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ORIGINAL RESEARCH

Correlation Between Xpert MTB/RIF Results and *rpoB* Mutations within Probe-Targeted Regions in *Mycobacterium tuberculosis* Isolates from Sichuan Basin

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Purpose: This study characterized mutation patterns within the rifampicin resistance-determining region (RRDR) of the *rpoB*gene (codons 507–533), targeted by Xpert MTB/RIF probes, in *Mycobacterium tuberculosis* isolates from Southwest China. Regional molecular profiles were and compared regional profiles against global datasets to elucidate implications for rifampicin resistance surveillance.

Methods: We conducted a retrospective study of 197 rifampicin-resistant tuberculosis cases involving 197 cases of rifampicin-resistant tuberculosis (comprising 194 pulmonary and 3 extrapulmonary cases) confirmed through the GeneXpert MTB/RIF assay at Suining Central Hospital during 2023–2024. Mutation characteristics across five RRDR probes (labeled A–E) were assessed, along with the semi-quantitative classification of bacterial loads.

Results: Single-probe mutations predominated (82.7%; 163/197), with hotspots at Probe E (S531L, 55.8%) and Probe A (Q510H/V511D, 12.2%). Dual-probe mutations (16.8%, 33/197) were primarily A+B combinations (13.7%, 27/197), showing significantly elevated prevalence in patients aged 46–60 years (OR = 2.31, 95% CI: 1.12–4.79, P< 0.05). Bacterial load stratification revealed strong diagnostic accuracy associations: smear positivity rates were 79.4% (high load) and 44.4% (medium), while culture positivity rates were 67.6% and 57.8%, respectively.

Conclusion: A unique double-peak mutation pattern (primarily involving probes E and A) was identified in Sichuan Province, which contrasts sharply with the single hotspot pattern observed in Europe and the epidemiological pattern in Beijing, China, where probe E predominates. The elevated Probe A mutation rate (12.2%) potentially reflects regional rifabutin usage in second-line regimens. These findings provide molecular epidemiological insights for optimizing diagnosis and drug resistance monitoring in southwestern China.

Keywords: *Mycobacterium tuberculosis*, *rpoB* gene, drug resistance

Introduction

Tuberculosis (TB) remains a leading global health threat, with an estimated 480,000 rifampicin-resistant TB (RR-TB) cases among 10 million new infections in 2023. In China, RR-TB accounts for 7.1% of new cases, with the southwest region facing unique challenges due to uneven healthcare resource distribution. Rapid and accurate detection of rifampicin resistance is critical for timely treatment initiation.

The Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA), a World Health Organization (WHO)-endorsed nucleic acid amplification test (NAAT), detects *Mycobacterium tuberculosis* and rifampicin resistance by targeting five overlapping probes (A–E) within the 81-bp RRDR of the *rpoB* gene (codons 507–533).[1] Geographic variation in *rpoB*

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mutation patterns significantly impacts treatment decisions. For example, the S531L mutation (probe E) is strongly associated with high-level rifampicin resistance, whereas Q510H (probe A) may retain susceptibility to rifabutin.[2] However, most global mutation data originate from high-income regions, with limited insights into regional patterns in resource-limited settings like Southwest China.

The Xpert *Mycobacterium tuberculosis*/*rifampicin* (MTB/RIF) system, produced by Cepheid in Sunnyvale, CA, USA, has serves as a critical diagnostic tool in the diagnostics of tuberculosis (TB). It facilitates the swift identification of TB and rifampicin (RIF) resistance. As a nucleic acid amplification test (NAAT), Xpert is endorsed as a primary diagnostic instrument for TB and RIF resistance by both the World Health Organization (WHO) and Singapore's clinical management guidelinesas a frontline diagnostic tool for TB and rifampicin resistance.[3] Xpert demonstrates a high specificity rate of 98% in identifying RIF resistance and boasts a positive predictive value (PPV) greater than 90% in environments where the prevalence of RIF resistance exceeds 15%.[4]

