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Detection of *KatG* Mutation in MDR *Mycobacterium tuberculosis* Isolates by PCR-RFLP and DNA Sequencing

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Abstract

Objective: Tuberculosis (TB) is among the widespread and rapidly growing infections in the world. Furthermore, TB is one of the major public health problems in Pakistan as every year 48,000 Pakistani dies due to this infection. Pakistan ranks fifth among high burden countries worldwide. The convergence of Tuberculosis (TB), Human Immunodeficiency Virus (HIV), COVID-19, and Multi-Drug Resistance (MDR) strains of *Mycobacterium tuberculosis* (*M. tb*) has created an unprecedented and threatening global scenario. The objective of this study was to elucidate the genetic mechanism underlying drug resistance in local TB isolates.

Methodology: PCR Amplification: The hotspot region of the *KatG* gene, responsible for Isoniazid (INH) resistance which encodes catalase peroxidase, is amplified using PCR. The amplified DNA is subjected to RFLP, where specific restriction enzymes were used to cleave the DNA at known restriction sites. The amplified DNA is further analyzed through Sanger sequencing, which determines the precise nucleotide sequence of the region.

Results: PCR-RFLP revealed forty-five out of eighty INH resistant *M. tb* strains had mutations in *KatG* (codon 315) which is 56.2% of all cases. Sequencing results revealed that this is substitution mutation; AGC to ACC (Ser315Thr).

Conclusion: It may be concluded that majority of INH resistance is due to the mutation in the codon 315 of *KatG* in local isolates. Furthermore, PCR-RFLP technique could be considered as a reliable method for the early detection of *KatG* mutations in MDR-TB.

Keywords: Tuberculosis; Multi-Drug Resistance (MDR); Isoniazid; PCR-RFLP

Introduction

Tuberculosis (TB) is a communicable disease caused by bacillus *M. tb*. This disease has latently infected 33% of the world's population, accounts for 9 million new cases each year and caused more deaths than any other single infectious disease [1]. It is transmitted through airborne droplets from patients with pulmonary TB, who expel bacteria through coughing. Natural history of TB shows that only 5% of the infected individuals progress into active TB disease [2]. However, the people infected with Human Immunodeficiency Virus (HIV) are more susceptible to TB infection. The incidences recorded in Pakistan were 0.5 million in 2020 [3]. The individuals most susceptible to Tuberculosis (TB) include those who reside with active TB patients, as well as individuals who are economically disadvantaged, homeless, or incarcerated in prisons [4].

Pakistan ranks fifth among high burden countries worldwide [5]. COVID-19 pandemic is another threat contributing to emergence of MDR-TB as Pakistan is estimated to have fourth highest prevalence of MDR-TB globally [6].

Multidrug resistance arises from improper use of antituberculosis drugs and the use of substandard drugs for the treatment of TB in certain setting [7]. The sequential acquisition of mutations in target genes results in drug resistant *M. tb* [8]. MDR-TB is those which are resistant to two or more first line antituberculosis drugs, like Rifampicin (RIF) and Isoniazid (INH) [9]. INH has been one of the most potent drugs in tuberculosis chemotherapy for almost half a century. It is a prodrug and converted to active drug for its antituberculosis activity by *KatG*, an enzyme with dual activities of catalase and peroxidase [10].

Active INH drug leads to the inhibition of mycolic acid synthesis, which is an integral part of the mycobacterial cell wall. It is believed that INH inhibits two enzymes namely an enoyl acyl protein reductase (InhA) and β ketoacyl acyl protein synthase, which elongate the fatty acid in biosynthesis

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process [11]. In addition, the research article highlights the role of *KatG* in the oxidative protection mechanism of *M. tb* specially; *KatG* functions as a catalase enzyme, facilitating the elimination of hydrogen peroxide. The catalytic activity of *KatG* is regarded as a significant virulence factor, as it enhances the bacteria's ability to survive within macrophages [12]. Keeping in view the importance of *KatG* gene, the present study aimed to detect mutation(s) in this gene within local Multidrug-Resistant Tuberculosis (MDR-TB) isolates.

Materials and Methods

Sample collection and processing

A total of 169 Sputum samples were processed at the National Reference Laboratory, National TB Control Program in Islamabad for the isolation of *M. tb*. All samples were collected and processed aseptically following the recommended protocol.

