Distribution and frequency of common mutations in rpoB gene of Mycobacterium tuberculosis detected by Xpert MTB/RIF and identification of residential areas of rifampicin resistant-TB cases: A first retrospective study from Mizoram, Northeast India

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Abstract

Background: Rifampicin resistance (RR) is a surrogate marker of multidrug-resistant tuberculosis. The aim of this study is to determine the frequency of the commonly mutated probes for rpoB gene and locate the residential areas of the Rifampicin Resistant-TB (RR-TB) patients in a high endemic zone of Northeast India.

Methods: Archived data of 13,454 Xpert MTB/RIF reports were evaluated retrospectively between 2014 and 2021. Socio-demographic and available clinical information were reviewed and analyzed for RR-TB cases only. Logistic Regression was used to analyze the parameters with respect to probe types. P-value \( \leq 0.05 \) was considered to be statistically significant.

Results: 2,894 patients had Mycobacterium tuberculosis infection out of which 460 were RR-TB, which was most prevalent among 25–34 years. The most common mutation occurred in probe A (25.9 %) followed by E (23.5 %), D (9.8 %), B (2.6 %) and the least in C (0.2 %). High prevalence (34.3 %) of probe mutation combinations were also found: probes AB (0.4 %), AD (32.8 %), AE (0.4 %), DE (0.2 %) and ADE (0.4 %). Seventeen patients (3.7 %) were found without any missing probes. RR-TB patients were mostly located in Aizawl North–III, South–II and East–II constituencies.

Conclusion: This study provides genetic pattern of drug resistance accountable for RR over the past years in Mizoram which will help local clinicians in the initiation of correct treatment. Also, our findings provide a baseline data on the magnitude of RR-TB within the state and identification of the residential areas can help local health authorities in planning surveillance programs to control the spread of RR-TB.

1. Introduction

Multidrug-resistant tuberculosis (MDR-TB) is a form of tuberculosis (TB) infection which is caused by a bacterium known as Mycobacterium tuberculosis. It is resistant to two of the most potent anti-TB drugs, rifampicin (RIF) and isoniazid (INH) [1]. Rifampicin Resistance (RR) is a key indicator of multidrug-resistant tuberculosis, since >90 % resistance observed are also isoniazid resistant [2,3]. According to WHO guidelines, the identification of MDR/RR-TB requires further employment of different tests including bacteriological confirmation of both TB along with drug resistance testing using rapid molecular tests such as Xpert/MTB RIF, other sequencing technologies or solid or liquid culture method [4].

Introduction of WHO approved Xpert/MTB RIF assay (Cepheid, USA)
has revolutionized the diagnosis of TB since it is capable of simultaneously detecting *M. tuberculosis* and the mutations that confer RR [5,6]. Rifampicin being the key first line anti-TB drug inhibits DNA-dependent RNA polymerase activity in susceptible cells. This appears to occur as a result of the drug binding in the polymerase subunit facilitating direct blocking of RNA elongation.

Mizoram is situated in the north eastern part of India and shares borders with Myanmar and Bangladesh. It is the least populous state in India after Sikkim, having a population estimate of 1.26 million in 2021 (https://www.populationu.com/in/mizoram-population). Currently, among the laboratory tests available for TB, smear microscopy along with Xpert MTB/RIF assay are the only diagnostic tools implemented in the state. Xpert MTB/RIF is being utilized by Mizoram since the end of 2014 and from September 2017, it is being used as a Universal Drug Susceptibility Test. Despite the tremendous efforts and progress achieved by National Tuberculosis Eradication Program (NTEP) (erstwhile Revised National Tuberculosis Control Program), TB is still a burden in certain peripheral areas of India such as Mizoram. A PubMed and Google Scholar search strategy using relevant keywords (such as Northeast India, Xpert MTB/RIF, Genexpert, Mizoram RR-TB) did not provide any information on rpoB gene mutations from North East India. Due to the unavailability of information on the underlying genetic pattern of drug resistance from the state, which can provide insights into the pattern of mutation accountable for RR over the past years, this retrospective study was taken up to understand the magnitude of Rif resistance TB (RR-TB) using molecular diagnostic tools.

