>katG-FTB86</td>
<td>GAAACACCGCGGCTGATCGT</td>
<td>209</td>
<td>60</td>
<td>64.3</td>
</tr>
<tr>
<td>katG-RTB87</td>
<td>GGTGTCCTATTCTGTCGGGG</td>
<td>209</td>
<td>60</td>
<td>62.8</td>
</tr>
</tbody>
</table>
were found to be culture positive & 5 (9.09%) samples were found to be culture negative. Conventional PCR was carried out using the primers targeting IS6110 sequence for detection of *Mycobacterium tuberculosis* (Table 1), 52 (94.55%) samples were found to be positive and 3 (5.45%) samples negative by PCR.

Drug susceptibility testing (DST) revealed 35 (70%) samples out of the total 50 culture positive samples were susceptible while 15 (30%) samples were resistant to both Isoniazid and Rifampicin. A total of 17 (34%) samples were found to be mono-resistant while 33 (66%) samples were found to be susceptible to isoniazid. Likewise, 18 (36%) samples were found to be mono-resistant and 32 (64%) samples were found to be susceptible to rifampicin phenotypically by DST. The results were considered as ‘true results’ when comparing with molecular methods, as DST is considered the gold standard for determination of drug resistance (Table 3).

Amplification of specific gene segments for detection of rifampicin and isoniazid resistance of MTB was done using suitable primers targeting the 158-bp fragment of *rpoB* gene spanning codons 526 and 531 and the 208-bp fragment of KatG gene spanning codon 315 of MTB (Table 2). Direct sequencing of PCR products of selected samples and standard strain was performed with a 96-capillary 3730xl DNA Analyzer on a third party commercial basis with M/s Ocimum Biosciences Ltd. PCR products of selected 15 samples of either type i.e., resistant and susceptible by both techniques (DST & Conventional PCR) were sent for sequencing.

<table>
<thead>
<tr>
<th>Drug Susceptibility profile</th>
<th>No of samples</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible to Isoniazid</td>
<td>33</td>
<td>66%</td>
</tr>
<tr>
<td>Resistant to Isoniazid</td>
<td>17</td>
<td>34%</td>
</tr>
<tr>
<td>Susceptible to Rifampicin</td>
<td>32</td>
<td>64%</td>
</tr>
<tr>
<td>Resistant to Rifampicin</td>
<td>18</td>
<td>36%</td>
</tr>
<tr>
<td>Susceptible to both drug</td>
<td>35</td>
<td>70%</td>
</tr>
<tr>
<td>Resistance to both drug (MDR)</td>
<td>15</td>
<td>30%</td>
</tr>
</tbody>
</table>

**Table 3. Drug Susceptibility Testing (DST) Result for Isoniazid and Rifampicin Drug**

**Fig. 1.** *M. tuberculosis* katG gene amino acid sequence showing single nucleotide polymorphism

**Fig. 2.** *M. tuberculosis* rpoB gene amino acid sequence showing single nucleotide polymorphism
Of these, seven samples failed during sequencing due to low template yield. The sequences for remaining eight samples were aligned with standard reference strain CDC1551 of *M. tuberculosis* (Gene Bank accession number AE000516) to detect point mutations depicting the AGC/ACC mutation in the katG gene and TCG/TTG, CAC/TAC or GAC/GTC mutation in the rpoB gene (Figure 1 & 2). It was found that six out of eight samples harbored the AGC/ACC mutation for the katG gene. This mutation exhibits a S315T substitution on the respective translated proteins. The remaining 2 strains were found to be drug sensitive. Five out of the eight samples showed the TCG/TTG mutation for the rpoB gene, the remaining three were found to be drug sensitive. This mutation exhibits a S531L substitution on the respective translated proteins.

**DISCUSSION**

Anti-tuberculosis drug resistance poses a significant threat to human health, which usually develops due to the alteration of drug targets by mutations in *M. tuberculosis* chromosomal genes. A large number of mutations in several genes that confer resistance to *M. tuberculosis* have been reported from different countries. However no study until now has managed to reveal the range of mutation in clinical samples from India, one of the countries with the highest TB prevalence. Hence, in the present study, we have attempted to identify the molecular basis of the drug resistance of *M. tuberculosis* in India specifically in and around Mumbai.

Multi-Drug Resistant Tuberculosis (MDR-TB) is based on the culture and Drug Sensitivity Testing (DST) for confirmation which is considered as the ‘gold standard’ test for identification of rifampicin and isoniazid resistance. But these phenotypic techniques require 6–8 weeks for a definitive diagnosis. Rifampicin, the most potent anti-tubercular drug, is bactericidal to *M. tuberculosis* as it inhibits mycobacterial DNA-dependent RNA polymerase by binding with its b-subunit which is coded by the rpoB gene. Mutations in rpoB gene indicate resistance to rifampicin and are often associated with resistance to other drugs also, most notably isoniazid.

Rifampicin resistance as reported in previous studies is due to mutation in the Rifampicin Resistance Determining Region (RRDR) of rpoB gene. The most frequent mutations reported in RRDR of rpoB gene include codon 531 followed by codon 526 and codon 516. In the present study, we have sequenced RRDR of rpoB gene in rifampicin resistant/sensitive samples (DST proven) and observed that codon 531 (TCG/TTG) is the most frequent site of mutation leading to rifampicin resistance. Five out of eight samples harbored the TCG/TTG mutation for the rpoB gene. This mutation exhibits a S531L substitution on the respective translated proteins.

