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Molecular Characterization of Multi Drug Resistant *Mycobacterium tuberculosis* Isolated from the Patients in the Hospital of Azad Jammu and Kashmir

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Key words
Rifampicin resistance, rpoB gene, Multi drug resistance, Isoniazid, katG mutation, *inhA* gene, Line probe assay, Multi drug resistant tuberculosis

ABSTRACT

Globally, Pakistan, with population of around 231.4 million, is at 5th spot in the list of countries with the highest number of TB patients in the world and it is further challenged by an increase in drug-resistant TB cases. The present study was conducted to estimate multi drug resistant (MDR) tuberculosis, to analyse mutations responsible for the drug resistant tuberculosis and to control the spread, better understanding of *Mycobacterium tuberculosis* in Azad Jammu and Kashmir state of Pakistan. A total of 463 suspected patients of AJK having mean age of 41.54 were included in this study from January 2014 to December 2015. These suspected patients were examined and their samples were tested through Gene Xpert to find out Rifampicin (Rif) resistance. A total of 3.8% Rif resistant cases were detected in Abbas Institute of Medical Science, AJK. Drug resistant genes analysis was done in National Reference Lab, Islamabad. Thirty seven TB patients were further processed through Line Probe Assay (LPA) to analyse common mutations responsible against first line drugs. Genes associated with drug resistance like katG and inhA for Isoniazid (INH), rpoB for RIF resistant isolates were checked for mutation using PCR amplification and LPA. It was observed that out of 37 MDR patients 25 patients (67.5%) had mutations in rpoB gene. Observed mutations were at codon 531 (72%) and 526 (16%) while 27 (72.9%) patients were resistant to INH. 22 (59.4%) patients had katG mutation at codon 315. The C to T transition at point -15 for inhA promoter region was also observed in 2 (0.054%) patients responsible to resist INH. Our study in Azad Jammu and Kashmir underscore the urgent need for targeted interventions in AJK to curb the spread of drug-resistant TB. There should be a large scale screening of communities for the timely detection of TB and MDR TB to reduce the disease burden and saving the lives of suffering human beings.

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Abbreviations
AIDS, acquire immune deficiency syndrome; AM-A, amplified mixes A; AM-B, amplified mixes B; AMK, Amikacin; ATT, anti-tuberculosis treatment; B +ive, bacteriology positive; bp, base pairs; CAT I, category I; CAT II, category II; CPR, capreomycin; DST, drug susceptibility testing; EMB, ethambutol; EPTB, extra pulmonary tuberculosis; FAS-I, fatty acid synthesis system I; FAS-II, fatty acid synthesis system II; FLQ, fluoroquinolone; Gyr, gyrase; HIV, human immunodeficiency virus; INH, isoniazid; KAN, kanamycin; LAM, lipo-arabinomannan; LJ, lowenstein-

jenson; LPA, line probe assay; M. tb, *Mycobacterium tuberculosis*; MDR, multi drug-resistance; MgCl2, magnesium chloride; MGIT, microbial growth indicating tubes; ml, milliliter; MTB, *Mycobacterium tuberculosis*; MTBC, *Mycobacterium tuberculosis* complex; MTBDR, *Mycobacterium tuberculosis* drug resistance; OFL, oflaxacin; PCR, polymerase chain reaction; PTB, pulmonary tuberculosis; PZA, pyrazinamide; RIF, rifampicin; RRD, rifampicin resistance detected; STR, streptomycin; TB, tuberculosis; WHO, world health organization; XDR, extensively drug resistant; µg, microgram; µl, microliter.

INTRODUCTION

In human history tuberculosis (TB) is one of the oldest infective disease. It most commonly affects the lungs and is an infectious bacterial disease (Mamta and Subodh, 2013). The causative agent of TB is *Mycobacterium tuberculosis (M. tb)* which is a slow mounting acid fast bacillus. *M. tb* having a G-C rich genome is a gram-positive bacterium. *M. tb* and the close lineages of *M. tb* Complex (MTBC) such as *M. bovis* and *M. africanum* have at least ten thousand strains. Current studies have revealed a significant relation between the genetic lineage of strain involved and the geographic origin of TB (Brudey *et al*., 2006; Hirsh *et al*., 2004). MTBC cause tuberculosis in humans and animals, consist of a group of five closely related sibling species (*M. tb sensu stricto* (s.s.), *M. africanum*, *M. microti*, *M. bovis*, and *M. canettii*) (Herrera-Leon *et al*., 2005; Kim *et al*., 2004; Sajduda *et al*., 2004; Kiepiela *et al*., 2000).

