PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF ISONIAZID RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

SOUNDHARI C & RAJARAJAN S
PG & Research Department of Microbiology & Biotechnology, Presidency College, Chennai, Tamil Nadu, India

ABSTRACT

Tuberculosis (TB) is a single infectious disease, caused by Mycobacterium tuberculosis and represent high mortality of people in many countries. It is estimated that over three million people die a year of tuberculosis. Isoniazid (isonicotinic acid hydrazide; INH) is one of the structurally simplest primary chemotherapeutic and prophylactic drugs used to treat M. tuberculosis since 1952. INH is the most widely administered to tuberculosis patients than any other drug, and among the antituberculosis drugs, it is against INH emergence of resistance have been frequently reported. INH-resistance is apparently controlled by a complex genetic system that involves several genes, namely, katG, inhA, oxyR, ahpC. The two predominant mutations of katG, and those most referred to, are found within codons 315 and 463. The primary target of activated INH is an NADH-dependent enoyl-acyl carrier protein reductase, designated InhA. The aim of this study is to confirm M. tuberculosis to PCR targeting MPB64 gene and IS6110 and to determine isoniazid resistance by in vitro phenotypic resistant to the drug by Broth Microdilution method and resistance associated mutation by DNA isolation, PCR amplification, DNA sequencing analysis to detect mutations within katG gene and inhA gene of the isolates to search further why the isolate become isoniazid-resistant. The study revealed INH-resistant isolates for which the INH MICs were 0.19 to 3.125 mg/ml had polymorphism in codon 463 (R463L), and insertions at 323, 719 and 720 with these mutations conferring low level INH resistance.

KEYWORDS: Isoniazid Resistant Mycobacterium tuberculosis, katG, inhA, MPB64, IS6110

INTRODUCTION

Tuberculosis (TB) is a single infectious disease, caused by Mycobacterium tuberculosis and represent high mortality of people in many countries. It is estimated that over three million people die a year of tuberculosis (World Health Organization, (http://www.who.int/mediacentre/factsheets/fs104/en/)). In 2011, there were an estimated 8.7 million incident cases of TB (range, 8.3 million–9.0 million) globally, equivalent to 125 cases per 100 000 population (Fig 1). In 2011, there were an estimated 8.7 million new cases of TB (13% co-infected with HIV) and 1.4 million people died from TB, including almost one million deaths among HIV-negative individuals and 430 000 among people who were HIV-positive (Fig. 2). TB is one of the top killers of women, with 300 000 deaths among HIV-negative women and 200 000 deaths among HIV-positive women in 2011. The five countries with the largest number of incident cases in 2011 were India (2.0 million–2.5 million), China (0.9 million–1.1 million), South Africa (0.4 million–0.6 million), Indonesia (0.4 million–0.5 million) and Pakistan (0.3 million–0.5 million). Resistance to primary antitubercular drugs complicates treatment and makes TB more difficult to manage

Isoniazid (isonicotinic acid hydrazide; INH) is one of the structurally simplest primary chemotherapeutic and prophylactic drugs used to treat M. tuberculosis since 1952. INH is the most widely administered to tuberculosis patients than any other drug, and among the antituberculosis drugs, it is against INH emergence of resistance have been frequently reported. (Nusrath Unissa et al 2008) A high prevalence of resistance to isoniazid (INH), a first line antituberculous
antibiotic, has been observed around the world (Ducasse-Cabanot et al 2004). Resistance to INH occurs more frequently than for most anti-tuberculosis drugs, at a frequency of 1 in 105-6 bacilli in vitro (Zhang and Yew 2009). A data from WHO sponsored Global project on Anti –TB drug surveillance has indicated that 36% of TB patients are infected with strain resistant to traditional antibiotics, such as, isoniazid and rifampin. (Ramesh et al 2012). Isoniazid has remarkable specificity for M. tuberculosis, with a minimum inhibitory concentration (MIC) of 0.02 mg/L (Zhang Y and Young D 1994).

