Early detection of multidrug resistant (MDR) *Mycobacterium tuberculosis* in a single tube with in-house designed fluorescence resonance energy transfer (FRET) probes using real-time PCR

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Rapid and correct diagnosis is crucial for the management of multidrug resistance (MDR) in *M. tuberculosis* (MTB). The present study aims at rapid diagnosis for identification of multidrug resistance tuberculosis (MDR-TB) using real-time PCR. FRET hybridization probes targeting most prominent four selected codons for rpoB 526 and 531 and katG 314 and 315 genes were designed and evaluated on 143 clinical MTB isolates and paired sputa for rapid detection of MDR-TB. The results of real-time PCR were compared with gold standard L-J proportion method and further validated by DNA sequencing. Of the 143 MTB positive cultures, 85 and 58 isolates were found to be ‘MDR’ and ‘pan susceptible’, respectively by proportion L-J method. The sensitivity of real-time PCR for the detection of rifampicin (RIF) and isoniazid (INH) were 85.88 and 94.11%, respectively, and the specificity of method was found to be 98.27%. DNA sequencing of 31 MTB isolates having distinct melting temperature (Tm) as compared to the standard drug susceptible H37Rv strain showed 100% concordance with real-time PCR results. DNA sequencing revealed the mutations at Ser531Leu, His526Asp of rpoB gene and Ser315Thr, Thr314Pro of katG gene in RIF and INH resistance cases. This real-time PCR assay that targets limited number of loci in a selected range ensures direct and rapid detection of MDR-TB in Indian settings. However, future studies for revalidation as well as refinement are required to break the limitations of MDR-TB detection.

**Keywords:** Drug resistance, katG gene, Isoniazid, Rifampicin, rpoB gene, TB.

The increasing spread of multidrug-resistant tuberculosis (MDR-TB), resistant to at least two drugs including isoniazid (INH) and rifampicin (RIF), and the recent emergence of extensively drug-resistant tuberculosis (XDR-TB), with additional resistance to a fluoroquinolone (FQ) and at least one of the three injectable second-line drugs, possess a significant threat to tuberculosis (TB) control1-5. The phenotypic drug susceptibility testing (DST) method causes serious delay in diagnosing drug resistant tuberculosis. Rapid diagnosis of drug resistance is essential for effective antibiotic therapies and prevent the transmission of drug-resistant strains⁶. Mycobacterium tuberculosis (MTB) acquires drug resistance mainly through mutations in specific genes⁷,⁸ and resistance to multiple drugs is the consequence of accumulation of such mutations⁹. Resistance to rifampicin is mainly due to the missense mutations in the β subunit of DNA-dependent RNA polymerase encoded by the rpoB gene. Any mutation in 81 bp hyper variable region of the rpoB gene results in failure of binding and subsequently, resistance⁹. Various studies have shown that more than 95% of all mutations are located in 81 bp core region of the rpoB gene between codons 507-533 with the most common changes in Ser531Leu, His526Tyr and Asp516Val⁹,¹⁰. Missense mutations at codons 526-531 possibly are crucial in conferring a high degree of resistance to rifampicin.

Isoniazid resistance in MTB is more complex and it involves more than one gene (katG, inhA and kasA, etc). However, mutations in katG have been reported to be associated with high level of isoniazid resistance⁵,¹⁰. The most frequently observed alteration in katG is a

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serine-to-threonine substitution at codon 315 located within the active site of the catalase moiety of katG, but the frequency with which these mutations are observed varies by geographic location.

Molecular genotypic methods have the advantage of a shorter turnaround time, no necessity of growth of the organism, possibility for direct application in clinical samples, less biohazard risks, and feasibility for automation. Of the various molecular methods, real-time PCR has been one of the most widely applied methods. Molecular assays for diagnosis of drug-resistant TB are available but not widely used. There is no information related to its use in the screening of MDR-TB in high-burden settings.

