

Mutation analysis for detection of drug resistance in mycobacterium tuberculosis isolates from Khyber Pakhtunkhwa, Pakistan

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Abstract

Objective: To examine the frequency and distribution of mutations in the 'hot spot regions' of drug-resistant genes.

Methods: The study was conducted at Provincial Tuberculosis Reference Laboratory, Peshawar, Pakistan, from April 2015 to March 2016, and comprised sputum samples. Isolates were tested for drug susceptibility and resistant isolates were investigated for mutations analysis in the 'hot spot regions' of *rpoB*, *rpsL*, *embB*, *pncA*, *gyrA* and *gyrB* genes.

Results: Of the 163 isolates, 47(28.8%) isolates were resistant to streptomycin, 56(34.36%) to rifampicin, 31(19%) to ethambutol, 16(9.82%) to pyrazinamide and 59(36.2%) isolates were resistant to ofloxacin. Resistant isolates were randomly selected for mutation analysis. Moreover, 14(25%) rifampicin-resistant isolates were analysed for mutation in *rpoB* gene. Ser450Leu, Asp435Gly, Ser450Gln, Gly455Asp and Pro454His mutations were detected in the selected isolates. Furthermore, 16(34%) streptomycin-resistant isolates were analysed for mutation in *rpsL* gene. Lys43Arg, Lys88Arg and Lys111Ile mutations were detected in *rpsL* gene of 6(37.5%) isolates. Besides, 16(51.6%) ethambutol-resistant isolates were analysed for mutations in *embB* mutation; Ala281val, Met306Leu and Met306Val mutations were detected in 10(10(62.5%) isolates. Also, 8(50%) pyrazinamide-resistant isolates were analysed for mutation in *pncA* gene.

Conclusion: Some novel mutations were found in *rpoB*, *rpsL* and *pncA* genes.

Keywords: Mycobacterium tuberculosis, Antibiotic resistance, Mutation, Sequencing. (JPMA 67: 1684; 2017)

Introduction

Tuberculosis is a global health problem common in developing countries and its early detection and proper treatment is important to save human life. Mycobacterium (*M.*) tuberculosis transfers from patient to healthy individuals through air.¹ In one year it can infect 10 to 15 people if left untreated. Tuberculosis (TB) could be easily acquired by people living in regions where TB is endemic, those in direct contact with TB patients and those having weak immune system.

As first treatment option, the first-line TB drugs are normally used against *M. tuberculosis*. Multiple drug-resistant tuberculosis (MDR-TB) strains have been evolved that are resistant to at least two first-line drugs. MDR-TB is treated with second-line drugs.

The first-line drug rifampicin binds to the beta (β)-subunit of ribonucleic acid (RNA) polymerase which blocks the

elongation of messenger RNA. Mutations in hyper-variable regions of *rpoB* gene result in rifampicin-resistant strains.² Streptomycin is an aminocyclitol glycoside antibiotic that was the first antibiotic used in the treatment of TB. Its mechanism of action is to bind with 16S ribosomal ribonucleic acid (rRNA) and inhibit translation initiation.³ Streptomycin resistance develops due to mutations in *rpsL* and *rrs* genes which are involved in the synthesis of ribosomal proteins.⁴ Ethambutol (EMB) targets the cell wall of *M. tuberculosis* through interfering with arabinosyl transferases which are encoded by *embB* gene and are involved in cell wall synthesis. *embB* gene mutations at codons 306, 406 and 497 are accountable for low and moderate levels of EMB resistance.^{5,6} Pyrazinamide (PZA) is a structural analogue of nicotinamide. It is converted to pyrazinoic acid (active form of pyrazinamide) by the enzyme pyrazinamidase/nicotinamidase that is coded by *pncA* gene. Mutations in *pncA* gene contribute to pyrazinamide resistance in *M. tuberculosis*. The second-line drug ofloxacin inactivates *M. tuberculosis* by binding to gyrase-deoxyribonucleic acid (DNA) complexes and inhibiting DNA replication. Ofloxacin-resistant strains develop through mutations in quinolone resistance-determining regions of *gyrA* and *gyrB* genes which code for DNA topoisomerase gyrase.