Multi drug resistant (MDR) TB is resistant to the two main anti-TB drugs, isoniazid (INH) and rifampicin (RIF). Worldwide 440,000 cases of MDR TB have been reported in 2008. Pakistan is amongst 27 countries with high load of MDR TB. It was estimated that there were about 15000 MDR TB patients in Pakistan in 2008 (WHO, 2010, 2011). Genomic mutation in *M. tb* produce resistance to antibiotics, like the *kat-G* gene is resistant for INH and the *rpo-B* gene for RIF resistance (Bakonyte *et al*., 2005; Leung *et al*., 2003; Garcia *et al*., 2002; Makorousev *et al*., 2002; Dvorska *et al*., 2001).

In presence of catalase-peroxidase enzyme INH, a pro-drug (in bacterial cell it became inactive), is changed into radical which is toxic, oxidising and stop the preparation of important component of bacterial cell wall i.e. mycolic acid. The molecular mechanism of RIF is less complex than of INH resistance (changes are observed commonly in *katG* gene, *inhA* gene and less common in *oxyR*, *ahpc* and *kasA* genes) (Bartfai *et al*., 2001). 50 to 95% of INH resistance all over the world is due to mutation in *katG* gene that codes for catalase-peroxidase (Ramaswamy and Musser, 1998). Extensively drug resistant tuberculosis (XDR-TB) needs an MDR phenotype with also resistance to any of fluoroquinolone (FLQ) and have minimum one of the second-line injectable drugs kanamycin (KAN) or amikacin (AMK), capreomycin (CPR) (WHO, 2006). The people belonging to AJK have scarce information about epidemics of TB due to lack of proper guidance, also there is insufficient data regarding the gene profiling related to the drug resistant tuberculosis in AJK.

The primary focus of the present study was aimed to determine the incidence of DR-TB in AJK, providing crucial insights into the prevalence and impact of drug resistance in tuberculosis cases within the region. Secondly, the research sought to analyze the mutations responsible for antibiotic resistance present in the genome of *Mycobacterium tuberculosis* found in AJK. This genomic analysis aimed to enhance our understanding of the molecular mechanisms contributing to drug resistance, facilitating the development of targeted interventions.

MATERIALS AND METHODS

Study population, sample collection and initial screening

This study was accomplished in Abbas Institute of Medical Science (AIMS) hospital Muzaffarabad as well as patients of AJK referred to National Reference Lab (NRL) Islamabad with suspected tuberculosis. The molecular characterization of MDR cases was carried out in NRL Islamabad.

Sputum samples of 463 susceptible patients belonging to AJK were collected from Abbas Institute of Medical Sciences, AJK. The samples collection initiated before the onset of Covid-19 pandemic, was continued for 3 years. For liquefaction and extraction of DNA, sample-reagent (sputum and sputolysin reagent) of 2:1 ratio was used. After incubation and vortex mixing, the sample was slowly dispensed into a cartridge to minimize the chances of aerosolization and biohazard.

The prepared cartridge containing the liquefied sample was loaded into the gene Xpert Dx software within five hours. The Xpert Assay required nearly two hours to detect both MTB and RRD (rifampicin-resistant) cases. Results from this assay were interpreted using the DX software system, categorizing cases into MTB detected, with levels ranging from very low to high. Subsequently, MTB cases identified as drug-resistant by gene Xpert underwent further examination to analyze the mutations responsible for drug resistance.

Sputum analyses and MTB culture

A total of 189 samples belonging to AJK were referred to National Reference Laboratory, Islamabad from January 2014 to December 2015. Of these samples 37 *M. tb* cultures were collected randomly for the analysis of common mutations responsible for two universally used drug resistance. Lowenstein–Jensen (LJ) medium and MGIT 7H9 was used for the isolation and culturing

of *M. tb* isolates from sputum and other specimens. The MTB isolates were then tested for drugs susceptibility against RIF, INH, EMB and STR on LJ and MGIT media. These samples were then further processed to analyse DR mutations by using LPA. Details of culturing and DST is given as under.