The objectives of this study were: 1) To determine the frequency of the commonly mutated probes for rpoB gene in the 81 bp Rifampicin Resistance Determining Region (RRDR) of *M. tuberculosis* via Xpert MTB/RIF and compare the frequencies of gene mutations in different geographical areas, 2) determine the association of the gene mutation with demographic factors, and 3) To understand the highly prevalent RR-TB residential areas for prediction of future epidemics.

2. Materials and methods

2.1. Ethical clearance

Approval for the study was obtained from the Institutional Ethics Committee (IEC) of Civil Hospital, Aizawl, Mizoram (B.12018/1/13-CH (A)/IEC/63). All the patients who participated had provided a written consent at the time of testing their samples.

2.2. Study population and data collection

As per the Health and Family Welfare Department, Government of Mizoram, during April 2021 to March 2022, 6,745 people were examined for TB out of which 1,698 were diagnosed and notified from both public and private sectors. In addition, 2,634 patients were tested for drug resistance of which 109 were diagnosed as RR-TB. For the current study, data were evaluated retrospectively from archived results for all the types of specimens received and tested using Xpert MTB/RIF assay at District TB Center, Falkawn and Synod Hospital, Durtlang, Mizoram, Northeast India from December 2014 to May 2021. A total number of 13,927 patients suspected for TB were subjected to Xpert/MTB RIF assay and out of which 473 had invalid, error, duplicates or no results and hence were removed. 13,454 patients were included in the study. RR result delivered through Xpert/MTB RIF was solely relied upon and no additional screening was performed. Socio-demographic and available clinical information were reviewed and analyzed for RR-TB cases only. Previous studies employing Xpert/MTB RIF system describing probe mutations conferring RR were retrieved from online sources and compared with the results of the present study to analyze the differences or similarities in the mutation pattern of the rpoB gene region.

2.3. Laboratory diagnosis

Previous studies have frequently stated the presence of up to 95 % of Rifampicin Resistance (RR) to be associated with mutations in the 81-bp hotspot region (codons 507–533 of the *E. coli* numbering system/ codons 426–452 of the *M. tuberculosis* numbering system) of the rpoB gene and is known RRDR. Xpert/MTB RIF is a cartridge-based, automated, hemi-nested real-time PCR system that utilizes five overlapping probes named as Probe A (codons 507–511), Probe B (codons 511–518), Probe C (codons 518–523), Probe D (codons 523–529) and Probe E (codons 529–533) [7,8]. To delineate the resistance associated mutations for *M. tuberculosis*, a numbering system based on *E. coli* sequence annotation circa 1993 was used. Whole genome sequencing is an alternative numbering system based on *M. tuberculosis* rpoB sequence [9]. However, the nomenclature used in this study follows the *E. coli* numbering system.

The assay for 13,454 patients included in the study was performed in accordance to the manufacturer’s instructions using Xpert MTB/RIF assay G4 (Cepheid, Sunnyvale, CA, USA) with a sensitivity of 94.4 % and specificity of 98.3 % for diagnosis of RR [10]. The Xpert MTB/RIF provides semi-quantitative results based on the probes’ Cycle Threshold (Ct) i.e., number of PCR cycles required to amplify the DNA of *M. tuberculosis* to a detectable level. Hence, the results are reported as “High” (Ct ≤ 16), “Medium” (Ct 16–22), “Low” (Ct 22–28) or “Very low” (Ct > 28). Presence of rpoB mutations can change the dynamics of hybridization between the amplexon and the probe(s) which corresponds to the mutated site thereby causing a difference between the Ct values of the probes and when hybridization is inhibited, it results in missing probes. However, in cases without mutated rpoB, all the 5 probes match exactly to the amplified MTB DNA. The G4 version of Xpert MTB/RIF software interprets sample results as resistant to Rif, if the difference between two probes (the first and last Ct) is > 4 cycles (ΔCt > 4) [11].