Sample inoculation and identification

The sputum samples were decontaminated with 4% Sodium hydroxide to prevent the growth of unwanted organisms. Subsequently, the samples were treated with N-acetyl-L-Cysteine to enhance mucolytic activity. These procedures were carried out in a Biosafety Level 3 (BSL-3) Laboratory. Following decontamination and mucolytic treatment, the samples were inoculated onto Lowenstein-Jensen (LJ) media and were incubated aerobically for 4 to 8 weeks.

For final identification, the colonies were characterized based on their physical appearance. Additionally, the Zeihl-Neelsen stain was employed to visualize acid-fast bacilli, aiding in the identification process. Furthermore, biochemical tests were conducted to confirm the identification of the *M. tb*.

Overall, these stringent procedures and tests were performed in a controlled environment to ensure accuracy and prevent contamination, ultimately leading to reliable identification of the *M. tb*. present in the samples.

Antimicrobial susceptibility test

An antimicrobial susceptibility test was performed to identify the MDR strains of *M. tb*. The susceptibility test utilized Isoniazid (INH), Rifampin (RIF), Streptomycin (STR), and Ethambutol (EMB), following the established protocol [13]. The method described by waler et al. 2022 was employed for susceptibility testing with slight modifications [14].

DNA extraction and quantification

DNA of *M. tb* isolates was extracted using CTAB method initially described by [15] with few modifications done by [16]. Extracted DNA was quantified spectrophotometrically at 260 nm and 280 nm wavelength. The ratio of 260 nm/280 nm was used to assess the quality of DNA.

PCR- RFLP

The online bioinformatics tool Primer 3 was used to design primers for the amplification of specific region of *KatG*. The sequence of forward and reverse primers was 5'AGCTCGTATGGCACCGGAAC3' and 5'ACGGGTCCGGGATGGTG3' respectively.

The Polymerase Chain Reaction (PCR) was performed using master mix after optimizing the conditions. An estimated fragment of 200 bp was amplified which contained *KatG* codon 315 from 904 to 1103 nucleotide using a master cycler gradient (Eppendorf, USA). The reaction mixture contains 15 p moles of primers in 30 ul of PCR mixture, 1.5 mM of $MgCl_2$, 1U recombinant *Taq* DNA polymerase (Fermentas, USA) and 200 uM of each deoxyribonucleotides triphosphate (Fermentas, USA). The reaction was performed by initial denaturation at 95°C for 4 min, 30 PCR cycles with denaturation at 95°C for 20 sec, annealing at 54°C for 33 sec and extension at 72°C for 1 min in each cycle and a final elongation at 72°C for 8 min.

Agarose gel of 1.5% was prepared in 1X Tris-Acetate-EDTA (TAE) buffer for the confirmation of PCR results. After electrophoresis, the gel containing PCR products was stained in 1% ethidium bromide staining solution for 1 minute, visualized and recorded using gel documentation system (Kodak, USA). The PCR-RFLP assay was carried to detect mutation, if any. The restriction digestion was carried out for 2 h in a mixture containing 18 ul DNAse and RNAse free water, 10 ul of PCR products, 2 ul of HapII and 2 ul of Tango buffer. The restriction digested and undigested products as control were electrophoresed using 3% agarose gel for 30 min at 100 voltages. The largest restriction product obtained after restriction digestion for Isoniazid Resistant Strains (INHr) was 132 bp and for wild type the *KatG* codon 315 digested products were 153 bp.

DNA sequencing

The desired *KatG* region of 200 bp was amplified *via* PCR for sequencing. The amplified PCR products were first analyzed by gel electrophoresis, and then purified by mixing 40 µl PCR products with 5 µl of 10 M ammonium acetate and 80 µL of ice-cold absolute ethanol in 0.2 ml Eppendorf tube. The products obtained were centrifuged at 14,000 rpm and 40°C for about 12 min. The supernatant was removed, and the pellet washed with 70% ethanol. The pellet was reconstituted in 21 µL distilled water after being air dried thoroughly. Thereafter, sequencing reaction was prepared. For which a mixture of dye terminator cycle sequencing 8 µl using big dye, forward primer 1 µl, distilled water 8 µl, 2 µl of already purified PCR products were added to a 0.2 ml sterilized microcentrifuge tube. All the ingredients were mixed properly and were amplified on Master Cycler Gradient (Eppendorf, USA) using optimized cycling parameters as described earlier. The 10 µl amplified product was then purified by mixing 2.5 µl of 125 mM sodium-EDTA in addition with 30 µl of absolute ethanol. Then centrifugation was done at 14,000 rpm for about 10 min, the supernatant was discarded, and pellet was washed with 70% ethanol. Then ethanol was removed, and pellet was, fully air dried and heated at 96°C for 1 min, and then cooled on ice and loaded to the sample plate wells. The plate of samples was placed in the 3130 Genetic Analyzer, Hitachi (Japan). The data obtained was subjected for comparison with database using Basic Local Alignment Research Tool (BLAST) program.

