Original article

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

Muhammad Ilyas[1], Falak Niaz[2], Rafaqat Ishaq[3], Rafiullah[4], Azra Khanum[5]

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. As the TB has become most threatening because of the epidemics of human immune deficiency virus (HIV), Covid-19 and the emergence of multi-drug resistance (MDR) strains of *Mycobacterium tuberculosis (M. tb*). This study was aimed to understand the genetic mechanism of drug resistance in local TB isolates. ***Methodology:*** For the genetic studies of INH resistance, *KatG* (encoding catalase peroxidase) hotspot region was amplified through PCR followed by RFLP and sequencing. ***Results:*** The study of PCR-RFLP showed that forty-five out 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 *Kat*G in local isolates. Furthermore, PCR-RFLP technique could be considered as a reliable method for the early detection of *Kat*G mutations in MDR-TB.

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

Bangladesh Journal of Medical Science Vol. 22 No. 04 October'23 Page : 804-808
DOI: https://doi.org/10.3329/bjms.v22i4.67114

Introduction:

Tuberculosis (TB) is a communicable disease caused by bacillus *Mycobacterium tuberculosis* (*M. tb*). This disease has afflicted approximately 33% of the population globally, causes 9 million new cases per year, and kills more people compared to any other infectious disease.[1]. It spreads via respiratory droplets from patients suffering from pulmonary tuberculosis who expectorate cough. According to the natural history of tuberculosis, only 5% of infected people develop active TB [2]. However, human immunodeficiency virus (HIV) infected patients are more vulnerable to TB. The incidences recorded in Pakistan were 0.5 million in 2020[3]. Most susceptible people are those living with active TB patients, are poor, homeless and prisoners[4]. Pakistan ranks fifth among high burden countries worldwide[5]. Covid-19 pandemic is another threat contributing to emergence of MDR-TB as Pakistan ranks fourth in the world in terms of MDR-TB prevalence.

Multidrug resistance develops because of inappropriate use of antituberculosis drugs and the use of poor-quality drugs for the treatment TB[6]. Mutations in a specific set of genes leads to emergence of MDR-TB[7]. MDR-TB refers to tuberculosis that is resistant to RIF and INH, the two most important

1. Muhammad Ilyas, University Institute of Biochemistry and Biotechnology, Pir Mehr Ali Shah, Arid Agriculture University Rawalpindi, Pakistan and Riphah International University, Islamabad, (Malakand Campus) Pakistan
2. Falak Niaz, Riphah International University, Islamabad, (Malakand Campus) Pakistan
3. Rafaqat Ishaq, University Institute of Biochemistry and Biotechnology, Pir Mehr Ali Shah, Arid Agriculture University Rawalpindi, Pakistan
4. Rafiullah, Riphah International University, Islamabad, (Malakand Campus) Pakistan
5. Azra Khanum, University Institute of Biochemistry and Biotechnology, Pir Mehr Ali Shah, Arid Agriculture University Rawalpindi, Pakistan

Correspondence: Muhammad Ilyas, University Institute of Biochemistry and Biotechnology, Pir Mehr Ali Shah, Arid Agriculture University Rawalpindi, Pakistan, e-mail: milyas.frahs@riphah.edu.pk

first-line antituberculosis drugs[8]. For nearly half a century, INH has been the most effective drug in tuberculosis pharmacotherapy. The prodrug INH is converted into a clinically effective antituberculosis drug by KatG (an enzyme with both catalase and peroxidase activity)[9].

INH works by inhibiting mycolic acid synthesis, which is a necessary component of mycobacterial cell wall (inhibitor of cell wall sythesis). INH is thought to inhibit enzymes enoyl acyl protein reductase (InhA) and β ketoacyl acyl protein synthase that synthesize/elongate β fatty acids during synthesis process [10]. KatG, on the other hand, acts as a catalase, remove hydrogen peroxide as oxidative protection system of *M. tb*. Its catalytic activity is thought to be a crucial virulence factor because it aids *M.tb* intramacrophage survival [11,21]. Keeping in view the importance of *KatG* gene, the present study was carried out to detect mutation(s) in this gene in local MDR-TB isolates.

Materials and methods:

Mycobacterium Tuberculosis MDR Strain Collection

Eighty MDR isolates and control H37Rv were collected from the National Reference Laboratory, National TB Control Program, Islamabad after conducting drug susceptibility test on sputum of the patients. The drugs used for susceptibility test were isoniazid (INH), rifampin (RIF), streptomycin (STR) and ethambutol (EMB) on Lowenstein-Jensen [Susceptibility Media] media[12].

