

Molecular characterisation of isoniazid resistant clinical isolates of *Mycobacterium tuberculosis* from Khyber Pakhtunkhwa, Pakistan

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Abstract

Objectives: To investigate the frequency of mutations in catalase-peroxidase and inhA genes in clinical isolates of isoniazid resistant *Mycobacterium tuberculosis* strains.

Methods: The study was conducted at Provincial Tuberculosis Reference Laboratory, Peshawar, Pakistan, from April 2015 to March 2016, and comprised sputum specimens obtained from patients of different ages. All the isolates were analysed for isoniazid resistance. Thirty resistant isolates were randomly selected for mutation analysis of the hotspot regions of catalase-peroxidase and inhA genes.

Results: Of the 163 positive isolates, 79(48.46%) were resistant to isoniazid. Among these, 21(70%) had mutation in catalase-peroxidase gene and 2(6.6%) had C15T mutation in inhA promoter region. Among the 21 catalase-peroxidase mutants, Ser315Thr mutation was detected in 15(71.4%) isolates. Gly316Ser mutation was detected in 3(14.2%) isolates. Ser315Arg mutation was identified in 2(9.5%) isolates. Double mutation of Ser303Trp and Lys274Arg was detected in 1(4.7%) isolate. Among the inhA promoter region mutations, 2(6.6%) of the thirty isolates had the most common C15T mutation in the promoter region.

Conclusion: One novel mutation at codon 303 in catalase-peroxidase gene was found in the study, and it could contribute to isoniazid resistance.

Keywords: inhA, katG, PCR, Sequencing. (JPMA 67: 1224; 2017)

Introduction

Mycobacterium tuberculosis (MTB) is the causative organism of the devastating tuberculosis (TB) disease. Tuberculosis is a global health problem and could be transmitted from one individual to another by droplet aerosol.¹ The disease is caused by a group of related bacterial species called mycobacterium tuberculosis complex (MTBC). These include mycobacterium (*M.*) africanum, *M. microti*, *M. cannetii*, *M. bovis* and *M. tuberculosis*. About 9.6 million people were infected with *Mycobacterium tuberculosis* infection and 1.5 million death cases were reported in 2014.² About 480,000 people developed multiple drug-resistant tuberculosis (MDR-TB) in 2014. Many new cases are arising rapidly in countries like China, Bangladesh, Pakistan, India and Indonesia, which are highly populated countries of the world. Pakistan ranked sixth amongst highest TB reported countries.³ According to World Health Organisation's (WHO) estimates, 43 million people were saved through advancement in TB diagnosis techniques and treatments during the last fourteen years.²

Antimicrobial susceptibility testing is performed invitro to measure *Mycobacterium tuberculosis* growth response

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against antimicrobial agents. BACTEC *Mycobacteria* Growth Indicator Tube (MGIT) 960 system is an automated instrument that has been reliably used for antimicrobial susceptibility testing. Antimicrobial resistance develops when microorganism acquire new mechanisms to overcome the effects of antimicrobial agents used for the treatment of various infections. *Mycobacterium* TB isolates develop resistance to the targeted drug as a result of modification in drug activating and target including genes and/or their promoter regions.⁴ The first-line TB drugs are generally used against mycobacterium tuberculosis as first treatment option. These include isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin. The bacterium could develop resistance to these drugs. The resistant strains are called MDR-TB strains, i.e. tuberculosis strains with resistance to a minimum of two (isoniazid and rifampicin) of the first-line drugs.⁵ To control these MDR-TB strains, second-line drugs which include capreomycin, kanamycin or amikacin are used; however, these drugs have more toxic effect and are more expensive. *Mycobacterium tuberculosis* strains resistant to both the first- and second-line drugs are known as totally drug-resistant TB strains or extremely drug-resistant TB(XDR TB) strains which need more advanced treatment options.⁶

Isoniazid (INH) is a first-line anti-tuberculosis antibiotic used for treatment of tuberculosis since its introduction in 1952.⁷