In the present study, we designed and evaluated potential real-time PCR based hybridization probes that target most frequent mutations associated with INH resistance (katG315 and a comparable less frequent katG314) and RIF resistance (rpoB531 and 526) for detection of MDR-MTB from culture isolates as well as direct from sputa. The selected mutations are also well known to have relationship with clinically relevant degree of resistance.

Materials and Methods
Collection of sputum and isolation of M. tuberculosis strains and their susceptibility testing

A total of 143 MTB cultures and also paired sputum samples obtained from cat II pulmonary tuberculosis cases belonging to Western U.P. and Bundelkhand region were included in the study. Samples were collected during October 2008 to June 2010 at Department of Microbiology and Molecular Biology, National JALMA Institute for Leprosy & Other Mycobacterial Diseases (NJILOMD), Agra with prior approval of the study by Institute Ethical Committee. As per standard operating procedure of Revised National Tuberculosis Control Program (RNTCP) clinical sputa samples of pulmonary TB cases were subjected to smear microscopy for confirmation of acid fast bacilli (AFB), and were cultured on Lowenstein-Jensen (LJ) media and incubated at 37°C up to 8 weeks. Biochemical identification of MTB colonies was done on the basis of niacin production and other standard criteria. Gold standard proportion method with LJ medium was used as per guidelines of RNTCP to define the MTB isolate as drug resistant. An isolate was considered resistant by its growth that at least 1% of the number of colonies grew on drug-free medium at critical concentrations of the drugs (i.e., 4, 40, 0.2 and 4 µg/mL of streptomycin (STR), rifampicin (RIF), isoniazid (INH) and ethambutol (EMB), respectively).

Isolation of genomic DNA from clinical specimen and MTB culture isolates

Genomic DNA from each MTB culture isolates were extracted using the CTAB method as described by van Soolingen et al. Whereas, DNA from sputum was extracted (soon after collection) using the method adapted from Boom et al. All respiratory specimens had been decontaminated previously by the N-acetyl-L-cysteine-NaOH conventional method. The purity and concentration of genomic DNA was checked by spectrophotometer and DNA from sputum samples were stored at −20°C till further analysis by real-time PCR (after the culture, biochemical and drug susceptible testing results became available).

In-house designed FRET probes and primers

The FRET probes spanning the region rpoB526 and 531 and katG314 and 315 codons were designed on the basis of wild type sequence to amplify 192 bp and 144 bp product (Table 1). Sensor probe was labeled with a Light Cycler® Red 640 (LC-Red 640) fluorophore at the 5′ end so that it cannot be extended (emission spectra-640 nm). The other anchor probe was labeled with fluorescein at the 3′ end. DNA as well as protein sequences of rpoB and katG gene were retrieved from MTB.
genome database server (Tuberculist: http://genolist.pasteur.fr/Tuberculist/). The FRET probes and primers were commercially synthesized from TIB Molecular Biology, Germany. The MDR-TB cases were detected by the deviation in melting temperature (Tm) of FRET hybridization probes targeting wild type sequence of rpoB and katG mutations as compared to wild type sequence using real-time PCR platform. Manual reaction determination method was used to detect the fluorescence and it was analyzed by plotting the fluorescence level against Tm of designed probes.

Hybridization probe (FRET) based Real-time PCR assay

The real-time PCR mixture was prepared in a final volume of 20 µL, including 2 µL ready to use reaction mix (Roche Diagnostic, Germany) which contained Taq DNA polymerase, reaction buffer, dNTP mix and 10 mM MgCl2. Further, a final concentration of 4 mM of MgCl2, 0.5 and 0.2 µM concentration of each primers and probes, respectively and 2 µL (~10ng) of template DNA was added in reaction mixture. The reaction was performed in a closed glass capillary after proper mixing and brief centrifugation. The cycling conditions were 95°C for 5 min followed by 45 cycles of 95°C for 10 s, 56 and 60°C (annealing temperature for katG and rpoB genes, respectively) for 15 s and 72°C for 20 s with continuous monitoring of fluorescence during the annealing phase, followed by a melting programme of 45-90°C at 0.1°C/s with continuous fluorescence monitoring in real-time detection system (Roche Diagnostics, Germany)12. A negative control without a DNA sample and a standard wild-type control with DNA of H37Rv strain were included for every real-time PCR experiment. The melting curve was analyzed using Light Cycler software 2.0 version (Roche Diagnostics, Germany).

