Research Article

FREQUENCY OF MUTATION IN ISONIAZID RESISTANT ISOLATES OF Mycobacterium tuberculosis COMPLEX FROM WESTERN MAHARASHTRA INDIA

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Abstract- Introduction: The alarmingly worsening scenario of Multi drug-resistant tuberculosis (MDR-TB) call for urgent need for a simple method for the rapid detection of drug-resistant TB in clinical settings. Testing of mycobacterial culture and drug susceptibility testing (DST) capacity are limited in resource-scares countries; leading to inadequate treatment and development of favoring resistance. [1].

Isoniazid (INH) is an effective first-line anti-tuberculosis drug. KatG, a catalase-peroxidase enzyme, converts INH to an active form in Mycobacterium tuberculosis, and katG mutations are major causes of INH resistance [1,2]. The usefulness of INH, a key component of short-course chemotherapy of tuberculosis, is threatened by the emergence of drug-resistant strains of MTB with mutations in the katG gene. This study is an effort to study the frequency of mutations in INH mono resistant isolates and in MDR strains of MTB complex with two different molecular methods, LiPA and Dideoxy sanger sequencing for single nucleotide polymorphism from Western Maharashtra.

Material and Methods

Study was conducted in tertiary care hospital from Dr. D. Y. Patil Medical College, Hospital & Research Centre, Pune India over a period of two years i.e. January 2014- December 2016.

Methods: Samples were processed for two molecular methods, GenoTypeMTBDRplus (LiPA) and Dideoxy Sanger Sequencing. Samples processed for DNA extraction, nested PCR reaction was done by annealing at 55°C with specific primers. After confirmation of band on Gel Doc, Sequencing was done with one primer. Sequencing was also done for inhA and inhA promoter region. Result: Major mutation found was S315T which is in katG gene of drug resistant strains of MTB complex. Two different methods were chosen as LiPA was available at institute and it gives information about MTBC complex & drug resistance in shorter time but could not provide information about exact Single nucleotide polymorphism (SNP). To confirm the exact SNP mutation and validation of LiPA results Sanger sequencing was the best method available in collaboration.

Inclusion criteria

Monodrug and Multidrug resistant isolates (growth cultures or excess amount availability of decontaminated sediment).

LiPA test was criteria along with clinical correlation from suspected cases of tuberculosis; clinical samples, Sputum, BAL Pleural Fluid, Tissue, Pus, CSF. Extracted DNA required for both molecular testing could be possible only with grown cultures or excess amount of decontaminated sediment

LiPA was performed as per manufacture’s instruction.

Dideoxy Sanger Sequencing

Isolation of genomic DNA

DNA was isolated using QIAGEN kit DNAeasy (CAT.NO. 56404) as per
manufacturer’s instructions.

**katG genotyping**

**PCR Primers and Amplicon size**

KatG gene amplification using nested PCR primers targeting for catalase-peroxidase gene of M. tuberculosis was performed. Nested primers included one outer set of primers and one inner set of primers. Upstream outer primer [9, 10] and downstream outer primer 5’TACGGCGGTACTGGAAGA3’, and inner primer set for the second round consisting of upstream primer 5’GTCCTTGGCCG GTGTATT3’ and downstream primer: 5’CATGACAGACGTCGAAAC 3’. Outer primer set codes for 547 bp region from KatG gene, while the inner primer set codes for a region 304 bp within the 547 bp. Analysis of the results on agarose gel electrophoresis and visualization of the amplified products over the GelDoc - XR (BioRad) was done for 2nd round amplification products.

