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Impact of *rpoB* gene mutations and Rifampicin-resistance levels on treatment outcomes in Rifampicin-resistant tuberculosis

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Abstract

Background Although many studies have examined the connection between mutations in the *rpoB* gene and drug resistance, the impact of common mutations on treatment outcomes for RR-TB is not yet fully understood.

Objectives This study explores the relationship between *rpoB* gene mutations and drug-resistant phenotypes, assesses their role in predicting RR-TB prognosis, and investigates the impact of disputed *rpoB* mutations in *M. tuberculosis* on treatment outcomes.

Methods 192 rifampicin-resistant isolates were retested for drug susceptibility and gene sequencing. Minimum inhibitory concentrations (MICs) were determined for 98 isolates with disputed *rpoB* gene mutations. These mutations can cause low-level resistance to rifampicin, leading to inconsistencies in drug susceptibility testing and impacting medication therapy decisions.

Results Of 192 cases, 116 (60.4%) achieved successful outcomes, while 76 (39.6%) were unsuccessful. Among the 98 isolates tested for phenotypic drug susceptibility testing (DST) based on minimum inhibitory concentration (MIC), 67 (68.4%) showed high-level resistance with a MIC of ≥ 1 µg/mL. In contrast, 31 (31.6%) drug-susceptible tuberculosis isolates exhibited low-level resistance with a MIC of < 1.0 µg/mL. Of the 31 isolates with low-level resistance, 14 (45.2%) had successful treatment outcomes, while 17 (54.8%) did not. Among the 67 isolates with high-level resistance, 41 (61.2%) achieved successful outcomes, whereas 26 (38.8%) did not. In analysing the 14 codons of the Rifampicin Resistance Determining Region (RRDR) of the *rpoB* gene, the Leu430Pro codon showed the highest odds ratio (OR) of 2.98 (95% CI: 0.96–9.27) with a *p*-value of 0.0591, indicating statistically not significant. However, this suggests a potential association with rifampicin resistance that requires further investigation, particularly in areas with high drug-resistant tuberculosis prevalence. Other reported variants had lower odds ratios: Asp435Val with 1.23 (95% CI: 0.32–4.75), Asp435Tyr with 1.86 (95% CI: 0.60–5.76), His445Tyr with 1.16 (95% CI: 0.47–2.91), and Ser450Leu with 1.44 (95% CI: 0.81–2.58).

Conclusions This study indicates that low-level rifampicin mono-resistance in tuberculosis (TB) patients is associated with poor clinical outcomes. A mutation at the Leu430Pro codon showed the highest odds ratio of 2.98 (*p*-value

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0.0591), suggesting a potential association with rifampicin resistance that warrants further research, especially in areas with high drug-resistant TB. It highlights the need for more aggressive treatment strategies for patients with low-level rifampicin resistance, even if they seem solely mono-resistant.

Keywords Mycobacterium tuberculosis, Disputed *rpoB* mutations, Low-level resistance, High-level resistance, Treatment outcomes, Minimal inhibitory concentration

Background

Drug-resistant Tuberculosis (DR-TB) is becoming a worldwide threat to Tuberculosis (TB) control, with over 410,000 estimated cases of Multi-drug-resistant TB/Rifampicin resistance (MDR/RR-TB) in 2022 [1]. DR-TB adds to the global challenge of antimicrobial resistance, often requiring a significant portion of the healthcare budget and resources in many heavily affected countries [2]. The challenge of preventing and controlling TB is increasing due to the emergence of drug-resistant strains, significantly impacting treatment outcomes. According to the 2023 global TB report, the successful global treatment outcomes among cases of drug-susceptible TB (DS-TB) and MDR/RR-TB was 88% and 63%, respectively [3]. Numerous studies conducted in different settings have highlighted the problem of drug resistance in various countries. These studies have shown that cases of DR-TB tend to have a higher rate of poor treatment outcomes compared to cases of DS-TB [4]. Host factors such as poor patient adherence, sex, age, diagnostic delay, co-infection with HIV, and TB treatment history are well-known to be associated with treatment failure. Still, the mycobacterial basis of this association is poorly understood [5, 6]. We are interested in investigating whether the genetics of the *M. tuberculosis* strain causing the infection may also play a role in poor treatment outcomes. Several previous studies have indicated a link between drug resistance and genetic mutations. However, the effects of common and borderline mutations on the treatment outcomes of MDR-TB remain unclear. Drug resistance is the primary cause of treatment failure in TB [7], but genomic studies indicate that other bacterial factors may also contribute. Research on DR-TB has yielded inconsistent results due to variations in study populations, treatment protocols, and healthcare environments. The resistance patterns observed in MDR-TB and RR-TB significantly affect treatment success. Additionally, variations in study designs, sample sizes, follow-up durations, and access to healthcare further contribute to these inconsistencies [8]. Successful treatment outcomes for tuberculosis not only achieve patient recovery, but also prevents disease transmission, and emergence of drug-resistant strains. Therefore, treatment outcomes are crucial metrics for assessing the effectiveness of TB control programs. Discordant results for RR caused by disputed *rpoB* mutations are frequently encountered and can create dilemmas in treatment and management. These disputed *rpoB* mutations may be clinically relevant and significantly affect the outcomes of RR-/MDR-TB [8]. The risk factors contributing to acquired drug resistance and their association with outcomes in patients with DR-TB are not well understood. This study explores the role of mycobacterial genomic determinants, apart from host factors, that may be linked to poor treatment outcomes.

