

## ORIGINAL ARTICLE

# **katG GENE ASSOCIATION WITH ISONIAZID RESISTANCE IN MULTIPLE DRUG RESISTANT TUBERCULOSIS IN PAKISTAN**

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## ABSTRACT

**Background:** The unfolding of multi-drug resistant Mycobacterium Tuberculosis (MDR-MTB) constitutes a grave threat to the current endeavors for controlling the tuberculosis outbreak. During TB treatment, the drug-resistance with 1st line drugs like Rifampicin (RIF) and Isoniazid (INH) has to be re-evaluated. It is assumed that gene mutation is underlying etiology for drug resistance during TB treatment. In Isoniazid resistant MTB, researchers have reported point mutation in katG gene at codon 315. The main objective of this study was to evaluate katG gene association with isoniazid (INH) resistance in our population.

**Methods:** Sputum specimens from suspected TB were inoculated in LJ Medium after proper decontamination. Identification of the organism colonies growth on slopes was done followed by drug sensitivity analysis. Isolates having resistance to Isoniazid (INH) and Rifampicin (RIF) with or without any other drug resistance were categorized as Multi-drug resistant (MDR). Kit method was used for DNA extraction. Then region from base 2714 to 3232 of katG gene was amplified through PCR followed by sequencing with the help of BLAST-alignment software. 24 Multi Drug Resistant MTB specimens were processed.

**Results:** DNA sequence analysis revealed the reported mutation Ser → Thr in katG codon 315 in five samples (21%). In this study, an authentic molecular analysis (test) was developed and validated for identification of INH resistant strains in Pakistani population.

**Conclusion:** Our study concludes that mutation of some genes particularly point mutation of katG gene at codon 315 is associated with development of resistance to the drug INH in Multi drug resistant TB.

**Keywords:** Isoniazid; Multi-Drug Resistant; Mycobacterium Tuberculosis.

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## INTRODUCTION

Mycobacterium Tuberculosis (MTB), the causative bacterial agent of tuberculosis, is the cause of health issues for about 10 million populations every year and is one of the leading causes of human deaths across the world. The prevalence of tuberculosis in Pakistan is reported to be 270/100,000 population<sup>1</sup>. Pakistan is graded 5<sup>th</sup> among 22 countries of the Eastern-Mediterranean territory. Prevalence of TB Multi drug-resistant (MDR) tuberculosis has emerged as a tenacious peril, with

490,000 million cases registered in 2016 and around 110,000 cases that were sensitive to the anti-tuberculosis drug isoniazid but resistant to rifampicin, which is the most efficacious 1st line anti-tuberculosis therapy (ATT) available in current era. Death due to MTB is graded above HIV infection in 2016 surveys<sup>2</sup>.

Multiple factors are responsible for spread of tuberculosis. It is due to penurious social, economic and political circumstances, absence of primary health facilities in many areas, malnourishment,

overcrowded population, and inundation of refugees from different countries.<sup>3</sup> It has been reported that an actual TB case detection and reporting rate is only 32% in our country.<sup>4</sup>

Tuberculosis bacilli, transmitted in the form of droplet nuclei, which are present as residues of dried air droplets. Each droplet nucleus frequently contains 1–10 TB bacilli and is usually 1–5µm in size. These droplets after remaining in the air for many hours can be inhaled into the lungs alveoli<sup>5</sup>, and enters the pulmonary terminal alveoli and are engulfed by macrophages. After replication inside the infected cells, MTB nuclei pass out of the alveoli and undergo dissemination leading to multiple body organ involvement. This entire phenomenon is observed before the start of immunity against MTB.<sup>6</sup>

The causative mycobacteria are seen microscopically as acid-fast naked bacilli, non-spore forming, and are not motile. These are obligate aerobes with slender and slightly curved architecture. Mycobacterial cell wall is exceptional and complicated with very high lipid load approximately 60% percent. This much high lipid stuff of mycobacterial cell wall make it arduous to stain giving it a reason of being acid fast.<sup>7</sup> Many researchers reported point mutation, which is frequently observed in katG gene at codon 315 in Isoniazid resistant MTB and the most common substitution is of Serine → Threonine.<sup>7,8</sup>