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Khyber Pakhtunkhwa (KPK) province of Pakistan is a high burden TB region of Pakistan. The current study was planned to examine the frequency and distribution of mutations in the 'hot spot regions' of *rpoB*, *rpsL*, *embB*, *pncA*, *gyrA* and *gyrB* genes among clinical isolates.

Materials and Methods

The study was conducted at Provincial TB Reference Laboratory, Peshawar, Pakistan, from April 2015 to March 2016, and comprised sputum samples. Informed consent was obtained from all participants. Approval was obtained by the ethics committee of the Centre of Biotechnology and Microbiology, University of Peshawar. BACTEC 960 was used for culturing *M. tuberculosis*. Drug susceptibility testing (DST) of positive isolates was performed using BD BACTEC MGIT 960 SIRE kit (Cat No: 245123, Becton, Dickinson).

Heat and sonication method for DNA extraction was used to isolate DNA from *M. tuberculosis*.^{7,8} Amplification of the hotspot regions of *rpoB*, *rpsL*, *embB*, *pncA*, *gyrA* and *gyrB* genes was carried out in a thermal cycler [Eppendorf AG 22331 Hamburg] using pre-made master mix (Solis BioDyne-5X FIREPol® Master mix) according to the manufacturer's instructions. Briefly, 4µL master mix (Solis BioDyne-5X FIREPol® Master mix), 0.5µL reverse and forward primer each and 15µL polymerase chain reaction (PCR) grade water were used to make 25µL reaction. The hotspot region of *rpoB* gene was amplified using primers described by Talenti et al.⁹ The reaction profile for *rpoB* gene consisted of: initial denaturation at 94°C for 5 minutes followed by 20 cycles of denaturation at 94°C for 1 minute, annealing at 52.9°C for 1 minute and extension at 72°C for 1 minute, and final elongation at 72°C for 8 minutes. The hotspot region of *rpsL* gene was amplified using primers described by Elif et al.¹⁰ The reaction profile for *rpsL* gene consisted of: initial denaturation at 94°C for 5

minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute and extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The reaction profile for *embB* gene consisted of: initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 56°C for 1 minute and extension at 72°C for 40 seconds, and final elongation at 72°C for 8 minutes. Primers for *gyrA* and *gyrB* genes amplifications were designed as shown in Table-1. The reaction profiles for both genes consisted of initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 40 sec and extension at 72°C for 50 seconds, and final elongation at 72°C for 8 min. Primers were designed for *pncA* gene amplification as given in Table-1. The reaction profile was similar to *gyrA* and *gyrB* genes except annealing temperature of 59°C. PCR products were analyzed by gel electrophoresis using 1.5% agarose concentration. Gel documentation system (SYNGENE Serial number SYDR/2138) was used for visualization.

PCR products of each gene were sequenced through Macrogen (Korea) using both forward and reverse primers. ExoSAP purification kit was used for PCR product purification. The purified products were sequenced both with forward and reverse primers using BigDye Terminator V3.1 kit and ABI 3730xl DNA analyser (96 capillary type). The sequencing results were compared with reference *M. tuberculosis* H37Rv sequence using BioEdit sequence alignment editor (version 7.2.5.0).

Results

Of the 794 samples, 163(20.53%) were positive. Of them, 56(34.36%) samples showed resistance to rifampicin, 47(28.8%) samples to streptomycin, 31(19%) to ethambutol, 16(9.82%) to pyrazinamide and 59(36.2%) to

Table-1: Primers used for amplifications and sequencing.

Genes	Primers	Sequences 5'→3'	Product size (bp)	Reference
<i>rpoB</i>	TR8	TGCACGTCGCGGACCTCCA	157	Talenti et al. (1997) ⁹
	TR9	TCGCCGCGATCAAGGAGT		
<i>rpsL</i>	rpsL1	GGCCGCAACAGAACGT	500	(Elif et al., 2005) ¹⁰
	rpsL2	GTTCACTCAACTGGGTGAC		
<i>embB</i>	emBF	CGTGGTGATATTCCGGCTCC	900	(Self designed)
	emBR	CTTGGTGGGCGTGAACATC		
<i>pncA</i>	H37Rv-F	GGCGTTGATCATCGTCGAC	528	(Self designed)
	H37Rv-R	TCAGGAGCTGCAACCAACT		
<i>gyrA</i>	gyrA. F	ACCGTTGACATCGAGCA	626	(Self designed)
	gyrA. R	CTTAAACCCGCCCATGAC		
<i>gyrB</i>	gyrB.F	CCCCTGGTTTGAAGCCAA	628	(Self designed)
	gyrB.R	TTGATCTTCTCCCGCCCTT		

Table-2: Multidrug resistance profiles of *Mycobacterium tuberculosis* isolates.