The tested patients were categorized into two (pulmonary and extrapulmonary TB). Results of the Xpert MTB/RIF assay were categorized into the following: 1) *M. tuberculosis* not detected. 2) *M. tuberculosis* detected; RR not detected. 3) *M. tuberculosis* detected; RR Detected. 4) Invalid. 5) Error. 6) No result. For the tests having *M. tuberculosis* detected along with RR detected, the missed probe types, bacteria load, sample type and available demographic data were assessed.

2.4. Household identification

The localities of patients belonging to Aizawl District were entered in excel sheet, coded, segregated, and mapped with the constituency they belong using the following link as a reference (https://ceo.mizoram.gov.in/state_profile). The frequencies were calculated (N/460x100) and was further arranged in descending order of RR-TB prevalence.

2.5. Statistical analysis

Data were entered, coded and analyzed using Statistical Package for Social Sciences (SPSS) version 20.0 (IBM Corp, USA). Bivariate logistic regression analysis was used to characterize clinical and demographic variables like frequencies of specimen type, DNA quantity, probe types and gender. Multivariate logistic regression analysis was used to characterize the demographic variables (age group) with respect to probe types. *P*-value ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. Socio-demographic and clinical information

Of the 13,454 patients that were included in the study, *M. tuberculosis* was detected in 2,894 (21.5 %) cases, out of which 460 (15.9 %) were RR-TB detected (i.e., presence of mutation in the rpoB gene either in the form of probe drop out or in the presence of hybridization with the
difference between the highest and lowest ct value as > 4) and 2,434 (84.1 %) were RR-TB not detected (i.e., absence of mutation in the rpoB gene). About 10,560 patients were Xpert/MTB RIF negative even though they presented with either of the following symptoms: prolonged cough, probable infection of the lungs, pyrexia of unknown origin and contact with TB patients (Fig. 1).

Among the 460 RR-TB cases, 57.8 % (n = 266) were males and 42.2 % (n = 194) were females. The maximum number of cases were observed in the age group of 25–34 years (34.6 %) followed by 35–44 years (20.4 %), 15–24 years (19.3 %), 45–54 years (11 %), 55–64 years (6.1 %), >65 years (4.8 %), 5–14 years (2.4 %) and the least in 0–4 years (1.3 %). These age groups are further correlated with gender (Table 1).

The mean age of RR-TB in this study was 34 years. The most prevalent age group among extrapulmonary TB was also between 25 and 34 years (n = 29, 44.6 %).

3.2. Sample type vs semi-quantitative analysis (Bacillary load)

Majority of the specimens were of pulmonary origin (n = 395, 85.9 % - sputum, broncho alveolar lavage (BAL), gastric lavage, tracheal aspirate) and the remaining were extrapulmonary origin (n = 65, 14.1 % - lymph node aspirate, pleural fluid, ascitic fluid, cerebrospinal fluid, pus/abcess) from various sites.

As Xpert MTB/RIF assay can semi-quantitatively quantify DNA present in the samples, the amount of DNA (bacillary load) among the 460 RR-TB cases were segregated as follows: High bacillary load (n = 107, 23.26 %) i.e., 104 from sputum, 1 each from gastric aspirate, liver abscess and pleural fluid, respectively. Medium bacillary load (n = 129, 28 %) i.e., 125 from sputum, 3 from BAL and 1 from Fine Needle Aspiration Cytology (FNAC) of lymph node. Low bacillary load (n = 126, 27.39 %) i.e., 92 sputum, 11 lymph node FNAC, 9 abscess, 7 pleural fluid, 2 tracheal aspirate, 2 gastric aspirate, 1 BAL, 1 ascitic fluid and 1 Cerebrospinal Fluid (CSF). Very low bacillary load (n = 98, 21.3 %) i.e., 3 abscess, 61 sputum, 1 ascitic fluid, 2 BAL, 6 CSF, 12 lymph node FNAC, 2 gastric aspirate, 11 pleural fluid.