The study was designed to evaluate katG gene mutations, which are associated with isoniazid resistance in MDR MTB cases. This can be beneficial for quick and correct diagnosis of MDR MTB patients and thus help in alternative treatment therapies without delay. Trivial limitations of delays due to culture and sensitivity for MTB (90 days) and ATT drug sensitivity testing (21 days) can be saved. The objective of the study was to evaluate katG gene association with isoniazid resistance in Mycobacterium Tuberculosis.

## MATERIALS AND METHODS

**Case Selection Criteria:** Suspected TB samples were collected by physicians of DHQ hospital Kot Khwaja Saeed Lahore, Govt. Mian Munshi DHQ teaching hospital Lahore and DHQ hospital Gujranwala and submitted to IPH; Institute of Public Health at TB reference lab (Punjab Provincial Reference Lab for TB control Lahore). Study cases included those having cough for 03 or more weeks, showing no response to antibiotic regime and cases having cough with blood-tinged sputum, marked weight loss and evening temperature rise. Cases responding to antibiotics were excluded from the study.

**Sample Collection:** Sputum specimens from 100 MTB cases were collected in transparent and wide-mouthed jar and submitted to IPH Lahore after informed consent from patients from Septem-

ber to March 2018. Sputum was then decontaminated.

After inoculation, the slopes were incubated at 37°C for about 8 weeks. Para-nitro benzoic acid was used to check growth of MTB colonies in LJ medium. Growth was inhibited in the presence of PNB (500µg/ml). The sensitivity test was performed on LJ medium carrying antibiotics isoniazid and rifampicin and then incubation was done at 37°C for 21 days. After incubation the MTB colonies growth was examined. Multi drug resistant (MDR) was tagged if it was still resistant to Isoniazid and Rifampicin. DNA extraction was done with help of QIAamp DNA mini-kit (Qiagen). This DNA was used directly or stored as per requirement. DNA quantification was done by using 0.8% agarose gel. The primers designed by Abdelaal et al.<sup>9</sup> and Caws et al.<sup>10</sup> for katG gene (Gene-Bank Accession no. X68081.1) were used. For checking non-specific binding at any other loci, these reported primers were aligned by NCBI Blast. Primers, katG3 (Forward) and (Reverse) were subjected to PCR optimization for their annealing temperatures (50 to 60°C). Total 24 DNA samples were processed for PCR amplification using katG3 (Forward) primer having 3'-5' priming direction and sequence GGTCGACATTCGC-GAGACGTT and (Reverse) primers having 5'-3' priming direction and sequence CGGTGGAT-CAGCTTGTAACAG. Amplification was confirmed by Electrophoresis (Agarose Gel). After the DNA amplification of the desired portion of the DNA, PCR products were purified and then subjected to sequencing. One complete set of sequencing primers were used to sequence katG gene completely. The dye chain termination process with the help of dideoxynucleotide chain terminators labeled with fluorescent dye was used for sequencing.

Mutations were detected from the aligned sequences by using ABI Genetic Analyzer (3100) with the help of BLAST alignment software and Bio-Edit software.

## RESULTS

Out of these 100 TB cases, 24 patients were MDR MTB (resistant to both isoniazid and rifampicin). These 24 MDR MTB cases were analyzed for presence of katG gene mutation. The mutation, in katG gene was detected in five samples of MDR MTB patients (5% of total and 20.8% of MDR MTB resistant cases (Table 1)).

### Mutational Analysis

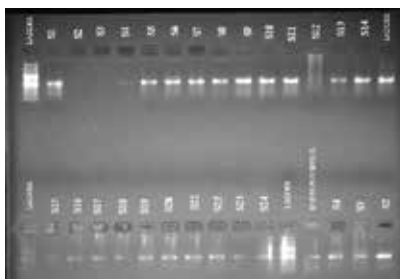
Five samples showed the reported Ser → Thr (AGC to ACC) mutation in the katG gene region of interest when the query sequences of this gene were compared with the reported sequence of MTB katG gene available on NCBI website.