Rifampicin-resistant tuberculosis (RR-TB) typically arises from mutations in the *rpoB* gene, which encodes the beta subunit of RNA polymerase. These mutations are primarily found within a specific region, known as the rifampicin resistance-determining region (RRDR), that is 81 base pairs long. According to the World Health Organization's 2023 classification criteria for the surveillance of drug-resistant tuberculosis (DR-TB), around 95% of cases classified as rifampicin-resistant tuberculosis (RR-TB) are linked to mutations in this rifampicin resistance-determining region of the *rpoB* gene8.[1,5] Xpert identifies rifampicin resistance using five overlapping probes that target the RRDR region of the *rpoB* gene. This gene encodes a total of 27 amino acids from sites 507 to 533. Specifically, probe A encompasses amino acids 507 to 511, probe B spans amino acids 512 to 518, probe C includes amino acids 518 to 523, probe D covers amino acids 523 to 529, and probe E addresses amino acids 529 to 533. The mutation locations of the *rpoB* gene are frequently defined by geographical factors, leading to variances in the documented *rpoB* gene mutation locations across various countries and areas. Moreover, specific mutation sites within the *rpoB* gene are linked to elevated resistance of MTB to rifampicin, including mutations at positions 531 and 526, which correlate with significant rifampicin resistance.[6]

Although global mutation patterns are well-documented, regional data from Southwest China remain scarce. Prior studies report Probe E mutation frequencies of 51.2% in Shanghai, 64.6% in Beijing, 51.5% in the United States, 51.6% in Iran, and 52.8% in India. Our observed rate of 55.8% underscores geographical heterogeneity.[7] This research intends to delineate the distinctive mutation characteristics present in Sichuan Province, which will support the improvement of rapid diagnostic methods locally. The objective of this investigation was to assess the prevalence of various *rpoB* gene mutation-related probes using the GeneXpert® MTB/RIF assay (Cepheid, GenXpert IV system). This study seeks to identify the mutation prevalence across different areas and establish a foundation for additional analysis and exploration of RR-TB resistance gene mutation hotspots in the region.

Materials and Methods

Data Collection

Inclusion Criteria

- Age ≥18 years
- Xpert® MTB/RIF positive with *rpoB* mutation
- Complete clinical records including prior TB treatment history

Exclusion Criteria

- Non-tuberculous mycobacteria (NTM) coinfection confirmed by culture

A total of one hundred ninety-seven patients diagnosed with tuberculosis (TB) who tested positive using the Xpert assay and exhibited mutations in the *rpoB* gene were recruited from our hospital between January 2023 and December 2024. Among these patients, there were 194 instances of pulmonary tuberculosis, which included 143 males and 54 females,

with ages ranging from 19 to 81 years and a median age of 60 years. In accordance with WHO guidelines, respiratory specimens were collected. The study involved 65 sputum samples and 129 specimens of bronchoalveolar lavage fluid. Additionally, there were 3 instances of extrapulmonary tuberculosis, which comprised 2 cases of pleural fluid (indicative of tuberculous pleurisy) and 1 case of cerebrospinal fluid (indicative of tuberculous meningitis). The demographic details of the 197 TB patients are depicted in Table 1.

Research Methodology

Xpert

Specimens were processed according to manufacturer protocols (Cepheid, USA). Xpert results reported MTB detection status, rifampicin resistance, and *rpoB* probe mutation profiles (A–E). Xpert evaluated the MTB load in specimens through a semi-quantitative analysis, categorizing the MTB levels based on the cycling threshold (Ct). The MTB content was classified into four groups: very low, low, medium, and high, depending on the Ct values. Specifically, a Ct value greater than 28 was deemed very low, values between 22 and 28 were classified as low, values ranging from 16 to 22 indicated medium levels, and values below 16 were associated with high levels of MTB.[8]

Acid-Fast Bacilli (AFB) Smear Microscopy

All 197 specimens underwent acid-fast bacilli (AFB) smear microscopy via Ziehl-Neelsen staining and mycobacterial culture. Smear results were categorized as negative or positive (+, ++, +++).[9]

Table 1 Demographic Characteristics of 197 Cases of Tuberculosis

Gender	**N**	**P**
Female	54	<0.001*
Male	143	
Age group (years)		
19-45	59	0.525
46-60	72	
>60	66	
Previous status		
New case	4	0.002*
Treatment failure	81	
Relapse	112	
Residence		<0.001*
Urban	57	
Rural	140	
Type of TB		
Extrapulmonary	3	
Pulmonary	194	
Nature of samples		0.001*
Sputum	65	
Broncho-alveolar aspirate	129	
Cerebrospinal fluid	1	
Pleural effusion	2	

Notes: Pearson's independence and Fisher's exact probability chi-square tests were used to compare percentages; *P values < 0.05 were considered statistically significant.

