Evaluation of Multiplex Polymerase Chain Reaction Assay for the Detection of katG (S315T) gene Mutation in Mycobacterium tuberculosis Isolates from Puducherry, South India

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To evaluate the Multiplex Allele Specific-Polymerase Chain Reaction (MAS-PCR) assay for the detection of katG315T mutations in Mycobacterium tuberculosis (MTB) clinical isolates. Totally, 78 MTB clinical isolates were analyzed by conventional Lowenstein–Jensen (LJ), BACTEC MGIT-960™ liquid cultures, MAS-PCR, PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) and DNA sequencing methods. Fifty one (65.4%) out of 78 isolates were drug resistant, and 27 isolates (34.6%) were drug susceptible. MAS-PCR assay identified katG315 mutations in 47 (60.2%) isolates, in which 41 (52.5%) isolates had mutation at codon S315T (AGC→ACC), 6 (7.7%) isolates had mutation at codon S315N (AGC→AAC), and none of the 27 susceptible isolates had mutation. Concordant result was obtained by PCR-RFLP and DNA sequencing method was used further to validate the results obtained by MAS-PCR and PCR-RFLP assays. Novel mutations were found at G305V (GGC→GTC) and Q295P (CAG→CCG) codons in combination with S315T codon in 5 (6.4%) and 4 (5.1%) drug resistant strains respectively. katG315T was the frequently mutated codon of katG gene in M. tuberculosis isolates, which can be used as a marker of choice for direct detection of isoniazid resistance by MAS-PCR assay in regions where katG315T codon mutation is predominant among drug resistant isolates.

Key words: Mycobacterium tuberculosis, Multi drug resistance, katG, multiplex-PCR, PCR-RFLP, Isoniazid resistance.

One-third of the world population carries an asymptomatic infection with Mycobacterium tuberculosis, (MTB) which results in 9.0 million people were infected with tuberculosis (TB) and 1.5 million deaths in 2013 1,2. Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) are major public health problems, especially in developing countries1. Rapid diagnosis and appropriate chemotherapy become the first priority in controlling the growing epidemics of TB infection1,2.

Isoniazid (INH), a mainstay of the first-line drug against TB and mutations in various genes namely, katG, inhA, oxyR, kasA, aphC and ndh are responsible for INH drug resistance1. Particularly, katG gene codes for catalase-peroxidase and inhA gene codes for enoyl-acyl carrier protein reductase enzymes are key factors in mycolic acid biosynthesis and presence of mutations in these
genes show INH resistance (INH)
Clinical isolates were identified with absence/complete
deletion of katG gene has also exhibited INH
phenotype7. Altogether, mutations occurring in
katG and inhA genes account for up to 80% in
resistance to INH drug5,8. Moreover, majority of
mutations are associated with katG gene at codon
315 in 30–90% of INHR isolates spans across the
world9,10.
Many conventional approaches were
used to detect drug resistance in isolates but, they
are mainly time depending. Hence, numerous
Genotypic assays have been developed in past
decades to precisely target the mutated codon and
to rapidly detect drug resistance among clinical
isolates5,6,11-13. The genotypic methods described
so far for the detection of mutations in katG gene
at different codons which includes katG315. More
specifically, the second base of the katG gene at
315 codon S315T (AGC → ACC) substitution is the

Hence, this study was attempted to
evaluate the efficiency of MAS-PCR and PCR-
RFLP techniques to rapidly detect katG315
mutations in MTB clinical isolates in this region.

MATERIALS AND METHODS

*M. tuberculosis* clinical isolates and susceptibility
testing
Totally, 78 *M. tuberculosis* (MTB) isolates
were isolated from hospitalised patients with newly
diagnosed pulmonary TB infection and were
admitted as advised by physician to the hospital
in Government Hospital for Chest Diseases,
Intermediate Reference Laboratory at Puducherry.
Species identification was performed as described
previously2,14. Conventional solid Lowenstein-
Jensen (LJ) medium was used for the identification
and culturing of isolates, and susceptibility testing
was performed by the proportion method as
followed previously6,15. A microbial suspension was prepared
according to the McFarland turbidity standards
(0.5, 1) and was diluted 1:10; then, 0.2 ml of the
dilution was added to LJ medium slant with or
without a drug. Drug resistance was considered as
if bacterial growth was occurred at a
concentration of 0.02µg/ml of INH and resistance
proportion was calculated for each isolate16,17. The

DNA extraction
Genomic DNA was isolated from all the
clinical isolates by Phenol:Chloroform extraction
method as described previously18. Isolated
Genotypic DNA was stored at -20°C for further PCR
analysis.