Methods

Study population and setting

This study employed the World Health Organization (WHO) methodology [9], focusing on 192 patients with MDR-TB and RR-TB. The cohort included 150 newly diagnosed patients and 42 previously treated cases from January 2020 to December 2023 in southern India. The research was conducted across ten sites within the National Tuberculosis Elimination Programme (NTEP), emphasizing the Programmatic Management of Drug-resistant Tuberculosis (PMDT).

Genotype MTBDRplus assay -Version 2

All 192 Rifampicin-monoresistant and multidrug-resistant isolates were tested using the Genotype MTBDRplus assay (Hain Lifescience, Nehren, Germany, version 2) to identify genetic factors associated with resistance to rifampicin. The extraction, amplification, detection, and interpretation of results were performed according to the manufacturer's instructions [10–12].

Determination of minimum inhibitory concentration using mycobacteria growth indicator tube

The Minimum Inhibitory Concentration (MIC) of rifampicin was determined for 98 isolates using the BACTEC MGIT 960 system. Stock solutions of rifampicin were prepared for two-fold serial dilutions, resulting in the following concentrations: 0.06, 0.125, 0.25, 0.5, 1, 2, 4, and 8 µg/mL. The rifampicin concentrations used in this study were specifically chosen to determine its minimum inhibitory concentration (MIC). In the Mycobacteria Growth Indicator Tube (MGIT) system, the critical concentration for rifampicin is 1 µg/mL. This concentration serves

as the threshold in susceptibility testing to differentiate between susceptible and resistant strains of *M.tuberculosis*, corresponding to levels achievable in patients and demonstrating clinical efficacy. For each MGIT tube representing eight different concentrations, ranging from 0.06 to 8 µg/mL, we added 100 µL of the drug solution corresponding to each concentration and 0.8 mL of OADC. The inoculum was diluted at a ratio of 1:5, and 500 µL of this dilution was transferred to the MGIT tube. Additionally, 0.5 mL of a 1:100 dilution of the inoculum was added to a drug-free growth control tube. Each isolate was tested in duplicate, and the H37Rv control strain was included in every MIC experiment. All tubes were placed in the MGIT instrument and were evaluated at the end of two weeks. The results were considered acceptable if the H37Rv strain demonstrated susceptibility to rifampicin at the critical concentration of 1 µg/mL [13–15].

DNA sequencing of drug resistance-related genes

Mutations in the RRDR region of the *rpoB* gene were analyzed using the Sanger sequencing method, which involves PCR amplification and sequencing of the resulting amplicons from selected Rifampicin-resistant samples with the corresponding oligonucleotide primers. *rpoB*-F (5'- GCGAGCTGATC CAAA ACCAG-3') and *rpoB*-R (5'- TCCAGGAAGGG AATC ATCGC-3') to detect the rifampicin resistance-associated mutations [16, 17]. PCR products were sent to Shrimpex Biotech Services Private Limited, a 2008 NABL-accredited laboratory located in Chennai, for sequencing. The sequences were then aligned to the H37Rv reference strain (GenBank accession no. NC 000962) using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/blast/Blast.cgi).

Clinical data

Standard treatment protocols were initiated in patients with RR and MDR tuberculosis. The treatment included fluoroquinolones, injectable agents, and other second-line medications such as bedaquiline, clofazimine, or cycloserine. The intensive phase of treatment typically lasted six months, followed by a continuation phase that ranged from twelve to eighteen months, depending on the patient's response to the treatment and the specific regimen outlined in the PMDT guidelines [18]. Treatment outcomes are classified according to standardized international consensus [19]. Successful outcomes include cure and treatment completion, while unsuccessful outcomes encompass death, treatment failure, and interruption or loss to follow-up.

Ethical consideration

The Ethics and Scientific Review Committee at the General Hospital Institute, part of the Directorate of Health and Family Welfare Services in Puducherry, approved this study (Ref. No/GHIEC/2020/243; June 2020) and granted a waiver for informed consent. All methods were conducted in accordance with the guidelines and regulations established by the WHO and the NTEP, and the Declaration of Helsinki.

Statistical analysis

Descriptive statistics were employed to summarize the minimum inhibitory concentration values of Multidrug-resistant and rifampicin-resistant *M. tuberculosis isolates*. For all statistical analyses, we used MedCalc software (version 22.026) [20]. Logistic regression analysis was performed to determine the odds ratio (OR) related to MDR/RR resistance transmission.