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Table 2 Range of Drugs Used and the Breakpoints and Drug-Susceptibility Patterns of RIF-Resistant *M. tuberculosis* Isolates (n=138)

Drug	Concentration Range (μg/mL)	Clinical Breakpoint (μg/mL)	Resistant Isolates (n)	Resistance Rate (%)
RIF	0.5–4	1.0	138	100.0%
INH	0.1–1	0.2	26	18.8%
RFB	0.25–2	0.5	19	13.8%
OFX	1-4	2.0	23	16.7%
LVFX	1-8	2.0	19	13.8%
MXF	0.25–2	0.5	20	14.5%
TH	1.25–5	2.5	5	3.6%
PAS	1-4	2.0	32	23.2%
AK	0.5–2	1.0	2	1.4%
KAN	2.5–10	5.0	4	2.9%
CPM	1-4	2	4	2.9%
EMB	2.5–10	5	4	2.9%
PZA	50-300	100	14	10.1%
SM	1-4	2	21	15.2%

Abbreviations: RIF, rifampin; INH, isoniazid; RFB, rifabutinl; OFX, ofloxacin; LVFX, Levofloxacin; MXF, moxifloxacin; TH, Protionamide; PAS, Para-aminosalicylic Acid; AK, amikacin; KAN, kanamycin; CPM, Capreomycin; EMB, ethambutol; PZA, pyrazinamide; SM, streptomycin.

Correlation Between Xpert Probe Mutations and Phenotypic Drug Susceptibility

138 samples underwent phenotypic drug susceptibility testing (pDST) system testing. Specific results are shown in Table 2.

A total of 138 isolates underwent phenotypic drug susceptibility testing (pDST) against 14 anti-tuberculosis drugs. As shown in Table 2, all 138 isolates (100%) were resistant to rifampicin (RIF), confirming the Xpert MTB/RIF results. Resistance rates to other drugs varied: isoniazid (INH) (18.8%), rifabutin (RFB) (13.8%), ofloxacin (OFX) (16.7%), levofloxacin (LVFX) (13.8%), moxifloxacin (MXF) (14.5%), thionamide (TH) (3.6%), para-aminosalicylic acid (PAS) (23.2%), amikacin (AK) (1.4%), kanamycin (KAN) (2.9%), capreomycin (CPM) (2.9%), ethambutol (EMB) (2.9%), pyrazinamide (PZA) (10.1%), and streptomycin (SM) (15.2%).

Mycobacterium tuberculosis Culture

Samples were treated with an equal volume of NALC-NaOH (4%) for 15 minutes at room temperature and then inoculated onto modified L-J medium. The L-J medium was checked daily for the first week and then weekly for visible colony growth. After 8 weeks of continuous observation, the culture is considered negative if no colonies are present.[10]

Phenotypic Drug Susceptibility Testing (pDST)

Minimum inhibitory concentrations (MICs) for 14 anti-tuberculosis drugs (including isoniazid, rifampicin, ethambutol, streptomycin, pyrazinamide) were determined using a microbroth dilution method (Zhuhai Beisuo Co., China). Isolates from solid cultures were homogenized and diluted to McFarland standard 1.0 ($\approx 1\times10^8$ CFU/mL).

Inoculation and Incubation

Drug susceptibility testing plates: A 96-well plate pre-coated with 14 anti-tuberculosis drugs (isoniazid, rifampicin, streptomycin, ethambutol, pyrazinamide, etc.) was utilized.

Procedure: Each well (excluding pyrazinamide controls) received 100 μL of Culture Medium I-diluted inoculum. Pyrazinamide wells received 100 μL of Culture Medium II-diluted inoculum. Plates were sealed and incubated at 36±1°C.

Result Interpretation

Reading Time: Plates were monitored daily for contamination (days 1–7) and MIC endpoints (days 14–21).

Criteria

Susceptible (S): No visible growth in drug-containing wells compared to growth controls.

Resistant (R): Visible growth in drug-containing wells.

Pyrazinamide (PZA): Growth inhibition ≥50% relative to PZA-positive controls was classified as susceptible.

Quality Control

Reference Strain: M. tuberculosis H37Ra (ATCC 25177) was included in each batch to validate assay performance.

Standards: H37Ra growth in positive controls and complete inhibition in drug-sensitive wells (eg, isoniazid 0.2 μg/mL, rifampicin 1 μg/mL) were mandatory.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (version 28.0, Armonk, NY), with categorical variables analyzed using Fisher's exact test and continuous variables by Mann–Whitney *U*-test, as appropriate for non-parametric distributions.