DNA sequencing and sequence analysis

In order to validate the result of real-time PCR, a total of 31 MTB isolates were randomly selected (on the basis of Tm variations in RIF and INH resistant strains as compared to control strain H37Rv showed in real-time PCR analysis) and were subjected to DNA sequencing. Of the total 31 MTB isolates, subjected to DNA sequencing, 16 and 15 isolates were analyzed for the presence/absence of INH and RIF mutations, respectively. The sequencing of amplicon was carried out using the ABI PRISM 310 (Applied Biosystems, USA) automated DNA sequencer as per manufacturer’s instructions. Sequences generated by DNA sequencer were compared with wild type sequences of H37Rv strain of MTB using ClustalW tool (www.ebi.ac.uk/services/clustalw)20. Identified mutations were then translated to detect amino acid changes at specific locations using Translate (www.ebi.ac.uk/translate)21. In this study, the sensitivity of real-time PCR assay (for the detection of drug resistant MTB) refers to the ability of assay to correctly identify drug resistant MTB, whereas, specificity of assay refers to the ability of the test to correctly identify drug susceptible MTB. Susceptibility pattern found by gold standard phenotypic proportion method was used to calculate the sensitivity and specificity of real-time PCR. In this study, concordance (which was calculated by overall percentage of agreement between two tests) of real-time PCR was also analyzed by comparing with gold standard phenotypic proportion method as well as genotypic DNA sequencing method.

Results

Of the total 143 biochemically characterized MTB isolates, 58 MTB isolates were found pan susceptible while remaining 85 MTB isolates were MDR which were further classified into four different groups as per drug susceptibility profile (Table 2).

Our real-time PCR method showed the deviation in Tm of hybridization probes with respect to mutations at targeted codons of rpoB and katG. The Tm for the control strain H37Rv was found to be 61.59 and 59.17°C for rpoB and katG (Fig. 1 A and B) gene analysis, respectively. While the change from wild type to mutant at 531 codon of rpoB gene resulted in the increase of Tm by 1-6°C, whereas, mutant at of rpoB 526 codon showed a drop in Tm by 5-10°C. In case of katG gene analysis, rise in Tm by 1-7°C and drop in Tm by 1-3°C was detected as a result of mutation at 315 and 314 codons, respectively. The susceptible clinical isolates showed Tm in accordance with that of the control drug susceptible H37Rv strain. With respect to detection of RIF resistant MTB, the sensitivity of our real-time PCR method was found to be 85.88% (73/85) by the detection of mutation at targeted codons of rpoB and katG genes, respectively (Fig. 1 A and B).

Table 2—MTB drug susceptibility testing pattern by phenotypic L-J proportion method

<table>
<thead>
<tr>
<th>L-J Proportion resistance pattern</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan susceptible</td>
<td>58/143</td>
</tr>
<tr>
<td>Resistance to MDR+ EMB + STM</td>
<td>11/143</td>
</tr>
<tr>
<td>Resistance to MDR + EMB</td>
<td>12/143</td>
</tr>
<tr>
<td>Resistance to RIF and INH (MDR)</td>
<td>49/143</td>
</tr>
<tr>
<td>Resistance to MDR + STM</td>
<td>13/143</td>
</tr>
</tbody>
</table>
at 531 and 526 codons of \( rpoB \) gene of MDR MTB in culture isolates as well as paired sputa. Of the total 85 MDR MTB isolates, 55 (64.70\%), 9 (10.59\%) and 9 (10.59\%) showed mutation at 531, 526 and at both 531-526 codons of \( rpoB \) gene, respectively. Therefore, overall 85.88\% of RIF resistant isolates had mutation covered by both the codons in \( rpoB \) gene region of MTB. The specificity of real-time PCR was found 98.27\% (57/58) for both specimen types. To check the accuracy, we compared the results of L-J proportion method and real-time PCR hybridization probe method that showed 90.90\% (130/143) of concordance for both the types of specimen (clinical sputa as well as their cultures).