Over the time mycobacterium TB strains have developed resistance to almost all antibiotics, however, resistant frequency to isoniazid is higher than other drugs.⁸ Isoniazid resistance develops as a result of gene mutations involving catalase-peroxidase gene (*katG*), inhibin alpha (*inhA*), *kasA*, *ahpC* and *ndh* genes.⁹ *KatG* code for enzyme catalase/peroxidase which activates isoniazid and loss or reduced activity of the enzyme is associated with isoniazid resistance which occurs due to mutations in *katG*.¹⁰ The *inhA* gene codes for enoyl-acyl carrier protein reductase that takes part in synthesis of mycolic acid.¹ Activated isoniazid inhibit mycolic acid synthesis by blocking nitrate reductase (NADH)-dependent enoyl-acyl carrier protein (ACP) reductase, *inhA* coded enzyme.¹¹ *InhA* promoter region mutation is responsible for higher expression of *inhA*.¹² The most common mutation in isoniazid resistance is at codon 315 (AGC to ACC) of *katG* gene.¹³ The predominant mutation in *inhA* promoter region occurs at -15 position (C15T).¹ *InhA* mutations are also responsible for ethionamide resistance.¹⁴ The present study was planned to find out mutations in the "hot-spot regions" of *inhA* and *katG* genes among clinical isolates.

Materials and Methods

The study was conducted at the Provincial TB Reference Laboratory, Peshawar, Pakistan, from April 2015 to March 2016, and comprised sputum specimens obtained from patients of different ages.

According to the prevalence of isoniazid drug resistance 33.33% sample size was calculated for a 95% ($p < 0.05$) confidence interval. Informed consent was obtained from the respective patients and approval was obtained from the ethics committee of the Centre of Biotechnology and Microbiology, University of Peshawar.

The sputum samples were collected in 50ml sterile plastic bottles. Some of the patients were newly treated and some were previously treated. All of the patients were human immunodeficiency virus (HIV) negative. The samples were processed for digestion and decontamination using standard N-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) method. Cultures were produced using BACTEC 960 system. Cultures were incubated at 37°C. For the identification of acid-fast bacillus (AFB), fluorescent microscopy was performed. For the confirmation of *Mycobacterium tuberculosis*, TBc identification kit (Catalogue No: 245159, Becton, Dickinson) was used.

BD BACTEC MGIT 960 SIRE kit (Catalogue No: 245123, Becton, Dickinson) was used to perform drug susceptibility testing (DST). The final concentration of isoniazid was 0.1 µg/ml.

Deoxyribonucleic acid (DNA) was extracted from mycobacterial colonies by combined heat and sonication

method.^{15,16} Eppendorf tube containing broth culture was kept in water bath at 95°C for 30 minutes to kill *Mycobacterium tuberculosis*. After heating in water bath cell lysate was sonicated in sonicator [ELMASONIC S 30] for 15 minutes. After centrifugation at 10,000 revolutions per minute (rpm) for 5 minutes, pellet was discarded and supernatant was collected. The extracted DNA (5 µL) was used as a template for amplification.

To find out mutations in isoniazid-resistant isolates, 30 isolates were randomly selected for amplification of *katG* and *inhA* genes using primers described by Telanti et al.¹⁷ A 209 bp fragment of *katG* and 248 bp fragment of *inhA* genes were amplified in a thermal cycler [Eppendorf AG 22331 Hamburg]. The amplified region covered "hot spot" region for mutation. The polymerase chain reaction (PCR) profiles were set as suggested by manufacturer (Solis BioDyne-5X FIREPol® Master mix). Briefly, 25 µL PCR reaction mixture consisted of 4 µL master mix, 0.5 µL reverse and forward primer, 5 µL template DNA and 15 µL molecular grade water. The PCR parameters used for *inhA* and *katG* gene were: 10 minutes initial denaturation at 95°C, denaturation at 95°C for 45 seconds, annealing at 62°C for 1 minute and elongation at 72°C for 40 seconds. The reaction was repeated 35 times. A final elongation at 72°C for 8 minutes was also included (Table-1). Amplified products were detected by gel electrophoresis. A 100bp DNA ladder (Catalogue No: DMOO3-R500) was used. The gel was studied in Geldoc system (SYNGENE Serial number SYDR/2138) and images were captured.

PCR products were sequenced through Macrogen (Korea) using both with forward and reverse primers. The sequencing results were compared with reference *Mycobacterium tuberculosis* H37RV sequence using BioEdit sequence Alignment Editor (version 7.2.5.0).¹⁸

Results

Of the 794 specimens, 462(58.2%) were from males and 332(41.8%) from females. Of all, 163(20.5%) samples yielded positive results by fluorescence microscopy and real-time PCR. Of them, 79(48.46%) samples showed resistance to isoniazid while 84(51.5%) isolates were sensitive to isoniazid. Among the 30 isolates randomly selected for amplification of *katG* and *inhA* genes, 21(70%) had mutation in *katG* gene. Of them, 15(71.4%) isolates had Ser315Thr mutation, 3(14.2%) isolates harboured Gly316Ser mutation, 2(9.5%) isolates had Ser315Arg mutation and 1(4.7%) isolate had double mutation at two different loci, i.e. Ser303Trp and Lys274Arg. Ser303Trp and Lys274Arg mutation in *katG* gene was identified for the first time in Pakistan. Among the thirty isoniazid resistant isolates, only 2(6.6%) isolates had C15T mutation in the

Table-1: Primers used for desired genes amplifications.