Results

Between 2020 and 2023, 192 cases of rifampicin-resistant TB were identified using MTBDRplus Ver. 2.0. Of 192 RR isolates, 122 were classified as truly resistant based on their mutation patterns. Additionally, 51(26.6%) isolates were found inferred resistant, while 19(9.9%) were identified as heteroresistant. According to the Line Probe Assay (LPA), inferred resistance occurs when both wild-type and mutant probes are absent. This absence indicates that the strain is resistant to the drug being tested. It means the assay fails to detect the presence of the mutant sequence associated with resistance, as well as the wild-type sequence that typically indicates susceptibility. Consequently, the strain is classified as resistant, and further investigation usually involves sequencing the target gene to confirm the resistance mechanism and identify specific mutations. On the other hand, heteroresistance occurs when both resistant and susceptible *M.tuberculosis* strains are present within the same isolate. This condition can be detected in the LPA by the presence of both wild-type and mutant probes. It indicates that while some bacteria in the population are resistant, others remain susceptible to the drug being tested. This mixed population can complicate treatment, as the resistant strains may survive despite the medication, potentially leading to unfavourable treatment outcomes if not properly managed [21]. Sequencing was performed for all 192 isolates to determine the specific mutations. A total of 14 distinct mutations were identified across 7 codons within the 81 base pair RRDR of the *rpoB* gene (Fig. 1). Among the RRDR mutants, the majority, accounting for 105 samples (54.7%), exhibited the single Ser450Leu mutation. Additionally, 35 samples (18.2%)

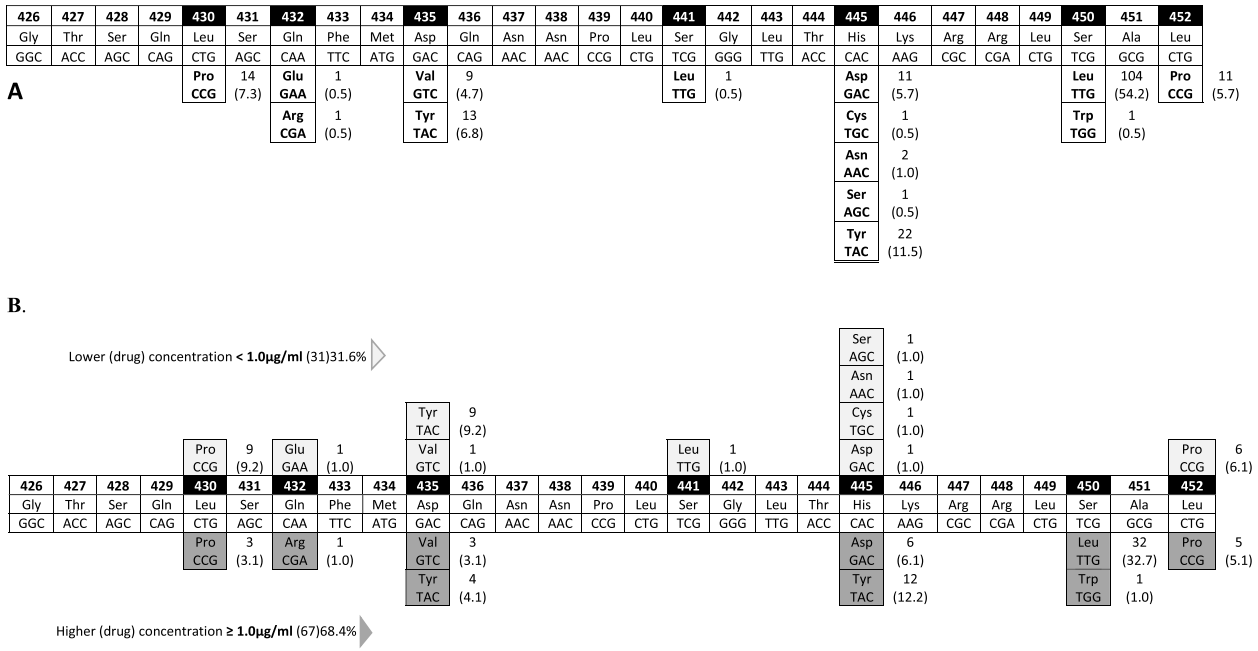

Fig. 1 **A** Schematic representations of mutations in Rifampicin Resistant Determination. Region (RRDR) of *rpoB* gene among 192 isolates. **B** Correlation of genotypic Resistant (Sequencing) against phenotypic drug. Concentrations (MIC) among 98 isolates

had mutations at the His445 codon. Of the 22, 15 samples (7.8%) presented a borderline mutation, Asp435Tyr. Notably, all 22 samples with the Asp435Tyr/Val mutation were detected at diagnosis either through the Xpert test ($n=22$) or Line Probe Assay (LPA, $n=22$). Still, only seven were identified using phenotypic drug susceptibility testing (pDST). Other mutated codons included 430 (7.3%), 432 (1.0%), 441 (0.5%), and 452 (5.7%).

