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Detection of Mutations in 81-bpRifampin Resistance Determining Region (RRDR) of *rpoB* gene in *Mycobacterium tuberculosis* using GeneXpert MTB/RIF in Clinical Specimens from Quetta, Pakistan

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MZM, FA, AS and TMA designed the experiments. MZM, MN, HA and JR performed the experiments. MZM, MN analyzed the data. All authors contributed in manuscript writing.

Key words
***Mycobacterium tuberculosis*, Rifampicin resistance determining region, RNA polymerase beta gene, Xpert MTB/RIF assay**

ABSTRACT

GeneXpert MTB/RIF has revolutionized the tuberculosis diagnosis by simultaneous detection of *Mycobacterium tuberculosis* and resistance to RIF (rifampicin), a surrogate marker for multidrug-resistant TB in less than two hours. The RIF-resistance pattern in Balochistan, Pakistan, is not documented. This study was aimed to detect RIF-resistant TB and mutations in RNA polymerase beta (*rpoB)* gene of *M. tuberculosis* within 81-bp RRDR in Quetta, Pakistan using GeneXpert® MTB/RIF assay. In total, 2300 clinical specimens were collected from suspected TB patients at Fatima Jinnah General and Chest Hospital Quetta, Pakistan between January and August 2017. These specimens were analyzed by GeneXpert® MTB/RIF assay. The data was statistically analyzed using SPSS software. Out of 2300 clinical specimens, *M. tuberculosis* was positive in in 899 (39.1%) cases by GeneXpert® MTB/RIF assay [positive respiratory cases 42.9% (871/2032) and non-respiratory 10.4% (28/268) with statistically significant difference (χ^2= 104.5, p<0.001)]. Among 899 MTB positive cases, 46 (5.1%) were RIF-resistance caused by various *rpoB* gene mutations within 81-bp RRDR. Most of the RIF-resistant isolates were observed to harbor mutationsin Probe E 78.3% (n=36) whereas mutations in Probe A, B, D were observed 2.2% (n=1), 4.3% (n=2), and 6.5% (n=3), respectively. However, none of cases had RIF-resistance associated with Probe C. Out of 46 RRD cases, 21 (45.7 %) were males and 25 (54.3 %) were females. Additionally, Xpert® test showed higher detection rate than fluorescent microscopy (39.1% vs 31.2%, P<0.05) and detected MTB in 186 (11.8%) smear-negative specimens. Among 42 confirmed TB patients had MDR contact and eight patients were co-infected with HIV. In conclusion, 5.1% of the TB patients showed rifampicin resistance. The most frequent *rpoB* genetic mutations were observed in codons 531/533 (Probe E, 78.3%) whereas the least within the sequence 511 (Probe A, 2.2%).

INTRODUCTION

The emergence of multi-drug resistant tuberculosis (MDR-TB) has become a significant obstacle to global TB control. With the worldwide spread of *M. tuberculosis* (MTB) strains resistant to both isoniazid and rifampicin (RIF), MDR-TB has become a major public health problem posing formidable challenges due to its complex diagnostic and treatment requirements (Kant *et al*., 2010). This highlights the need for having rapid molecular diagnostic techniques which could facilitate early diagnosis and appropriate delivery of anti-tubercular therapy. GeneXpert MTB/RIF (Cepheid, USA), a real-time automated nucleic acid amplification system is one such technique which detects *M. tuberculosis* as well as mutations which confer resistance to rifampicin (most effective first-line drug) in less than two hours.

The detection of RIF-resistance serves as a surrogate marker for the detection of MDR-TB (resistance to at least isoniazid and rifampicin) because >90% of RIF-

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resistant MTB strains also exhibit resistance to isoniazid (Drobniewski *et al*., 1998; Sadri *et al*., 2016). Rifampicin binds the β-subunit of MTB RNA polymerase (*rpoB*) and thus inhibits RNA synthesis. RIF-resistance mechanisms usually involve missense mutations in the 81 base pairs (codons 507 to 533) hot-spot region of the *rpoB* gene referred to as Rifampincin-Resistance-Determining Region (RRDR) and 95% of RIF-resistant strains have mutations within this 81-bp RRDR (Ramaswamy and Musser, 1998; Van Der Zanden *et al*., 2003). The rapid spread of MDR/RR-TB particularly in new patients is challenging the success of tuberculosis control programs mostly in low-income countries, including Pakistan.