PCR Amplification
PCR amplification of IS6110 element in
Mycobacterium genome was performed in an
authorized thermal cycler (Eppendorf Gradient
Cycler). The primers and PCR reaction conditions
were used this assay is listed in table 1 (2,19).
Totally 50 µl PCR mixture was used which consists,
10 pmol of each primer, 1.5 mM MgCl₂, 1U of Taq
DNA polymerase (New England Biolabs), 200µM
deoxynucleotide triphosphate. Amplified PCR
product was run on a 2% agarose gel and bands
were photographed using a Geldoc (Bio-Rad,
Germany).

MAS-PCR Assay
MAS-PCR assay was performed by using
two outer primers (Table 1)11 to amplify a product
of 435-bp (mutated allele) in the absence of the
katG315 AGC codon, and one inner reverse primer,
binds at katG315 AGC second base and amplifies
a product of 292-bp, (wild-type allele) in an
authorized thermal cycler (Eppendorf Gradient
Cycler). Totally 30 µl PCR mixture was prepared
which consists, 10 pmol of each primer, 1.5 mM
MgCl₂, 1U of Taq DNA polymerase (New England
Biolabs), 200µM deoxynucleotide triphosphate. The amplified fragments were electrophoresed in
2% agarose gels and photographed by Geldoc
system (Bio-Rad, Germany).

PCR amplification for PCR-RFLP analysis
A primer pair, katG1F and 4R (Table 1),
was used to amplify a 200-bp katG gene fragment,
as described14. Totally 30 µl PCR mixture was carried
out which consists, 10 pmol of each primer, 1.5 mM
MgCl₂, 1U of Taq DNA polymerase (New England
Biolabs), 200µM deoxynucleotide triphosphate. The amplified fragment was assessed by
electrophoresis in a 2% agarose gel.

Restriction Enzyme digestion
In a PCR tube, 10 µl of the PCR amplified
Table 1. PCR primers and reaction conditions for amplifying and DNA sequencing of *M. tuberculosis* drug resistance genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’–3’)</th>
<th>PCR product (bp)</th>
<th>PCR reaction condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS6110*</td>
<td>GTGAGGGCATCGAGGTGG CGTAGGCGTGCAGTCACAAA</td>
<td>123</td>
<td>94°C 5min, 35x (94°C 1min, 57°C 1min, 74°C 1min) 74°C 10min</td>
<td>Helen et al. 2003</td>
</tr>
<tr>
<td>mtp40</td>
<td>GGTTCACACACACCGTTAG CCAACATCGACGCGTACCC</td>
<td>332</td>
<td>95°C 5min, 35x (94°C 1min, 60°C 1min, 72°C 1min, 72°C 5min)</td>
<td>Alexis et al. 1996</td>
</tr>
<tr>
<td>katGb</td>
<td>GAAACAGCGCGCTGATGCTTGG TACCGGACCGGAGCCGGG</td>
<td>206</td>
<td>94°C 5min, 30x (94°C 1min, 60°C 1min, 72°C 1min, 72°C 5min) 72°C 5min</td>
<td>Somoskovi et al. 2006</td>
</tr>
<tr>
<td>katG1Fc</td>
<td>AGCTCGATGTCACGGGAGCAATGTCACGGGAGCAATGGG</td>
<td>200</td>
<td>96°C 3min, 20x (94°C 1min, 60°C 1min, 72°C 1min, 72°C 5min) 72°C 5min</td>
<td>Mokrousov et al. 2002a</td>
</tr>
<tr>
<td>katG4Rc</td>
<td>AAGCGGTGTCGAGGAGGATGTCAG</td>
<td>435</td>
<td>96°C 3min, 5x (95°C 1min, 64°C 1min, 5x (95°C 1min, 62°C 1min, 72°C 1min, 20x (95°C 1min, 60°C 1min, 72°C 1min, 72°C 5min)</td>
<td>Mokrousov et al. 2002a</td>
</tr>
</tbody>
</table>

a, Species identification  
* katG amplified products for DNA sequencing  
* PCR-RFLP for *katG* gene (132bp for the mutated (315 AGC→ACC) allele, the INH* strain, 153bp for the *katG* 315 wild-type or differently mutated alleles by *MspI* digestion)  
* MAS-PCR for *katG* gene (292-bp in the absence of mutation at *katG* 315 and 435-bp in the presence of mutation at *katG* 315)

