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Pattern of Mutations in Rifampicin Resistance (rpoB) Gene and Analysis of Rifampicin Indeterminate Result in Tuberculosis Detected by Xpert MTB/RIF Assay in Kalaburagi, India

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Abstract

Tuberculosis (TB) is a serious disease that has been observed since ancient times. In the mid-to late-20th century, the main clinical approach to this disease involved focusing on its diagnosis, prevention, and treatment. However, in the 21st century, the focus has shifted toward the diagnosis and treatment of drug-resistant TB. With the use of the Xpert MTB/RIF assay at the frontlines in India, interpreting indeterminate results to treatment with rifampicin, an antitubercular drug, can be challenging. This is further exacerbated by a lack of knowledge regarding mutation frequency in antitubercular drug-resistant genes in this region. Among antitubercular drugs, rifampicin is the most potent and effective drug for the treatment of tuberculosis; hence, understanding the pattern of rifampicin resistance (rpoB) gene mutations will provide insights into the genetic basis of this resistance, which may help in the prevention and treatment of TB. This retrospective observational study presents sociodemographic details, sample types, *Mycobacterium tuberculosis* load, types of probe mutations detected, and rifampicin indeterminate results from the Xpert MTB/RIF assay. Of the 314 samples analyzed, 258 showed rifampicin resistance as detected by MTB, with 56 samples of MTB-detected rifampicin indeterminate results. Type E probe mutation (58.9%) was the most common type, while the least frequent mutation was Type C probe (1.5%). No missing probe was observed in approximately 8.9% of samples. Among the 56 rifampicin indeterminate results, the maximum Cycle threshold value did not cross 34.5 in six samples.

Keywords: Xpert MTB/RIF, rpoB Mutation, Rifampicin Indeterminate

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INTRODUCTION

India is currently the leading country in tuberculosis (TB) cases and deaths worldwide, accounting for one-fifth of the global TB cases, and the second largest number of multi-drug resistant TB (MDR-TB) cases, after China.[1] Most recently, with an increase in the utilization of the Xpert MTB/RIF assay, a rapid molecular test for the identification of TB and its resistance to rifampicin (RIF), a greater number of rifampicin-resistant TB (RRTB) cases have been reported.[2] MDR-TB is resistant to isoniazid and rifampicin, two of the most potent antitubercular drugs. However, only rifampicin resistance (RR) can be detected by the Xpert MTB/RIF assay, due to the fact that over 90% of these cases are also resistant to isoniazid.[3] rpoB mutations are observed in 96.1% of RRTB strains throughout the world, typically observed in between the 507th and 533th amino acid residuals (81 bp) in the rpoB gene, denoted as the rifampicin-resistance determining region (RRDR).[4] The Xpert MTB/RIF assay uses five various overlapping probes (A, B, C, D and E) to identify the mutations in codons from 507-511, 511-518, 518-523, 523-529, and 529-533, respectively. These probes can be used to differentiate the wild type from the mutated type, the latter of which is related to rifampicin resistance. Since the effects of mutations in this gene on bacterial physiology and survival depend on the different types of mutations, understanding the types, patterns, and geographical tally of these mutations could benefit future interventions.[5] The Xpert MTB/RIF assay is a rapid, automated molecular test that detects multi-drug-resistant tuberculosis using rifampicin as a predictor. However, it has limitations. For example, when the bacilli load was very low, indeterminate results for *M. tuberculosis* were observed, indicating that the test could not determine whether the bacteria were resistant to rifampicin. This represents a challenge for clinicians wishing to initiate antitubercular treatment (ATT) in their patients based on indeterminate rifampicin results. To address this, this study determined the type of rpoB gene mutation common in the geographical area of Kalaburagi, India, in relation to rifampicin indeterminate results.

Objectives
1. To determine the most common type of rpoB gene mutation and its frequency
2. To analyze rifampicin indeterminate results in the Xpert MTB/RIF assay

MATERIALS AND METHODS

This retrospective study was conducted at a tertiary care center at the ESIC Hospital Kalaburagi, Karnataka, India. The district tuberculosis office was informed when the present study was conducted.