Results

Semi-Quantitative Results of MTB Content in Xpert-Positive Specimens

Of the 197 specimens that tested positive for Xpert and exhibited *rpoB* gene mutations, High semi-quantitative MTB levels were observed in 17.2% (34/197) of specimens, while 32.0% (63/197) showed a moderate level, 32.5% (64/197) had a low level, and 18.3% (36/197) were classified as having a very low level. The detection levels of MTB—high, medium, low, and very low—were found to be more prevalent in specimens from pulmonary TB compared to those from extrapulmonary TB, as illustrated in Figure 1.

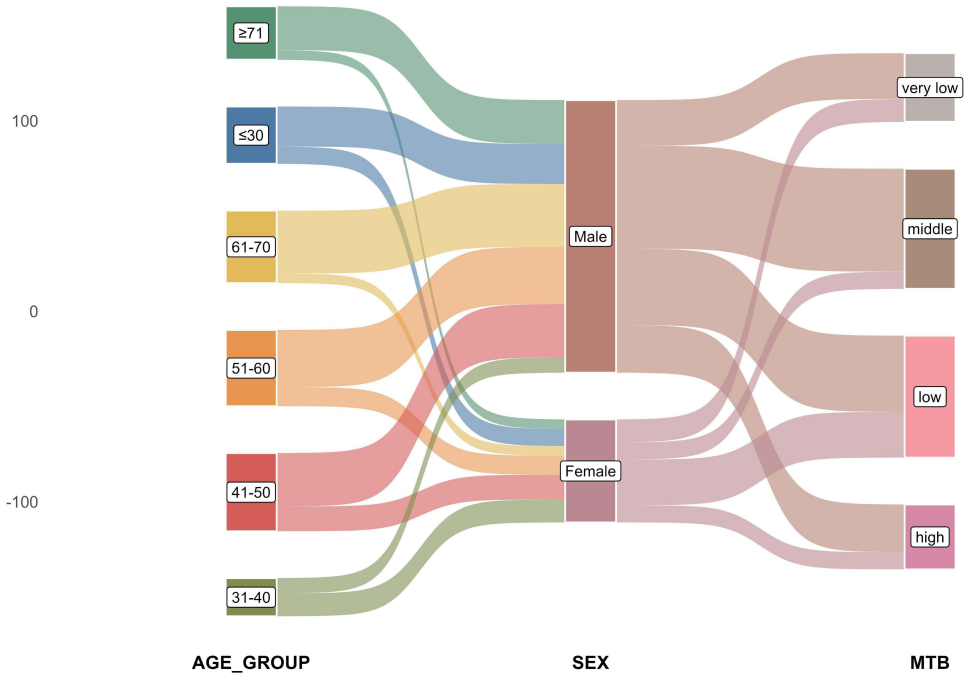

Figure 1 Distribution of MTB levels in *rpoB* gene mutation specimens from different patients.

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Correlation of Xpert-Positive MTB Semiquantitative Levels with Smear-Positive Results

A total of one hundred ninety-seven specimens were subjected to AFB smear microscopy for staining of antacid bacillus, resulting in 61 (31.0%) positive and 136 (69.0%) negative findings. The rates of smear positivity among the specimens were as follows: 79.4% (27 out of 34) for the high MTB semiquantitative level, 44.4% (28 out of 63) for the medium level, 9.4% (6 out of 64) for the low level, and 0.0% (0 out of 36) for the very low level. Additionally, specimens that tested positive for Xpert exhibited a higher smear-positive rate.

Correlation Between Xpert Semi-Quantitative Levels and Auxiliary Assays

As shown in Figure 2, smear positivity rates significantly differed across MTB load categories: 79.4% (high), 44.4% (medium), 9.4% (low), and 0% (very low)(χ^2=72.79, P<0.001). Similarly, culture positivity rates were 67.6% (high), 57.8% (medium), 23.1% (low/very low) (χ^2 = 30.04, P< 0.001). Among Xpert-high/medium samples, 73.5% were smear-positive versus 26.5% of low/very low samples.

Distribution and Frequency of Mutations in Xpert-Positive rpoB Gene Probes rpoB

The mutation rate of the gene was 82.7% (163/197) for single probe, 16.8% (33/197) for double probe and 0.5% (1/197) for triple probe. Among the single-probe mutations, the highest mutation rate was found in probe E (55.8%, 110/197), followed by probe A (12.2%, 24/197); among the double-probe mutations, the highest mutation rate was found in probe A+B (13.7%, 24/197), and there was one case of triple-probe mutation in which the mutation was found in probe A+D+E. Notably, no mutations were detected in Probe C or specific combinations some of the combinations were not found at all. The distribution and frequency of mutations in the *rpoB* gene probes were not statistically significant between pulmonary and extrapulmonary TB specimens, see Table 3. Mutation data for different probes for different genders, for rifampicin resistant patients were made in Table 4.