Nucleic acid amplification using IS6110 primers to detect M. tuberculosis has been extensively used as a laboratory tool for the diagnosis for tuberculosis. The insertion sequence IS6110 is a mobile genetic element and has universal acceptance, since it is found only in M. tuberculosis complex group of mycobacteria. M. tuberculosis strains typically carry multiple copies of the element, although a minority strains with only a single copy have been identified (Gunisha et al 2000). Also detection of M. tuberculosis genome with primers targeting the MPB64 gene for the immunogenic protein found only in culture filtrates of M. tuberculosis and occasional isolates of M. bovis BCG have been reported. These are absolutely specific and sensitive to detect M. tuberculosis.

INH enters the mycobacterial cell through passive diffusion and kills only dividing bacteria and lack of killing is found when mycobacteria are in stationary phase. Mycolic acid are long-chain α- alkyl β-hydroxy fatty acids, which are essential component of the mycobacterial cell wall. INH induces the loss of mycobacterial acid fastness and also induces morphological changes in mycobacteria like wrinkles and bulging.

INH-resistance is apparently controlled by a complex genetic system that involves several genes, namely, katG, inhA, oxyR-ahpC. The katG gene codes an 80-kDa hemoprotein of 744 aminoacids for expression of catalase and peroxidase activity (Slayden and Barry 2000). The catalase –peroxidase activates INH to generate free radicals, which attack multiple targets in the cells. The two predominant mutations of katG, and those most referred to, are found within codons 315 and 463. The primary target of activated INH is an NADH-dependent enoyl-acyl carrier protein reductase, designated InhA. Mutations within the inhA structural gene or within the inhA promoter have been identified and are associated with INH resistance.

Min Zhang et al 2005 in their investigation found five novel mutations within katG. They were mutations Gly3Cys (GGC3TGC) at codon 491, Arg3Tyr (CGC3TAC) at codon 515, and Gly3Arg (GGC3CGC) at codon 685, deletion of G at position 1559, and a 64-bp fragment insertion at position 1559.

Bostanabad SZ 2008 detected the following isoniazid associated mutations by performing katG amplification and DNA sequencing. Most mutations were found in katG codons 315,316 and 309.

Bolotin et al 2009 carried out molecular characterisation of drug resistance in studies from Mycobacterium tuberculosis isolates of Ontario and found 5 types of mutations at codons 304,315,336,414 and 457


Zakerbostanabad S et al 2009 observed predominant nucleotide changes were observed in 315, 316 and 309. Mutations were also found in codon 357. In addition two mutations which were also observed in codons 463 and 454 (MIC≤2). Three silent mutations were identified in four isolates in codons 306, 309 and 314. These silent mutations did not show an effect on the susceptibility testing pattern.
Lily Therese K et al. 2012 confirmed the isolates in their study as M. tuberculosis by PCR targeting MPB64 and IS6110 region from different clinical specimen.

The aim of this study is to confirm M. tuberculosis to PCR targeting MPB64 gene and IS6110 and to determine isoniazid resistance by in vitro phenotypic resistant to the drug by Broth Microdilution method and resistance associated mutation by DNA isolation, PCR amplification, DNA sequencing analysis to detect mutations within katG gene and inhA gene of three isolates to search further why the isolate become isoniazid-resistant.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Ten clinical resistant isolates of M. tuberculosis were obtained from patients with pulmonary tuberculosis from the Tuberculosis Research Centre (NIRT), Chennai.

Strain Determination

Specificity of M. tuberculosis was done by using the isolated DNA with specific primers for IS6110 and MPB64. The isolate was subjected to PCR targeting MPB64 gene and IS6110.

Drug Susceptibility

Phenotypic drug susceptibility was performed by Broth Micro dilution Method. INH MICs were determined for the resistant isolates. The isolates were cultured in Middlebrook 7H9 broth containing two fold concentrations of INH ranging from 25 to 0.01 μg/ml. The MIC was defined as the lowest concentration of INH that prevented growth. M. tuberculosis strain H37Rv was used as a susceptible control.

Inoculum Preparation for BMM

Freshly grown colonies from LJ medium were transferred to a tube containing 3-4 ml phosphate buffered saline and 6 to 9 sterile glass beads. Tubes were vigorously agitated on a vortex mixer and clumps were allowed to settle for 30 min. The supernatant was transferred to sterile tubes. The supernatant was then adjusted with phosphate buffer saline to equal the density of 0.5 McFarland standards for use as the standard inoculum in the Broth Micro dilution Method (BMM) (Clarice Queico Fujimura Leite et al. 2000; Coban et al. 2004).