Genes	Primers	Sequences 5'→3'	Product size (bp)	Reference
Kat G	TB86	AAACAGCGGCGCTGGATCGT	209	Talenti et al .(1997)
	TB87	GTTGTCCCATTTTCGTCGGGG		
InhA	TB92	CCTCGCTGCCAGAAAGGGA	248	Talenti et al .(1997)
	TB93	ATCCCCGGTTTCTCCGGT		

KatG: Catalase-peroxidase

InhA: Inhibin alpha.

Table-2: Mutations in katG and inhA genes of Mycobacterium tuberculosis isolates.

Gene	Codon/nucleotide change(s)	Amino acid/nucleotide change(s)	Total no of isolates
katG	AGC to ACC	Ser315Thr	15
	GGC to AGC	Gly316Ser	3
	AGC to CGC	Ser315Arg	2
	AAG to AGG and TCG to TGG	Lys274Arg and Ser303Trp	1
InhA	C to T	At position -15	2

KatG: Catalase-peroxidase.

InhA: Inhibin alpha.

promoter region of inhA gene (Table-2).

Discussion

Worldwide different mutations have been reported to be responsible for resistance in mycobacterium. However, little knowledge is available about the molecular basis of mycobacterium resistance in the Khyber Pakhtunkhwa (KPK) province of Pakistan, one of the high burden TB prevalent regions. Hence, the present study was under taken to know some of the molecular mechanisms responsible for resistance in Mycobacterium. To our knowledge, current research reports for the first time the molecular characterisation of isoniazid resistance among TB isolates from the province. Mutations in the "hot-spot" region of inhA and katG genes among resistant isolates were analysed. Worldwide a number of studies have reported mutations in katG gene to account for 50-95% isoniazid resistance.¹⁹⁻²² Among these, the most prevalent mutations involve the Ser315Thr substitutions in the katG gene both regionally and globally.^{1,12,23-26} Our finding showed that mutations in katG gene were correlated with 70% isoniazid resistance. The predominant mutation (50%) was Ser315Thr in katG gene as reported globally.^{1,12,25,26} A study by Gonulaslan et al. reported Ser315Thr mutation as the most prevalent with a frequency of 60%.²⁵ Another study involving 348 isoniazid-resistant isolates from four geographically diverse countries reported 86% frequency of Ser315Thr mutation slightly higher than our findings.²⁶ A study conducted in Jiangxi, China, reported 51% frequency of Ser315Thr mutation in katG gene.²⁷ Ser315Thr mutation frequency in Belarusian

patients was found to be 32.4%.²⁸ Differences in Ser315Thr mutation frequencies in katG gene might be due to different geographical distribution of the isolates.

Distribution of various mutations associated with isoniazid resistance in katG gene has been widely studied around the world. Mutations observed in our study included Gly316Ser, Ser315Arg, Lys274Arg and Ser303Trp. The Ser315Arg mutation was also reported in one isolate in a study conducted in Jiangxi province, China.²⁷ The frequency of Gly316Ser mutation was reported to be 16.2% in a previous study conducted in Belarus while in our study the frequency was found to be 14.2%.²⁸ A novel double mutation of Lys274Arg and Ser303Trp was also observed in one isolate which could have a role in isoniazid resistance.

Mutation frequency in the inhA promoter region has been reported to range from 10%-34%.^{29,30} The C15T mutation frequency was found to be 6.6% isolates in inhA promoter region. Higher C15T mutation frequencies (34% and 16.6%) have been reported in previous studies.^{26,27} A study from the Punjab province of Pakistan reported 12% frequency of C15T mutation in inhA promoter region.¹ Comparison of different mutations reveals that strains present in different geographical regions have different mutation profiles. Our finding revealed the existence of mutations both in katG and inhA in local Mycobacterium tuberculosis strains that contribute to isoniazid resistance.

Conclusion

One novel mutation at codon 303 in katG gene was found

in this study, and it could contribute to isoniazid resistance. Moreover, the findings of the current study will assist to understand molecular mechanisms of drug resistance and in the use of molecular-based techniques for rapid diagnosis of MDR tuberculosis in high burden TB regions like Pakistan.

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Conflict of Interest: None.

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