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MYCOBACTERIUM TUBERCULOSIS;

FREQUENCY OF RIFAMPIN RESISTANCE MUTATIONS IN 81-BP RRDR OF RPOB GENE IN MYCOBACTERIUM TUBERCULOSIS ISOLATES IN QUETTA, PAKISTAN.

Ghazala Siddique[1], Muhammad Shafee[2], Ferhat Abbas[3], Muhammad Naeem[4], Abdul Raziq[5], Shafique Ahmad[6], Khan Muhammad Babar[7], Ziauddin[8]

1. M.Phil. Scholar
Center for Advanced Studies in Vaccinology & Biotechnology, University of Balochistan, Quetta, Pakistan.
2. PhD.
Assistant Professor
Center for Advanced Studies in Vaccinology & Biotechnology, University of Balochistan, Quetta, Pakistan.
3. PhD.
Professor
Center for Advanced Studies in Vaccinology & Biotechnology, University of Balochistan, Quetta, Pakistan
4. M.Phil.
Lecturer
Center for Advanced Studies in Vaccinology & Biotechnology, University of Balochistan, Quetta, Pakistan.
5. M.Phil.
Assistant Professor
Department of Statistics, University of Balochistan, Quetta, Pakistan.
6. M.Phil.
Scientific Officer
Balochistan Agriculture & Development Center, (BARDC), Quetta.
7. MBBS, FCPS
Bolan Medical Complex Hospital, Quetta.
8. PhD
Lecturer
Assistant Professor
Center for Advanced Studies in Vaccinology & Biotechnology, University of Balochistan, Quetta, Pakistan.

Correspondence Address:
Dr. Ghazala Siddique
Western Bypass Hassan Colony, House No. 2 Samangli Road. Quetta, Pakistan.
ghazalasiddique99@gmail.com

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ABSTRACT.... Objectives: The primary objective of this study is to determine the frequency of rpoB gene mutations within 81-bp RRDR in isolates from Quetta, Pakistan using GeneXpert® MTB/RIF assay. **Background:** Multi drug resistance is one of the major obstacles in the control of tuberculosis throughout the globe. **Study Design:** A cross sectional experimental study was designed. **Setting:** A total of 500 clinical specimens obtained from suspected TB patients at Provincial TB Reference Laboratory, Fatima Jinnah General and Chest Hospital Quetta, Pakistan **Period:** From January to July 2017. **Methodology:** Were analyzed by GeneXpert® MTB/RIF assay. Statistical analysis of the data was performed using SPSS version 20. **Results:** Out of total 500 samples, MTB was detected in 211 (42%) cases by GeneXpert® MTB/RIF assay [positive pulmonary cases 48.8% (206/422) and extra-pulmonary 6.4% (5/78)]. Among 211 MTB positive cases, the assay detected 11 (5.2%) cases with RIF-resistance caused by various rpoB gene mutations within 81-bp RRDR. All the eleven RIF-resistant isolates were found to have mutations only in Probe E and none of cases had RIF-resistance associated with probes A, B, C, and D. Out of 11 RRD cases, 4 (2%) were males and 7 (3.3%) were females. New TB cases were 3 (1.4%) and previously treated cases were 8 (3.8%). **Conclusion:** In our settings 11/211 (5.2%) of the TB patients showed rifampicin resistance. Probe E mutations (also called codons 531 and 533) were the only rpoB gene mutations detected by GeneXpert® MTB/RIF assay. No mutations were detected in the codons 511, 513, 516, 518, 522 and 526 sequences.

Key words: GeneXpert, MDR, Quetta, RIF-resistance, RRDR.