Bostanabad et al. in 2008 demonstrated mutations in the ‘hot spot’ region (RRDR) of rpoB gene leading to rifampicin resistance with predominant nucleotide changes in codon 510, 523, 526 and 531. Study by Deepa et al. in 2005 reported dominant presence of mutations at codon 531 (TCG/TTG). Meera et al. in 2003 and Mani et al. in 2001 studied strains of rifampicin resistant *M. tuberculosis* by Line Probe Assay (LiPA) have reported codon 531 (TCG/TTG) and codon 526 (CAC/TAC) to be the most frequent site of mutation in RRDR of rpoB gene. Mechanisms such as a permeability barrier or membrane proteins acting as drug efflux pumps may also confer resistance to rifampicin. In our study, we characterized only the RRDR of rpoB. A second formal possibility to account for the inability of detecting rpoB mutations in these rifampicin-resistant cases is that changes might have occurred in one or more genes whose products participate in antibiotic permeability or metabolism. Bostanabad et al. (2008) in their study reported that 5 rifampicin resistant isolates did not show any mutations in RRDR of rpoB gene. Mani et al. in 2001 from Tuberculosis Research Centre (TRC), Chennai reported that 13.6% of mutations occur outside RRDR of rpoB gene.

The frequency of mutation in katG gene is 100 fold higher than rpoB gene and is regarded as first step in evolution of MDR-MTB. Also it creates more possibility for acquiring mutations in other genes. The combination of INH resistance and maintained virulence might make it possible for some INH resistant strains, especially those with the katG Ser315Thr mutation, to acquire extra drug resistance and become MDR-TB. The molecular basis of resistance to isoniazid is more
complex and is caused by a variety of mutations in four different genes of MTB i.e. katG encoding catalase peroxidase, inhA encoding the enoyl acyl carrier protein (ACP) reductase, kasA encoding â-ketoacyl ACP synthase and ahpC encoding alkyl-hydroperoxide reductase. Even then, nearly 5-10% of isoniazid-resistant *M. tuberculosis* isolates do not have an identifiable mutation. This study was conducted to gain further insight into molecular basis of INHr MTB circulating in India specifically in and around Mumbai. We found that mutation in *katG* gene (Ser315Thr substitution) was responsible for six of INH resistant strains. Out of the eight samples sequenced, two samples were found to be sensitive to isoniazid. We compared our result of mutation at *katG* codon 315 associated with INH resistance and results of demographic findings with those of earlier studies done worldwide.

Several studies have revealed that mutation in *katG* gene is responsible for 60-70% of INH resistant strains. A study by Marahatta S.S *et al.* in 2006, India revealed *katG* S315T mutation in 74.19% of strains of MTB from Delhi. In Nepal where incidence of all forms of TB is 173 cases per 1,00,000 population the Ser315Thr mutation found was not as high as those of north-western Russia (93.6%) and Germany (86.4%). Similarly, Wang Y.C *et al.* from China reported 68.6% of INHr strains associated with mutation in *katG* due to S315T substitution. Our finding of mutation in the *katG* Ser315Thr substitution (i.e. 75%) is similar to those reported in other parts of the world, which reflects a global pattern. Since all the INHr isolates were resistant to RIF as well so we concluded that all the strains under study are MDR-MTB isolates. Thus, chances that Mumbai may carry extensive transmission rate of MDR-TB with *rpoB* and *katG* gene mutation cannot be ignored.

The advantage of DNA sequencing method opted in the current study is the time between sampling and availability of the test results for clinical decision making. In general, Conventional methods of culture and DST take 6-8 weeks for definitive diagnosis. Consequently, clinical decisions cannot be taken until then. In comparison, the results of rifampicin and isoniazid susceptibility of *M. tuberculosis* based on genetic alteration in the RRDR of *rpoB* and *katG* gene are available within 3-5 days. This period is based on 1-2 days for DNA extraction from clinical sample followed by PCR and 2-3 days for amplification and sequencing of gene.

**CONCLUSION**

To conclude, early detection of MDR-pulmonary TB cases is of immense clinical importance. DNA sequencing is rapid, conclusive and more advantageous over conventional drug susceptibility testing (DST). It determines the exact site and frequency of mutation of *rpoB* and *katG* gene which lead to rifampicin and isoniazid resistance in different geographical areas. This DNA sequencing serves two fold functions. First, it provides an accurate and rapid prediction of rifampicin and isoniazid resistant *M. tuberculosis* which is clinically useful. Secondly it can serve as useful data in developing a screening protocol for detection of MDR-TB in future. Early detection of the resistant strains will facilitate the modification of treatment regimen for patients and appropriate infection control measures can be taken in time to reduce the risk of further development and transmission of MDR-TB. An understanding at the molecular level of the mechanism of drug resistance in MTB will enable us to develop improved tools. It deserves further investigation to determine which mechanism may play the critical role in the epidemic of MDR-TB, since the implication behind it could be meaningful to evaluate the performance of local TB control as well as to determine the MDR-TB strategies.

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