N-acetyl L-cysteine (NALC-NaOH) was used to decontaminate the sputum samples. 2 ml sputum sample was mixed with equal volume of NALC-NaOH solution. Vortexed for 15-20 seconds and kept at 20-25°C for 10-15 min. Then tubes were filled with phosphate buffer, vortexed and kept into centrifuge at 3000g for 15 min. Supernatant was poured off, while the residue was re-suspended in 0.3 ml phosphate buffer. It was then inoculated onto two labeled slopes of LJ medium and one tube of MGIT, and incubated for up to eight and six weeks respectively, or until the appearance of any visible colonies at 37°C. 250µl residue was used to inoculate into each slope of LJ and 500µl for MGIT. By using 1-2 drops of this re-suspended sample marked slide was used for smear preparation and microscopic examination. For drug sensitivity testing, the confirmed isolates of M. tb were checked by using LJ medium. This in vitro test is used for rifampicin, isoniazid, streptomycin and ethambutol. One percent proportion method as per standard guideline was used for drug susceptibility testing (Hiza *et al*., 2017).

MTB DNA extraction

We utilized the Genolyse approach for DNA extraction as used by Kaswabuli *et al*. (2022), following the manufacturer's instructions provided in the GenoQuick® MTB kit (Hain Lifescience, GmbH, Hardwiesenstrasse 1/ 72147 Nehren/ Germany). Initially, a 500µl decontaminated sample was transferred into a labeled 1.5 ml screw cap tube and centrifuged at 10,000xg for 15 min. The resulting sediment was re-suspended in 100 µl lysis buffer (A-LYS), vortexed, and incubated at 95°C in a water bath. Subsequently, the re-suspended sample was mixed with 100 µl of neutralization buffer (A-NB), vortexed, and centrifuged for 5 min. For PCR amplification, 5µl of the supernatant was directly used. PCR amplification involved the use of two optimized solutions: Amplified Mix A (AM-A), which contained Taq polymerase, and Amplified Mix B (AM-B), comprising a mixture of primers, nucleotides, buffer, and $MgCl_2$.

Line probe assay (LPA) for mutation detection

The line probe assay for mutation detection was conducted manually using a Twincubator. Initially, hybridization buffer and stringent solution were preheated to 45°C in a water bath. A labeled plastic tray with 12 wells was prepared, and 20µl of denaturation solution was added to one corner, followed by gentle mixing of 20 µl of amplified solution or PCR products into the denaturation solution. The mixture was then incubated at room temperature for 5 min. Preheated hybridization buffer was added to each well of the tray, and one pre-labeled strip was added to each well. The tray with test strips and solution was then incubated in a Twincubator at 45°C for 30 min. Following incubation, stringent wash solution was added to each well and incubated at 45°C for 15 min. Subsequently, rinse solution was added to each well and incubated for 1 min before removal. Pre-diluted conjugate was then added to each well and incubated at 25°C for 30 min.

After incubation, all solutions were removed, and labeled strips were washed twice with rinse solution for 1 min. each. Additionally, 1 ml of water was used for a third-step wash. Substrate was added to each strip and incubated for 5-7 min, protected from light. Finally, all solutions were removed, and the reaction was stopped by rinsing the strips twice with distilled water. The strips were then gently removed from the tray and allowed to dry.

Interpretation and storage

The genotype MTB-DR-plus LPA strip consists of 27 reaction bands, including 6 control bands and various wild-type and mutant probes. Outcomes were interpreted according to the manufacturer's directions. Remaining processed sample deposits, DNA extracts, and amplified solutions were stored at -20°C. Each laboratory had -20°C deep freezers with accurate labeling for storing PCR products and DNA extracts separately. Any invalid results on LPA were repeated using processed stored deposits. Additionally, sterile molecular-grade water and MTB H37Rv strain were included in each step as positive and negative controls, respectively.

RESULTS

Of the 463 patients included in this study, 18 (3.88%) showed resistance to RIF. Table I shows the details of 189 patients from NRL. During 2014, 27 cases were B-positive cases, 18 cases were culture positive but only 8 cases (29.63% of B-positive cases) were available for phenotypic drug susceptibility testing (DST). During 2015, 87 of of 137 were B-positive cases, 49 were culture positive but only 32 cases (36.78% of B-positive cases) were available for phenotypic drug susceptibility testing (DST). A total of 8 cases from 2014 and 29 cases from 2015 were considered for LPA to analyze mutations (Table I).