**PCR Reaction setup**

PCR mix typically consisted of 50 uL of final reaction volume containing 10mm Tris-HCl (pH 8.3), 50mm KCl, 2.5mm MgCl₂, 0.01% (w/v) gelatin, 50 pmol of respective primers (mentioned above), 2.5 nmol of each of the four deoxy nucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 1U of Taq DNA polymerase (Invitrogen, USA). The PCR cycle conditions were: Initial denaturation at 95°C for 5 minutes, 95°C for 1 minute to denature the DNA, then cooled to 55°C for 45 sec, heating to 72°C for 1 min for extension, cycle repeated 30 times with final incubation at 72°C for 10 min, for nested PCR 94°C for 30 s to denature the DNA, then cooled to 52°C for 30s, heating to 72°C for 30 sec for extension, cycle repeated 35 times with final incubation at 72°C for 10 min. Amplicons from first PCR were used as template for second round nested PCR.

![PCR Reaction setup](https://umr5558.bibiuniv.lyon-fr/mubii-select.cgi)

**Table-1 PCR REACTION /PRIMERS**

<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR REACTION /PRIMERS</th>
<th>Amplicum size</th>
<th>Annealing Temp</th>
<th>Mutation position</th>
</tr>
</thead>
<tbody>
<tr>
<td>katG</td>
<td>Fw: 5’ CGGTAGCGCCGCTCATAGAT3’</td>
<td>547 bp</td>
<td>55 °C</td>
<td>S315T</td>
</tr>
<tr>
<td>(nested)</td>
<td>Rx: 5’ TACGGCGGTACTGGAAGA3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>katG</td>
<td>Fw: 5’ GTCCTTGGCCG GTGTATT3’</td>
<td>304 bp</td>
<td>52 °C</td>
<td>S315T</td>
</tr>
<tr>
<td>(nested)</td>
<td>Rx: 5’ CATGACAGACGTCGAAAC 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>inhA promoter</td>
<td>Fw: 5’ GGCACTGACAGCTGTATATGTA 3’</td>
<td>479 bp</td>
<td>55 °C</td>
<td>C15T / A16G</td>
</tr>
<tr>
<td></td>
<td>Rx: 5’ GGTCCTTGGCCG GTGTATT3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>inhA</td>
<td>Fw: 5’ AAGCCGATTCGTTGGAAGC 5’</td>
<td>300 bp</td>
<td>55 °C</td>
<td>T8C / T315A</td>
</tr>
<tr>
<td></td>
<td>Rx: 5’ GGGTTGATGCCCATCCCGG 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table-2 Association of mutation pattern in LIPA and Sequencing**

<table>
<thead>
<tr>
<th>Gene</th>
<th>LIPA falling Wild type band</th>
<th>Codon analyzed</th>
<th>Developing mutation band</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>katG</td>
<td>katG WT 1</td>
<td>315</td>
<td>KatG MUT 1</td>
<td>S315T</td>
</tr>
<tr>
<td></td>
<td>KatG MUT 2</td>
<td></td>
<td>S315T 2</td>
<td></td>
</tr>
<tr>
<td>inhA</td>
<td>inhA, WT 1</td>
<td>-15</td>
<td>inhA, MUT 1</td>
<td>C15T</td>
</tr>
<tr>
<td></td>
<td>inhA, MUT 2</td>
<td>-16</td>
<td>inhA, MUT 2</td>
<td>A16G</td>
</tr>
<tr>
<td></td>
<td>inhA, WT 2</td>
<td>-8</td>
<td>inhA, MUT 3A</td>
<td>T8C</td>
</tr>
<tr>
<td></td>
<td>inhA, MUT 3B</td>
<td></td>
<td>inhA, MUT 3B</td>
<td>T315A</td>
</tr>
</tbody>
</table>

**Results**

**Polymerase Chain reaction result:**

Each sample show PCR amplicons of desired size on agarose gel

![Fig-1 Kat-G gene PCR IMAGE: 1% (WV) Agarose Gel electrophoresis: Lane 1: 500 bp DNA marker; Lane 2: NTC; Lane 4 and 8: sample PCR Products](https://tbdreamdb.ki.se/Info/Default.aspx)

**DNA sequencing**

Sequences were aligned with katG REF or wild type sequence gene using-http://www.genome.jp/tools-bin/dustalw/Kyoto University Bioinformatics Center) [11]

Following are the genes sequenced and are analyzed for drug resistance using different online available tools and databases. https://umr5558.bibiuniv.lyon-fr/mubii-select.cgi [12], https://tbdreamdb.ki.se/Info/Default.aspx [13], http://tuberculist.epfl.ch/index.htm [14].