These data were further stratified by age group and probe type and we observed highest number of *rpoB* RRDR mutations detected by probe E region in >60 years age group. Dual probe mutations (A+B) accounted for 51.9% (14/27) of the patients in the 46–60 age group, which was significantly higher than in other age groups (OR=2.31, 95% CI 1.12–4.79), and may be associated with a more frequent history of second-line drug exposure in patients in this age group. Data distributions are visualized in Figure 3.

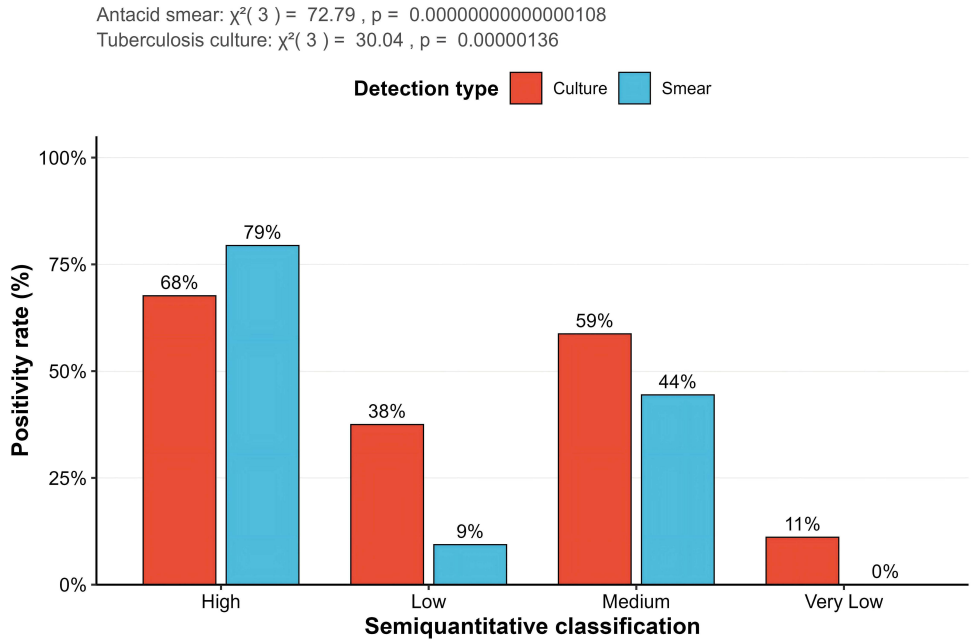

Figure 2 Comparison of smear and culture dual assay results based on concentration grading.

Table 3 Distribution of Probe Mutations in the *rpoB* Gene Used in the 197 Specimens

Mutant-Probe Type	The Amino Acid Site	Total (n=197)	Tuberculosis Specimen (n=194)	Extrapulmonary TB Specimens (n=3)*
One-probe				
A	507~511	24(12.2%)	24(12.4%)	0(0.0%)
B	512~518	1(0.5%)	1(0.5%)	0(0.0%)
C	518~523	6(3.0%)	6(3.1%)	0(0.0%)
D	523~529	22(11.2%)	22(11.3%)	0(0.0%)
E	529~533	110(55.8%)	107(55.2%)	3(100.0%)
Twin probe				
A+B	507~518	27(13.7%)	27(13.9%)	0(0.0%)
A+E	507~511, 529~533	1(0.5%)	1(0.5%)	0(0.0%)
B+E	512~523, 529~533	2(1.0%)	2(1.0%)	0(0.0%)
D+E	523~533	3(1.5%)	3(1.5%)	0(0.0%)
Three-point probe				
A+D+E	507~511, 523~533	1(0.5%)	1(0.5%)	0(0.0%)

Notes: *Analysis of extrapulmonary TB (n=3) showed limited statistical power due to small sample size. The values outside in parentheses represent the number of specimens, and the values in parentheses represent the mutation frequency (%).