Gender base area-wise population distribution

The gender base area-wise patient distribution are

shown in the Table II. A total of 189 patients from various districts of AJK were included in the study. Among them, 103 patients (54.4%) were males, while 86 (45.5%) were females. Among the various districts, Bagh had the highest number of included patients, with 39 males (37.9%) and 28 females (32.6%), totaling 67 participants. Kotli followed with 16 male patients (15.5%) and 9 female patients (10.5%), accounting for 25 participants. Mirpur had 11 male patients (10.7%) and 9 female patients (10.5%), totaling 20 participants. Muzaffarabad had 16 male patients (15.5%) and 12 female patients (14%), making up 28 participants. Poonch included 7 male patients (6.8%) and 10 female patients (11.6%), totaling 17 participants. Other districts, such as Bhimber, Jhelum Valley, Neelum, and Sudhnoti, also contributed to the patient distribution, even though with smaller numbers.

Table I. Details of the samples included in the present study.

Number of	2014	2015	Total
Total specimen received	52	137	189
B +ve cases	27	87	114
Culture positive	18	49	67
Pheno-DST available	8	32	40
LPA	8	29	37

History of anti-tuberculosis treatment in different districts of AJ and K

Anti-tuberculosis treatment (ATT) comprises of category I (CAT-I) and category II (CAT-II) treatments. The CAT-1 treatment is actually the combination of five different drugs like rifampicin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB) and pyrazinamide (PZA) whereas in the CAT-II treatment kanamycin (KAN), amikacin (AMK), capreomycin (CPR), oflaxacin (OFL) and floroquinolone (FLQ) are used in combination. Some of these patients were never treated before that, some of these were previously treated. It was found that 51 patients were having no treatment before that and 138 patients were those who were previously treated with CAT I and CAT II (Table II). The history of anti-tuberculosis treatment varied across different districts of AJ and K. Bagh district accounted for the highest number of participants, with 67 individuals included in the study. Among them, 52 participants (35.4%) had never been treated for tuberculosis before, while 15 participants (10%) had a history of previous treatment. Bhimber district had a smaller number of participants, with 11 individuals included. Among them, 8 participants (5.8%) were previously treated, while 3 participants (2%) were new to tuberculosis treatment. In Jhelum Valley, only one participant was included, who had never been treated for tuberculosis before. Kotli district had 25 participants, with 19 (13.2%) having received previous treatment and 6 (4.2%) being new to treatment. Similarly, in Mirpur, out of 20 participants, 16 (10.6%) had a history of previous treatment, while 4 (2.6%) were new to treatment. Muzaffarabad had 28 participants, with 22 (14.8%) having received previous treatment and 6 (4%) being new to treatment. Neelum district had 4 participants, all of whom were new to tuberculosis treatment. Poonch district had 17 participants, with 14 (9%) having received previous treatment and 3 (2%) being new to treatment. Sudhnoti district had only one participant, who had never been treated for tuberculosis before.

Table II. Patients included in the study from different districts of AJK and their previous history of treatment.

AJK Districts	Number of patients included			Previous treatment history		
	Females	Males	Total	Never treated	Previously treated	Total
Bagh	28 (32.6%)	39 (37.9%)	67	15	52	67 (35.4%)
Bhimber	6 (7%)	5 (4.9%)	11	3	8	11 (5.8%)
Jhelum Valley	0 (0%)	1 (1%)	1	0	1	1 (0.5%)
Kotli	9 (10.5%)	16 (15.5%)	25	6	19	25 (13.2%)
Mirpur	9 (10.5%)	11 (10.7%)	20	4	16	20 (10.6%)
Muzaffarabad	12 (14%)	16 (15.5%)	28	6	22	28 (14.8%)
Neelum	3 (3.5%)	1 (1%)	4	2	2	4 (2.1%)
Poonch	10 (11.6%)	7 (6.8%)	17	3	14	17 (9%)
Sudhnoti	1 (1.2%)	0 (0%)	1	1	--	1 (0.5%)
No specific area mentioned	8 (9.3%)	7 (6.8%)	15	--	--	--