The real-time PCR based mutation analysis of \( katG \) gene (at 314 and 315 codons) showed the sensitivity of method as 94.11\% (80/85) for detection of INH resistant MTB from the culture isolates as well as from the paired clinical sputa. Overall, MDR-TB isolates showed 77.64\% (66/85) mutation at 315 codon and 16.47\% (14/85) mutation at 314 codon. Therefore, both codons together cover 94.11\% mutation in \( katG \) gene of MDR MTB MDR-TB. The specificity of real-time PCR was 98.27\% (57/58) for both types of specimens. Overall, there were 95.80\% (137/143) concordance of results of real-time PCR and L-J proportion method for detecting the MDR and drug susceptible MTB from culture and sputa (Table 3). Interestingly, comparison
Table 4—Sequence analysis for identification of mutation at katG gene (16) in INH resistant cases and rpoB gene (15) in RIF resistant cases

<table>
<thead>
<tr>
<th>Mutated gene codon</th>
<th>Nucleotide substitution</th>
<th>Amino acid change</th>
<th>Mutation at codon</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutated katG</td>
<td>AGC-ACC</td>
<td>Ser→Thr</td>
<td>codon 315</td>
<td>4</td>
</tr>
<tr>
<td>Mutated katG</td>
<td>ACC-CCC</td>
<td>Thr→Pro</td>
<td>codon 314</td>
<td>3</td>
</tr>
<tr>
<td>Wild type</td>
<td>No change</td>
<td>No change</td>
<td>No mutation</td>
<td>9</td>
</tr>
<tr>
<td>Mutated rpoB</td>
<td>CAC-GAC and TCG-TTG</td>
<td>His526Asp and Ser531Leu</td>
<td>526 and 531</td>
<td>1</td>
</tr>
<tr>
<td>Mutated rpoB</td>
<td>TCG-TTG</td>
<td>Ser→Leu</td>
<td>531</td>
<td>5</td>
</tr>
<tr>
<td>Mutated rpoB</td>
<td>CAC-GAC</td>
<td>His→Asp</td>
<td>526</td>
<td>1</td>
</tr>
<tr>
<td>Wild type</td>
<td>No change</td>
<td>No change</td>
<td>No mutation</td>
<td>8</td>
</tr>
</tbody>
</table>

of the results of real-time PCR with respect to types of samples (clinical sputa as well as their cultures) analyzed showed 100% concordance.

Of the 16 isolates, subjected to DNA sequencing based validation of real-time PCR for ‘INH susceptibility testing’, 9 were sensitive to INH (6 pan susceptible and 3 susceptible to INH, EMB and STR) and remaining 7 were resistant to INH as determined by proportion method and real-time PCR hybridization probe method. As per the results derived from DNA sequencing, no mutation in all 9 INH sensitive MTB isolates was found whereas, of the 7 INH resistant MTB isolates (which showed mutation by FRET probes targeting 315 and 314 codons in real-time PCR), 4 showed change in amino acid Ser-Thr (AGC-ACC) at 315 codon and 3 isolates showed change in amino acid Thr-Pro (ACC-CCC) at 314 codon of katG gene region of INH.

