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Epidemiology of Molecular Probes in Xpert MTB/RIF Assay in AJK, Pakistan

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Author`s Contribution

[1,3]Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work, manuscript writing.
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ABSTRACT

Objective: This study aimed to detect rifampicin-resistant tuberculosis cases and assess the frequency of missing probes in different study populations in Azad Jammu and Kashmir (AJK), Pakistan.

Methodology: The study was conducted at the State TB Reference Laboratory, District Headquarters Teaching Hospital, Mirpur, AJK. A total of 2,790 specimens collected between March 2016 to August 2019 were analyzed. Pulmonary TB (PTB) accounted for 94% of the cases, while 6% were classified as extra-pulmonary cases. All respiratory and non-respiratory samples underwent fluorescence smear microscopy (AFB) and a real-time PCR test (Xpert MTB/RIF assay) to detect Mycobacterium tuberculosis (MTB) and rifampicin resistance.

Results: Among the 2,790 suspected MTB patients, 734 (26%) were confirmed to have MTB using the Xpert MTB/RIF assay, while 564 (20%) tested positive by fluorescence microscopy. Of the MTB-positive patients, 720 (98%) were diagnosed with pulmonary TB, and 14 (2%) had extra-pulmonary TB. Rifampicin resistance (RR) was detected in 66 (9%) cases, with 97% of the resistant cases being pulmonary and 3% extra-pulmonary. The most frequently missing probe was E (Codon 529-533), accounting for 34% of the cases, followed by probe D (Codon 523-529) at 26%. The least frequently missing probe was C (Codon 523-529), observed in 3% of the cases. Probe B (Codon 512-518) was missing in 15.4% of cases, while probe A (Codon 518-523) was missing in 9.4% of cases.

Conclusion: The utilization of molecular diagnostic techniques, such as the Xpert MTB/RIF assay, enables rapid identification of MTB and detection of rifampicin resistance. This study provides valuable baseline data on the prevalence of 81 bp mutations in the rpoB gene, highlighting the need for further evaluation of mutation patterns in AJK.

Keywords: Antibiotics, Analgesics, Irreversible Pulpitis, Prescription, Management.

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Introduction

Mycobacterium tuberculosis is a causative agent of TB and it is one of the top 10 causes of death globally.[1] According to an estimation of Global Report 2020, about 10.0 million people were infected with TB.[1] Among HIV-negative people, 1.2 million people died of tuberculosis and additionally 208,000 people died among HIV-positive people (range 177 000-242 000). Both Sex categories and all age groups are affected by tuberculosis, but adult men have the highest burden, accounting for 56% of all tuberculosis cases in 2019; in contrast, adult women accounted for 32% and children accounted for 12%. Geographically, in 2019, the majority of tuberculosis cases occurred in the World Health Organization's (WHO) Southeast Asia (44%), Africa

(25%) and Western Pacific (18%) regions. The Eastern Mediterranean region accounted for a relatively small proportion (8.2%), America (2.9%) and Europe (2.5%). In 2019, multidrug-resistant tuberculosis was indicated as a public health crisis and a threat to health and safety. [1] In 2018, a total of 206,030 people worldwide had MDR or RR TB. Approximately half of the global burden of MDR-TB comes from three countries, i.e. India, China, and the Russian Federation.[1] Pakistan is ranked 5th among 30 countries with a high burden of tuberculosis and 4th in multidrug-resistant tuberculosis (MDR-TB). Approximately more than 5 lac people were infected with tuberculosis, and 15,000, unfortunately, died of multidrug-resistant tuberculosis. According to the NTP report in 2019, AJK has 5,639 notified cases, the estimated number of cases is 8,300, and the case determination rate (CDR) is 68%.[2]

Fast, thoughtful, and precise identification and drug susceptibility testing technology can ensure the correct treatment and control of tuberculosis. Many phenotypes (DST) and genotype (PCR) tests can be used to diagnose Mycobacterium tuberculosis (MTB) and rifampicin-resistant tuberculosis (RR-TB). The Xpert MTB/RIF test is a fully computerised and automatic system for the identification of Mycobacterium tuberculosis complex and rpoB gene mutations. It is a cartridge-based real-time PCR with molecular beacon probes, 5 probes for wild-type RRDR in rpoB, and 1 probe for amplification control (Bacillus globigii). Because all steps of decontamination, digestion, DNA extraction, amplification, and detection are carried out at the same time, limited biological safety is required.[3] It is a user-friendly random access instrument with minimal manual operation and can provide results in about 2 hours.[3]

Rifampicin is a powerful antibiotic that binds with the β-subunit of bacterial DNA-dependent RNA polymerase enzyme and inhibits protein synthesis. About 95-97% of cases of rifampicin resistance (RR) are linked with the mutations in the 81 bp region in the rpoB gene region (codon 507-533) of MTB which is referred to as hot spot region/Rifampicin resistance-determining region (RRDR).[4] The Xpert MTB/RIF assay uses a hemi-nested real-time PCR reaction to target the 81 bp rpoB gene.