Amplification by PCR using forward and reverse primers is shown in Figure 1 while sequencing done and mutation at codon 315 in Sample 1 with

highlighted bases showing the position of mutation is shown Figure 2. Similar results were obtained in other four samples.

**Table 1: Frequency of MDR TB and katG gene Mutation.**

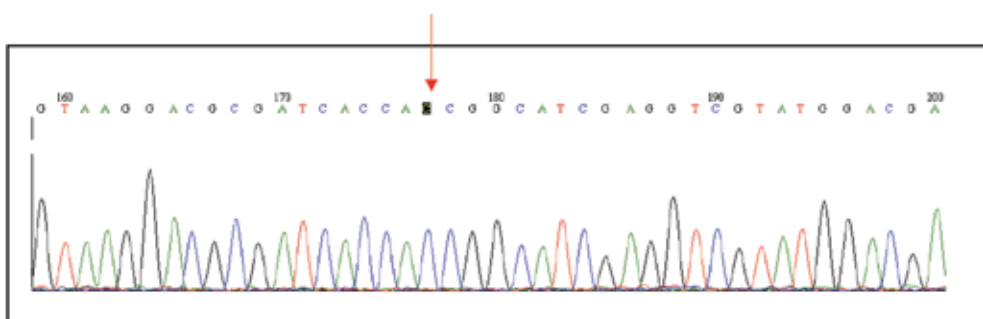
Tuberculosis Patients	MDR-MTB Resistant to Isoniazid and Rifampicin	KatG Gene mutation present in MDR TB resistant Patients	Frequency of KatG Gene Resistant
N = 100	24 (24%)	5 (5%)	20.8%



**Figure 1: PCR amplification of katG3 gene.**

**Lane M:** Marker 100bp (Fermentas Catalogue No. SM0241)

**Lane 1-24:** Amplified fragments of katG gene isolated from sputum of patients with MDR TB



**Figure 2: Chromatogram from sample 1.**

## DISCUSSION

Our study showed point mutation of katG gene at codon 315 that leads to development of isoniazid resistance in MDR MTB in five MDR samples. This confirmed katG Gene mutation with Serine being substituted by Threonine at codon 315. This accounted for 21 percent of the total number of samples. This study proves the possible association of katG gene with first line ATT drugs resistance such as isoniazid in multidrug resistant tuberculosis in Pakistan. Abdelaal et al. worked on 26 tuberculosis specimens resistant to Isoniazid (INH) in Egypt. The katG gene was studied to detect any relationship of this gene with development of isoniazid resistance in MTB patients. 24 samples (92.3%) out of 26 patients revealed katG gene point mutation with Serine substituted by

Threonine. Their results proved that katG gene is associated with progression of isoniazid resistance in tuberculosis patients.<sup>9</sup>

Caws et al. in Vietnam did another work regarding katG gene in MDR MTB. The data revealed that nearly 71% showed guanine→cytosine (G→C) mutation with serine being substituted by threonine. This is in agreement with our study that point mutation of katG gene at codon 315 leads to development of isoniazid resistance in MDR MTB cases<sup>10</sup>.

Many factors are involved that impart resistance to isoniazid. katG gene mutation can lead to isoniazid resistance most commonly but the other genes mutations are also documented. The literature revealed various genes which encode katG - cata-

lase and peroxidase both enzymes, inhA enzyme which is enoyl acyl carrier protein reductase, ahpC enzyme which is alkyl hydroperoxidoreductase, kasA enzyme chemically named as  $\beta$ -ketoacyl-acyl carrier protein synthase and ndh enzyme - abbreviated due to NADH dehydrogenase. There may be complete absence of katG gene as well.<sup>11</sup>