Table 4 Gender-Wise Distribution of Rifampicin-Resistance Patients in Relation to Different Regions of *rpoB* Gene Detected Through Probes A, B, C, D & E

Probe	Male (n=143)	Female (n=54)	φ (95% CI)	p
E	83 (58.0%)	27 (50.0%)	0.08 (−0.07–0.23)	0.301
A	15 (10.5%)	9 (16.7%)	0.09 (−0.04–0.22)	0.232
AB	21 (14.7%)	6 (11.1%)	0.05 (−0.07–0.17)	0.447
B	0 (0.0%)	1 (1.9%)	0.072 (−0.034–0.178)	0.301*
C	6 (4.2%)	0 (0.0%)	0.173 (0.014–0.332)	0.187*
D	14 (9.8%)	8 (14.8%)	0.068 (−0.081–0.217)	0.412
AE	1 (0.7%)	0 (0.0%)	0.051 (−0.044–0.146)	1.000*
BE	1 (0.7%)	1 (1.9%)	0.050 (−0.087–0.187)	0.541*
DE	1 (0.7%)	2 (3.7%)	0.102 (−0.068–0.272)	0.298*
ADE	1 (0.7%)	0 (0.0%)	0.051 (−0.044–0.146)	1.000*

Notes: Labeled* p-values were tested using Fisher's exact test (due to the presence of expected frequencies <5). The φ coefficient is calculated using Cramer's V equation.

As delineated in Figure 4, significant heterogeneity in mutation profiles was observed across patient treatment categories. Treatment failure cases exhibited the highest frequency of Probe E mutations (61.7%, 50/81), whereas relapse cases demonstrated increased complexity with 16.1% (18/112) dual AB mutations and 6.3% (7/112) triple-probe mutations (AE/BE/DE/ADE). New cases showed distinct predominance of Probe D mutations (50.0%, 2/4). Statistical analysis revealed significantly higher rates of complex mutations (≥2 probes) in relapse (25.9%) and treatment failure (20.9%) groups compared to new cases (0%) (Fisher's exact test, P<0.001).

Discussion

Global burden and regional significance: Tuberculosis remains a leading global health threat, with rifampicin-resistant TB (RR-TB) posing critical challenges to disease control.[11] Drug-resistant tuberculosis (DR-TB) represents a severe public health burden, particularly in resource-limited settings.[12]

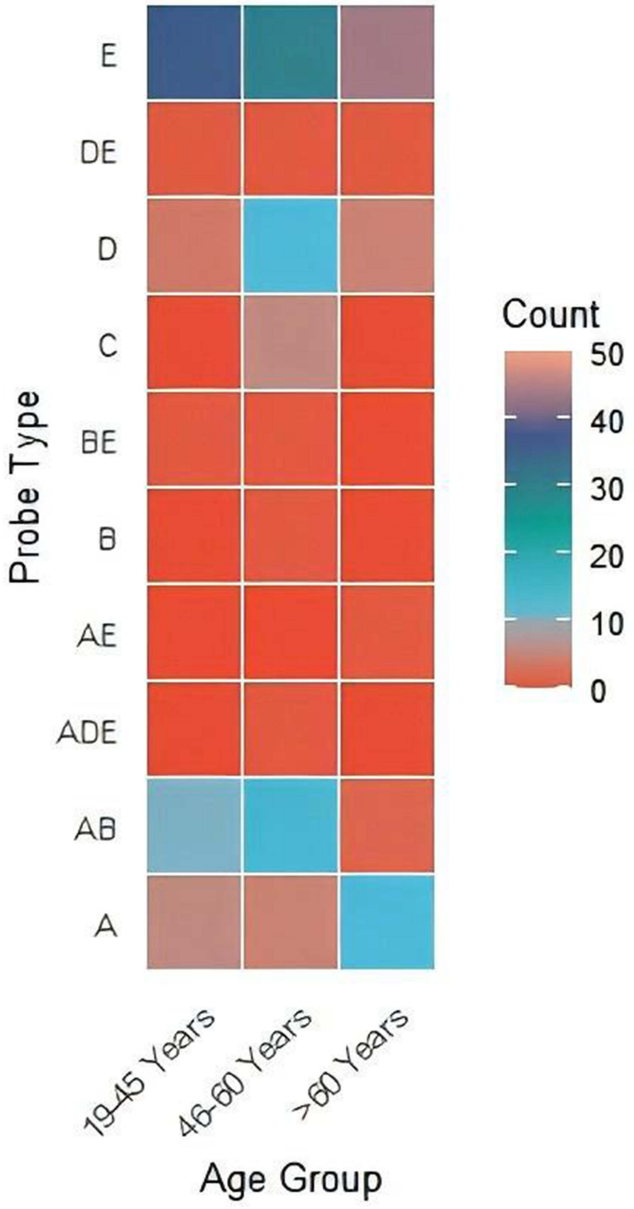

Figure 3 Thermal stratification of probe distribution across aging cohorts.