The Xpert MTB/ RIF assay uses three specific primers and five unique molecular beacon probes namely, Probe A binds with codon no.507-511, Probe B with codon no. 512–518, Probe C with 518-523, Probe D with 523-529, and Probe E bind with codon no. 529-533 in the 81bp region of the rpoB gene, to guarantee the highest level of specificity, which is vital for detecting mutations related to RRD.[5] The purpose of the current study was the precise detection of rifampicin resistance cases and rate of mutation (missing probes) which target 81 bp regions of the rpoB gene in the population of Azad Jammu and Kashmir, Pakistan. This study was helpful to find out polymorphisms in the drug target genes and reflected as molecular markers for drug resistance identification in MTB isolates in AJK, Pakistan. Information about missing probes (mutation pattern) present in rifampicin resistance isolates might offer an idea into the epidemiology of RIF-resistant MTB isolates of AJK region.

Methodology

The work presented in the study comprises a retrospective study and pattern of missing probe detection in rpoB gene of RRD specimens collected from different areas of Azad Jammu and Kashmir, Pakistan. The study was conducted from March 2016 to August 2019 in the State Reference Laboratory (SRL) for TB, DHQ Teaching Hospital, Mirpur, AJK.

Individuals with signs and symptoms of pulmonary and extra-pulmonary tuberculosis such as coughing for > two weeks, Sputum containing pus and blood, appetite loss, weight loss, weakness, night sweats, fatigue and fever, indoor and outdoor patients treated at DHQ Teaching Hospital, Mirpur, as well as other TB centres of AJK were included in this study. The patient's data were also recorded, including gender, age, history of multi-drug resistance exposure, and history of tuberculosis.

A total of 2,790 samples from patients with suspected pulmonary and extrapulmonary tuberculosis were included in the study. These specimens comprised 94% (2,626/2,790) pulmonary specimens (sputum, gastric aspirate/lavage) whereas 6% (164/2,790) extrapulmonary specimens. Extrapulmonary specimens further included 8/164 cerebrospinal fluid (CSF), 58/164 pleural fluid, 1/164 pericardial fluid, 12/164 ascetic fluid, 14/164 lymph node, 28/164 pus, 5/164 urine, 3/164 stool, 2/164 bone marrow, 3/164 skin scraping, 12/164 FNAC, 2/164 tissue biopsy, 12/164 synovial fluid, 2/164 cyst fluid, and 2/164 cervical fluid (Table I).

All specimens were processed for AFB microscopy and Xpert MTB/RIF (Cepheid) assay to diagnose TB and rifampicin resistance tuberculosis (RR-TB). The slides were stained according to standard procedures.6 Air-

dried slides were placed on the staining rack over a sink around one cm separated and were totally loaded up with 0.1 % fluorescent auramine-rhodamine solution, and were given 20 minutes for color penetration. The slides were gently washed with water until all the noticeably apparent stains were washed away and next decolourized with

0.5% acid alcohol for two minutes. At this point, the slides were gently washed again with water. Now 0.3% methylene blue dye was loaded for 1 minute which gives a blue colour to the smear background (counter stain dye). Now slides were again washed with water thoroughly. Place the slides on racks for air drying and then observe under the microscope. For Xpert MTB/ RIF assay; 2 ml of clinical sample and 4 ml of Xpert MTB/ RIF sample reagent were placed in a 50 ml falcon tube, shaken strongly and incubate at room temperature for 15 minutes. Then 2 ml of this mixture was transferred to the Xpert MTB/RIF cartridge and then install the cartridge into the device according to the manufacturer's instructions. Lastly, the result was interpreted by the Xpert MTB/RIF system and automatically presented after 1 hour and 50 minutes.[7]