Table 2. Prevalence of nucleotide and amino acid change among drug resistant isolates with *katG* mutations

<table>
<thead>
<tr>
<th>Codon(s)</th>
<th>Change of Nucleotide(s)</th>
<th>Change of amino acid(s)</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>315</td>
<td>AGC→ACC</td>
<td>Ser→Thr</td>
<td>33</td>
</tr>
<tr>
<td>315</td>
<td>AGC→AAC</td>
<td>Ser→Asn</td>
<td>5</td>
</tr>
<tr>
<td>305</td>
<td>GGC→GTC</td>
<td>Gln→Val</td>
<td>1</td>
</tr>
<tr>
<td>295</td>
<td>CAG→CCG</td>
<td>Gln→Pro</td>
<td>1</td>
</tr>
<tr>
<td>com. 315, 305</td>
<td>AGC→ACC</td>
<td>Ser→Thr</td>
<td>4</td>
</tr>
<tr>
<td>com. 315, 295</td>
<td>AGC→ACC</td>
<td>Ser→Thr</td>
<td>2</td>
</tr>
<tr>
<td>com. 315, 275</td>
<td>AGC→ACC</td>
<td>Ser→Thr</td>
<td>1</td>
</tr>
<tr>
<td>com. 315, 295</td>
<td>AGC→ACC</td>
<td>Ser→Thr</td>
<td>1</td>
</tr>
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<td>com. 315, 295</td>
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<td>Ser→Thr</td>
<td>1</td>
</tr>
</tbody>
</table>
product, 2U MspI restriction endonuclease (New England Biolabs), 2µl of 10X buffer4™, 18µl of nuclease free water were added and incubated at 37°C for 1h in an authorized thermal cycler as described11. The restriction fragments obtained were electrophoresed in a 2% agarose gel and documented.

DNA Sequencing

PCR reaction of katG gene was performed in all MTB isolates, spanning codon 315 (206-bp fragment) with the primers listed in Table 16,20 and subjected to direct DNA sequencing. Obtained sequences were analysed using the BLASTn and BLASx bioinformatics tools21 available at the National Centre for Biotechnology Information (NCBI) to determine the specificity of PCR amplification and to identify the nucleotide and amino acid changes in comparison with wild type M. tuberculosis (H37Rv). Moreover, MAS-PCR and PCR-RFLP assay results were further confirmed with DNA sequencing data.

Results

Drug susceptibility

Totally, 27 (34.6%) isolates were susceptible and 51 (65.4%) isolates were isoniazid resistant. Out of 51 drug resistant, 17 (26%) were isoniazid mono-resistant and 34 (52%) were MDR-TB isolates. 36 out of 51 (70.5%) drug-resistant clinical isolates showed a high level of resistance against the INH drug (1µg-10µg/mL) had S315T mutations. However, strains having S315N and Q295P mutations were susceptible at 1µg/mL. Drug susceptible strains were used as controls in this study to avoid discrepancy of MAS-PCR and PCR-RFLP results.

MAS-PCR results

A 292-bp PCR amplified product was observed in drug susceptible isolates indicating the absence of mutation in the target katG315 codon (katG315AGC) (Fig. 1, Lanes 5,6 and 8). A 435-bp amplified product was observed in drug-resistant isolates with the presence of katG315 AGC→ACC (Fig. 1, Lanes 2–4, 7, 9 and 10) and 292-bp amplified fragment was observed in isolates harboured katG315 AGC→AAC codons (Fig. 1, Lanes 11 and 12).