RR-TB was more prevalent among pulmonary TB as compared to extra-pulmonary TB among the males. On the contrary, RR-TB was more in females suffering from extrapulmonary TB (Fig. 2).

3.3. Mutation in rpoB probes between pulmonary and extrapulmonary TB

In this study, probes A, B and D had higher mutation among extrapulmonary samples as compared to pulmonary samples. Whereas, probes C and E had higher mutation among pulmonary samples (Fig. 3). The mutations in different probes with the different types of samples used for TB diagnosis is shown in Suppl. Table 1.

3.4. Mutation in rpoB probes between male and female TB patients

The mutation frequencies in males showed (i) Single probe mutation: probe A – 24.4 % (65/266), probe B- 3.8 % (10/266), probe C- 0.4 % (1/266), probe D- 7.9 % (21/266) and probe E- 28.2 % (75/266). (ii) Multiple probes mutation: probes AD- 29.3 % (78/266), probes AE- 0.8 % (2/266), probes DE- 0.4 % (1/266) and probes ADE – 0.4 % (1/266). All probes positive RR-TB were found in 5 samples. On the other hand, the mutation frequencies in females showed (i) Single probe mutation: probe A- 27.8 % (54/194), probe B- 1 % (2/194), probe C- 0 %, probe D- 12.4 % (24/194) and probe E – 17 % (33/194). (ii) Multiple probes mutation: probes AB – 1 % (2/194), probes AD – 37.6 % (73/194) and probes ADE – 0.5 % (1/194). All probes positive were found in 12 samples. The results showed that mutations in probes A, B, C, E, AD, AE and DE were more frequent among males whereas probes D and AB mutations were more among females. In addition, different age groups among males and females displayed varied probe mutations (Suppl. Fig. 3A and 3B).

Fig. 1. Flowchart for analysis of RR-TB patients by Xpert MTB/RIF.
In this retrospective study, it was observed that throughout the seven years (2014 to May 2021), single probe mutations were observed as follows: probe A (codons 507–511), n = 119 (25.9 %) had the most common mutation as compared to the other four probes. This was followed by probe E (codons 529–533), n = 108 (23.5 %), probe D (codons 523–529), n = 45 (9.8 %), probe B (codons 512–518), n = 12 (2.6 %) and probe C (codons 518–523), n = 1 (0.2 %). There was no probe B mutation in the year 2017 as well as in 2021 (till the study time period). Also, mutation in probe C (codons 518–523) was observed only once (in 2015) within these 7 years. Within these seven years, mutation combinations were also observed as follows: Probes AB (n = 2, 0.4 %), probes AD (n = 151, 32.8 %), probes AE (n = 2, 0.4 %), probes DE (n = 1, 0.2 %) and probes ADE (n = 2, 0.4 %) (Suppl. Fig. 2). However, 17 (3.7 %) RR-TB cases had no missed probe types (all probes positive) and the ΔCt for these samples were all > 4 cycles. Including all the mutations (single and multiple), the Xpert/MTB RIF probe mutations throughout seven years are shown (Fig. 4). Among the 460 RR-TB cases, 98 (21.3 %) samples had “very low” DNA quantity which is correlated with the missing probes as (i) single probe mutation: Probe A (n = 23, 23.5 %), B (n = 0), C (n = 1, 1 %), D (n = 9, 9.2 %), E (n = 15, 15.3 %), (ii) multiple probes mutation: Probes AD (n = 44, 44.9 %), AE (n = 1, 1 %) and ADE (n = 2, 2 %) and all probes positive (n = 3, 3 %).

Twelve studies employing Xpert MTB/RIF were retrieved from 2014 to 2021. These studies reported probe mutations conferring RR and were compared with the current study as shown in Fig. 5.

### 3.6. Association of socio-demographic data and mutations present at each probe among all RR-TB cases

The study of the association of demographic characteristics of patients to RR showed the following: Using bivariate analysis, the frequency of mutation at probes A (p = 0.009; OR = 1.670; CI = 1.137–2.453) and D (p = 0.007; OR = 1.668; CI = 1.146–2.426) were statistically significant for female gender. For sample type, the frequency of mutation at probes A (p = 0.047; OR = 1.782; CI =
Fig. 4. Comparison of rpoB probes mutation over a span of seven years.