When the cycle threshold is reached and at least two of the five probes provide a positive signal (CT) ≤ 38 cycles, sample is categorized as MTB positive. The bacterial load depends on the cycle threshold range (high, <16; medium, 16-22; low, 22-28; very low, >28).[8, 9]

RIF resistance is detected by either rpoB mutations that entirely prevent probe hybridization and leads to probe missing (absence of probes) or allow partial probe hybridization and results in dissimilarity between the first (early CT) and the last (late CT) MTB-specific beacon probes (ΔCT) of more than 3.5 cycles.[4,5] Frequencies of mutant probes were examined in all RR- TB patients based on CT readings. [8,9]

Results

About 54.7% of the study participants were males and 45.3% were females. The age of the patients ranged from 0 year to 96 years. Among the notified cases, 2626/2790(94%) were Pulmonary TB (PTB) whereas 164/2,790 (6%) were extra pulmonary cases shown in Table I. Xpert MTB/ RIF assay detected MTBC in 734(26%) patients. The age of these TB-positive patients ranged from 1 to 90 years with the highest MTB percentage (34%) and RRD (47%) in the youngster age group (15-29) followed by the older age group (>60) 22.6% as shown in Table II. However, the second RRD group is (45-59) 28% followed by group 2 (15-44)17%.

The paediatric age group (<14) have the lowest percentage (3.26%) as shown in Figure 1. The overall

Table I: List of pulmonary (P) and extra pulmonary (EP) specimens

Specimen Name P and EP	Specimen Type	Specimens (n=2790)%	MTB Detected (n=734)%	RRD (n=66)%
Sputum	Pulmonary	2560 (91%)	702(95%)	64(97%)
Gastric aspirate	Pulmonary	57(2%)	2(0.3%)	
Gastric lavage	Pulmonary	9(0.3%)	1(0.1%)	
Pleural fluid	EP	58(2%)	3(0.1%)	
Ascetic fluid	EP	12(0.43%)	1(0.1%)	
Pericardial fluid	EP	1(0.03%)	0	
Synovial Fluid	EP	12(0.43%)	0	
Cyst Fluid	EP	2(0.07%)	0	
Cerebrospinal Fluid (CSF)	EP	8 (0.07%)	0	
Cervical Fluid	EP	2(0.07%)	1(0.1%)	
Pus	EP	28(1%)	16(2.2%)	1(1.5%)
Lymph Node	EP	14(0.5%)	6(0.8%)	1 (1.5%)
Skin scraping	EP	3(0.1%)	0	
Fine Needle Aspiration Cytology (FNAC)	EP	12(0.43%)	1(0.6%)	
Bone Marrow	EP	2(0.07%)	1(0.1%)	
Tissue Biopsy	EP	2(0.07%)	0	
Urine	EP	5(0.2%)	0	
Stool	EP	3(0.1%)	0	

Table II: Rifampicin resistant tuberculosis in different age groups and gender of study population positive cases (n=734)

Study Groups	Male MTB detected	Female MTB detected	Total	Male RRD	Female RRD	Total RRD
Paediatrics						
(<14)	5(0.7%)	19(2.5%)	24(3.26%)	2(3%)	0	2(3%)
Adults						
15-29	102(14%)	158(21.5%)	250(34%)	19(28.7%)	12(18%)	31(45.4%)
30-44	87(12%)	45(6%)	132(18%)	6(9%)	6(9%)	12(47%)
45-59	104(14%)	58(8%)	162(22%)	14(21%)	4(6%)	18(27%)
Geriatric(>60)	100(13%)	66(9%)	166(22.6%)	2(3%)	1(1.5%)	3(4.5%)
Total specimens	389(53%)	345(47%)	734(26%)	43(65%)	23(35%)	66

Table III: Correlation of different missing probes of the rpoB gene in the rifampicin resistance case in sample types

Probe Missing	A	B	C	D	E	E ,D	E ,C	Total
Codon no.	507-511	512-518	518-523	523-529	529-533	523-533	518-523 529-533	
Sample type								
PTB	6(9, 4%)	10(15.6%)	2(3%)	17(26.6%)	23(34.4%)	4(6.2%)	2(3%)	64(9%)
E PTB					1(1.5%)	1(1.5%)		2(3%)

percentage of the adult age (15-44) group is (74.11%). In positive patients 389 (53%) cases were males and