S.No	Conc (µg/mL)	Drugs	Resistant strains	Sensitive strains
1	1.0	rifampicin	56	107
2	1.0	streptomycin	47	116
3	5.0	ethambutol	31	132
4	100	Pyrazinamide	16	147
5	2.0	Ofloxacin	59	104

Table-3: Mutations in *M. tuberculosis* genes that confer resistance to 1st and 2nd line drugs.

Gene	Codon/nucleotide change(s)	Amino acid/nucleotide change(s)	Total. No of isolates
rpoB	TCG to TTG	Ser450Leu	6
	GAC to GGC	Asp435Gly	2
	TCG to CAG	Ser450Gln	2
	TCG to TTG and CCC to CAC	Ser450Leu1 and Pro454His	2
	TCG to TTG and GGC to GAC	Ser450Leu and Gly455Asp	2
	rpsL	AAG to AGG	Lys43Arg
AAG to AGG		Lys88Arg	2
AAA to ATA		Lys111Ile	2
embB	ATG to CTG	Met306Leu	2
	GCC to GTC	Ala281Val	4
	ATG to GTG and GCC to GTC	Met306val and Ala281Val	2
	ATG to CTG and GCC to GTC	Met306Leu and Ala281Val	2
pncA	CAG to CCG	Gln141Pro	1
	TCC to TCT and GGT to AGT	Ser65Ser* and Gly132Ser	1
	G insertion	Frame shift+392G	2
	TCC to TCT and TGT to TGA	Ser65Ser* and Cys138Stop	1
	gyrA	GAC to GGC	Asp94Gly

ofloxacin (Table-2).

Moreover, 14(25%) rifampicin-resistant isolates were randomly selected and analysed for mutation in *rpoB* gene. All of them showed mutations in *rpoB* gene. Ser450Leu mutation was detected in 6(42.9%) isolates, Asp435Gly mutation in 2(14.3%) isolates, Ser450Gln mutation in 2(14.3%) isolates, double mutation of Ser450Leu and Pro454His in 2(14.3%) isolates, and double mutation of Ser450Leu and Gly455Asp was detected in 2(14.3%) isolates.

Furthermore, 16(34%) streptomycin-resistant isolates were analysed for mutation in *rpsL* gene. Of them, 6(37.5%) isolates showed *rpsL* gene mutations. Lys43Arg mutation was observed in 2(12.5%) isolates. Lys88Arg mutation was detected in 2(12.5%) isolates. A novel

Lys111Ile mutation was observed in 2(12.5%) isolates.

Also, 16(51.6%) ethambutol-resistant isolates were analysed for mutation in *embB* gene. Of them, 10(62.5%) isolates showed *embB* mutation at either codon 281 or 306 or both. Ala281val mutation was observed in 4(25%) isolates and Met306Leu mutation was observed in 2(12.5%) isolates. Double mutation of Met306val and Ala281val was observed in 2(12.5%) isolates. Another double mutation of Met306Leu and Ala281val was also observed in 2(12.5%) isolates.

Besides, 8(50%) pyrazinamide-resistant isolates were analysed for mutation in *pncA* gene. Of them, 6(75%) isolates had mutations. Insertion of G at position 392 was observed in 2(25%) isolates. Gln141Pro mutation was observed in 1(12.5%) isolate. Double mutation of

Ser65Ser* and Gly132Ser was observed in 1(12.5%) isolate. Double mutation of Ser65Ser and Cys138Stop was observed in 1(12.5%) isolate.