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INTRODUCTION

The incidence of TB has increased after the emergence of drug resistant TB. When M. tuberculosis shows resistance to at least two of the essential first-line anti-TB drugs, rifampicin (RIF) and isoniazid (INH), is known as multi-drug resistant TB (MDR-TB).[1] RIF-resistance has a distinct epidemiological significance and is an essential identical marker for MDR-TB strains. Although ≥ 90% of RIF-resistant strains also exhibit resistance to INH.[2] RIF binds to the β-subunit of bacterial RNA polymerase (rpoB) enzyme and thereby prevents RNA synthesis. Generally, genetic mutations in rpoB gene occur in 95-97% of RIF-resistant M. tuberculosis strains globally. These mutations are found at the 507 to 533 amino acid residues (81-bp), a region known as Rifampicin-Resistance-Determining Region (RRDR).[3] Although more than fifty mutations in this region have been identified by DNA sequencing. While point mutations in codons 531 or 526 are primarily regarded to confer high resistance to RIF.[4] On the contrary, mutations at codons 511, 516, 518, 522, and 533 cause lower levels RIF

resistance.[5]

Prompt detection of MTB and RIF resistance is necessary for the competent control of drug-resistant tuberculosis infection.[6] Conventional diagnostic methods for TB are slow, less sensitive and are unable to find drug resistance. Currently, the WHO recommended GeneXpert® MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) have changed the scenario in the diagnosis of tuberculosis. The assay tests for MTB and also mutations associated with rifampicin resistance directly from clinical samples. It is a semi-quantitative nested real-time PCR designed to amplify 81-bp hot-spot region of the rpoB gene within RRDR and probing the region subsequently for RIF- resistance related mutations.[1]

Tuberculosis incidence was approximately 10.4 million worldwide in 2016. India, Indonesia, China, Philippines and Pakistan were the top five countries that together contributed to 56% of global incidence. Drug-resistant TB is a massive threat, with an estimated 490,000 million MDR-TB cases (19% cases previously treated and 4.1% new cases) emerging in 2016. China, India and the Russian Federation together accounted for 47% of the global MDR-TB cases[1]. Pakistan ranks 5th among thirty high-burden countries and 4th among 27 countries with high burden of MDR-TB.

The drug resistance TB and biological characteristics of M. tuberculosis usually differs in various geographical areas.[7] The detection of rpoB gene mutations is most useful for diagnosing RIF-resistance in Mycobacterium. In Pakistan, limited data is present concerning frequency of rpoB gene mutations. Hence, this study was designed to determine the prevalence of RIF-resistant M. tuberculosis strains in clinical samples obtained from suspected TB patients in Quetta as well as to detect frequency of various rpoB gene mutations in RRDR in RIF-resistant isolates using Xpert® assay.

METHODOLOGY

Study Area

This study was carried out at Provincial TB Reference Laboratory, Fatima Jinnah General and Chest Hospital, Quetta, Balochistan, Pakistan from January to July 2017. It is the provincial capital and largest city of the province.[8]

Ethical Information's

The study was approved by the Ethics Review Committee of CASVAB, University of Baluchistan (UOB), Pakistan, as well as Fatima Jinnah General and Chest Hospital, Quetta. All patients were given consent form and only those patients were included in the study who gave their written/ verbal informed consent.

Patients' Inclusion Criteria

All the patients at outpatient department (OPD) of Provincial TB Reference Laboratory with symptoms suggestive of TB (both pulmonary and extra-pulmonary) referred by physicians for TB diagnostic tests were included in the study. Patients' information about age, gender, previous history of TB, MDR-contact etc. were collected through a preformed standardized questionnaire.

Collection of the Specimens

In total, 500 specimens were collected from patients suspected for tuberculosis. The specimens (sputa, bronchoalveolar lavage (BAL), gastric aspirate, cerebral spinal fluid, plural fluid, pus, colon biopsy, urine, and ascetic fluid). Sputum specimens were directly collected in sterile containers from patients visiting hospital, while extrapulmonary samples were sent from indoor patients.

Fluorescent Microscopy (FM)

Smears were prepared from direct sputum specimens and concentrated specimens other than sputum using standardized protocol.[9] Briefly, a drop of each sample was smeared on a glass slide, air-dried and heat-fixed. The slides were flooded with 0.1% auramine-O dye and allowed for 20 minutes. The slide were then rinsed with water and flooded with 0.5% acid alcohol and allowed for 2 minutes to decolorize. The slides were again rinsed with water and flooded with 0.5% $KMnO_4$ to counter-stain for 2 minutes. Finally, the slides were rinsed and allowed to air dry. The slides were visualized under fluorescent microscope using either 20 or 40X objective lens.