345(47%) were female. Using fluorescence microscopy, only 564/2790 (20%) samples were positive while 734/2790 (26%) were MTB positive on Xpert MTB/ RIF assay. Out of the genexpert-positive patients, 705/734(96%) had pulmonary TB and 29/734(4%) were EPTB cases (Table I). These positive EPTB cases included 6 lymph nodes, 1 FNAC, 1 cervical fluid, 1 bone marrow, 1 peritoneal fluid, 3 pleural fluids and 16 pus specimens. Resistance to RIF was detected in 66/734 (9%) cases of which 64/66 (97%) pulmonary TB cases whereas 2/66 (3%) were extra-pulmonary cases (pus, lymph node). However, one previously treated bone marrow (extrapulmonary) case became pulmonary RRD. RIF resistance was more in males 43/66(65%) patients as compared to females 23/66 (35%). RRD was detected in 30 (47%) youngsters (15-29), 29(45%) adults (30-59), 3(5%) geriatric patients (>60% and 2(3%) paediatric patients (<14). The youngster age group (15-29) have the highest ratio of rifamcin resistant than other age groups. Of these, 64(8.7%) RRD specimens, 19(28%) had a very low bacillary load (CT value >28) on Xpert MTB/ RIF assay. High and medium load of bacilli was observed in 13 (18.7%) and 27(42%) sputum specimens respectively. Only 7(11%) specimens have low bacillary load as shown in Table IV. Extra-pulmonary TB is considered paucibacillary in nature.

Table IV: Specimen wise bacterial load in RRD patients using Xpert assay

Specimen name	Very Low	Low	Medium	High
Sputum	18	7	27	12
Pus				1
Lymph Node	1			

The frequency of the probes missing in RRD cases was as follows: A 9.4%, B 15.6%, C 3 %, D 26.6%, E 34.4%, and combined mutation of E and D were 6.2% whereas E and C were 3%. The frequency of probe E was highest (34 %) followed by probe D (26.5%). The details of missing probes in various subpopulations are given in Table III. History-wise prevalence shows that 43.7% of RRD patients were previously treated, 50% were new cases whereas 4.5% were on treatment/defaulter.

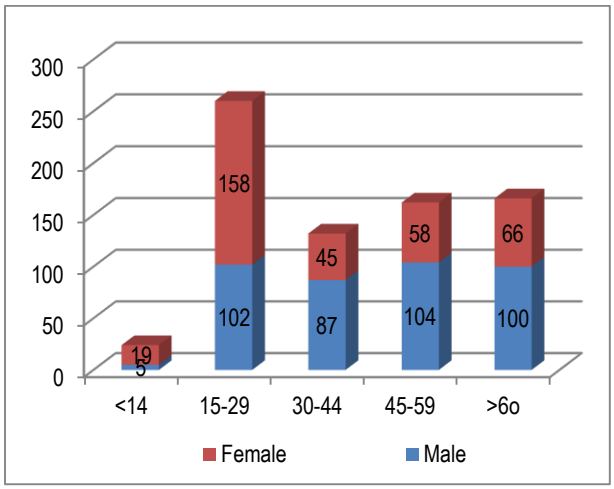

Figure 1. Age and gender-wise distribution of TB Cases

Discussion

In the current observations, attempts have been made to understand the causes of rifampicin resistance by using Xpert MTB/RIF assay and on the basis of these results probe mutation frequency is also detected. In these investigations, about 26.34% of cases were diagnosed with TB out of which 53% were male and 47% were female. Our outcomes are parallel to other investigations conducted in the neighbouring areas and in developing countries.[10-12] According to this study, the adult age group (15-45 years) has more tuberculosis (74.11%) than others which is in close covenant with earlier studies from Pakistan. India, Nepal and Ethiopia.[13-15] According to the Global TB Report 2020, around 10.0 million people (range, 8.9–11.0 million) developed TB disease in 2019 out of which 56% were men belonging to the adults (aged ≥15 years) age group, 32% were women and remaining 12% were child (aged<15).[1] The reason for the high incidence of TB in these subpopulations may be more outdoor activities related to their occupation. The high proportion of men with tuberculosis can be attributed to deficient and unsatisfactory reporting and diagnosis of tuberculosis in women, or gender differences in access to health care.[16] In our study, 9% of the cases developed

resistance. We found presumptive resistance RRD cases 7% which was lesser than other studies from Pakistan. In Khyber Pakhtunkhwa, 29% presumptive rifampicin resistance TB was reported.[17]