DNA Extraction and Quantification

DNA of *M. tb* isolates was extracted using CTAB method initially described by Somerville *et al.,* 2005[13] with few modifications done by[14]. Extracted DNA was quantified spectrophotometrically at 260 nm and 280 nm wavelength. The ratio of 260/280 nm was used to assess the quality of DNA.

PCR- RFLP

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

The polymerase chain reaction (PCR) was run using master mix after optimizing the conditions. An estimated fragment of 200bp was amplified which contained *KatG* codon 315 from 904 to 1103 nucleotide using a master cycler gradient (Eppendorf, USA). The reaction mixture contains 15 pmoles 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 minutes, 30 PCR cycles with denaturation at 95˚ C for 20 second, annealing at 54˚ C for 33 seconds and extension at 72˚ C for 1 minute in each cycle and a final elongation at 72˚ C for 8 minutes.

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 hours 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 largest restriction product obtained after restriction digestion for isoniazid resistant strains (INH^r) were 132 bp and for wild type the *KatG* codon 315 digested products were 153 bp. The restriction digested and undigested products as control were electrophoresed using 3% agarose gel for 30 minutes at 100 voltages.

DNA Sequencing

The desired *KatG* region of 200bp was amplified via PCR for sequencing. The amplified PCR products were first analyzed by gel electrophoresis, then purified by mixing 40 µl PCR product 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 4^0C for about 12 minutes. The supernatant was removed, pellet was washed with 70% ethanol. The pellet was reconstituted in 21 µL distilled H_2O 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 plus 30 µl of absolute ethanol. Then centrifugation was done at 14,000 rpm for about10 minutes, the supernatant was discarded,

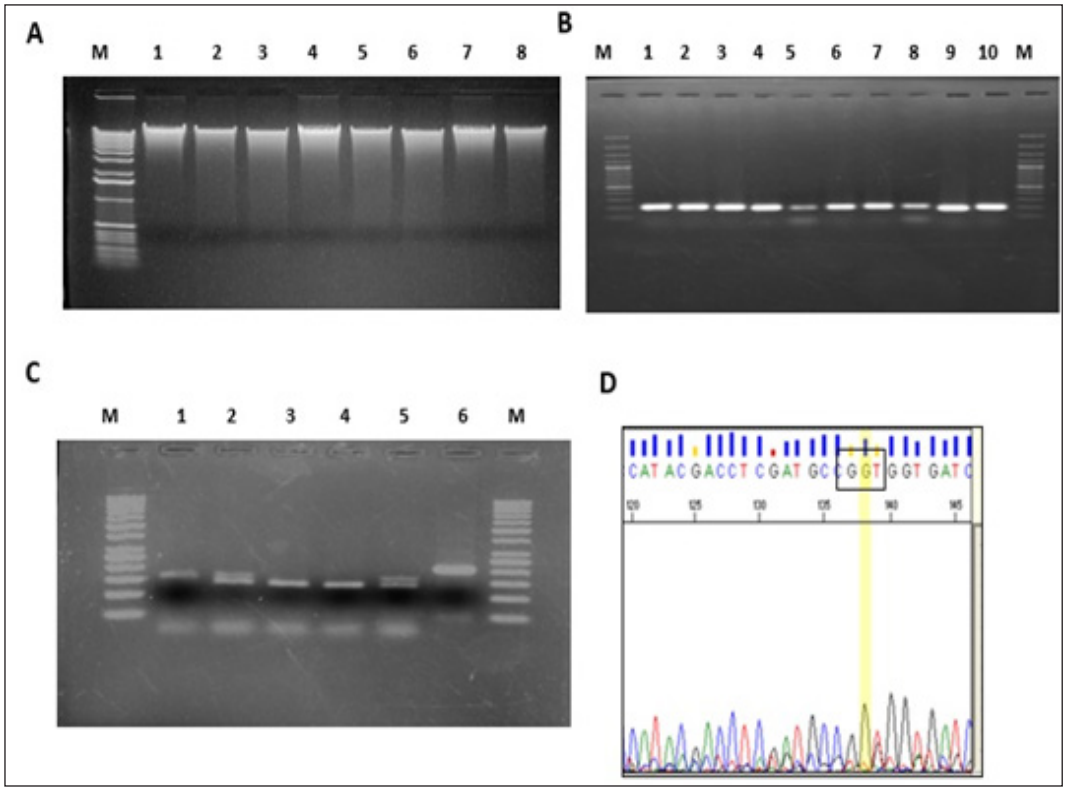

Figure A: DNA extracted from *M. tb* isolates run on 1 % agarose gel. Lane 1- 8: samples of DNA and M: 100bp DNA marker Fermentas, USA. **Figure B**: PCR products with *KatG* forward and reverse primers run on 1% agarose gel. M: 100 bp DNA ladder Fermentas, USA, Lane 1-10: *M. tb* PCR products. **Fig. C:** PCR-RFLP for MDR *M. tb* using *HapII* restriction enzyme. M: 50bp DNA ladder Fermentas, USA. Lane 1: 153bp restriction products. Lane 3 and 4 containing 132 bp restriction products. Lane 2 and 5 mixed digested restriction products **Fig. D:** DNA sequencing of PCR-RFLP products showing mutation in *KatG* codon 315. The encircled sequence shows GGT which is reverse complement of ACC.