To characterize the molecular basis of RIF and INH resistance in MDR-TB cases, the result profiles of real-time PCR hybridization probe method were compared with sequencing analysis and randomly (selection was on the basis of melting Tm variation showed by some RIF and INH resistant strains as compared to the melting Tm of control strain H37Rv showed in real-time PCR analysis) selected 16 MTB isolates were subjected to PCR sequencing of katG gene (Table 4). Some INH resistant isolates selected for sequencing on the basis of rise in melting temperature (Tm) up to <7°C (to confirm mutation at 315 codon) and drop in melting temperature (Tm) up to <1-3°C (to confirm mutation at 314 codon) as compared to the wild type control strain H37Rv (Tm 59.17°C).

Out of these, 9 INH sensitive isolates (6 were pan susceptible isolates and 3 susceptible to INH, EMB and STR only as determined by proportion method and real-time PCR hybridization probe method) did not show any mutation and they were wild type according to DNA sequencing and remaining 7 INH resistant M. tuberculosis isolates (which showed mutation by FRET probes targeting 315 and 314 codon in real-time PCR), 4 showed change in amino acid Ser-Thr (AGC-ACC) at 315 codon and 3 isolates showed change in amino acid Thr-Pro (ACC-CCC) at 314 codon of katG gene region of INH by DNA sequencing, so the results obtained by sequencing were in concordance with DST.

In order to characterize the molecular basis of RIF resistance and validating the results of real-time PCR, 15 randomly selected MTB isolates were subjected to DNA sequencing. Out of these, 8 RIF sensitive isolates (6 were pan susceptible and 2 were sensitive to RIF, EMB and STR only as determined by proportion method and real-time PCR hybridization probe method) did not show any mutation and were found as wild type by DNA sequencing. Whereas, of the remaining 7RIF resistant MTB isolates (which showed mutation by FRET probes targeting 531 and 526 codon in real-time PCR), one isolate showed change in amino acid in both codons His526Asp (CAC-GAC) and Ser531Leu (TCG-TTG) and 5 isolates showed change in amino acid TCG-TTG Ser→Leu at 531 codon and remaining one isolate showed change in amino acid CAC-GAC His→Asp at 526 codon by DNA sequencing (Table 4). Overall, the results of real-time PCR was concordant to that obtained by sequencing.

Discussion

Tuberculosis is a global public health problem. In recent years, various approaches have been employed to develop effective diagnostics and vaccines for the management of TB and other important mycobacterial infections. Several real-time PCR-based assays, including both commercial and in-house assays having high sensitivities and specificities have been
developed during the last few years[27-31]. However, they had problems such as: (i) faulty assay design based on a limited number of genes and sites that allowed some mutations go un-detected; (ii) the mixed population of resistant and susceptible bacilli could be detected, since both drug resistant and susceptible bacilli present in the patient during the early stages of the development of drug resistance; and (iii) no use of molecular tests for post-treatment efficacies as the test detects dead organisms, and so a positive test does not indicate treatment failure. In addition, high cost of equipment and reagents, laboratory space and need of trained manpower also limits the use of such molecular assays in routine practices in resource constrained settings.

Currently, a revolutionary equipment, Cepheid GeneXpert System (a single use sample processing cartridge system with integrated multicolor real-time PCR capacity) endorsed by WHO is available in public sector. Although, it simplified nucleic acid amplification tests and offer tests for both TB and drug resistance, issues such as high cost, requirement of stable electric supply and limited shelf-life of the diagnostic cartridges restrict its practical application in the developing countries including India. GenXpert only detects the mutations responsible for RIF resistance and not for INH resistance. Indeed, RIF resistance is surrogate marker for determining the MDR but the prevalence of RIF mono-resistance and INH mono-resistance is high and such information are important for programmatic management of drug resistant TB in the country. Recently, an Indian study observed low performance of GeneXpert in detection of RIF resistance (in RIF mono-resistant cases), thus putting question on implementation of this technology in national TB control programme in India[10]. Therefore, still there is plenty of opportunity to develop some indigenous molecular technology that can efficiently detect both RIF and INH resistance directly from clinical specimen. In the present study, we used real-time PCR hybridization probe method for rapid detection of MTB-DR MDR-TB cases directly from the clinical sputum samples and compared the results with their cultures for the genotypic analysis of RIF and INH resistance in tuberculosis. Despite its high sensitivity and speed, this method was seldom applied to evaluate clinical samples in India.