Our data showed that MTB was detected at high, medium, low and very low levels in pulmonary TB specimens than in extrapulmonary TB specimens. This finding indicates significantly higher bacillary loads in pulmonary specimens MTB levels were significantly higher in pulmonary TB specimens than in extrapulmonary TB specimens. Our data showed that the higher the semiquantitative level of MTB in Xpert-positive specimens, the higher the smear-positive rate of the specimens as well as the positive culture rate of *Mycobacterium tuberculosis*. This is consistent with Blakemore et al.[13] The results of the study were similar. A portion of the specimens from TB patients in our data were Xpert-positive smear-negative, which may be due to the fact that the lowest limit of detection for smear microscopy is approximately 1000 CFU/m1 and the lowest limit of detection for the Xpert technique is 112.6 CFU/mL.[14] Both Xpert semi-quantitative level and smear classification reflect the load of *Mycobacterium tuberculosis* in samples, but they have different principles: The amount of bacteria was quantified indirectly by detecting the amplification signal intensity of *rpoB* gene, which was affected by nucleic acid extraction efficiency; The smear was directly observed under a microscope for acid-fast bacilli, depending on the integrity of the bacterial morphology. This study found that Xpert high/medium levels

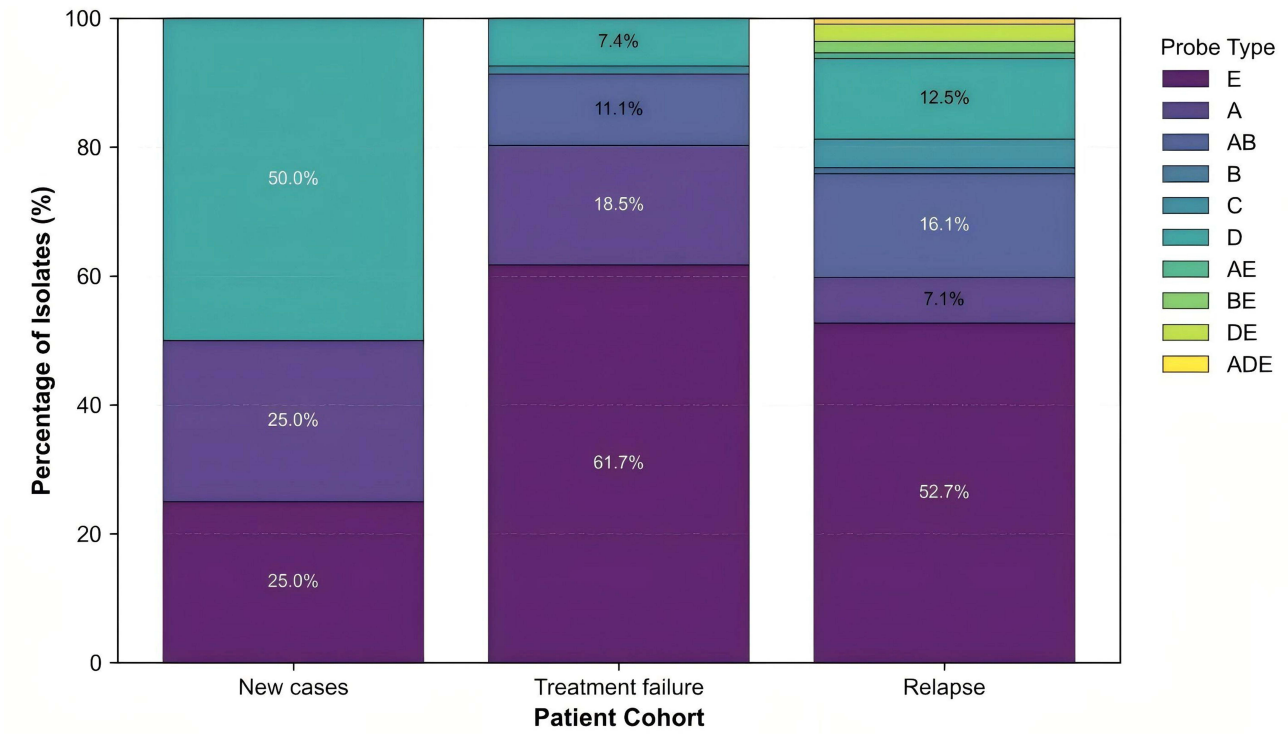

Figure 4 Distribution of *rpoB* probe mutations stratified by treatment history.

were highly overlapping with smear positive (++/+++)(73.5%), suggesting that the two are consistent in assessing bacterial load, but Xpert is more sensitive in samples with very low bacterial load (11.1% culture positive vs smear negative).