According to WHO report, 2018, the global incidence of TB was 10.0 million in 2017 with 160,684 cases of MDR/RR-TB. An estimated 558,000 people developed TB who showed RIF-resistance (RR-TB), and among these 82% had MDR-TB (WHO, 2018). Pakistan ranks fifth among 30 most tuberculosis affected countries and forth among the 27 countries with high burden of MDR-TB. In Pakistan 525,000 people developed TB and 54,000 deaths occurred among HIV-negative and 2200 among HIV-positive in 2017. Approximately 27,000 MDR/RR-TB cases emerged in Pakistan of which 16% were previously treated cases and 4.2% were new cases (WHO, 2018).

Studies conducted in diverse geographical areas have shown that the burden of MDR-TB and the mutations responsible for drug resistance vary from country to country and region to region (Purwar *et al*., 2011). However, data regarding prevalence of RR/MDR-TB and *rpoB* gene mutations is scarce at Quetta, Balochistan. Therefore, this study was conducted to determine the prevalence of rifampicin resistance and *rpoB* gene mutations among the suspected TB cases in this region using GeneXpert MTB/RIF. Knowledge of the pattern of mutations present in RIF-resistant isolates could provide insight into the epidemiology of RIF-resistant MTB isolates of this particular area.

MATERIALS AND METHODS

Ethical consideration

The current study was approved by the Research and Ethics Review Committee of University of Balochistan, Quetta. All the participants gave their informed consent.

Patients selection

The individuals with the symptoms of pulmonary and extra-pulmonary tuberculosis attending outpatient department (OPD) of Fatima Jinnah General and Chest Hospital, Quetta were recruited in the study. Patients' data including gender, age, MDR-contact, TB history etc. were also recorded.

Clinical specimens

A total of 2300 specimens were obtained from patients suspected to have TB infection. These specimens were screened for the routine mycobacteriological diagnostic tests at Provincial TB Reference Laboratory (BSL-3), Fatima Jinnah General and Chest Hospital, Quetta. All the specimens were examined for infection of *M. tuberculosis*, resistance to rifampicin as well as detection of mutations in the RRDR *rpoB* gene by GeneXpert MTB/RIF.

The patients' data and samples' characteristics (consistency and volume) were recorded. The samples collected were sputum, bronchoalveolar lavage (BAL), cerebral spinal fluid, plural fluid, gastric aspirate, ascetic fluid colon biopsy, pus, and urine. The specimens were directly collected in sterile containers and labeled with patient ID while other forms of specimens were collected by specialists and sent to the Laboratory. All the precautionary measures were adopted during sample collection. Specimens were processed immediately for laboratory testing.

Laboratory procedures

All samples were decontaminated using *N*-acetyl-L-cysteine (NALC)-NaOH technique except the CSF samples and followed by concentrating the samples by centrifugation for 15 minutes at 3000g using the standard protocol recommended by Center for Disease Control and Prevention (Kent and Kubia, 1985). For fluorescent microscopy, concentrated smears were prepared from decontaminated specimen sediments using standard protocol (Kent and Kubia, 1985), stained with Auramine-O and visualized under fluorescent microscope using 200X and 400X magnification for the presence of *M. tuberculosis.*

GeneXpert MTB/RIF (Cepheid) was performed in accordance with the manufacturer's guide. Briefly, the Sample Reagent (SR) was mixed with the decontaminated specimen in a 3:1 ratio and agitated for 15 minutes. The mixture was then introduced into the cartridge and loaded into the GeneXpert instrument. *M. tuberculosis* genomic DNA was extracted by sonication with subsequent DNA amplification by PCR. Moreover, the Xepert detects RIF-resistance cases conferred due to mutations in the *M. tuberculosis rpoB* gene using fluorescent probes know as molecular beacons (Fig. 1). The RIF-resistance detection is based on the amplicon hybridization with 5 overlapping probes complementary to 81-bp core region (RRDR) of *rpoB*MTB gene (El-Hajj *et al*., 2001).

Statistical analysis

Data analysis was performed using SPSS-20. Descriptively, the percentages and frequencies were

calculated for various variables. Chi-square test was used to investigate the associated factors of TB infection and RIF-resistance. *P* value <0.05 was considered statistically significant.