Comparison between gene Xpert, DST and LPA

Out of the 189 samples sent to the National Research Lab (NRL), 37 were checked more with line probe assay (LPA). Before this, another test called phenotypic drug susceptibility testing (DST) was done to confirm drug resistance. The results of these 37 samples were compared using three methods: gene Xpert, LPA, and phenotypic DST. Eleven suspects were resistant to all three tests. None were resistant to both Xpert and LPA but not to DST. Only one sample was resistant to Xpert and DST but not to LPA. None showed sensitivity with Xpert but resistance with both LPA and DST. One sample was sensitive to both Xpert and DST but not to LPA. Only one sample was sensitive to Xpert and LPA but not to DST; however, seven were sensitive to all three. Sixteen samples were not tested with Xpert but were with LPA and DST. Out of these, 12 were resistant to both LPA and DST. Additionally, one was resistant to LPA but not to DST, one was sensitive to LPA and resistant to DST, and two were sensitive to both LPA and DST. Table III presents a comparison between Gene Xpert, phenotypic drug susceptibility testing (DST), and line probe assay (LPA) results for rifampicin (Rif) resistance.

Table III. Comparison between Gene Xpert, DST and LPA.

Xpert	LPA	Phenotypic DST	
		Resistant	Sensitive
Rif R	Rif R	11	0
	Rif S	1	0
Rif S	Rif R	0	1
	Rif S	1	7
NA	Rif R	12	1
	Rif S	1	2

Correlation between RIF resistant and RIF sensitive samples

The 37 MTB samples analyzed through LPA were from 2014 and 2015. In 2014, 8 samples were processed, with 6 samples (75%) being RIF resistant and 2 samples (25%) RIF sensitive on LPA. Similarly, in 2015, 29 samples were processed, with 25 samples (86.2%) being RIF resistant and 12 samples (41.4%) RIF sensitive on LPA. Table IV illustrates the resistance and sensitivity patterns of RIF in MDR samples from 2014 and 2015.

Correlation between INH resistant and INH sensitive samples

Additionally, the 37 samples from AJK also exhibited patterns of isoniazid (INH) resistance and sensitivity, as mono-resistance to rifampicin alone is not possible. The pattern of INH resistance and sensitivity was closely associated with the pattern of RIF resistance/sensitivity. Out of the 8 samples from 2014, 6 samples (75%) showed INH resistance, while 2 samples (25%) showed INH sensitivity on LPA. Similarly, out of the 29 samples from 2015, 20 samples (69%) exhibited INH resistance, and 9 samples (31%) showed INH sensitivity on LPA. In total, 27 samples showed INH resistance on LPA. Among these 27 samples, 2 samples exhibited mono-resistance to INH. Table IV showed resistance and sensitive pattern of INH in MDR samples of 2014 and 2015.

Table IV. Correlation of RIF resistant - RIF sensitive and INH resistant and INH sensitive samples of 2014 and 2015.

Year	Rifampicin		Isoniazid	
	Resistant	Sensitive	Resistant	Sensitive
2014	6	2	7	1
2015	19	10	20	9
Total	25	12	27	10

Comparison between gene Xpert and LPA

Out of these 37 MDR samples, 21 were selected for the comparison between gene Xpert and LPA. Among these 21 samples, 12 (57.1%) were resistant and 9 (42.9%) were sensitive on LPA. Of the 12 resistant samples, 11 (91.7%) were identified as RRD, and 1 sample (8.3%) was marked as ND on gene Xpert. Among the 9 LPA-sensitive samples mentioned above, 1 (11.1%) was classified as RRD, while 8 samples (88.9%) were categorized as ND (not detected) on Xpert Assay (Table V).

Table V. Comparison between gene Xpert and LPA results.

Diagnostic tools	No. of Rif resistant participants	No. of Rif sensitive participants	Total participants
Gene Xpert	11 (91.7%)	1 (8.3%)	12
LPA	1 (11.1%)	8 (88.9%)	9

Comparison between phenotypic DST and LPA

RIF-resistant samples were obtained from various regions or districts of AJK. Among the 28 RIF-resistant samples identified through DST, 1 sample (3.57%) was from Bagh, 1 sample (3.57%) was from Bhimber, 5 samples (17.86%) were from Kotli, 4 samples (14.29%) were from Mirpur, 14 samples (50%) were from Muzaffarabad, 1 sample (3.57%) was from Neelum, 1 sample (3.57%) was from Poonch, and 1 sample (3.57%) was from AJK

but lacked specific area information. Out of the 25 RIF-resistant samples identified through LPA, 1 sample (4%) was from Bagh, none were from Bhimber, 5 samples (20%) were from Kotli, 2 samples (8%) were from Mirpur, 13 samples (52%) were from Muzaffarabad, 1 sample (4%) was from Neelum, 1 sample (4%) was from Poonch, and 2 samples (8%) were from AJK but their specific district/area was not mentioned (Table VI).