Zeka et al[15] prior studies associate S531L/H526D/D516V mutations with high-level rifampicin resistance mutations in S531L, H526D, and D516 V result in high levels of resistance to rifampicin; DSl6Y, M515I, and Q510H mutations are associated with low-level resistance to rifampicin; and double mutations in S512I and D516G are associated with low-level resistance to rifampicin. In this study, we analyzed the distribution and frequency of mutations in the *rpoB* gene probes in 197 Xpert-positive tuberculosis patients, with the highest mutation rate in probe E (locus 529–533, 55.8%), followed by probe A (locus 523–529, 12.2%). The five probes used for this purpose in studies in other parts of the world showed different frequencies of mutations leading to rifampicin resistance, and the highest mutation was found in probe E among all studies, which is in agreement with our results, but it is worth my difference with what our study data showed is that other studies showed that mutations in the *rpoB* gene were mostly clustered in probes E, D, and B, which accounted for 86% to 94% of the mutations, however, The unexpectedly high probe A mutation rate (12.2%) may reflect including frequent use of rifabutin in second-line regimens. As rifabutin remains a common second-line agent in Sichuan Province, selective pressure on codon 511 could drive this distinct pattern. However, Xpert's inability to detect non-RRDR mutations (5% of cases) may underestimate true mutation diversity.[16–19] The increased mutation rate of probe A (12.2%) likely reflects selective pressure from regional rifabutin usage resulting from the use of rifabutin in second-line treatment regimens in the region, as mutations at positions 510/511 are associated with the retention of rifabutin sensitivity.[20] This hypothesis is supported by our pDST data, which showed a rifabutin resistance rate of 13.8%. However, the inability of Xpert to detect non-RRDR mutations (such as I491F) may underestimate the true diversity of resistance. Thus knowing the specific mutation site of the *rpoB* gene is also clinically relevant to the rational use of rifamycin drugs.

The stratification by treatment history provides critical clinical context for mutation patterns. The elevated frequency of dual AB mutations (16.1%) and multi-probe combinations exclusively in relapse cases supports the hypothesis that repeated drug exposure drives cumulative genetic alterations.[21] This aligns with our demographic finding of increased A

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+B mutations in older patients (46–60 years), who likely experienced longer treatment durations. Notably, the absence of complex mutations in treatment-naïve cases underscores that probe multiplicity is an acquired resistance marker.

Limitations of This Study

There are two major limitations of this study: first, the insufficient sample size of extrapulmonary tuberculosis (n=3) and the lack of common types such as osteoarticular tuberculosis may affect the completeness of the mutation spectrum. Second, Xpert's blindness to the detection of RRDR extra-regional mutations (eg, I491F) may miss some low-level drug-resistant cases. These limitations suggest the need for a mechanism to review regional resistance mutations.

Conclusions

We identified a bimodal *rpoB* mutation profile (Probe E:55.8%; Probe A:12.2%) among RR-TB isolates in Sichuan Province. The predominance of dual A+B mutations in patients aged 46–60 years (OR = 2.31) suggests associations with prolonged drug exposure to prolonged drug exposure. While Probe A's elevated frequency may reflect regional rifabutin utilization, future studies integrating drug exposure records are warranted. These insights advance molecular epidemiological understanding for optimizing diagnostics in Southwest China.

Data Sharing Statement

All pertinent data utilized in the analysis and the subsequent generation of findings presented within this study are fully encompassed within the content of this manuscript.

Ethics Approval and Consent to Participate

Written informed consent was waived by the Ethics Committee of Suining Central Hospital (Approval No. KYLLKS20250078). The strains analyzed were derived from routine laboratory diagnostics and did not involve the use of any human genetic material. The research was conducted strictly following the ethical principles laid down in the Declaration of Helsinki.

Consent for Publication

All authors have thoroughly reviewed the manuscript and have provided their explicit consent for publication.

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Disclosure

The authors hereby certify that there are no conflicts of interest, financial or otherwise, associated with this research work.

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