Of 192 isolates, the MIC of rifampicin was performed for 98 isolates. The selection of isolates for phenotypic drug susceptibility testing (pDST) was based on inferred resistance ($n=51$), hetero-resistance patterns ($n=19$), and true resistance patterns ($n=31$), all detected through line probe assay (LPA) and genetically confirmed via Sanger sequencing. In cases of true resistance, priority was given to isolates with specific mutant codons D435V, H445Y, and H445D. Among the 105 isolates with the S450L mutation, only 24 isolates, which exhibited a mix of favourable and unfavourable treatment outcomes (representing 23% of the total s450L mutant isolates), were selected for further analysis. This selection contributed to a total of 101 isolates used for conducting the MGIT minimum inhibitory concentration (MIC) testing of eight different concentrations of Rifampicin, ranging from 0.06 to 8 µg/ml. Of 101 isolates tested for phenotypic drug susceptibility testing (pDST), 67 isolates (68.4%) demonstrated resistance at higher drug concentrations (≥ 1.0 µg/ml). In contrast, 31 isolates (31.6%) showed resistance at lower drug concentrations (<1.0 µg/ml). Additionally, 3 isolates (2.97%) were inferred to be resistant; these isolates exhibited resistance in the Line Probe Assay (LPA) but were found to be susceptible in pDST. Sequencing results revealed that two isolates contained a specific mutation L430P, while one isolate had a different mutation H445N, both of which may contribute to drug resistance. Therefore, it is crucial to further analyze MGIT-sensitive isolates using advanced sequencing techniques to confirm resistance and guide treatment decisions for effective patient management. To investigate the relationship between minimum inhibitory concentrations and various *rpoB* mutations, we analysed the distribution of MICs, ranging from ≤ 0.06 to ≥ 8 µg/mL. Among the 98 isolates, 6 strains (6.12%) had MICs of ≤ 0.06 µg/mL; 4 strains (4.08%) had MICs of 0.125 µg/mL; 13 strains (13.27%) had MICs of 0.25 µg/mL; 8 strains (8.16%) had MICs of 0.5 µg/mL; 4 strains (4.08%) had MICs of 1.0 µg/mL; 5 strains (5.10%) had MICs of 2.0 µg/mL; and 58 strains (59.18%) had MICs of ≥ 8.0 µg/mL. Out of the 98 cases, 31 isolates exhibited mutations associated with codons at Leu430Pro (29.0%), Gln432Glu (3.2%), Asp435Tyr (29.0%), Asp435Val (3.2%), His435Val (3.2%), Ser-441Leu (3.2%), His445Asn (3.2%), His445Cys (3.2%), His445Ser (3.2%), and Leu452Pro (19.4%). These isolates had MICs of ≤ 1 µg/ml. In contrast, 67 isolates

exhibited mutations at codons Leu430Pro (4.5%), Gln432Arg (1.5%), Asp435Val (4.5%), Asp435Tyr (6.0%), His445Tyr (17.9%), His445Asp (9.0%), Ser-450Leu (47.8%), Ser450Trp (1.5%), and Leu453Pro (7.5%). These isolates had a MIC of ≥ 1 µg/ml, as shown in Table 1.

Drug concentrations associated with Rifampicin-resistant treatment outcomes

Figure 2 illustrates the forest plot analysis of seven drug concentration factors linked to unfavourable clinical outcomes in patients with rifampicin resistance. An unfavourable outcome in tuberculosis (TB) treatment

Table 1 *rpoB* mutation types and amino acid changes linked to rifampicin resistance in 101 Mycobacterium tuberculosis

Type of Resistance	Amino acid changes	low-level resistance MIC < 1 µg/ml	high-level resistance MIC ≥ 1 µg/ml[a]	Total no mutant (*n* = 101)	Successful outcomes *n* = 58 (57.4%)	Unsuccessful outcomes *n* = 43 (42.6%)
Inferred resistant (*N* = 51)	L430P	**9**	3 + **2**[d]	14	5	9
	S441L	1	0	1	0	1
	Q432E	1	-	1	1	0
	Q432R	-	1	1	1	0
	D435Y	**9**	4	13	6	7
	H445C	1	-	1	0	1
	H445N	1	**1**[d]	2	2	0
	H445S	1	-	1	1	0
	H445Y	-	1	1	0	1
	S450L	-	4	4	3	1
	S450W	-	1	1	0	1
	L452P	**6**	5	11	8	3
True resistant (*N* = 31)	D435V	1	2	3	2	1
	H445D	1	2	3	1	2
	H445Y	-	1	1	1	0
	S450L	-	**24**	24	19	5
Hetero-resistant (*N* = 19)	D435V	-	1	1	0	1
	H445D	-	4	4	2	2
	H445Y	-	**10**	10	5	5
	S450L	-	4	4	1	3

DST Drug susceptibility test, *MIC* Minimum Inhibitory Concentration; [a]Epidemiological cut-off value for rifampicin (µg/ml), d-Discordance observed in L430P and H445N