Table VI. Comparison between phenotypic DST and LPA results regarding MDR TB.

Name of districts	MDR on	
	Phenotypic DST	LPA
Bagh	1	1
Bhimber	1	0
Kotli	5	5
Mirpur	4	2
Muzaffarabad	14	13
Neelum	1	1
Poonch	1	1

Mutation pattern in different districts of AJ and K

Our study revealed that out of 37 MDR cases, 25 (67.6%) were resistant to RIF as detected by LPA, which can identify common mutations such as those in the rpoB, InhA, and katG genes. Mutations in the rpoB gene, in particular, are associated with RIF resistance, a crucial drug in TB treatment. The mutation pattern of the rpoB gene varies across different regions of AJK. Among the 25 RIF-resistant samples, 18 (72%) exhibited the rpoB MUT3 mutation, while 4 (16%) exhibited the rpoB MUT2A mutation, and 3 (12%) had unknown mutations. This mutation pattern also differs among the various districts of AJK. Specifically, among the 18 samples with the rpoB MUT3 mutation, 5 (27.8%) were from Kotli, 1 (5.6%) from Mirpur, 10 (55.6%) from Muzaffarabad, 1 (5.6%) from Neelum, and 1 from AJK with no specific district mentioned. Among the 4 samples with the rpoB MUT2A mutation, 1 (25%) was from Mirpur, 2 (50%) from Muzaffarabad, and 1 (25%) from the Poonch district. Additionally, among the 3 samples with unknown mutations, 1 (33.3%) was from Bagh, 1 (33.3%) from Muzaffarabad, and 1 from AJK, but the area/district was not specified (Table VII).

Possible mutations and their relevant amino acids

The 18 samples with the *rpoB* MUT3 mutation had mutations at position 531 of the *rpoB* gene, where the amino acid serine was converted into leucine. Similarly, the 4 samples with the rpoB MUT2A mutation had mutations at position 526 of the *rpoB* gene, where the amino acid histidine was converted into leucine. The other 3 mutations were also present but were unidentified by LPA. This was because LPA can only detect mutations controlled by probes on the nitrocellulose strips. These mutations were identified due to the absence of rpoB wild-type (WT) bands at positions 3 and 4 on the nitrocellulose strips, hence they are termed as "unknown" mutations due to the absence of their mutated bands on the LPA strip.

Table VII. Mutation pattern in different districts of AJK.

Districts	*rpoB* MUT3	*rpoB* MUT2A	UK MUT
Bagh	-	-	1
Kotli	5	-	-
Mirpur	1	1	
Muzaffarabad	10	2	1
Neelum	1	-	-
Poonch	-	1	-
Total	18	4	3

Similarly to RIF, INH is also a potent drug used against TB. Out of the 37 MDR samples, 27 were resistant to INH, while the remaining 10 showed sensitivity to INH. Among the 27 INH-resistant samples, 22 were resistant due to the katG MUT1 mutation, where at position 315 of the katG gene, the amino acid serine was converted into tyrosine. INH resistance can also result from mutations in another gene, InhA, where at position 15 of the InhA gene, the base pair cytosine is converted into thymidine. Three additional mutations are responsible for INH resistance, but these mutations were unidentified by LPA (Table VIII).

Table VIII. Possible mutations and their relevant amino acids.

Rif resistance	No. of mutations	AA/ Codon
	25	**--**
ropB MUT3	18	S531L
rpoB MUT2A	4	H526Y
Unknown	3	--
INH Reistance	27	--
KatG MUT1	22	S315T
inhA MUT1	2	C-15T
Unknown	3	--

DISCUSSION

TB disproportionately affects low-income groups in poorly ventilated and congested areas. The study addresses the global rise in MDR TB cases, a challenge prevalent in Pakistan. To improve TB diagnosis, particularly MDR cases, traditional techniques like microscopy and drug susceptibility testing (DST) fall short in sensitivity and time efficiency. Hence, the study employs advanced technologies like the Gene Xpert Assay and LPA for suspected MDR TB patients, recommended by the WHO for rapid detection of *Mycobacterium tuberculosis* (MTB) and MDR TB (Helb *et al*., 2010).