Sample Preparation
The specimens were prepared for GeneXpert® MTB/RIF assay as per the instructions of manufacturer.

Preparation of Sputum Specimens
Briefly, the Sample Reagent (SR) was added to untreated sputum specimen in a 2:1 ratio and to each decontaminated sputum specimen pellets in a 3:1 ratio. The lids were replaced tightly to avoid any spill or leakage. The closed sputum containers were briefly vortexed and kept at room temperature for ten minutes. After 10min incubation, the container was re-vortexed and incubated for additional 5 minutes at room temperature. After incubation, the sputum specimens were completely liquefied, inactivated and ready for testing with GeneXpert® MTB/RIF assay.

Preparation of Specimens other than Sputum
The specimens other than sputum (bronchoalveolar lavage, gastric aspirate, pleural fluid, ascitic fluid, cerebrospinal fluid and pericardial fluid) were first concentrated by centrifugation for 15 minutes at 3000×g because of their high volumes (10-30 ml). The supernatant was carefully decanted and the concentrated pellets were processed further similarly like sputum specimen by adding SR in a 3:1 ratio to each concentrated pallet.

GeneXpert® MTB/RIF Assay
Each GeneXpert® MTB/RIF assay cartridge was labeled with the specimen ID. Two ml of the prepared specimens were transferred to the test cartridge and closed firmly. The GeneXpert® platform was turned on and test was created. Cartridge barcodes were scanned with barcode reader and cartridges were inserted into the automatically selected GeneXpert® platform module and the assay was initiated. The assay provides automated results within two hours which is displayed on screen from measured fluorescent signals by the GeneXpert® DX software.

Data Analysis
The data were analyzed descriptively and inferentially using statistical software SPSS version 20. Data were presented as frequencies and percentages. Chi-square test was used to check significant difference between test results and also to determine association between risk factors and TB disease. P-value less than 0.05 was considered as statistically significant.

RESULTS
A total of 500 clinical specimens including 84.4% (n=422) pulmonary and 15.6% (n=78) extra-pulmonary samples were obtained during the study period from patients with suspected TB. The respiratory specimens included sputum, broncoalveolar lavage and gastric aspirates whereas non-respiratory were pericardial fluid, plural fluid, ascetic fluid, colon biopsy, cerebrospinal fluid, pus, urine. The mean age of the TB suspects was 41.56 ± 23.14 years (rang: 3 months to 95) while males and females were 241 (48.2%) and 259 (51.8%), respectively (sex ratio 1:1.075).

Out of 500 cases, MTB was detected in 211 cases by GeneXpert® MTB/RIF assay detected. Among these 211 cases, RIF-resistance was detected in 11 cases. RIF-resistance is caused by different mutations in rpoB gene within the 81-bp RRDR which is overlapped by five probes in GeneXpert® MTB/RIF assay, namely, probe A, B, C D, and E. All the eleven RIF-resistant cases were detected to have mutations only in Probe E and none of cases had RIF-resistance associated with other probes. Out of 11 RIF-resistant cases, 4 (2 %) were males and 7 (3.3 %) were females. New TB cases were 3 (1.4 %) and previously treated cases were 8 (3.8 %) and none had MDR contact.

Out of 500 cases GeneXpert® MTB/RIF assay detected 211 (42.23%) MTB positive specimens and 289 (57.8%) MTB negative specimens. Xpert yielded positive results in 206/422 (48.8%) respiratory and 5/78 (6.4%) non-respiratory samples. This difference was statistically significant (χ^2= 48.5, p<0.001, Table-I).

Among 422 respiratory samples, 195/360 (54.2%) sputum samples, 10/32 (31.3%) broncoalveolar lavage specimens and 1/30 (3.3%) gastric aspirates were Xpert-positive. Among 78 non-

respiratory MTB samples, 2/41 (4.9%) plural fluid specimens, 1/24 (4.2%) CSF samples, 2/8 (25%) pus were Xpert–positive whereas no MTB was detected in other specimens (ascetic fluid, colon biopsy, pericardial fluid and urine) (Table-II).