Mutations in katG gene at 315 codon still cause the gene to continue performing its role but on the lower side and thus cause resistance to isoniazid.<sup>12-14</sup> MTB cases with isoniazid resistance have shown reduced activity of both enzymes catalase and peroxidase after the launch of isoniazid as 1st line ATT drug for treating TB.<sup>15</sup> Aktas et al. analyzed 29 samples of MTB in Turkey that were resistant to isoniazid and rifampicin. Thus, 63% samples with Isoniazid resistance revealed mutation at katG at codon 315. This is in line with our study that katG gene mutation can cause isoniazid resistance in MTB.<sup>16</sup>

The katG 315 mutation occurrence varies in different parts of the world. In Madrid (Spain) resistant to isoniazid due to katG gene mutation observed was 34.6% MTB whereas; in Northwest part of Russia 93.6% katG gene mutation was seen. This indicates that katG gene mutation occurrence in various geographical regions of the world is not same.<sup>17</sup> Isoniazid can be converted to its active form only by the KatG enzyme (catalase and peroxidase). The mutations in KatG gene results in development of resistance to isoniazid due to defective underlying enzyme.<sup>18</sup>

An important work regarding KatG gene mutation and InhA gene in isoniazid resistant MTB was done by Sadri et al. 34 out of 125 MTB samples were found to be Isoniazid resistant strain and the remaining 91 strains were susceptible to isoniazid. Of these 34 samples labeled as INH resistant strains, 32% cases had KatG 315 (Serine→Threonine substitution). This study is also in agreement with our study.<sup>19</sup>

Another interesting study was done by Bollela et al. in two areas; Brazil and Mozambique. They picked up 466 MTB clinical isolates, 311 samples were from Brazil and 155 clinical specimens were from Mozambique. Out of these selected 311 clinical Brazilian isolates, 22 isolates were INH-resistant (7.1%), 15 samples of these isolates were also found to be rifampicin-resistant (MDR:MTB). The seven samples were resistant only to isoniazid. On the other hand out of 155 clinical samples taken from Mozambique, 38 clinical isolates were resistant to isoniazid (24.5%), 25 clinical samples of these were also resistant to rifampicin (65.8%) accounting for (MDR:MTB) and 13 isolates were mono-resistant to isoniazid (34.2%). They concluded that katG mutation was detected in 12/22 (54.5%) of the clinical isolates in Brazil and 32/38 (84.2%) clinical isolates had mutation in Mozambique.<sup>20</sup>

The focus of this research was the identification of INH resistant MTB and to find their association with katG gene mutations in codon 315. This will not only rapidly identify the MDR strains but will also drastically reduce the turnaround time of MTB culture and sensitivity from 90 days to less than a week. Drug resistance while treating tuberculosis (TB) is biggest challenge faced by the world in this century. The drug resistance to the 1st line ATT drugs such as isoniazid and rifampicin leads to increase in morbidity and mortality in TB patients across the globe.<sup>21</sup> Even though isoniazid resistance in TB patients and its association with katG gene have been studied in Pakistan, limited work has been done on katG gene sequencing and detection of mutations in isoniazid resistant strains found in Pakistan. Further larger clinical trials are needed to explore katG gene and other mutations association with drug resistance in TB patients in Pakistan.

## CONCLUSION

The study concludes that point mutation in codon 315 of katG gene is associated with development of resistance to the drug INH in Multi drug resistant TB. However, this study had number of limitations such as firstly the sample size was relatively small, mutations in promoter region of some other genes such as inhA gene were not included in our study; these mutations are also reported to be responsible for INH drug resistance in multi-drug resistant tuberculosis.

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## CONFLICT OF INTEREST

There is no conflict of interest in the study.

## AUTHORS CONTRIBUTION

Dr. Faruq Qmar Malick: Data collection; Dr. Imran Ali: Writing the manuscript; Dr. Sana Hafeez: Tables and figures; Prof Dr. Anila Jaleel: Statistical analysis; Dr. Amna Iqtidar: Data compilation; Ali Raza Awan: Supervision of the research.

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