Fig. 5. Comparison of the prevalence of probes mutation conferring rifampicin resistance in different countries.
1.099–3.149) and D (p = 0.001; OR = 2.450; CI = 1.432–4.189) were statistically significant for extrapulmonary TB. The frequency of mutation at probe D site alone was also statistically significant for samples with low (p = 0.000; OR = 2.915; CI = 1.685–5.042) and very low (p = 0.000; OR = 3.283; CI = 1.837–5.867) DNA quantity (Table 2). Multi-variate analysis showed that among the various age groups, the frequency of mutation at probe A site was statistically significant for younger age groups: 15–24 years (p = 0.015; OR = 2.394; CI = 1.183–4.847) and 25–34 years (p = 0.032; OR = 2.009; CI = 1.061–3.802) (Table 3). Single and mutation combination of the rpoB probes observed were statistically significant (p = 0.000) for ≤ 40 years of age.

3.7. Geographical distribution of RR-TB cases in various residential areas

There are 40 constituencies in Mizoram out of which 14 are in Aizawl District (the capital). The localities of RR-TB patients belonging to Aizawl District (n = 344, 74.8 %) after segregation and mapping are given in Fig. 6. Four constituencies had multiple probe mutation combinations: Aizawl West-I (AD and DE), Aizawl North -II (AD, AB and ADE), Aizawl West-III (AD and AB), Aizawl East-I (AD, AE and ADE). Detailed information about the distribution of single and multiple probe mutations present in different constituencies is shown in Suppl. Table 2. Patients belonging to districts other than Aizawl were also enrolled and diagnosed as RR-TB in the District TB Center-Falkawn, Aizawl and Synod hospital, Durtlang, Aizawl. Among these patients, the prevalence of RR-TB as per the archived record of Xpert/MTB RIF instrument are also shown in Suppl. Fig. 3. As a whole, the geographical distribution of RR-TB in Mizoram is shown in a map (Fig. 7).

4. Discussion

TB was declared as a ‘global public health emergency’ in 1993 [12]. As per India TB Report 2020, diagnosis via Xpert/MTB RIF is offered to all patients with smear positivity upon follow up, which includes treatment failures of first line anti-tuberculosis therapy (ATT) [13]. MDR-TB arises in clinical field either when therapy is inadequate or because of poor compliance of patients to adhere fully to an appropriate regimen or due to administration of counterfeit drug. In spite these, TB remains a huge burden especially in remote hilly areas such as Mizoram and till date reliable published information on the extent of MDR/RR-TB within the state is unavailable.

In the present study, there were 2,894 TB cases detected via Xpert MTB/RIF from December 2014 to May 2021. Among these, the proportion of RR-TB diagnosed was 15.9 % (n = 460). Our findings are similar with the 17 % report from Central India [14]. However, higher prevalence has been reported in other parts of India such as New Delhi (17.9 %) [15] and Lucknow (27.8 %) [16]. Among the RR-TB cases, males (57.8 %) are predominant compared to females (42.2 %). A systematic review from Ethiopia reported that male gender is an identified risk factor for MDR-TB [17]. The most affected age groups observed were productive individuals between 25 and 34 years, which is in concordance with a study from China and Ethiopia [18,19]. This can be attributed to the specific age group being more exposed to open cases of TB especially males being more susceptible to TB associated risk factors. Among the 460 RR-TB cases, 85.9 % were from pulmonary TB while 14.4 % from extrapulmonary TB. Higher DNA amount was observed in pulmonary cases (sputum) while low/very low amount of DNA was common among extrapulmonary cases which is in concordance with a study from Ethiopia [19]. Xpert MTB/RIF also has the added advantage of detecting TB in extrapulmonary cases where smear microscopy using Ziehl-Neelsen stain is often negative.