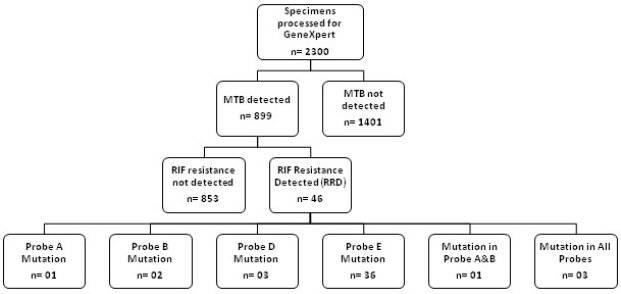

Fig. 1. Flow chart showing the specimen analysis.

RESULTS

Total of 2300 clinical specimens were collected from suspected TB patients during the study period. Of whom, 2032(88.3%) specimens were pulmonary and 268 (11.7%) were extra-pulmonary. The respiratory samples comprised sputum, gastric aspirates and bronchoalveolar lavage (BAL) while the non-respiratory samples included pleural fluid, pericardial fluid, ascetic fluid, cerebrospinal fluid (CSF), colon biopsy, urine and pus. The age of the patients ranged from 2 months to 107 years with the mean of 42.2 ± 22.8 years. Of the total, 1175 (51.1%) were males and 1125 (48.9%) were females with the sex ratio of 1:1.1.

From 2300 TB suspected cases, *M. tuberculosis* was found to be in 899 (39.1%) cases as detected by GeneXpert MTB/RIF. Among 899 confirmed patients, the rifampicin resistance was detected in 46 (5.1%) cases. RIF-resistance is conferred by *rpoB* gene mutations present within 81-bp RRDR overlapped by 5 different Probes A, B, C D, and E in GeneXpert. Of the 46 RIF-resistant patients, most of the cases (78.3%, n=36) harbored mutations in Probe E whereas mutations in Probe A, B, D were observed 2.2% (n=1), 4.3% (n=2), and 6.5% (n=3) cases, respectively. Three cases had mutations in all Probes and one had mutations in 2 Probes A and B. No patient with RIF-resistance was found to have Probe C related mutations. Among the RIF- resistant patients, 45.7 % were males and 54.3% were females. About half of the proportions of RIF-resistant patients were aged 40-59 (Table I). 42 patients had MDR contact and eight patients were co-infected with HIV.

GeneXpert® MTB/RIF detected TB-positive cases in 39.1% (899/2300) cases and negative in 60.9% (1401/2300) cases. Of these, GeneXpert detected MTB in 871/2032 (42.9%) respiratory and 28/268 (10.4%) non-respiratory specimens. Statistically significant difference (χ^2= 104.5, p<0.001) was observed in the *M. tuberculosis* positivity between pulmonary and extra-pulmonary tuberculosis (Table II).

Table I. Distribution of mutations in *rpoB* gene of RIF-resistant determining region (RRDR) with different variables.

Variable	Probe types						Total (%)
	A	B	D	E	A & B	All +ve	
Probe mutation	1	2	3	36	1	3	46 (5.1%)
Gender							
Female	0	1	0	21	0	3	25 (54.3)
Male	1	1	3	15	1	0	21 (45.7)
Age (year)							
<20	0	0	0	5	0	0	5 (10.9)
20-39	0	0	0	15	1	0	16 (34.8)
40-59	1	2	3	13	0	2	21 (45.7)
60-79	0	0	0	3	0	1	4 (8.7)

Table II. GeneXpert result for the detection of MTB in pulmonary and extra-pulmonary TB cases.

GeneXpert	N	Type of TB		P value
		Pulmonary (n=2032)	Extra-pulmonary (n=268)	
MTB detected	899	871(42.9%)	28(10.4%)	<0.001*
Not detected	1401	1161(57.1%)	240(89.6%)	

Chi-square, 104.5; *P, significant.

Among 2032 respiratory samples, 817/1802 (45.3%) sputum samples, 51/152 (33.6%) bronchoalveolar lavage specimens and 3/78 (3.8%) gastric aspirates were Xpert-positive. Among 268 non-respiratory MTB samples, 11/148 (7.4%) plural fluid specimens, 4/68 (5.9%) CSF samples, 10/28 (35.7%) pus, 2/3 (66.7%) lymph node, 1/2 (50%) pericardial fluid were GeneXpert-positive while no *M. tuberculosis* were found in ascetic fluid, urine and colon biopsy (Table III).