Determination of Minimal Inhibitory Concentration (MIC) by Broth Microdilution Method

The BMM was performed in 96-well microtitreplates with U-shaped wells. Wells were filled with 0.1 ml amounts of Middlebrook 7H9 broth, supplemented with oleic acid, albumin, dextrose and, catalase (ADC) enrichment. The stock suspensions of drugs were diluted in Middlebrook 7H9 medium and seven serial dilution for each drug were prepared and 0.1 ml volumes were dispensed into plates (Clarice Queico Fujimura Leite et al. 2000; Coban et al. 2004).

Inoculation

Each well was inoculated with 5 μg/ml of bacterial suspension (0.5 MacFarland standard). Medium without antimicrobial agents was inoculated with same suspension and with a 100 fold diluted suspension, as a growing control. The plates were sealed put in plastic bags and incubated at 37°C for 28 days in a moisturized incubator. The bacterial growth (turbidity) was examined 2, 8, 14, 20 and 28 days after incubation. (Clarice Queico Fujimura Leite et al. 2000; Coban et al. 2004)
Polymerase Chain Reaction for *M.tuberculosis* Genome

PCR for detection of *M.tuberculosis* was carried out by nested amplification using the primers targeting IS6110 region and MPB64 gene at VRF, Chennai.

Amplification of DNA for IS6110

Amplification of *M. tuberculosis*--specific 200-bp DNA was done in a 50-ml reaction mixture, which consisted of 5 µl of 10X buffer (500 mM potassium chloride, 100 mM Tris chloride, 15 mM magnesium chloride, gelatin 0.1%, pH 8.3), 50mM each of primers, 200 mM of each deoxyribonucleotide triphosphate, 1 U Taq DNA polymerase and 5 µl of DNA template. Distilled water was added to make the volume to 50 µl.

The reaction mixture was overlaid with 50 µl of sterile mineral oil. Amplification was performed programmable thermal cyclers (Perkin–Elmer model no. 0480 Omni Gene Hybaid).

The primers used are listed in table 1 Amplification was done by (i.e., denaturation for 5 minutes at 94°C, followed by a three step profile: denaturation at 94°C for 2 minutes, annealing for 2 minute at 68°C, and extension for 2 minute at 72°C for a total of 30 cycles. Extension time was increased by 5 seconds with each subsequent cycle. Analysis of the results on agarose gel electrophoresis and visualization of the amplified products over the UV transilluminator was done (Figure 1) (Gunisha et al 2000)

![Figure 1: Gel Photo for PCR Amplified Product of IS6110](image)

Ethidium bromide stained 2% agarose gel with amplification product IS6110 gene from Isoniazid resistant isolates. lane 1- NC – Negative control, lane 2- H37Rv, lane 3- sample 485, lane 4-sample 567, lane 5- sample 597, lane 6- H37Rv standard positive control, lane 7 -100 bp molecular weight ladder.

<table>
<thead>
<tr>
<th>Primers(1:25 Diluted)</th>
<th>Primer Sequence : IS6110</th>
<th>Amplified Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I ROUND</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1 5’GTGAGGGGCATCGAGGTGG 3’</td>
<td></td>
<td>240 bp</td>
</tr>
<tr>
<td>R1 5’CGTAGGCGTCGTCGACAAA3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>II ROUND</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2 5’GATGCACCGTCAACCG 3’</td>
<td></td>
<td>200 bp</td>
</tr>
<tr>
<td>R2 5’CCACGGTAGGCGAACCCCT3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Amplification of DNA for MPB64 Gene

Amplification of *M. tuberculosis*-specific 200-bp DNA was done in two rounds. First step of amplification was done in a 50-ml reaction mixture, which consisted of 5 µl of 10X buffer (500 mM potassium chloride, 100 mM Tris chloride, 15 mM magnesium chloride, gelatin 0.1%, pH 8.3), 100 ng each of primers, 200 mM of each deoxyribonucleotide triphosphate, 1 U Taq DNA polymerase and 5 µl of DNA template. Distilled water was added to make the volume to 50 ul. The reaction mixture was overlaid with 50 µl of sterile mineral oil. PCR was performed in a Perkin–Elmer automatic thermal cycler. The primers used are listed in Table 2. Amplification was done by using a three step profile (i.e., denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C, and extension for 1 minute at 72°C for a total of 35 cycles). Subsequently, a second step amplification was performed using 5 µl each of the first-round products. A reaction mixture was constructed in the same way, except that the outer sets of primers were replaced by the inner sets.