and pellet was washed with 70% ethanol. Then ethanol was removed, and pellet was, fully air dried and heated at 96 ^{0}C for 1 minute, 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.

Ethical Approval: This study was approved by the Research Ethical Committee, Riphah International University Malakand Campus.

Results:

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 Fig: A.

Amplification of KatG Specific Region through PCR

The genomic DNA was subjected to PCR amplification of the specific region of *KatG* gene using forward and reverse primers. Two hundred base pairs region of *KatG* spanning codon 315 was successfully amplified through PCR and analyzed on 1% agarose gel (Fig: B).

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 as shown in Fig. 3. Digested PCR product was compared with 50bp ladder and a negative control i.e., the undigested PCR products (Fig: C.).

DNA 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 (Fig: D).

Discussion:

Tuberculosis is one of the most lethal human infections. It is one of the major killers amongst the 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 incorrigible disease[13]. In the present study clinical isolates of *M. tb* were used to detect molecular drug resistance patterns *M. tb* isolates locally in Islamabad, Pakistan. Total confirmed 80 MDR-TB samples were collected and their DNA was extracted using CTAB method. Extracted DNA was amplified using PCR, restriction of amplified DNA product was done by *Hap II* restriction enzyme that yielded 132 and 153bp product for drug resistant and wild type isolates respectively. Each digested PCR product's position was tested along with a 50bp ladder and an undigested PCR product as a negative control. According to RFLP, 45 out of the 80 isolates, or 56.2% of the total, were mutants. The incidence of patients diagnosed with MDR-TB in the Netherlands (55%) and Kuwait (65%) was similar to our findings for the KatG Ser315 mutation. Frequency of KatG mutation in Pakistan, on the other hand, is lower than Russia's, which is 95%[15,16]. Recent studies also support the notion that INH resistance is most commonly caused by a mutation in the KatG gene at codon 315.[17, 18,19]. DNA sequencing was also carried out to confirm the *KatG* gene mutation in INH resistant isolates. The DNA sequencing results in these isolates showed only the Ser/Thr315 mutation but no other mutations. These results were similar with already reported results by[20]. PCR-RFLP is a reliable, cost-effective, and specific method, but it needs a restriction site for the mutant allele detected by the enzyme, particularly when more than one allele is associated with the mutation. Although sequencing is currently regarded as the gold standard, with benefits such as rapid and precise determination of the location and nature of mutations, its high costs and requirement for sophisticated infrastructure limit its potential application in low-income setups.

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 confirmed by DNA sequencing, which confirm that most of the drug resistance is due to *KatG* Ser315Thr in the local isolates.

Source of fund: Nil

Conflict of Interest: The authors declare that there is no conflicts of interest among them.

Authors Contribution:

Data collection and analysis: Muhammad ilyas

Writing and submission: Muhammad Ilyas, Rafaqat Ishaq

Editing and approval of final draft: Muhammad Ilyas, Dr. Rafiullah

Proofreading: Falak Niaz

Supervision: Dr. Azra Khanum

References

1. Rahim Z, Nakajima C, Raqib R, Zaman K, Endtz HP, Van Der Zanden AGM, et al. Molecular mechanism of rifampicin and isoniazid resistance in Mycobacterium tuberculosis from Bangladesh. *Tuberculosis.* 2012 Nov 1**;92**(6):529–34.
2. Global tuberculosis report 2014 [Internet]. [cited 2022 Sep 16]. Available from: https://apps.who.int/iris/handle/10665/137094
3. Global tuberculosis report 2021 [Internet]. [cited 2022 Sep 15]. Available from: https://www.who.int/publications/i/item/9789240037021
4. Hussain H, Akhtar S, Nanan D. Prevalence of and risk factors associated with Mycobacterium tuberculosis infection in prisoners, North West Frontier Province, Pakistan. *Int J Epidemiol.* 2003;**32**(5):794–9.
5. Global tuberculosis report 2014 [Internet]. [cited 2022 Sep 15]. Available from: https://apps.who.int/iris/handle/10665/137094
6. Zumla A, Abubakar I, Raviglione M, Hoelscher M, Ditiu L, Mchugh TD, et al. Drug-Resistant Tuberculosis Current Dilemmas, Unanswered Questions, Challenges, and Priority Needs. *J. Infect. Dis*.2012; **8**(205):228–40.
7. Riska PF, Jacobs Jr WR, Alland D. Molecular determinants of drug resistance in tuberculosis. IJTLD. 2000; 4(2 Suppl 1):S4-10.
8. Traore H, Fissette K, Bastian I, Devleeschouwer M, Portaels F. Detection of rifampicin resistance in Mycobacterium tuberculosis isolates from diverse countries by a commercial line probe assay as an initial indicator of multidrug resistance. *IJTLD*. 2000;**4**(5):481-4.
9. Lei B, Wei CJ, Tu SC. Action mechanism of antitubercular isoniazid: activation by Mycobacterium tuberculosis KatG, isolation, and characterization of InhA inhibitor. *JBC*. 2000;**275**(4):2520-6.
10. Bang D, Bengård Andersen A, Thomsen VØ. Rapid genotypic detection of rifampin-and isoniazid-resistant Mycobacterium tuberculosis directly in clinical specimens. *JCM*. 2006;**44**(7):2605-8.
11. Mo L, Zhang W, Wang J, Weng XH, Chen S, Shao LY, Pang MY, Chen ZW. Three-dimensional model and molecular mechanism of Mycobacterium tuberculosis catalase-peroxidase (KatG) and isoniazid-resistant KatG mutants. *MDR*. 2004;**10**(4):269-79.
12. Heifets L. Qualitative and quantitative drug-susceptibility tests in mycobacteriology. *Am. Rev. Respir. Dis.* 1988;**137**(5):1217-22.
13. Somerville W, Thibert L, Schwartzman K, Behr MA. Extraction of Mycobacterium tuberculosis DNA: a question of containment. *JCM.* 2005;**43(**6):2996-7.
14. Khan SN, Niemann S, Gulfraz M, Qayyum M, Siddiqi S, Mirza ZS, et al. Molecular Characterization of Multidrug-Resistant Isolates of Mycobacterium tuberculosis from Patients in Punjab, Pakistan. *Pakistan J Zool.* 2013;**45**(1):93–100.
15. van Doorn HR, de Haas PEW, Kremer K, Vandenbroucke-Grauls CMJE, Borgdorff MW, van Soolingen D. Public health impact of isoniazid-resistant Mycobacterium tuberculosis strains with a mutation at amino-acid position 315 of katG: a decade of experience in The Netherlands. *Clin Microbiol Infect.* 2006**;12(**8):769–75.
16. Lipin MY, Stepanshina VN, Shemyakin IG, Shinnick TM. Association of specific mutations in katG, rpoB, rpsL and rrs genes with spoligotypes of multidrug-resistant Mycobacterium tuberculosis isolates in Russia. *Clin Microbiol Infect.* 2007;**13(**6):620–6.
17. Khan SN, Niemann S, Gulfraz M, Qayyum M, Siddiqi S, Mirza S, et al. Molecular Characterization of Multidrug-Resistant Isolates of Mycobacterium tuberculosis from Patients in Punjab , *Pakistan. Pak. J. Zool*. 2013;**45**(1):93–100.
18. Ajbani K, Rodrigues C, Shenai S, Mehta A. Mutation detection and accurate diagnosis of extensively drug-resistant tuberculosis: report from a tertiary care center in India. *JCM.* 2011;**49**(4):1588-90.
19. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, Hooks DP, Cowan LS, Plikaytis BB, Posey JE. Molecular detection of mutations associated with first-and second-line drug resistance compared with conventional drug susceptibility testing of Mycobacterium tuberculosis. Antimicrob. Agents Chemother. 2011;**55(**5):2032-41.
20. Mokrousov I, Filliol I, Legrand E, Sola C, Otten T, Vyshnevskaya E, et al. Molecular characterization of multiple-drug-resistant Mycobacterium tuberculosis isolates from northwestern Russia and analysis of rifampin resistance using RNA/RNA mismatch analysis as compared to the line probe assay and sequencing of the rpoB gene. *Res Microbiol. 2*002;**153**(4):213–9.
21. Comparative studies evaluating macrophage activity of pulmonary tuberculosis patients with and without diabetes mellitus (tb-dm and non tb-dm). BJMS. 2018; 17(1), 107–111. https://doi.org/10.3329/bjms.v17i1.35290