Inclusion criteria
All *M. tuberculosis* detected rifampicin resistance/rifampicin indeterminate TB cases diagnosed by the Xpert MTB/RIF, including all age groups and suspected, symptomatic, referred, pulmonary, and extrapulmonary cases of tuberculosis.

Table 1. Age-Gender distribution and MTB Bacillary Load vs Sample type

Age group	Males (%)	Females (%)	Total	Bacillary Load	Pulmonary (%)	Extra Pulmonary (%)
1-10	0.00	1.80	2	High	28.63	10.00
11-20	4.76	18.92	28	Medium	33.47	20.00
21-30	29.93	30.63	78	Low	20.56	30.00
31-40	21.09	9.91	42	Very Low	17.34	40.00
41-50	20.41	18.92	51	Total	100.00	100.00
51-60	14.97	10.81	34			
61-70	6.80	8.11	19			
71-80	2.04	0.90	4			
Total	100.00	100.00	258			

Exclusion criteria

Cases of *M. tuberculosis* detected with rifampicin sensitivity.

Data were collected retrospectively from the last five years for all types of specimens received and tested using the Xpert MTB/RIF assay at tertiary care hospitals in the CBNAAT Laboratory from March 2018 to February 2023. The collected data included sociodemographic details, sample types, MTB loads, types of probes where the mutation was detected, and indeterminate results for rifampicin.

Using hemi-nested real-time PCR, the Xpert MTB/RIF assay (Cepheid, CA, USA) four module version was used to simultaneously identify *M. tuberculosis* and rifampicin resistance in patient samples. Targeting the 81-bp rpoB gene, five partially overlapping fluorescent probes (A, B, C, D, and E) were used. Based on the cycle threshold (CT) value, the probes were semi-quantitatively graded as High, Medium, Low, or Very Low. In cases with *M. tuberculosis* that were sensitive to rifampicin, all five probes matched the amplified DNA with similar CT values. Samples resistant to rifampicin were indicative of a mutation that caused differences in the CT values among the probes. A difference ≥4 between the maximum CT value and minimum CT value among the five probes was considered to indicate rifampicin resistance.[6]
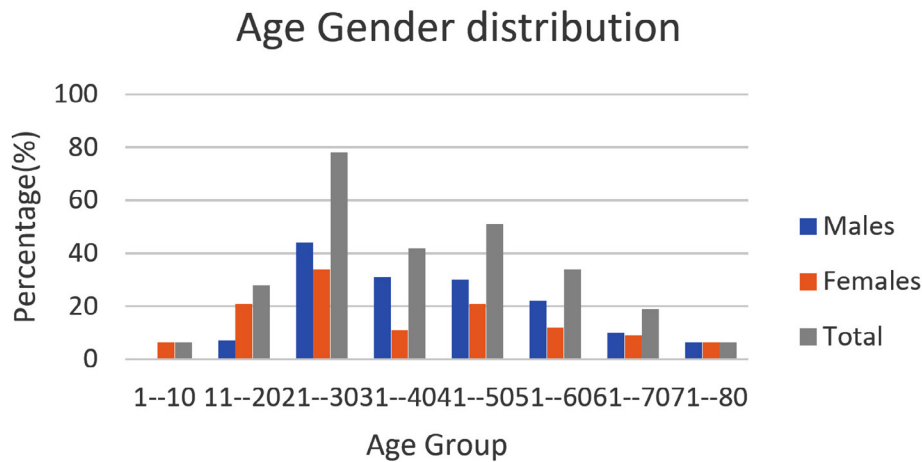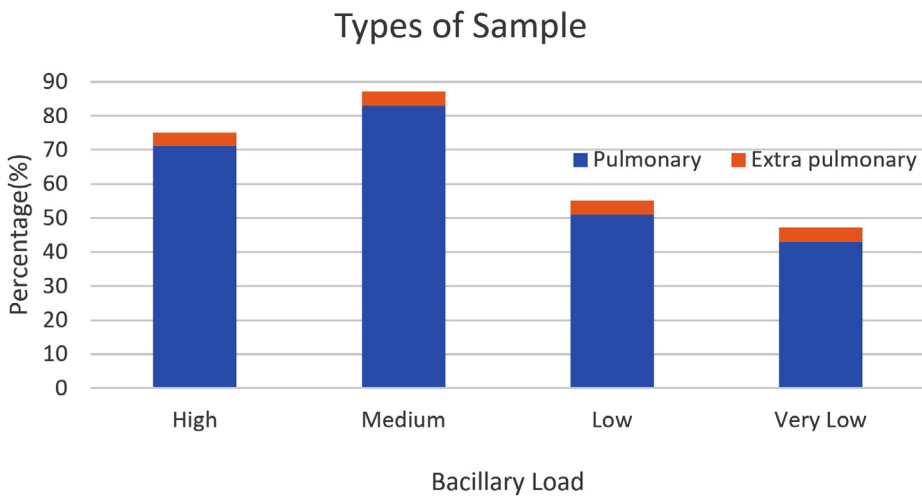