A cycle count of 25 cycles (i.e., total of 60 cycles) was adopted (Tables : 4,5,6) Each set of amplification was done in the presence of negative controls— one for sample extraction and another as a reagent control— and a positive control, which consisted of 5 µl *M. tuberculosis* H37Rv DNA. The n PCR for each specimen was repeated at least twice to confirm the reproducibility of the results. Analysis of the results on agarose gel electrophoresis and visualization of the amplified products over the UV transilluminator was done for both the 1st and 2nd round amplification products. (Figure. 2) (Madhavan HN et al 2000; Madhavan HN et al 2002)

![Gel Photo for PCR Amplified Product MPB64 Gene](image)

Ethidium bromide stained 2% agarose gel with amplification product MPB 64 gene from Isoniazid resistant isolates.

<table>
<thead>
<tr>
<th>Primers(1:25 Diluted)</th>
<th>Primer Sequence : MPB64</th>
<th>Amplified Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I ROUND</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>5’ TCCGCTGCCAGTCTTTCC 3’</td>
<td>240 bp</td>
</tr>
<tr>
<td>R1</td>
<td>5’ GTCTCGCGACTCTAGGCA 3’</td>
<td></td>
</tr>
<tr>
<td><strong>II ROUND</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>5’ ATTGTGCAAGGTAAGGCTAG 3’</td>
<td>200 bp</td>
</tr>
<tr>
<td>R2</td>
<td>5’ AGCATCGAGTCTCGCGGGA 3’</td>
<td></td>
</tr>
</tbody>
</table>
DNA Extraction and PCR Based DNA Sequencing Targeting *Katg* and *Inha* Gene

Molecular Characterization of Resistant Strains

It included three main steps:

- DNA extraction to release DNA from mycobacterial cells
- Amplification of the target part of gene by PCR and detection by agarose gel electrophoresis.
- Automated DNA sequencing to detect mutation in comparison with the similar region of a wild strain

DNA Extraction

Chromosomal DNA was extracted from those resistant isolates with MIC higher by keeping the suspension at 80°C for 20 minutes. Genomic DNA was prepared using QIAamp DNA Mini kit (qiagen) as per manufacturer’s instruction.

DNA Amplification

PCR Amplification of *KatG* Genes

PCR was carried out targeting *katG* and *inha* genes. Table 3 lists the sequences of different primers used in the study with the thermal profile and amplified product size.

A 1215-bp fragment of *katG* gene, was amplified by using the primers (F- AGC TCG TAT GGC ACC GGA AC R- TCA GCG CAC GTC GAA CCT G) targetting codon 320-740 and 200 bp fragment *katG* gene was amplified with primers (F -AGC TCG TAT GGC ACC GGA AC R- AAC GGG TCC GGG ATG GTG) targetting codon 315. The amplified products were subjected to electrophoresis on 2% agarose gel incorporated with 0.5 μg/ml ethidium bromide for visualisation by UV transilluminator (Figure 3)

![Figure 3: Gel Photo for PCR Amplified Product for KatG Gene](image)

Ethidium bromide stained 2% agarose gel with amplification product of *KatG* gene from Isoniazid resistant isolates.

PCR Amplification of *Inha* Genes

PCR was carried out targeting *inha* genes. Table 3 lists the sequences of different primers used in the study with the thermal profile and amplified product size. A 200-bp fragment of *inha* gene was amplified using the primers (F- CCT CGC TGC CCA GAA AGG GA R- ATC CCC CGG TTT CCT CCG GT) targeting the regulatory region of the gene. The
amplified products were subjected to electrophoresis on 2% agarose gel incorporated with 0.5 μg/ml ethidium bromide for visualisation by UV transilluminator (Figure 4)

![Figure 4: Gel Photo for PCR Amplified Product for Inha Gene](image)

Ethidium bromide stained 2% agarose gel with amplification product of KatG gene from Isoniazid resistant isolates.