This study focuses on AJK, an underdeveloped region in Pakistan with limited data on molecular epidemiology and drug-resistant genes of *M. tb*. The research aims to estimate the number of MDR TB suspects and identify prevalent drug-resistant genes in AJK, utilizing advanced techniques like Gene Xpert and LPA. Conducted at Abbas Institute of Medical Sciences (AIMS) in Muzaffarabad, where Gene Xpert Assay is exclusively available, the study includes patients from AJK referred to NRL Islamabad.

From January to December 2015, 463 susceptible TB patients at AIMS AJK were selected for MDR case estimation. Among them, 18 (3.8%) patients exhibited rifampicin (RIF) resistance on Xpert assay. RIF resistance, considered a surrogate marker for MDR-TB, is rare when occurring alone. The study emphasizes the high sensitivity of Gene Xpert compared to conventional methods. Data for further analysis of drug-resistant genes were collected from NRL Islamabad, involving 189 TB suspects referred from 2014 and 2015. The mean age of participants was 41.54 years, with 54.4% males and 45.5% females. Suspects exhibited common symptoms such as prolonged cough, fever, night sweats, weight loss, and anorexia. Out of 189 participants, 138 were taking anti-TB treatment (ATT), including first-line drugs (RIF, INH, EMB, PZA, STR) and second-line drugs (KAN, AMK, OFL, CPR, FLQ). Notably, 51 suspects had no history of ATT, indicating they were never treated with any anti-TB drugs.

A study demonstrated that among 108 smear-negative extra-pulmonary samples, the Xpert MTB/RIF assay (GX) for *M. tb* yielded positive results in 58.3% (63 cases) (Moure *et al*., 2011). Additionally, in a related investigation by Vadwai *et al*. (2011), the Xpert assay exhibited a sensitivity of 81% (228 out of 283 specimens), with 64% sensitivity for smear-negative cases and 96% for smear-positive cases, along with a remarkable specificity of 99.6%. It is important to note that the high detection rates observed in these studies were influenced by the inclusion of diagnosed TB cases, whereas our study specifically focused on TB suspects (Vadwai *et al*., 2011).

The Xpert assay not only detects TB but also identifies MDR TB through nested real-time PCR. The rapid identification of rifampicin (RIF) resistance is a unique advantage of the Xpert assay, providing results within two hours. In our study involving 189 suspects, 37 TB-positive samples underwent further analysis using LPA to investigate common mutations. Among these, 19 TB patients exhibited RIF-detected status on the Xpert assay, while the remaining patients were sensitive. The Xpert assay, known for its 100% sensitivity in detecting MTB DNA, was instrumental in evaluating 19 distinct and frequently occurring mutations in the rpoB gene (Helb *et al*., 2010). The genetic basis of antibiotic resistance in DR MTB isolates has been extensively researched. Point mutations in crucial resistance genes such as *katG, rpoB, embB*, and *rpsL* are commonly implicated in conferring resistance (Ramaswamy and Musser, 1998).

In our study, 67.5% of TB strains exhibited mutations in the RRDR of the *rpoB* gene, affecting two amino acid codons: 531 (72%) and 526 (16%). Interestingly, 3 mutations (0.081%) remained unknown by LPA due to the probes being labeled for the most common codons (531, 526) of the *rpoB* gene. Notably, the absence of rpoB wildtype 3 and 4 bands on LPA strips indicated a common mutation in codon 516, consistent with reports from other researchers. At position 531 of the *rpoB* gene, a serine amino acid was converted into leucine, represented by *rpoB* MUT3 on LPA. Similarly, at position 526, a histidine amino acid was converted into tyrosine, represented by *rpoB* MUT2A on LPA. Surprisingly, among the 37 TB suspects, 32.4% (12 individuals) were found to be sensitive on LPA.