Overall, in this study, the most common RRDR rpoB gene mutation in the 81 bp were observed in codons 507–511 (25.9 %), followed by codons 529–533 (23.5 %), codons 523–529 (9.8 %), codons 511–518 (2.6 %) and the least in codons 518–523 (0.2 %) which corresponds to probes A, E, D, B and C, respectively which strongly contradicts with findings of the studies from Ethiopia, Nepal and Madhya Pradesh [19–21] where they reported no mutation in probe A region. Our results are also discordant with the findings of the studies from Madhya Pradesh, India where the commonest mutation was found in probe B followed by probe C and E, while no mutations were found in probes A and D [21]. Though Sharma et al reported probe B to be the most mutated site, other previous studies conducted within and outside India had reported probe E (codon 531–533) as the commonest mutated site in the region of the RRDR rpoB gene. This might be due to higher mean relative fitness (Darwinian fitness) [22] and resistant mutants have a better ability to survive. The countries/cities reporting probe E as the most mutated site are Punjab, Andhra Pradesh, Ethiopia, Nepal, Mumbai, Nigeria, Pakistan, Bangladesh, Zimbabwe, Himachal Pradesh and Uganda [7,8,19,20,23–29]. However, next to probe E mutation, the order of the prevalence of probe mutation varies from region to region wherein E is followed by B, D, A and C [8,30] and by D and B [19,23,24,31]. A small proportion of probe C mutation was reported from Punjab, Andhra Pradesh, Nepal, Madhya Pradesh, Mumbai, Pakistan, Bangladesh, Zimbabwe and Himachal Pradesh [7,8,20,21,23,25–28] while no probe C mutation was reported from Ethiopia, Nigeria and Uganda [19,24,29]. This study also reports probe C mutation occurring only once within a span of seven years. The least mutations detected by Xpert MTB/RIF in probe C might be due to this specific site of RRDR being probably less susceptible to mutations conferring the resistance or because of less selection pressure in this region.

The present study reports the most frequent mutation occurring at probe A followed by probe E. Probably due to geographical differences in M. tuberculosis lineage, the mutations may also vary. Previous studies had mostly reported the mutations observed for probe E as S31L [30,32], probe B as D516V and D516Y and probe C as S522L [33] . Our previous sanger sequencing study from Mizoram reported the mutation at probe A region as L511P and probe D region as H526Q and H526L [34]. This study also reports high number of RR-TB detected using cycle threshold > 4 cycles (all probe positive RR-TB).

Like other studies [9,23,24], rpoB probe mutation combinations that were also found in this study presumably occurred due to the intrinsic or acquired ability of the bacteria to adapt to drug exposure. A study from

Table 2

| Categories (Age group) | Probe A | Probe B | Probe D | Probe E |
|-----------------------|--------|--------|--------|--------|
|                       | p-value | OR (95% CI) | p-value | OR (95% CI) | p-value | OR (95% CI) | p-value | OR (95% CI) |
| 0-4 years             | 0.820   | 1.217 (0.224–6.615) | -        | -        | -        | -        | -        | -        |
| 5-14 years            | 0.108   | 3.246 (0.771–13.661) | -        | -        | -        | -        | -        | -        |
| 15-24 years           | 0.015   | 2.394 (1.183–4.847) | 0.948    | 0.952 (0.218–4.161) | 0.415    | 1.335 (0.666–2.677) | 0.686    | 0.847 (0.380–1.891) |
| 25-34 years           | 0.052   | 2.090 (1.061–3.802) | 0.157    | 0.308 (0.060–1.575) | 0.780    | 1.095 (0.578–2.077) | 0.962    | 0.983 (0.476–2.028) |
| 35-44 years           | 0.156   | 1.643 (0.827–3.265) | 0.256    | 0.348 (0.056–2.153) | 0.831    | 0.927 (0.463–1.857) | 0.985    | 1.059 (0.486–2.307) |
| 55-64 years           | 0.472   | 1.405 (0.557–3.543) | 0.657    | 0.593 (0.059–5.981) | 0.154    | 0.476 (0.172–1.322) | 0.962    | 0.974 (0.337–2.819) |
| >65 years             | 0.074   | 2.699 (0.910–7.478) | -        | -        | -        | -        | -        | -        |