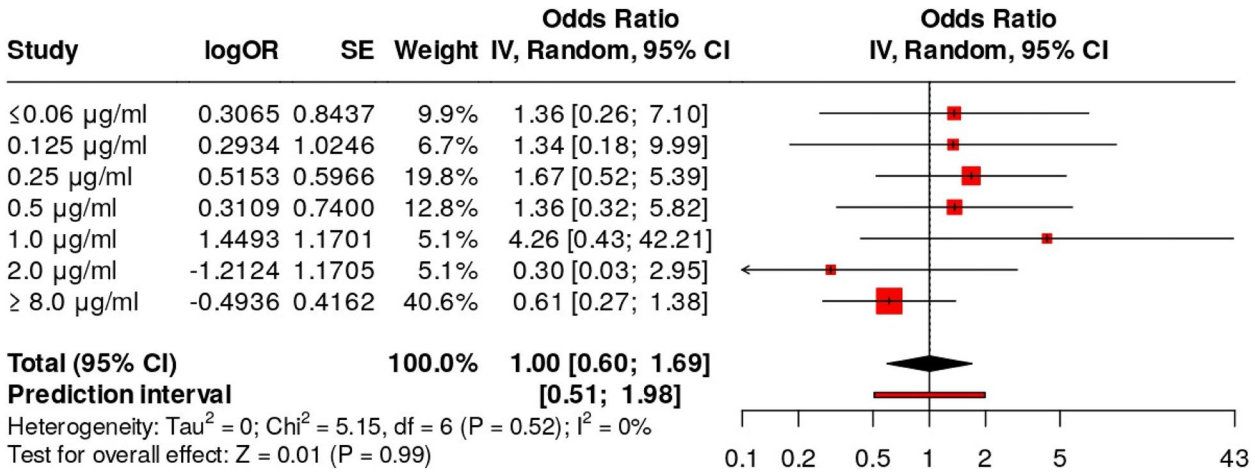

Fig. 2 Low-level resistance associated with increased unfavourable outcomes in patients with drug-resistant Tuberculosis

refers to any result that is not a cure or completion of treatment. This includes treatment failure (indicated by consistently positive sputum tests or clinical signs of TB), death, loss to follow-up, relapse, or the development of drug resistance. These outcomes are important indicators for assessing the effectiveness of treatment and informing improvements in TB control efforts. Among the 98 rifampicin-resistant isolates tested for MIC, 31 isolates (31.6%) displayed borderline resistance (1 µg/mL), while 67 isolates (68.4%) exhibited high levels of resistance (≥ 1 µg/mL). Among the 31 borderline resistance, 14 (45.2%) achieved successful outcomes, whereas 17 (54.8%) experienced unsuccessful outcomes (Table 3). In contrast, of the 67 high levels of resistance, 41 (61.2%) had successful outcomes, while 26 (38.8%) had unsuccessful outcomes. The factors influencing drug concentration that affect the treatment outcomes of RR-TB were assessed using a multivariable logistic regression analysis. Seven variables (Fig. 2) demonstrated some association with treatment outcomes in the analysis. After further evaluation, it was determined that two of these variables were independently linked to successful treatment outcomes. This study found that patients with a MIC of the drug against *M.tuberculosis* resistant isolates greater than 1.0 µg/mL had lower odds of treatment success. Specifically, the odds ratios for drug concentrations of 2.0 µg/mL were 0.32 (95% CI: 0.03–2.95), and for ≥ 8.0 µg/mL, the OR was 0.61 (95% CI: 0.27–1.38). Conversely, lower concentration of the drug (< 1 µg/mL) had higher odds of unfavourable treatment outcomes were ≤ 0.06 µg/mL (OR: 1.36; 95% CI: 0.26–7.10), 0.125 µg/mL (OR: 1.35; 95% CI: 0.18–9.99), 0.25 µg/mL (OR: 1.67; 95% CI: 0.52–5.39), and 0.5 µg/mL (OR: 1.37; 95% CI: 0.32–5.82). Additionally, isolates with a critical drug concentration of 1.0 µg/mL exhibited significantly higher odds of unfavourable treatment outcomes, with an OR of 4.23 (95% CI: 0.43–42.21). This study's findings indicate that isolates with a low drug concentration (< 1.0 µg/mL) had higher odds of experiencing unfavourable treatment outcomes, with an odds ratio of 1.91 (95% CI: 0.81–4.53). Seven study variables were analysed using the forest plot method. Employing a random effects model with the inverse variance method to compare the odds ratios (OR), we found no statistically significant differences. The summarized odds ratio was 1, with a 95% confidence interval ranging from 0.6 to 1.69. The overall effect test did not indicate a significant impact. Additionally, we did not observe significant heterogeneity, suggesting that the effect sizes across the studies were consistent in both magnitude and direction. Among the 101 rifampicin-resistant isolates tested for sequencing, low-level rifampicin resistance was linked to a higher likelihood of unfavourable treatment outcomes, with an odds ratio of 1.91 (95% CI: 0.81–4.53). Among the 24 identified mutation codons associated with low-level resistance, mutations at codon Leu430Pro were correlated with even greater odds of unfavourable treatment outcomes, yielding an odds ratio of 2.80 (95% CI: 0.36–21.73), as indicated in Table 2. Additionally, of the 55 identified high-level mutation codons, mutations at codon His445Asp were associated with significantly higher odds of unfavourable treatment outcomes, showing an odds ratio of 21.33 (95% CI: 0.11–15.7). Table 3 represents the individualized treatment outcomes of rifampicin low-level resistance. Out of 31 isolates, 54.8% ($n = 17$) had unsuccessful outcomes while 45.2% ($n = 14$) had successful outcomes.