PCR-RFLP

PCR-RFLP reaction was performed and 200-bp amplified product was observed in all the strains (Fig. 2, Lane 2-7) Amplified PCR products were further digested by MspI enzyme. Totally, 41 (52.5%) isolates had ACC at codon 315 (S315T substitution) (Fig. 3, Lane 2, 3 and 5) and resulted in a 132-bp small fragment, INH1 isolates had wild type codon at 315 (AGC) produced band at 153-bp

Fig. 1. Examples of MAS-PCR amplification

Fig. 2. Examples of PCR-RFLP amplification

Fig. 3: Examples of Restriction digestion by MspI Enzyme
large fragment (Fig. 3, Lane 4) after enzyme digestion. Clinical isolates had S315N (AGC→AAC) and other mutations produced 153-bp large fragment (Fig. 3, Lane 6 and 7).

MAS-PCR amplification of either 292-bp or 435-bp fragment provided a PCR quality confirmation with results that were consistent with the results generated by PCR-RFLP analysis. This ruled out the eventual false-negative results obtained because of lack of amplification.

Sequencing results

A clear band at 206-bp region was observed in 2% agarose gel and confirmed the amplification of the katG gene. Sequencing results of the amplified katG fragment showed common mutations were displayed in Table 2. katG S315T (AGC→ACC) mutation alone was found majority in 33 strains (42.3%) followed by katG S315N (AGC→AAC) mutation alone was found in 5 strains (6.4%). One strain (1.3%) had G305V (GGC→GTC) mutation; one strain (1.3%) had Q295P (CAG→CCG) mutation. Combination of mutations were observed as follows, 4 strains (6.4%) had mutations at S315T and G305V codons, 2 strains (2.5%) had mutations at S315T and Q295P codons, one strain (1.3%) had mutations at S315T and Q275P codons, one strain (1.3%) had triple mutations at S315T, G305V and Q295P codons. Two INH<sup>a</sup> strains (2.5%) showed resistance in LJ and BACTEC MGIT culture methods, but those strains showed INH drug resistance in LJ and BACTEC methods. Therefore, this result is supportive to the hypothesis that the presence of mutations in other gene namely, inhA, aphC, oxyR are also responsible for INH drug resistant among drug resistant isolates<sup>9,17</sup>.

In accordance with previous reports, almost 92.1% (47 out of 51 MDR-TB) of the multidrug resistant strains had S315T substitution. Hence, the data suggests that, the presence of a similar mutation at codon 315 of the katG gene was found in drug-resistant MTB isolates in South India during 2011-2012<sup>24</sup>. Although a point mutation (AGC→ACC) resulting in 315 (Ser→Thr) substitutions is the most common, other mutations involving at codon 315 in the katG gene also occur in INH R strains<sup>25</sup>. In this regard, katG gene harbouring S315N (AGC→AAC) mutation was found as less frequent event in this region. 34 (66.7%) of the 51 drug-resistant strains with the katG315 substitution showed resistance to other first-line drugs such as rifampicin, ethambutol and streptomycin. In this respect it is then supporting the concept of strains already have the S315T mutation have more possibility of acquiring mutations in other genes too<sup>16</sup>.

In this study, katGS315T mutation was found in totally 47 INH<sup>a</sup> strains and katG315 wild-type allele (AGC) was identified in all INH<sup>a</sup> strains. Of the 47 out of 78 isolates, 41 isolates had S315T, 6 had S315N, 1 had G305V, and one had G295P mutations. Two strains, mutation was not identified by any of these methods used in this study, but those strains showed INH drug resistance in LJ and BACTEC methods. Therefore, this result is supportive to the hypothesis that the presence of mutations in other gene namely, inhA, aphC, oxyR are also responsible for INH drug resistant among drug resistant isolates<sup>9,17</sup>.

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The result of this study was highly corroborated with recent Indian data. The specificity of the proposed method was 92.1% (47 out of 51 isolates), since all the results agreed with the conventional drug susceptibility test and with the sequence analysis performed in the isolates under investigation. Strains harbouring katG S315N (AGC→AAC) mutation and other mutations such as, 305, 295 which produced discrepancy results with MspI digestion which, required further attention like digestion by MspAIH would be of interest.

CONCLUSIONS

In conclusion, the katGS315T mutation can be serve as a reliable marker for the detection of INH resistance in M. tuberculosis clinical isolates among drug-resistant strains. The MAS-PCR and PCR-RFLP assays that are described herein are rapid, making easy for the detection of resistance to INH in clinical isolates where this mutation is prevalent.

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Conflicts of interest

The authors declare that they have no conflict of interest.

REFERENCES