Results

Growth on LJ media

Among the 169 samples, 118 tested positive for the growth of *M. tb*, exhibiting colony characteristics typical of *M. tb* on LJ medium. *M. tb* colonies appear rose, rough, and dry. They often have a buff-colored or pale yellowish tint. These colonies are non-pigmented or lightly pigmented, presenting as whitish, cream, or pale yellow in color. The lack of pigmentation is a distinguishing feature that sets them apart from other mycobacterial species. Furthermore, upon Zeihl-Neelsen staining, *M. tb* colonies exhibited red-colored rods. Biochemically M. tb. tested positive for Niacin, citrate, and Tween 80. Additionally, it displayed nitrate-reducing activity.

Antimicrobial susceptibility test

Out of the 118 samples collected, 80 samples were multidrug

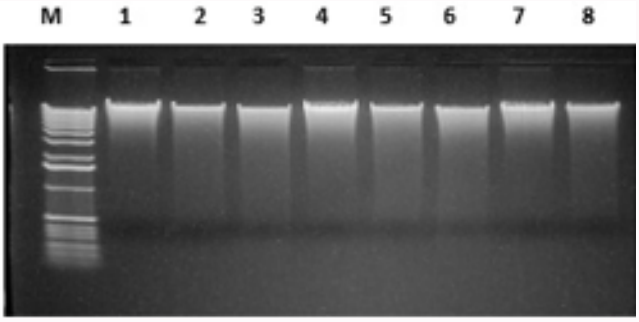

Figure 1: DNA extracted from *M. tb* isolates run on 1% agarose gel. Lane 1-8: Samples of DNA and M: 100 bp DNA marker Fermentas, USA.

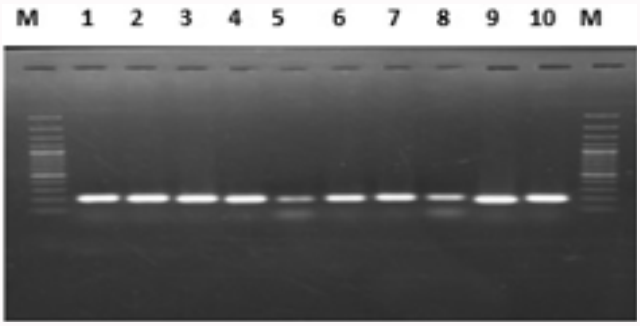

Figure 2: PCR products with *KatG* forward and reverse primers run on 1% agarose gel. M: 100 bp DNA ladder Fermentas, USA, Lane 1-10: *M. tb* PCR products.

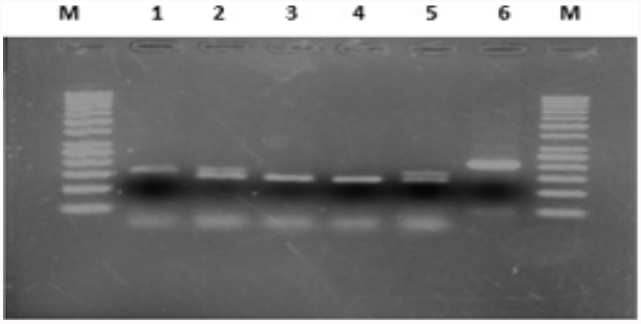

Figure 3: PCR-RFLP for MDR *M. tb* using *HapII* restriction enzyme: 50 bp DNA ladder Fermentas, USA. Lane 1: 153 bp restriction products. Lanes 3 and 4 contain 132 bp restriction products. Lane 2 and 5 mixed digested restriction products.

resistant. This means that these samples displayed resistance to two or more first-line drugs, which are typically the first line medications used for treating TB.