This method is based on the FRET probes which help in hybridization between amplicons and exposes the variability in the melting temperature (Tm) of mutant and wild type MTB strains on the basis of melting curve analysis. In-house designed probes and primers spanning the most prominent 4 selected codon (of wild type) covering region 531 and 526 of rpoB gene and 314 and 315 of katG gene have been used. This array of codons (except katG 314[13]) is reported in the literature as the most frequently mutated loci[3,4,10]. The information related to frequency of katG 314 mutation is limited in Indian MTB isolates. This method completes the whole analysis within 45-60 min, such closed-well system protocol has been reported to be helpful in making this technique 100% specific[33]. Other widely used Line probe assay are more time consuming and laborious than real-time PCR, mutation regions of the rpoB and katG genes that are not captured properly on the strips of the line probe assay may also be a possible limitation in the use of line probe assay[33].

In this study, the real-time PCR hybridization probe method correctly identified 85.8894.11 and 84.70% of the RIF, INH and combined resistance (MDR) in clinical sputum samples and their MTB cultures, respectively and had 98.27% specificity for both RIF and INH. Only one pan susceptible MTB sample showed mutation by real-time PCR analysis, this may be due to the coexistence of susceptible and resistant organism to anti-TB drugs in the same patients which is considered as preliminary stage of full resistance this phenomenon is referred to as hetero-resistance[36]. However, the relevance of hetero-resistance in TB was highly underestimated[37].

Interestingly, there was 100% concordance in the results profile of both specimen types (sputum samples as well as their MTB cultures), which support the reliability of clinical sputum samples for rapid detection of EMB susceptibility testing. The absolute concordance as observed, provides considerable evidence to support further studies of rapid detection using sputa for pulmonary TB cases. Superior sensitivity for molecular assay over gold standard culture method has been reported earlier for detection of MTB from clinical sputa media[38,39]. In this study, the validity of real-time PCR hybridization probe method is confirmed by sequencing of randomly selected 31 isolates in which MDR-TB isolates showed mutations at Ser531Leu, His526Asp of rpoB gene and Ser315Thr, Thr314Pro of katG gene. The distribution of mutations at rpoB531, rpoB526 and katG315, katG314 identified in our study is significantly different from what has
been reported earlier including the panel of MTB isolated in this study. In previously reported Indian studies the frequencies of these mutations were reported to be 41-43% in codon 531, 32-36% in codon 526 with most common changes in Ser 531 Leu, His 526 Tyr and these changes occur in more than 70-74% of RIF resistant isolates.\textsuperscript{3,4,10,40} In our study, we found that 85.88% of RIF resistant isolates had mutation at rpoB531, rpoB526 with distribution of 64.70% mutation at Ser531Leu, 10.58% at His526Asp and 10.6% at both codons. With respect to \textit{katG} mutation analysis, our study showed that 94.11% mutation occurs at \textit{katG}315, \textit{katG}314 codon among INH resistant strains with distribution of 77.64% mutation at Ser315Thr and 16.47% mutation at Thr314Pro substitutions. Overall, we demonstrated that higher percentage of INH and RIF associated mutations are covered by selected targets.

This study has ascertained the use of FRET probes hybridization probe real-time PCR method for rapid detection of MDR-TB clinical samples in India. Further, it provides scope for comparing the clinical usefulness of a genotypic method to that of a culture based phenotypic DST under routine conditions. Further studies may optimize the real-time PCR assay for evaluating MTB clinical samples effectively.

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