Table 2 Evaluation of borderline resistance via genotypic assays and phenotypic drug susceptibility tests, and classification of rpoB gene mutations

Resistance level of *rpoB* mutation	Total $n = 101$(%)	Successful outcomes n (%)	Unsuccessful outcomes n (%)	OR	95% CI	*p*-Value
High level Rif resistance	70(69.3)	43(61.4)	27(38.6)	Ref		
Low level Rif resistance	31(30.7)	14(45.2)	17(54.8)	1.93	(0.82–4.54)	0.1308
Key Mutations associated with High-level Rif Resistance $n = 55$						
Asp435Val	4(7.2)	2(3.6)	2(3.6)	Ref		
His445Asp	7(12.7)	3(5.4)	4((7.2)	1.33	(0.11- 15.7)	0.8192
His445Tyr	12(22.0)	6(11.0)	6(11.0)	0.75	(0.11- 4.9)	0.7638
Ser450Leu	32(58.1)	23(41.8)	9(16.3)	0.39	(0.09- 1.53)	0.1792
Key Mutations associated with low-level Rif Resistance $n = 24$						
Asp435Tyr	9(37.5)	4(16.6)	5(21.0)	Ref		
Leu430Pro	9(37.5)	2(8.3)	7(29.2)	2.8	(0.36–21.73)	0.3247
Leu452Pro	6(25.0))	4(16.6)	2(8.3)	0.14	(0.01–1.44)	0.0092

OR *Odds* Ratio, *CI* Confidence Interval, *Rif* Rifampicin drug

Table 3 Individualized Treatment outcomes analysis of low-level rifampicin resistance n [22]

Codon	Age	Isolates n(31)	Treatment type	Unsuccessful outcomes n(17)54.8%	Regiment followed	Successful outcomes n(14)45.2%	Regiment followed
Asp435Tyr (*n* = 9)	< 45	3	treated n(0)	0	NA	0	
					NA		
			New n(3)	3	Shorter regimen n(2)	0	
					Longer regimen n(1)		
	≥ 45	6	treated n(2)	1	Shorter regimen	1	Shorter regimen
			New n(4)	1	Shorter regimen	3	Longer regimen n(2)
							Shorter regimen n(1)
Leu430Pro *n* = 9	< 45	5	treated n(0)	0	NA	0	
					NA		
			New n(5)	3	Shorter regimen n(3)	2	Shorter regimen n(2)
	≥ 45	4	treated n(3)	3	Shorter regimen	0	Shorter regimen
			New n(1)	1	Shorter regimen	0	
Leu452Pro *n* = 6	< 45	2	treated n(0)	0	NA	0	
					NA		
			New n(2)	1	Shorter regimen	1	Shorter regimen
	≥ 45	4	treated n(0)	0	NA	0	
					NA		
			New n(4)	1	Shorter regimen	3	Shorter regimen n(3)
S441L	< 45	1	New n(1)	1	Shorter regimen	0	
Q432E	≥ 45	1	treated n(1)	0	NA	1	Shorter regimen
H445C	≥ 45	1	New n(1)	1	Shorter regimen	0	
H445N	< 45	1	New n(1)	0	NA	1	Shorter regimen
H445S	≥ 45	1	New n(1)	0	NA	1	Shorter regimen
D435V	< 45	1	New n(1)	0	NA	1	Shorter regimen
H445D	< 45	1	treated n(1)	1	Shorter regimen	0	

Rifampicin-resistant-conferring mutations associated with treatment outcomes

Figure 3 presents a forest plot analysis of seven mutations at various codons linked to poor outcomes in patients with rifampicin resistance. Out of 192 cases analyzed, 116 (60.4%) achieved successful outcomes, while 76 (39.6%) had unsuccessful outcomes. Among the 14 codons at the ten *rpoB* locus, higher odds ratios were at codons Leu-430Pro with OR of 2.98 (95% CI: 0.96–9.27), Asp435Val with 1.23 (95% CI: 0.32–4.75), Asp435Tyr with 1.86 (95% CI: 0.60–5.76), His445Tyr with 1.16 (95% CI: 0.47–2.91), and Ser450Leu with 1.44 (95% CI: 0.81–2.58). The mutation at Leu430Pro codon displayed the highest odds ratio of 2.98, with a p-value of 0.0591. Although this result is not statistically significant, it suggests a potential association with rifampicin resistance that warrants further investigation, especially in regions with a high prevalence of drug-resistant tuberculosis. In contrast, codons His445Asp and Leu452Pro exhibited lower odds ratios of 0.87 (95% CI: 0.24–3.06) and 0.55 (95% CI: 0.14–2.16), respectively. Seven study variables were analyzed using a forest plot analysis. The results from a random effects model that employed the inverse variance method to compare the odds ratio (OR) revealed no statistically significant differences. The summarized odds ratio (OR) was 1.37, with a 95% confidence interval ranging from 0.95 to 1.97. The overall effect test did not show any significant impact. Additionally, no notable variability was detected, indicating that the effect sizes across different cohorts were consistent in magnitude and direction.