Figure 1. Age Gender distribution

Figure 2. MTB Bacillary load vs Type of sample

Table 2. Mutation Frequency of different Probes of rpoB gene

Codon site	Number	Percentage
Probe		
A	18	6.98
B	18	6.98
C	4	1.55
D	31	12.02
E	152	58.91
A&B	4	1.55
A&D	1	0.39
A&E	1	0.39
C&D	1	0.39
B&D	1	0.39
D&E	4	1.55
No missing probe	23	8.91
Total	258	100.00

RESULTS

Of the 314 samples analyzed, 258 showed rifampicin resistance by MTB, with indeterminate rifampicin resistance detected in the other 56 samples. The incidence of MTB rifampicin resistance in this region was 2.36%, whereas that of indeterminate rifampicin resistance was 0.5%. Among the 258 rifampicin-resistant patients, 147 (57%) were males and 111 (43%) were females. The age group between 21 and 30 years (30.2%) was comprised of the largest number of cases, followed by the age groups 41-50 (19.7%), 31-40 (16.2%), 51-60 (13.1%), 11-20 (10.8%), 61-70 (7.3%), 71-80 (1.5%), and 1-10 (0.7%) (Table 1 and Figure 1).

Maximum type of clinical sample was from Pulmonary 248 and extrapulmonary samples were 10.

The grading of bacillary load according to CT value showed maximum cases of medium load followed by High, Low and very low. (Table 1, Figure 2).

The most common probe that was positive for mutations was probe E, followed by probes D, A, B, and C (Figure 3). Mutation combinations were also observed, as follows: AB, DE, AD, AE, CD, and BD (Table 2). No missing probe was observed in 23 samples.

Table 3. Rifampicin Indeterminate Result as shown

Maximum	Minimum CT	ΔCT	Remarks
38.5	32.6	5.9	
36.5	32.7	3.8	
35.8	33.2	2.6	
36.5	32.7	3.8	
33.2	28.1	4.1	No probe has crossed 34.5
36.5	34.1	2.4	
36.8	0	36.8	
37.8	33	4.8	
39.1	32.7	6.4	
40.5	0	40.5	
40.3	0	40.3	
35.8	0	35.8	
38.8	32.2	6.4	
40	35.3	4.7	
36.8	33.6	3.2	
38.3	32.7	5.6	
38.1	32.8	5.3	
38.8	34.9	3.9	
40.6	0	40.6	
34.6	0	34.6	
36.2	33	3.2	
38.1	35	3.1	
36.2	33.4	2.8	
36.2	32.7	3.5	
40.3	0	40.3	
38.5	33	5.5	
38.3	34.4	3.9	
40.8	35	5.8	
40.2	33.8	6.4	
36.2	33.1	3.1	
36.5	32.9	3.6	
36.5	32.3	4.2	
36.1	33.4	2.7	
37.4	32.6	4.8	
38	35.3	3.3	
37	32.8	4.2	
36.9	32.7	4.2	
40.8	0	40.8	
36.7	32.8	3.9	
36.8	33.4	3.4	
35.2	0	35.2	
33.3	0	33.3	Not Crossed 34.5
36.9	32.6	4.3	
36.4	32.4	4	
39.1	36.4	2.7	
41	32.4	8.6	
36.7	34	2.7	
35.7	0	35.7	

Table 3. Cont...