Among 2300 specimens, 718 (31.2%) showed smear-positivity whereas 1582 (68.8%) were smear-negative. Smear-negativity was mostly observed in pulmonary TB cases (1321/2032 = 65%) than extrapulmonary TB (261/268 = 97.4%) (χ^2= 115.6, p<0.001, Table IV).

Among 1582 FM-negative cases, GeneXpert yielded positive result in 186 (11.8%) cases while fluorescent microscopy (FM) detected *M. tuberculosis* in 5 (0.7%) cases of GeneXpert-negative samples. The detection rate of GeneXpert was statistically higher (χ^2= 1589.8, p<0.001) as compared with fluorescent microscopy (Table V).

Table III. Result of GeneXpert based on specimen type.

Clinical Specimen	No. of specimens (%)	GeneXpert (n=2300)	
		GX^+ (899) N (%)	GX^- (1401) N (%)
Sputum	1802 (78.3)	817 (90.9)	985 (70.3)
Broncho-alveolar lavage	152 (6.6)	51 (5.7)	101 (7.2)
Gastric aspirates	78 (3.4)	03 (0.3)	75 (5.3)
Plural fluid	148 (6.4)	11 (1.2)	137 (9.8)
Cerebral spinal fluid	68 (3.0)	04 (0.4)	64 (4.6)
Pus	28 (1.2)	10 (1.1)	18 (1.3)
Ascetic fluid	12 (0.5)	00 (0.0)	12 (0.9)
Urine	6 (0.3)	00 (0.0)	06 (0.4)
Lymph node	3 (0.1)	02 (0.2)	01 (0.1)
Pericardial fluid	2 (0.09)	01 (0.1)	01 (0.1)
Colon biopsy	1 (0.04)	00 (0.0)	01 (0.1)
Total	2300 (100)	899 (39.1)	1401 (60.9)

Table IV. Result of smear microscopy based on pulmonary and extra-pulmonary specimens.

Smear microscopy	Sample n	Type of TB		P value
		Pulmonary (n=2032)	Extra-pulmonary (n=268)	
Positive	718	711(35%)	7 (2.6%)	<0.001*
Negative	1582	1321(65%)	261 (97.4%)	

Chi-square, 115.6; *P=significant.

Table V. Comparison of GeneXpert and fluorescent microscopy.

Fluorescent microscopy	GeneXpert (n=500)		P value
	GX+(n=211)	GX– (n=289)	
Positive (n=718)	713 (99.3%)	5 (0.7%)	<0.001*
Negative (n=1582)	186 (11.8%)	1396 (88.2%)	

Chi-square, 1589.8; *P=significant.

Comparing the infection rate between the sexes, it was observed that more females 41.9% (471/1125) were infected by MTB than males showing the infection rate of 36.4% (428/1175). Xpert result differed significantly between males and females (χ^2= 7.147, p= 0.008). Half proportion of TB patients were aged between 20-39 years (49.2%), followed by 40-59 years (40.4%), 60-79 years (39.4%), above 80 years (35.8%) whereas least frequency of patients were aged below 20 years (25.6%). The difference in the age wise prevalence of TB was statistically significant (χ^2= 58.38, p<0.001, Table VI).

Table VI. GeneXpert result based on gender and age.

Variable	N (%)	GeneXpert		P value
		GX^+	GX^-	
Gender				
Male	1175(51.1%)	428(36.4%)	747(63.6%)	0.008^{ns}
Female	1125(48.9%)	471(41.9%)	654(58.1%)	
Age				
(year) <20	449 (19.5%)	115 (25.6%)	334 (74.4%)	<0.001*
20-39	543 (23.6%)	267 (49.2%)	276 (50.8%)	
40-59	579 (25.2%)	234 (40.4%)	345 (59.6%)	
60-79	620 (27.0)	244 (39.4%)	376 (60.6%)	
80<	109 (4.7%)	39 (35.8%)	70 (64.2%)	

Chi-square: gender, 7.147; Age, 58.38; ^{ns}P, non-significant; *P, significant.