Discussion

The relationship between drug resistance and gene mutations has been reported in several studies. Still, the impact of common mutations on treatment outcomes for RR/MDR-TB has yet to be clearly defined. This study compares the frequency of mutations in common drug resistance-related genes with the results from in vitro phenotypic drug susceptibility tests (pDST). We assessed their roles in predicting treatment outcomes for RR/MDR-Tuberculosis. The findings of this study may provide insights into how mutations in the *rpoB* gene are

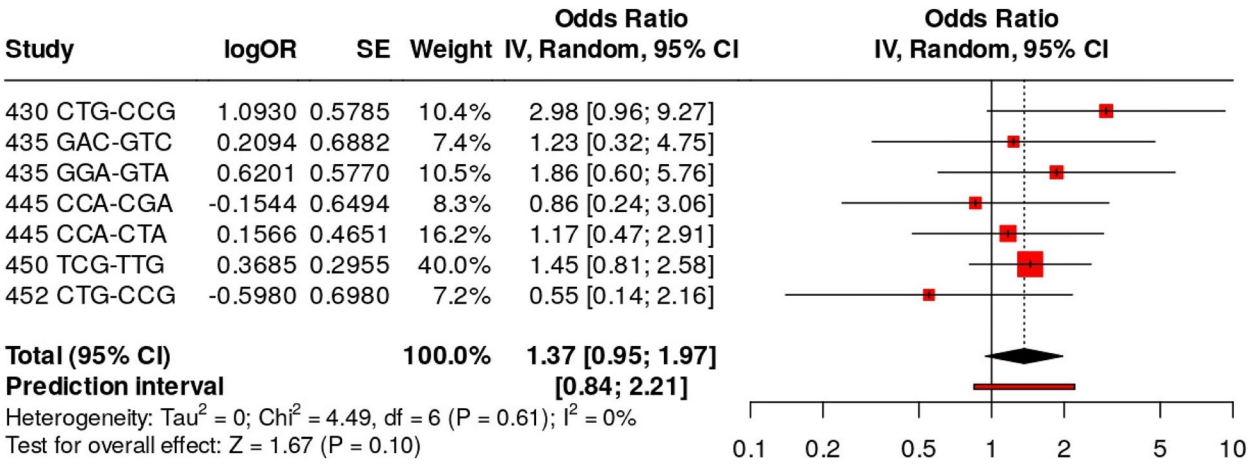

Fig. 3 Disputed *rpoB* mutations associated with increased unfavourable outcomes in patients with drug-resistant Tuberculosis

associated with poor prognosis and could be used to predict treatment outcomes for patients with RR/MDR-TB. The most frequently mutated codons associated with rifampicin in the *rpoB* gene are Asp435, His445, and Ser450, with mutation frequencies of 11.5%, 19.8%, and 54.5%, respectively. Li et al. [23] reported 16.1%, 28.0%, and 47.5% mutation frequencies for RR isolates at codons 435, 445, and 450, respectively. In contrast, Hameed et al. [24] reported mutation frequencies of 8.2%, 10.9%, and 47.9% for RR at the same codons.Additionally, 7.3% of the mutations were observed at the Leu430Pro codon, which was not reported in the previous study. The levels of rifampicin resistance in isolates with *rpoB* mutations at these codons depended on the specific amino acid changes. In multivariate analysis, the mutations Ser450Leu, Leu452Pro, His445Asp, and His445Tyr were strongly associated with high-level MIC of rifampicin resistance. In contrast, the mutations Asp435Val and Ser-441Leu were linked to moderate-level rifampicin resistance. Isolates with the mutations Ser430Pro, Asp435Tyr, His445Asn, His445Cys, His445Ser, and Gln432Glu were associated with low-level MIC of rifampicin resistance. Several mutations, including Gln432Arg and Ser450Trp, were found exclusively in high-level rifampicin-resistant isolates. These findings align closely with the reports by Shea et al. [25].

Borderline mutations are consistently linked to low levels of rifampicin resistance, exhibiting slightly elevated MICs that remain below the critical threshold of current drug susceptibility testing systems. Liu et al. [26] reported that the proportion of borderline *rpoB* mutations in RR *M. tuberculosis* isolates in China was 20.4%, which is lower than the findings of our study at 31.6%. Xia et al. [27] reported that borderline resistance in the *rpoB* gene is significantly associated with poorer clinical responses to treatment. Van Deun et al. [28] reported 24.3% borderline resistance in the *rpoB* gene associated with poorer treatment outcomes in their study. This study found that treatment outcomes for low-level drug resistance were 45.2%, which is lower than the 61.2% outcomes associated with high drug concentrations. Resistance due to borderline mutations may arise from fitness costs and a partial reduction in rifampicin's binding affinity to the *rpoB* protein [29]. Xia et al. [27] reported 48.2% low-level drug resistance in China, which is higher than our findings. The prevalence of low-level drug resistance in our study was higher than the 18.9% of low-level Rifampicin resistance reported by Shea et al. [25] in New York. Additionally, Getahun et al. [30] indicated that borderline rifampin resistance treatment outcomes have been associated with treatment failures. The findings of this study indicate that isolates with low drug concentrations were more likely to experience unfavourable treatment outcomes, with an odds ratio of 1.91. Notably, mutations at codon Leu430Pro were associated with even higher odds (2.98) of unfavourable treatment outcomes, which has also been reported by Xia et al. [27]. Cuella-Martin et al. [22] stated that low-level mutations have the same clinical impact and population distribution as high-level resistance mutations.