Table-III shows that among 500 MTB samples, 194 (38.8%) were smear-positive while 306 (61.2%) were smear negative. Smear-negative were predominantly obtained from pulmonary samples (230/422 = 54.5%) in comparison with extra-pulmonary samples (76/78 = 97.4%) (χ^2= 51.1, p<0.001).

Of the 306 FM-negative samples, Xpert detected MTB in 19 (6.2%) specimens whereas, FM yielded positive result in 2 (1%) Xpert-negative samples. Chi-square test revealed a highly significant difference (χ^2= 418.83, p<0.001) in the detection rate of MTB between GeneXpert assay and FM (Table-IV).

Based on gender distribution, 102/241 and 109/259 samples were Xpert positive among males and females, respectively. Xpert result could not differ significantly between males and females (χ^2= 0.003, p=0.96). Most MTB cases were detected in patients aged 21-40 years (55.5%), followed by 41-60 years (46.8%), >60 years (35.2%) while least number of cases were of age <20 years (31.2%, χ^2= 17.7, p=0.001, Table-V).

GeneXpert	No of samples n=500	Type of TB		Chi-square	P value
		Pulmonary (n=422)	Extra-pulmonary (n=78)		
MTB + ve	211	206 (48.8%)	5 (6.4%)	48.5	<0.001*
MTB - ve	289	216 (51.2%)	73 (93.6%)		

Table-I. Total samples subjected to GeneXpert analysis in the target area

Clinical Specimen	No. of Specimens (%)	GeneXpert (n=500)	
		GX+ (211)	GX− (289)
Sputum	360 (72%)	195	165
BAL	32 (6.4%)	10	22
Gastric aspirates	30 (6%)	1	29
Plural fluid	41 (8.2%)	2	39
CSF	24 (4.8%)	1	23
Pus	8 (1.6%)	2	6
Others	5 (1%)	0	5
Total	500 (100%)	211	289

Table-II. Result of GeneXpert based on specimen type

Microscopy	Sample, n	Type of TB		Chi-square	P value
		Pulmonary (n=422)	Extra-pulmonary (n=78)		
Positive	194	192 (99.0%)	2 (1.0%)	51.1	<0.001*
Negative	306	230 (75.2%)	76 (24.8%)		

Table-III. Result of smear microscopy based on pulmonary and extra-pulmonary specimens
P*=significant

Microscopy	GeneXpert (n=500)		Chi-square	P value
	GX+ (n=211)	GX− (n=289)		
Positive (n=194)	192 (99%)	2 (1%)	418.83	<0.001*
Negative (n=306)	19 (6.2%)	287 (93.8%)		

Table-IV. Comparison of GeneXpert and fluorescent microscopy
P*=significant

Variable	N (%)	GeneXpert		Chi-square	P value
		GX+	GX−		
Gender Male	241 (48.2%)	102 (42.3%)	139(57.7%)	0.003	0.96ns
Female	259 (51.8%)	109 (42.1%)	150 (57.9%)		
Age <20	128 (25.6%)	40 (31.2%)	88 (68.8%)	17.7	0.001*
21-40	110 (22%)	61 (55.5%)	49 (44.5%)		
41-60	154 (30.8%)	72 (46.8%)	82 (53.2%)		
60<	108 (21.6%)	38 (35.2%)	70 (64.8%)		

Table-V. GeneXpert® MTB/RIF results based on gender and age.
P*=significant; Pns= Non-significant

DISCUSSION

Extensive studies based on genetic causes of anti-TB drug-resistant M. tuberculosis strains have been carried out worldwide, which are widely considered to be caused by point mutations in essential genes such as rpoB, embB, rpsL, katG etc.[10] Concurrently global MDR-TB presents a huge threat to humans mainly in developing countries, this is the reason the current study was designed to investigate the anti-TB drug resistant MTB isolates for rpoB gene point mutations causing RIF-resistance since above 90% of RIF-resistant strains also show resistance to isoniazid.[11-12]