Among 20(33.9%) ofloxacin-resistant isolates analysed for mutation in *gyrA* gene, 9(45%) isolates showed Asp94Gly mutation. All isolates showed Ser95Thr mutation.

No mutation was found in *gyrB* gene among the 20(33.9%) ofloxacin-resistant isolates analysed for mutation in *gyrB* gene (Table-3).

The mutation results are shown in Table-3.

Novel sequences determined in the current study were recently deposited in the gene bank database (accession no. KX501217, KX501218 and KX501219).

Discussion

Mutations in *M. tuberculosis* genome play an important role in the development of resistance against antibiotics used for treatment of tuberculosis. Reports across the globe have highlighted this aspect of *M. tuberculosis* adaptability to resist various treatment options. The KPK province is a high-burden TB region of Pakistan, but little information is available about the molecular characteristics of *M. tuberculosis* strains prevailing. Current research reports for the first time the molecular characterisation of extensive drug resistance among TB isolates from Khyber Pakhtunkhwa province of Pakistan.

Among all the available anti-tuberculosis drugs, molecular mechanisms of rifampicin resistance mechanisms is most completely understood and it is recognised that mutations within the hotspot region of *rpoB* occur in 95% or more of rifampicin-resistant isolates.^{7,8} Rifampicin resistance due to common mutation at codons 531, 516, and 526 of the *rpoB* gene is consistent with data from Pakistan and other countries.¹¹⁻¹⁵ We observed a high frequency of mutations at codon 450, 435, 454 and 455 of *rpoB* gene. Mutations reported in the current study are not commonly observed mutations, however, they have been reported in studies conducted in South Africa and Mexico.^{16,17} One novel mutation, i.e. Gly455Asp, in *rpoB* gene was reported in the current study. Phenotypic resistance to rifampicin was 100% correlated with mutations in the hot spot region of *rpoB* gene indicating a need for studying molecular characteristics of the isolates along with conventional drug susceptibility testing.

Streptomycin-resistant strains are developed due to mutations in *rpsL* and *rrS* genes. We found 37.5% mutation frequency in *rpsL* gene in streptomycin-resistant isolates, similar to a study performed in

Barcelona which reported 24.6% *rpsL* gene frequency.¹⁸ The most common (Lys43Arg and Lys88Arg) mutations in *rpsL* gene reported in our study have also been reported in isolates from patients in Punjab, Pakistan, and from Barcelona.^{1,18} Lys111Ile novel mutation detected in streptomycin-resistant isolates could have a role in streptomycin resistance.

The importance of mutations in *embB* gene, particularly those at codon 306, had been controversial because similar kinds of mutations have been detected in both ethambutol-resistant and susceptible isolates.¹⁹ The most common mutations in *embB* gene observed in our study were Met306Leu, Met306Val and Ala281Val. The results of the current study are in line with previous studies from Poland and India.²⁰⁻²²

We also observed different types of *pncA* mutations associated with PZA resistance. Mutations in *pncA* gene found in the current study have also been reported worldwide.²³⁻²⁶

Resistance to fluoroquinolones in *M. tuberculosis* has been mostly attributed to *gyrA* mutations. Asp94Gly mutation frequency was 48.7% in a study conducted in China which is similar to our study (45%).²⁷ Asp94Gly mutation has been reported both regionally and globally.^{11,28} All the isolates studied showed Ser95Thr mutation which is a naturally occurring mutation that is not linked to fluoroquinolone resistance, as it occurs in both fluoroquinolone-susceptible and fluoroquinolone-resistant strains. In the current study no mutation was found in *gyrB* gene among ofloxacin-resistant isolates.

Distributions of various mutations associated with drug resistance in the studied population are somehow different from those reported globally. These variations in mutations coupled with drug resistance might be due to geographical distribution of the distinct strains.

Conclusion

Distributions of various mutations associated with drug resistance were somehow different from those reported globally. Some novel mutations in *rpoB*, *rpsL* and *pncA* genes were also observed.

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Disclaimer: None.

Conflict of Interest: Dr. Bashir Ahmad, the author of the manuscript, signed the ethical statement. He was the head of ethical committee. He was the senior most

professor and according to rules and regulations the senior most professor should be the head of ethical committee.

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