**DNA Sequencing and Data Analysis**

DNA sequencing was carried out using an ABI prism automated DNA sequencer. The sequences generated were compared with the wild type sequence by using Lalaign software to identify the presence of mutation.

<table>
<thead>
<tr>
<th>Target Gene Primer</th>
<th>Primer Sequence (5'-3')</th>
<th>Thermal Profile</th>
<th>No. of Cycles</th>
<th>Amplicon (Bp)</th>
<th>Accession Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>KatG (S)</td>
<td>F-AGC TCG TAT GGC ACC GGA AC R-AAC GGG TCC GGG ATG GTG</td>
<td>94°C – 5 min 60°C – 1 min 72°C – 5 min</td>
<td>30</td>
<td>202</td>
<td>KC197358</td>
</tr>
<tr>
<td></td>
<td>KatG (S)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F-AGC TCG TAT GGC ACC GGA AC R-TCA GCG CAC GTC GAA CCT G</td>
<td>94°C – 5 min 60°C – 1 min 72°C – 5 min</td>
<td>30</td>
<td>1215 1211 1210</td>
<td>JX995365 JX995364 JX995363</td>
</tr>
<tr>
<td>InhA (R)</td>
<td>F- CCT CGC TGC CCA GAA AGG GA R-ATC CCC CGG TTT CCT CCG GT</td>
<td>94°C – 5 min 55°C – 1 min 72°C – 7 min</td>
<td>35</td>
<td>211 211 248</td>
<td>JX975615 JX975616 JX995366</td>
</tr>
</tbody>
</table>

**RESULTS**

In an effort to study molecular insights into INH resistance at the genomic level three phenotypically isoniazid resistant clinical isolates obtained from TRC, Chennai were studied. Analysis of the results of PCR amplified MPB 64 gene and IS6110 of M. tuberculosis DNA on agarose gel electrophoresis with positive and negative control and visualization of the amplified products over the UV transilluminator confirmed the isolates genotypically as M. tuberculosis.

All the three isolates used in the study were resistant to isoniazid. MICs were generally read when the organisms had reached good growth in control wells after 14-17 days but the reading was repeated after 20 and 28 days. Strains had no change in MIC between 17 to 28 day of incubation. The MICs greater to critical concentrations were considered resistant. Differences were seen amongst the isolates with MIC values ranging between 0.19 to 3.125 mg/ml. Among ten
isolates 3 isolates showed MIC values higher than 1 mg/ml which included two INH monoresistant isolates (485 & 597) and a polyresistant isolate (567). Subsequent studies were restricted to these isolates.

PCR targeting different regions of katG gene of M. tuberculosis was carried out. The study detected the following isoniazid associated mutations by performing amplification of katG and inhA gene and DNA sequencing. The most commonly encountered aminoacid mutation S315T however was not found in any of the three isolates examined. Compared with catalase peroxidase of H37Rv polymorphisms at codon 463(CGG-CTG) mutations were found in katG (R463L) and insertion at positions 323, 719 and 720. These mutation were associated with relatively low levels of drug resistance (MIC of 1.5 to 3.125 mg/ml)

Compared with 3 o xoacyl carrier protein reductase of H37Rv no mutation was observed at the nucleotide level in inhA gene.

DISCUSSIONS

INH-resistant isolates for which the IHN MICs were 1.5 to 3.125 mg/ml had mutations in codon 323, 463 (R463L), 719 and 720. This is a low level mutation recorded. Polymorphisms at codon 463 positions resulting in Arginine to Leucine conversion has been recorded by many researchers. The mutations in codon 323 (insertion), 719 (insertion) and 720 (insertion) have been recorded perhaps for the first time.

The absence of mutation in studied regulatory region inhA of INH resistant isolates could be attributed to possible involvement of other codon positions at the same gene or other genes. Our study showed a correlation between the MICs of INH at these codons confers low levels of mutation in Mycobacterium tuberculosis.

ACKNOWLEDGEMENTS

Our sincere thanks to the Dr. N.Selvakumar, Department of Bacteriology, Tuberculosis Research Center (NIRT) for providing Mycobacterium tuberculosis clinical isolates.

REFERENCES


Phenotypic and Genotypic Characteristics of Isoniazid Resistance in *Mycobacterium tuberculosis*