![Fig-2 Mutation site for S315T Mutation S315T/GCT-GGT](https://tbdreamdb.ki.se/Info/Default.aspx)

Total 50 samples processed for Line Probe assay. Male to Female ratio was 26:24. Out of 50 samples, 38 were Pulmonary samples and 12 Extra-Pulmonary samples.ZN Staining was positive in 34 samples and 16 were Negative. From these 50 samples 30 samples were further processed for sequencing which were observed to be resistant for isoniazid by LIPA. From 21 MDR sequenced samples 19 showed only S315T mutation and 2 samples showed more than one mutation in addition to S315T. One sample showed N218K, V230A and T108C at promoter site. Another sample showed only G99T promoter mutation. From 9 mono resistant sequenced samples 5 showed S315T mutation, one sample showed only C101T promoter site mutation and rest did not show any mutations.

**NCBI Accession numbers**

Bankit 2051477 - MG019932 - MG019937
Bankit 2051485 - MG019938 - MG019947
Bankit 2051500 - MG019948 - MG019955

**Discussion**

The distribution and frequency of drug resistance mutations are variable across regions and countries. The frequency of katG mutation ranged from 58.5% to 93.7% and for inhA is 4.7% to 79.4%. The mostly commonly mutated katG locus was katG315 and for inhA was 15 [15]. Genotypic MTBDRplus were relatively rapid and simple method for detection of drug resistant TB but the disadvantage of this test is resistance detection is available for limited number of mutations. LIPA is also very much useful in Extra pulmonary TB also Other rapid molecular method as GeneXpert cannot detect the INH resistance. GenProbe can detect MTB complex for extra pulmonary samples but cannot detect drug resistance [16,17]. Phenotypic studies were not possible if there was contamination or less colony number for preparation of inoculum. Challenge is to diagnose extra-pulmonary tuberculosis even for the most practiced clinicians as clinical manifestations are vague, non-specific and typical chest radiograph findings may not be evident till late in the disease. Phenotypic methods for mycobacteriological culture and drug susceptibility testing are slow and cumbersome. Newer techniques for rapid detection of MBL and its anti-TB drug resistance have therefore become a priority hence with the development of molecular tests e.g., LIPA are most advanced [18]. S315T mutations are the common katG mutations observed in this study correlates with the other studies and commonly associated with high level INH resistance [19]. Studies have revealed that mutation in katG gene is responsible for 60-70% of isoniazid resistant strains. A study by Negi et al., in India reported 74.19% of S315T KatG mutation in MTB strains from Delhi [20]. But in contrast the...
Ser315Thr mutations accounted for 52.64% of strains in Central Asia. There is need for development of kits targeting more resistant gene mutations, possibly targeted for each specific geographic region. Two different mutations of katG observed in this study were N218K, V230A and different promoter mutations were also observed as C101T, T108C, G99T. This insists for more mutation studies to be done [21]. A study done by Hazbon et al. indicated that most studies examined relatively small numbers of isolates or failed to include sufficient number of drug susceptible controls to demonstrate statistically significant associations [22].

Conclusion: S315T can be potential genetic marker for isoniazid resistance. These mutations can be rapidly evaluated by rapid molecular diagnostic methods as LIPA.

Application of the research: Early detection of resistant strains of Mycobacterium tuberculosis infection will help in the institution of suitable therapy and also helps to reduce treatment failure and increase of resistant strains.

Research category: MDR Mycobacterium tuberculosis

Abbreviation:
MDR: Multidrug resistant

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Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

References