Collection and quantification of genomic DNA of MDR *M. tb* strains

Eighty isolates of MDR-*M. tb* were collected from National Reference Laboratory, National TB Control Program, Islamabad. DNA concentration was analyzed spectrophotometrically at 260 nm and 280 nm and run on 1% agarose gel. A representative result is shown in Figure 1.

Amplification of *KatG* specific region through PCR

The genomic DNA was subjected to PCR amplification of the specific region of *KatG* using forward and reverse primers.

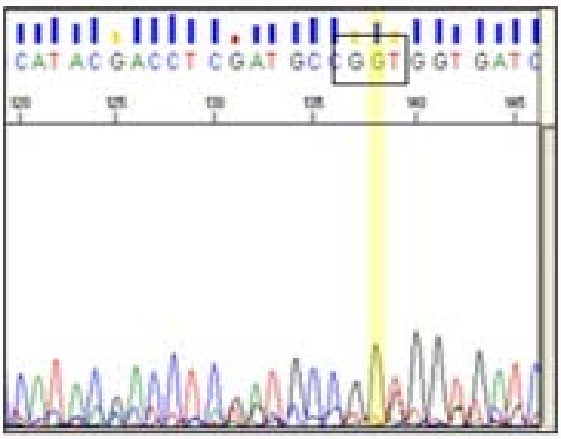

Figure 4: DNA sequencing of PCR-RFLP products showing mutation in *KatG* codon 315. The encircled sequence shows GGT which is reverse complement of ACC.

Two hundred base pairs region of *KatG* spanning codon 315 was successfully amplified through PCR and analyzed on 1% agarose gel (Figure 2).

RFLP analysis of the amplified PCR products

Thereafter, the PCR products of *KatG* specific region were subjected to HapII digestion for two hours. The RFLP products were examined on 2% agarose gel. The largest product obtained after the RFLP digestion was 153 bp for wild type and 132 bp for mutant type. Digested PCR products were compared with 50 bp ladder and a negative control i.e., the undigested PCR products (Figure 3).

Sanger sequencing of PCR-RFLP products

After confirmation of band shifts by RFLP the apparently mutant strains were analyzed by DNA sequencing technique to get more information. The results of DNA sequencing revealed that there is no mutation other than Ser/Thr at position 315 in these isolates which is shown by one of the representative results in Figure 4. Out of the eighty INH-resistant strains analyzed, 45 (56.2%) exhibited a substitution mutation from AGC to ACC at codon 315 of the *KatG* gene, whereas 35 (43.75%) isolates did not show any mutation in this region.

Discussion

Tuberculosis ranks among the deadliest human infections and remains a leading cause of mortality among infectious diseases. Although the use of a live attenuated vaccine and antibiotics can limit the infection. But the progress of drug resistance in the population has augmented concern that TB again can become an incurable disease [15]. In the current study we identified 45 resistant strains of *M. tb* out of 80 isolates i.e., 56.2% based on RFLP and sequencing. Our results for *KatG* Ser315 mutation was similar to the earlier reported rate of patients diagnosed with MDR-TB in Netherland 55% and Kuwait 65%. However, the frequency in Pakistan is less in comparison to Russia which has 95% frequency [17,18]. The recent data also support the fact that INH resistance is most common due to mutation in *KatG* gene at codon 315 [16,19,20]. DNA sequencing was also carried out to confirm the *KatG* gene mutation in INH resistant isolates. DNA sequencing revealed that there was no mutation other than Ser/Thr315 in these isolates. These results were similar with already reported results by [21]. Although PCR-RFLP is a robust,

economic, and specific method, but requires a site for mutant allele detected by the enzyme especially in instances when more than one allele is associated with the mutation. Although, at present sequencing is considered as gold standard with its advantages as rapidity and precise determination of the location and nature of the mutations.

Conclusion

Among eighty INH resistant strains 45 (56.2%) had substitution mutation of AGC to ACC in *KatG* codon 315 while 35 (43.75) isolates had no mutation in this region, which was further confirmed by DNA sequencing, which confirm that most of the drug resistance is due to *KatG* Ser315Thr in the local isolates.

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