In our study, 11 cases were found to be RIF-resistant out 211 cases detected by GeneXpert® MTB/RIF assay. All the 11 RIF-resistance mutations were detected in probe E overlapping the codons from 528 to 533 of the rpoB gene in 81-bp RRDR, whereas, we did not find any mutations in probe A, B, C and D (overlapping the codons from 507 to 527) in RIF-resistant isolates in over study. Another study in Khyber Pakhtoonkhwa, Pakistan found that Probe E mutation was the most common rpoB genetic mutation (77%).[13] However, they also found less common mutations in Probe B (10.8%), D (8.3%), A (1.2%), and C (1.5%). Similarly, in China reported mutations were in codons 531 (41%), 526 (40%), and 513 (4%) in 81-bp.[14] In a study from Kampala, Uganda it was found 7 out of 12 (58%) mutations in probe E.[15] Concurrently, in Punjab, Pakistan also observed the most frequent mutation in codon 531 (52%), followed by codon 516 (15%), 512 (7%) and 526 (7%).[16] These studies indicate that probe E related mutations are the most common associated with rifampicin resistance in the current study.

The absence of mutations within the RRDR of rpoB gene associated with probes other than probe E in this study probably recommends that these particular sites of RRDR are less susceptible to genetic mutations contributing drug resistance, or it may mean that the small sample size has limited the likelihood.

MDR-TB threatens global TB prevention and care, and is a great challenge of public health in many countries. WHO suggests the implementation of GeneXpert® MTB/RIF assay to detect MTB and rifampicin resistance simultaneously in TB patients.[1] In this study out of 500 samples, GeneXpert® MTB/RIF assay detected MTB in 211 (42.2%) samples. This result is in line with the finding of a study reporting that GeneXpert® MTB/RIF assay detected 125 (35%) MTB out of 350 sputum specimens[17], and is also comparable with the study from Pakistan in which MTB was detected in 49.8% pulmonary TB suspects by GeneXpert® MTB/RIF assay.[18] On the other hand, Xpert detected lower MTB cases as compared with our finding in studies in Bangladesh 10.6% (45/421), 20.15% (51/253) and in Malaysia (6.4% (8/125).[19-21]

MTB detection was comparatively lower by fluorescent microscopy (38.8%) than GeneXpert® MTB/RIF assay (42.2%) in this study. The GeneXpert® MTB/RIF assay also detected 6.2% FM-negative specimens in addition to 99% FM-positive samples. Statistically significant difference between both techniques was observed ($p<0.05$).

The current study showed no significant association of gender with TB infection ($p>0.05$).

However, statistically significant difference in the TB prevalence was observed between different age groups ($p<0.05$). The study demonstrated most of MTB cases (55.5%) between 21-40 years of age which in agreement with a study by Ndungu et al., 2013 that reported highest TB infection (66.7%) in age group 18-34 years.[22] Similarly, Shafee et al. 2014 also reported higher frequency of tuberculosis in more productive age group.[8] A from Pakistan reported that most (62.3%) of the patients are in the most productive age of 21-50 years.[23]

World Health Organization in 2012 extended the MDR-TB diagnosis program in countries with high TB burden and utilization of GeneXpert was endorsed for effective detection of resistance against anti-TB drugs.[24] Hence, this assay would be a beneficial tool in the World's fight against MDR-TB/TB disease predominantly in countries with high TB burden like Pakistan.

CONCLUSION

The GeneXpert® MTB/RIF assay detected the rifampicin resistance in 11 out of 211 MTB cases caused by mutations within 81-bp RRDR region. All the 11 RIF-resistant isolates had rpoB mutations in codons (528-533, most probably in codon 531) overlapped by Probe E. It is suggested that studies involving very large sample size should be conducted to find the occurrence of mutations in rpoB gene in our setting. Data on these mutations could be beneficial in the development of novel therapeutics for the treatment of TB disease.

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AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Ghazala Siddique	Experimentation.	
2	Muhammad Shafee	Manuscript writing.	
3	Ferhat Abbas	Design & conception.	
4	Muhammad Naeem	Sample collection.	
5	Abdul Raziq	Statistical analysis.	
6	Shafique Ahmad	Data interpretation.	
7	Khan Muhammad Babar	Data interpretation.	
8	Ziauddin	Critical review.	