Probe C was not significant for all the categories analyzed.
removal of uracil from DNA were reported to have a higher spontaneous
initiation of ATT drugs prior to testing the patient
resistance (low, moderate, high) depending on the position and the
can also predispose the bacteria to develop mutation(s) [37]. These
mulating damaged guanine nucleotides since it resides in the oxidative
mutation rate [35].

teracted by uracil-
combinations could be due to the fact that
gain rational understanding whether a specific codon mutation in the
tion combinations (n
in this study might also be retreatment cases as information on the
mutations in retreatment TB cases [30]. The mutation combinations observed
- M. tuberculosis
rpoB gene probes mutation.

| Categories (Age group) | p-value OR (95 % CI) | p-value OR (95 % CI) | p-value OR (95 % CI) | p-value OR (95 % CI) |
|-----------------------|----------------------|----------------------|----------------------|----------------------|
| 0-4 years             | 0.820 1.217 (0.224-6.615) | – | – | 0.183 2.500 (0.649-9.635) |
| 5-14 years            | 0.108 3.246 (0.771-13.661) | – | – | 0.415 1.335 (0.666-2.677) |
| 15-24 years           | 0.015 2.394 (1.183-4.847) | 0.948 0.952 (0.218-4.161) | 0.415 2.500 (0.649-9.635) | 0.610 0.650 (0.124-3.405) |
| 25-34 years           | 0.032 2.009 (1.061-3.802) | 0.157 0.308 (0.060-1.575) | 0.780 1.095 (0.578-2.077) | 0.962 0.983 (0.476-2.028) |
| 35-44 years           | 0.156 1.643 (0.827-3.265) | 0.256 0.348 (0.056-2.153) | 0.831 0.927 (0.403-1.857) | 0.885 1.059 (0.486-2.307) |
| 55-64 years           | 0.472 1.405 (0.557-3.543) | 0.657 0.593 (0.059-5.981) | 0.154 0.476 (0.172-1.322) | 0.962 0.974 (0.337-2.819) |
| >65 years             | 0.074 2.609 (0.910-7.478) | – | – | 0.983 0.989 (0.358-2.733) |

Reference Category: 45-54 years; Positive (No mutation).

Bangladesh had reported the occurrence of probes mutation combina-
tions in retreatment TB cases [30]. The mutation combinations observed
in this study might also be retreatment cases as information on the
initiation of ATT drugs prior to testing the patient’s sample is unknown.
Unlike other studies, this study reports high prevalence of probe mutation
combinations (n = 158, 34.3 %) which demands deeper insights to
gain rational understanding whether a specific codon mutation in the
probe is being favored evolutionarily. However, these mutation com-
binations could be due to the fact that M. tuberculosis is G + C rich and is
at a high risk for cytosine deamination, the process of which is coun-
teracted by uracil-N-glycosylase. Organisms that are defective in the
removal of uracil from DNA were reported to have a higher spontaneous
mutation rate [35]. M. tuberculosis is also at an increased risk of accu-
cumulating damaged guanine nucleotides since it resides in the oxidative
environment of the host macrophages. Some strains of this species might
undergo hypermutability resulting in multiple rpoB gene mutations
through down-regulation or inactivation of mutT genes, which plays a
crucial role for the removal of oxidized guanines and in turn preventing
errors during replication or transcription [36]. Non-adherence to the
drug due to its long-term usage or underdosage or counterfeit drugs etc.,
can also predispose the bacteria to develop mutation(s) [37]. These
mutation combinations could also be held accountable for the increase
in MDR cases in the study area.