DISCUSSION

Drug resistance in *M. tuberculosis* seems to result from the stepwise acquisition of new mutations in the genes for various drug targets (Heym*et al*., 1994). Emergence of MDR-TB is a serious challenge for clinicians; it arises mostly due to *rpoB* gene mutations (Miller *et al*., 1994). The frequency of mutations in MTB *rpoB* gene varies geographically (Adikaram *et al*., 2012). Mutations in *rpoB* gene have been reported earlier in Asian countries, which are generally related with a high level of RIF-resistance (Bahrmand *et al*., 2009). Despite Pakistan being a highly TB endemic area, few studies are available regarding molecular characterization of rpoB mutations in MDR-TB patients. The information regarding the prevalence of these mutations might be helpful for better therapy and management of MDR-TB patients. In the current study, we determined the prevalence of *rpoB* gene mutations conferring RIF-resistance in *M. tuberculosis* strains among TB patients.

Rifampicin resistance was mainly related with mutations in the 81-bp region within *rpoB* gene (Van Rie *et al*., 2001; Telenti *et al*., 2003). Studies based on DNA sequencing have indicated that above 95% of the RIF-resistant strains harbor mutations within the *rpoB* gene in 81-bp core region (Cavusoglu *et al*., 2002). Automated DNA sequencing has characterized greater than 50 mutations within this region, most of which possess point mutations at codons 516, 526 or 531 (Ahmad *et al*., 2002).

In our study the predominant genetic mutations in the 81-bp RRDR of *rpoB* gene were found in codons 531 (78.3%), 526 (6.5%), 513 (4.3%), 511 (2.2%), whereas no mutations was observed in codon 522. On the GeneXpert MTB/RIF assay these codons were represented by Probes E, D, B, A and C, respectively. A study by Mboowa *et al.*

(2014) in Kampala, Uganda found these frequencies 531 (58%), 513 (25%), 526 (8%), 511 (8%), and none for codon 522, while Ullah *et al*. (2016) in Khyber Pakhtoonkhwa, Pakistan also reported the most common mutations in codon 531 (77%) followed by codons 513 (10.8%), 526 (8.3%), 511 (1.2%), and 522 (1.5%). Khan *et al*. (2013) in Punjab, Pakistan found mutations in codons 531 (52%), 516 (15%), 512 (7%) and 526 (7%). These studies indicate that the most common mutation conferring RIF-resistance is associated with codon 531. Previous studies have shown the sensitivity of GeneXpert MTB/RIF to be 94.4-100% and that of specificity 98.3-100% for the detection of RIF-resistance (Moure *et al*., 2011).

RIF-resistance related with Probe C was not detected in this study, it could be attributed to the fact that this specific site within RRDR may probably be less susceptible to genetic mutations conferring drug resistance or Probe C related RIF-resistance is absent in our setting.

We found that GeneXpert detected 46 (5.1%) RIF-resistant TB cases out of 899 confirmed TB patients in Balochistan which is similar with the study by Masenga *et al*. (2017) who reported 5.9% RIF-resistant cases in Zambia. Several studies from different regions of Punjab, Pakistan reported 6%, 11.3% and 11.5% isolates resistant to at least one TB drug that is higher in comparison with our finding (Javaid *et al*., 2008; Qazi *et al*., 2014; Ullah *et al*., 2016). These variations in results could be due to different sample sizes in these studies.

In this study, the prevalence of RIF-resistance was almost similar in males and females (45.7 % *vs* 54.3 %). These findings are consistent with the study by Nair *et al*. (2016) in India who reported the similar risk of RIF-resistance among men and women. This could be due to the fact that both males and females are equally exposed to factors which cause RIF-resistance. However, we observed that more females were infected with MTB as compared with males with statistically significant difference (41.9%*vs* 36.4%, p= 0.008). In our study the frequency of TB patients was highest in the age group 20-39 years, which is in agreement with other studies conducted in Nairobi (Ndungu *et al*., 2013) and Pakistan (Munir *et al*., 2015), where the highest TB infection was observed in age groups of 18-34 and 21-50 years, respectively.

Balochistan, a province of Pakistan, has over thirty million population and shares a common border with Iran and Afghanistan. To the best of our knowledge, it is the first study to report the prevalence of rifampicin resistant TB cases and determine the frequency of *rpoB* gene mutations within 81-bp RRDR in this province.

In conclusion, GeneXpert MTB/RIF detected 46 rifampicin resistant cases out of 899 TB patients caused by *rpoB* gene mutations in the 81-bp RRDR. The most frequent *rpoB* gene mutation was observed in codon 531/533 (Probe E, 78.3%) while the least was detected within the sequence 511 (Probe A, 2.2%). Such studies on mutations can be useful for the development of novel therapeutics for the TB treatment.

Statement of conflict of interest

The authors declare there is no conflict of interest.

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