Maximum	Minimum CT	ΔCT	Remarks
33.2	0	33.2	Not Crossed 34.5
37.3	0	37.3	
34.8	0	34.8	
35.1	0	35.1	
33.2	0	33.2	Not Crossed 34.5
36.1	32.7	3.4	
32.5	0	32.5	Not Crossed 34.5
32.9	0	32.9	Not Crossed 34.5

Among the 56 Rifampicin indeterminate results, 6 samples had shown that the maximum CT value did not cross 34.5 (Table 3).

DISCUSSION

Knowing the frequency and type of mutations associated with drug resistance in MTB in different settings helps in the development and implementation of rapid, large-scale genetics-based assays for the diagnosis of drug resistance.[7]

In the present study, 258 MTB-detected rifampicin-resistant cases and 56 MTB-detected rifampicin indeterminate cases were analyzed. Among the 258 rifampicin-resistant cases, 147 (57%) were Male predominant, with 111 (43%) in females. Huge variation was observed in the total samples of different groups (only two samples in the 1-10 age group compared to 78 in the 21-30 age group). A study in Ethiopia found that males are at greater risk for MDR-TB.[8] In the present study, the 21–30 age group was found to be more affected by rifampicin resistance, which is consistent with previous studies from Mizoram, China, and Ethiopia.[3,9,10] This could be due to the fact that this age group is more active and exposed to tuberculosis. Furthermore, more pulmonary samples (248) than extrapulmonary samples (10) were observed.

A greater number of cases presented with a medium bacillary load, followed by high, low, and very low loads among 248 sputum samples, whereas extrapulmonary cases (10) presented with very low (4), low (3), medium (2), and high (1) loads.

Among the five probes evaluated, most common mutations were observed with probe E (58.91%), which corresponds to codon 529-533, followed by probe D (12.02%) at codon 523-529, probe A (6.98%) at codon 507-511, probe B (6.98%) at codon 511-518, and probe C (1.55%) at codon 518-523, consistent with a previous study by NIRT Chennai.[2] These results highlight the fact that this pattern is prevalent in south India, particularly when compared to studies conducted in Mizoram, Madhya Pradesh, which showed a different pattern.[1,3] Some degree of correlation
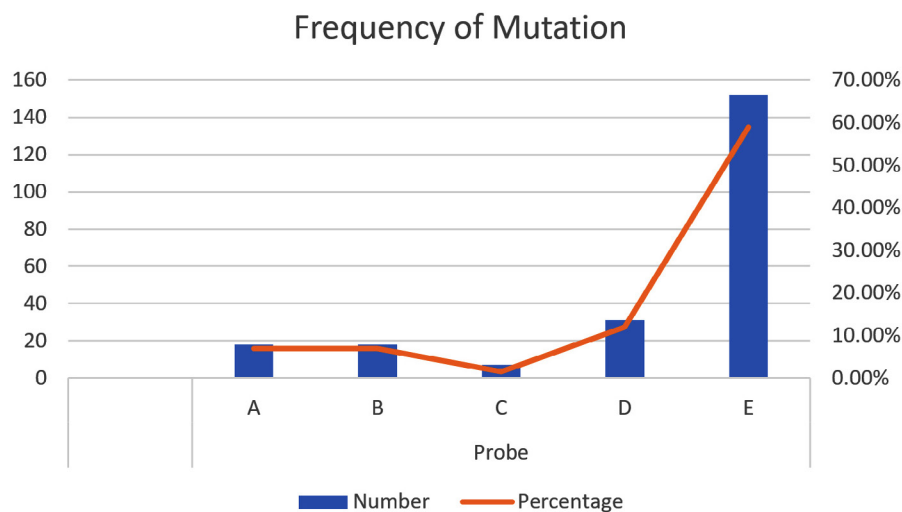