Mutations found in the rpoB gene can result in different levels of RIF
resistance (low, moderate, high) depending on the position and the
amino acid change. This is attributed to a consequence of receptor-
ligand interaction changes such as steric hinderance, electrostatic
repulsion, loss of hydrogen bond and hydrophobic interaction [38].
Mutations like H526D, H526Y and S531L (corresponding to probes D
and E) are associated with high level of drug resistance, while L511P,
D516Y, D516V (corresponding to probes A and B) and H526L,
L533P (corresponding to probes D and E) favours moderate and low-
level resistance respectively. In addition, L511P, D516Y, H526L,
H526S, H526N, H526C and L533P amino acid changes were reported to
be responsible for disputed rpoB mutations (genotypic resistant,
phenotypic sensitive) which could be associated with low-level rifam-
picin resistance leading to poorer treatment outcomes and disease
severity using standard-dosing rifampicin-based regimens [39-43].
This highlights the importance of sequencing to locate the exact amino-acid
change within a specific probe region as probe mutations alone detected
in Xpert MTB/RIF is not sufficient to determine the nature of drug
resistance.

Among the 460 patients tested, this study also reports a “very low”
bacillary load in 98/460 (21 %) cases. Ngabonziza et al. reported that
having a “very low” bacillary load on Xpert/MTB RIF test was signifi-
cantly correlated with false RIF resistance where they found that on
repeated Xpert MTB/RIF testing, 54/63 (86 %) of patients with a “very
low” bacillary load were falsely diagnosed as RIF resistance and further
proved by sequencing [44]. The RIF resistant cases with “very low”
bacillary load in this study were not re-tested with Xpert MTB/RIF. This
calls for the need to re-test Xpert MTB/RIF in instances where RIF
resistance arises with a “very low” bacillary load. In this study, due to

Fig. 6. Geographical distribution of RR-TB cases in Aizawl district, Mizoram, India. Mutation frequency (in percentage) is represented in the X-axis.
could not be determined and hence it is difficult to elucidate the implications of this finding. In this study, the proportion of extrapulmonary TB was more among females aged 25–34 years which is in concordance with a study from China where younger female patients are more likely to have extrapulmonary TB [45]. The predilection of extrapulmonary TB for women may be linked to the limited facilities for access to healthcare apart from the less prevalence of other risk factors such as habit of smoking. Previous studies have reported the increased risk of mortality with smoking in men but not in women [46]. In general, smoking is less common in women and hence they may be relatively protected from the hazardous pulmonary effect of smoking. This may be one of the factors for the differences in distribution [47]. This suggests that female gender and younger age could be an independent risk factor for TB especially in a high burden area. Bangladesh had reported the geographical distribution of RR-TB within their country [30]. Similarly, within Aizawl District, Aizawl North –III (11.9 %) had the highest proportion of RR-TB followed by Aizawl South –II (7.4 %) and Aizawl East –II (7.2 %) which warrants further investigation of the affected areas. The location of the residential areas might also prove useful for future epidemiological survey as well as for understanding the transmission pattern and severity of the disease.

5. Conclusion

In Mizoram where TB diagnostic infrastructures are limited, this retrospective study being the first molecular data on *M. tuberculosis* from the state provides genetic pattern of drug resistance accountable for RR over the past years, which will help local clinicians in the initiation of correct treatment. Unlike previous reports, the pattern of mutations observed in the 81 bp RRDR region of *M. tuberculosis* isolates were in codon 507–511 (probe A). Majority of the RR-TB were found in 25–34 years age group. Also, our findings provide a baseline data on the magnitude of RR-TB within the state and identification of the residential areas can help local health authorities in planning surveillance programs to control the spread of RR-TB.

6. Limitations

Our study has several limitations, being a retrospective study, it lacks relevant information such as previous history of TB treatment which could result in multiple probes mutation, HIV, diabetes and BCG vaccination status. In addition, *rpoB* gene sequencing could have established the specific *rpoB* mutation. As there is no scientific evidence yet, for the association of probe mutations with pulmonary or extrapulmonary sites, it is a very important objective to be considered for future studies by researchers.

7. Ethical clearance

Approval for the study was obtained from the Institutional Ethics Committee (IEC) of Civil Hospital, Aizawl, Mizoram (B.12018/1/13-CH (A)/IEC/63). All the participants had provided a written consent at the time of testing the samples.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CRediT authorship contribution statement

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