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 population1. Pakistan is graded 5th 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 first-line anti-tuberculosis therapy (ATT) available in current era. Death due to MTB is graded above HIV infection in 2016 surveys2.

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,
Tuberculosis bacilli, transmitted in the form of droplet nuclei, which are present as residues of dried air droplets. Each droplet nucleus 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, 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.6

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.7 Many researchers reported point mutations, which are associated with isoniazid resistance in Mycobacterium Tuberculosis. It has been reported that an actual TB case detection and reporting rate is only 32% in our country.4

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.9 and Caws et al.10 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 with the help of BLAST alignment software and sequences by using ABI Genetic Analyzer (3100) and 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.

**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.9

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 cases10.

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-

<table>
<thead>
<tr>
<th>Tuberculosis Patients</th>
<th>MDR-MTB Resistant to Isoniazid and Rifampicin</th>
<th>KatG Gene mutation present in MDR TB resistant Patients</th>
<th>Frequency of KatG Gene Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 100</td>
<td>24 (24%)</td>
<td>5 (5%)</td>
<td>20.8%</td>
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</tbody>
</table>

**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: Chromatograph from sample 1.**
 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.\textsuperscript{12-14} 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.\textsuperscript{15} 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.\textsuperscript{16}

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.\textsuperscript{17} 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.\textsuperscript{18}

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.\textsuperscript{19}

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.\textsuperscript{20}

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\end{acknowledgments}

\begin{conflict}

There is no conflict of interest in the study.

\end{conflict}

\begin{authorcontribution}

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.

\end{authorcontribution}

\begin{references}


\end{references}

\begin{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.

\end{conclusion}