Our study's findings align with global reports on mutations in the RRDR of the *rpoB* gene. Studies from various parts of the world, including Sun *et al*. (2008), indicate similarities in mutations at codons 531, 526, and 516 in RIF-resistant isolates. Consistent data from Pakistan and India (Ali *et al*., 2009; Ajbani *et al*., 2011) also support our results. However, it is noteworthy that 32% of isolates in our study did not show any mutation in the RRDR, contrasting with data showing only 4% of RIF-resistant isolates lacking RRDR changes (Yue *et al*., 2003). This discrepancy may stem from differences in prevailing genotypes across different regions globally. Additionally, studies have indicated that mutations associated with RIF resistance can occur outside the RRDR, although infrequently, aligning with the findings of our study and reinforcing similar observations by other scientists (Caws *et al*., 2006; Somoskovi *et al*., 2001). Their work consistently highlights that mutations in codons 516, 526, and 531 are most commonly linked with RIF resistance in the majority of studies.

In our study, the comparative effectiveness of gene Xpert and LPA was assessed using 21 samples. LPA demonstrated notable efficacy, revealing resistance in 57.1% of samples and sensitivity in 42.8%, while Xpert detected 52.3% and identified 0.047% as not detected. Importantly, LPA successfully identified 42.8% of samples deemed sensitive by Xpert, offering a superior DST profile and aiding in drug regimen decisions, particularly in patients with INH mono-resistance. Despite the rapidity of gene Xpert, it exhibits limitations in detecting INH resistance.

Contrasting our findings with broader research on TB drug resistance, Zheng *et al*. (2021) emphasized specific resistance patterns and genetic mutations, Li *et al*. (2022) revealed a high prevalence of drug resistance, especially in cases with prior treatment, and Yadav *et al*. (2023) underscored the increasing prevalence of M. tuberculosis and resistance to specific drugs.

Within our study conducted at AIMS, AJK, 3.8% of cases exhibited resistance to rifampicin. Subsequent analysis at the National Reference Lab in Islamabad uncovered common mutations in TB patients. Among 37 MDR patients, 67.5% displayed mutations in the *rpoB* gene (codons 531: 72%, 526: 16%), and 72.9% exhibited resistance to INH, primarily linked to the katG mutation at codon 315. Additionally, a C to T transition at position -15 in the inhA promoter indicated INH resistance in 0.054% of patients. These findings contribute to our nuanced understanding of the genetic mutations influencing TB drug resistance.

CONCLUSIONS AND RECOMMENDATIONS

Our study conducted in AJK revealed a concerning 3.8% resistance rate to rifampicin (RIF) among suspected TB cases. Analyzing 37 MDR patients, we found that 67.5% exhibited mutations in the rpoB gene, particularly at codons 531 (72%) and 526 (16%). High resistance rates to isoniazid (INH) (72.9%) and katG mutation (59.4% at codon 315) were observed. A C to T transition at position -15 in the inhA promoter region was noted in 0.054% of patients, indicating INH resistance. These findings underscore the urgent need for targeted interventions in AJK to curb the spread of drug-resistant TB. One of the objectives of the study was to raise awareness within the AJK community regarding TB transmission, preventive measures, and the impact of MDR TB on individuals' lives. By disseminating information, the study aimed to empower the community to take proactive measures in combating the spread of drug-resistant TB and mitigating its impact on public health in AJK. Gene sequencing should be employed to clarify mutations unidentified by LPA. Large-scale screening of communities is imperative for the timely detection of TB and MDR TB, thus reducing the disease burden and saving lives. A comprehensive preventive and awareness campaign is essential to eradicate this deadly disease. Proper surveillance and rehabilitation of affected populations across AJK are also crucial components of an effective strategy.

DECLARATIONS

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IRB approval, ethical approval and consent to participate

The study was approved by the Directorate of Advanced Studies and Research under approval number DASR/Zoo-9742/13-15, which have authority to approve research topics and it also deals with ethical issues in University of Azad Jammu and Kashmir, Muzaffarabad. The molecular characterization of MDR cases was carried out in National Research Lab, Islamabad. Permission was taken from the Hospital Ethical Committee. A filled and signed participation consent was collected from each participant and from their guardians before the sampling. All the information of the participants kept confidentially and laboratory testing and results provision were free of cost.

Availability of data and materials

The data is present in soft copy form (Excel sheet) as well as in the form of paper document and all the amplified samples are also present in our diagnostic centre, which will be provided to the journal on demand. Any audio and video data did not obtain during the study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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