Figure 3. Frequency of different Probe Mutation of rpoB gene

was also observed with studies conducted in Andhra Pradesh, Mumbai and Punjab, wherein the predominance of Probe E mutation was reported (Table 2).[11-13]

With respect to other countries, similar studies in Pakistan (Munir *et al.*, Alimgir *et al.*), Uganda (Mboowa *et al.*), and Ethiopia (Alemu *et al.*) found that the predominant mutation was observed using probe E, consistent with the results of the present study, indicating that probe E mutation is the most common type worldwide.[4,5,10,14] The least frequent mutation was that of probe C, which is also consistent with the majority of the aforementioned literature. This may be due to the lower frequency for this mutation in this region. The frequency of combined mutations was 4.65%, which is comparable to that reported in Nigeria (Ochang *et al.*). This highlights the non-adherence of patients to antitubercular treatment (ATT), which may have given rise to more than one mutation.[15] A lack of missing probes was consistent with the results reported by Munir *et al.*, who found that 8.3% of samples were missing probes, compared to other studies in which a comparatively lower percentage was reported.[14]

In the Xpert MTB/RIF assay, the criteria used to denote a rifampicin indeterminate result is if the Ct of the first probe is >34.5 cycles and the Ct of the last probe is >38 cycles. However, in the present study, among 56 rifampicin indeterminate results, six sample Ct values crossed neither >34.5 nor >38. This implies that the programmed/configured software was not completely accurate in interpreting the rifampicin sensitivity reports. Furthermore, in all six samples, a mutation was observed in one of the probes, which could be interpreted as rifampicin resistance. These results study emphasize that, along with the report, the Ct value should be analyzed and interpreted for resistance according to the interpretive guidelines (missing probe or >4 cycle difference between the minimum and maximum Ct values, provided that neither of the Ct values is >34.5). The rifampicin indeterminate results can also be analyzed by using Shewart control charts, as described by Abaate *et al.* (preprint).[16] To overcome this, the World Health Organization (WHO) has endorsed the Xpert MTB/RIF Ultra, which relies on melt curve analysis to detect rifampicin sensitivity.

The Xpert MTB/RIF Ultra is more specific for the diagnosis of rifampicin resistance than the Xpert MTB/RIF assay.[17,18]

CONCLUSION

This study was conducted in a high-TB-burden region of India, in which the highest prevalence of TB has been reported (the state of Karnataka, Kalaburagi). Data on rifampicin resistance and its pattern within this locality from the last five years was evaluated. As a result, probe E mutation was found to be the most common type, while probe C mutation was the least common. Approximately 8.3% of probes were missing, which may indicate a partial mutation. These results provide insights that will help clinicians understand the genetic basis of antitubercular drug resistance and initiate the right treatment. In addition, this study also highlights the importance of reading CT values in order to interpret rifampicin indeterminate results in cases where the minimum and maximum CT values do not cross 34.5. The latter can be overcome using the Xpert MTB/RIF Ultra or Shewhart control charts. Xpert MTB/RIF Ultra is superior to Xpert MTB/RIF in diagnosing rifampicin resistance because its results indicate either rifampicin sensitivity or rifampicin resistance rather than rifampicin indeterminate result. This highlights how the principle of detecting rifampicin resistance using Xpert MTB/RIF Ultra differs from that of Xpert MTB/RIF.

Limitation of the study
No standard comparative tests were used. Compared with the Xpert MTB/RIF assay, Xpert MTB/RIF Ultra was warranted.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION
All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, ESIC Medical College and Hospital, Kalaburagi, India, with reference number ESICMC/GLB/IEC(2)/05/2023.

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