In various regions of Punjab, Ullah et al.[18] and Javaid et al.[19] documented resistance to at least one anti-tuberculosis (TB) drug at rates of 11.5% and 11.3% respectively. A recent drug resistance survey conducted in Pakistan aimed at addressing the challenge of multidrug-resistant TB (MDR TB) revealed that among newly reported TB cases, the estimated percentage of MDR TB was 4.3%, and 19.4% among previously treated (PT) cases.[1] The observed disparity in these percentages may be attributed to factors such as geographical location, sample size, and the methodology employed to select presumptive patients for drug-resistant tuberculosis. Globally, among presumptive drug-resistant TB patients, the rate of rifampicin (RIF) resistance varies from 2.7% in the Americas and Africa to 27% in Southeast Asia.[1]

Two Asian countries, Bangladesh and Nepal, have reported very high RIF resistance rates of 50%and 86.5% respectively which is an area of concern.[13,16] The samples in the Nepal study came from a reference centre that referred to culture-positive cases from different geographic regions, while the Bangladesh study was conducted in hospitals that referred to suspected MDR-TB cases from all over the country. This may be the possible reason for such a high rate of resistance. In our study, we found that the presumed RR of drug-resistant tuberculosis was 9.30%. More than 50% of high RR has been reported from India and include studies by Raizada et al,[20] Tripathi et al,[21] and Singhal et al.[22] The reported resistance rates of rifampicin in these three studies were 51%, 56.32%, and 55.2%, respectively. The reason may be the selection bias of patients in these studies.

However, other studies in India by Kumar et al.,[23] Sharma et al.,[24] and Desikan et al.,[25] by using line probe detection (LPA) as a method to detect drug resistance, 25.8%, 22%, and 10.6% of MDR-TB have been reported respectively.

Divergence in drug resistance rates could be recognized as variances in the consciousness of studied populations about drug resistance, access to healthcare, incoherent patient diagnosis, treatment, and follow-up and poor observance of long treatment regimes. The excessive degree of rifampicin resistance is probably due to the fact rifampicin is presently used for the treatment of many different infectious diseases.

In our study, only 4% of EPTB cases were detected, out of which only 7% were RIF resistant which is very low rate when compared to other countries. Research conducted in India has demonstrated that rifampicin (RIF) resistance is present in approximately 9.94% to 19% of cases of extra-pulmonary tuberculosis (EPTB).[29,30] Similarly, studies conducted in Nepal and Bangladesh have reported resistance to rifampicin in 60% and 40.57% of cases, respectively, within the context of extra-pulmonary tuberculosis.[33]

Xpert MTB/ RIF assay indicates rifampicin by absence or mutation in at least any one of the five probes targeting the 81 bp-RRDR region of the rpo B gene. Probe E was the most common missing probe in our study (34.4%) followed by probe D (26.6%) which is similar to other studies from Pakistan. Earlier Pakistanis studies showed that there were 77% in Khyber Pakhtunkhwa, 58% in Punjab, 78.3% probe E mutations in providence Balochistan and 66.48% in Sindh respectively. [17,18,31,32] Our neighbouring country India[28,29] has also reported high mutation in the probe E, i.e. 77.39% whereas 64.83% probe E mutation was reported in Bangladesh.[33]

Comparable observations have been documented in various African nations.[34-36] In investigations conducted by Tripathi and Anupurba, Thakur et al., and Maurya et al. in India, utilizing the Line Probe Assay (LPA) for the identification of rifampicin resistance (RR), it was revealed that the prevailing mutations within the 81 base pairs predominantly occurred at codon 531 (designated as probe E in the Xpert MTB/RIF assay).[12,9,37] Correspondingly, Yue et al. reported analogous outcomes, where the mutation frequency was notably highest at codon 531 (41%) as determined through DNA sequencing.[38]

Conclusion

For the effective diagnosis and treatment of drug-resistant tuberculosis, precise drug susceptibility testing (DST) is required. In MTB, drug resistance arises due to point mutations in rpoB gene of the bacterial genome. Molecular assays such as Xpert MTB/RIF and Line probe assay are progressively being used for quick investigation. These findings emphasize the importance of ongoing surveillance and the development of targeted interventions to effectively manage and control RR-TB in

the region. This research stresses the need to further assess the mutation arrays in different areas of AJK.

Limitations of our study: The false positivity and false negativity of Xpert MTB/RIF assay were not calculated because we did not perform the DST (drug susceptibility testing). Further, we could not estimate the specificity and sensitivity of gene xpert test because sequencing (a gold standard technique) for rpoB gene was not performed.

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