Li et al. [23] analysed the crystal structure of the RIF-RNA polymerase (RNAP) complex to investigate how mutations affect the interactions between *rpoB* mutants and rifampicin. Resistance to rifampicin typically arises from mutations within the RRDR of *rpoB*, which play a crucial role in forming the Rifampicin-binding pocket. This may be the first outcome analysis study focusing on a specific mutation in the *rpoB* gene locus. The Leu430Pro mutation involves the substitution of leucine, a flexible and hydrophobic amino acid, with rigid proline, which can disrupt protein structure. Such a change may affect the protein's folding or secondary structure, particularly

if Leu430 is part of an alpha-helix or beta-sheet. This alteration can impair the protein's function by modifying its active site or its interactions with other molecules, potentially leading to loss of function, structural instability, and misfolding or aggregation of the protein [23, 31]. Gopie et al. [32] reported that low-level resistance had the same poor clinical prognosis as the more commonly recognized high-level resistance. Our study found that isolates with a mutation at codon Asp435Val were 1.23 times more likely (95% CI 0.32 to 4.75) to experience unsuccessful treatment outcomes. Most of the isolates (88.8%) exhibited high-level resistance mutation, which likely causes steric hindrance, leading to changes in the enzyme's three-dimensional structure, which affects its function and may contribute to the organism's drug resistance [23].

In this study, isolates with a mutation at codon Asp435Tyr were 1.86 times more likely (95% CI 0.60 to 5.76) to experience unsuccessful treatment outcomes. Second-line medications, such as fluoroquinolones (including moxifloxacin and levofloxacin), linezolid, clofazimine, and bedaquiline, are commonly used to treat patients with RR-TB. The treatment regimen is customized based on the specific resistance patterns and the patient's overall health. This regimen typically lasts between 18 and 24 months and consists of an intensive phase followed by a continuation phase. During this time, multiple second-line agents are combined to prevent the development of further resistance. The alteration at Asp435Tyr changes the protein's amino acid sequence by substituting a negatively charged aspartic acid with a bulky, neutral tyrosine. This change can impact the protein's structure, interactions, and overall function, potentially disrupting charge-based interactions, creating steric clashes, or affecting its ability to form specific bonds. The exact consequences depend on the protein's role and the mutation's location within its structure. This leads to potential outcomes ranging from loss of function to altered or even enhanced function, depending on the biological context [23].

In this study, isolates with a mutation at codon His445Tyr were 1.16 times more likely (95% CI 0.47 to 2.91) to experience unsuccessful treatment outcomes. This mutation can disrupt enzymatic activity if histidine is part of a catalytic site involved in proton transfer or metal ion coordination. Additionally, it may lead to changes in protein stability or structure, particularly if the mutation introduces steric clashes or alters protein folding. Furthermore, this mutation could modify signaling and protein interactions; the presence of tyrosine might create new phosphorylation sites or influence how the protein interacts with other molecules [23, 33]. Our study found that isolates with a mutation at codon Ser450Leu were 1.44 times more likely (95% CI: 0.81 to 2.58) to result in unsuccessful treatment outcomes. This mutation disrupts protein function by causing the loss of hydrogen bonding, altering hydrophobicity, or creating steric clashes. Additionally, it can lead to structural instability, misfolding, or decreased protein stability due to the introduction of the bulky, hydrophobic leucine in an otherwise hydrophilic region. These changes may also affect protein–protein interactions, potentially altering cellular signaling, enzyme activity, or the formation of protein complexes Furthermore, there could be disease associations, particularly if the mutation occurs in a critical region tied to enzyme activity, signaling, or structural maintenance. Serine is also needed for mediating T-cell function [23, 34].

This study has several limitations, including the evaluation of MIC for only 98 MDR/RR-TB isolates from ten sites in southern India, which may limit the applicability of our findings to other regions. Additionally, treatment adherence data were incomplete, highlighting the need for accurate recording in patient management portals to support clinical decisions. Robust data collection is essential for optimizing treatment outcomes, especially for low-level rifampicin-resistant tuberculosis. A larger study is necessary to inform guidelines for Rifampicin use in treating MDR/RR-TB, focusing on treatment outcomes and adverse events. Our sampling method, which involved 24 samples from 101 isolates of the S4350L variant, may also affect the generalizability of our results. Although low-level resistant mutations are not statistically significant, they warrant further attention due to their association with adverse outcomes. There is currently a lack of data on low-level Rifampicin resistance in *M. tuberculosis,* and discordant mutations may need re-evaluation in relation to poor clinical outcomes. Sequencing could help identify resistance mechanisms in isolates showing inferred resistance on the Genotype MTBDRplus assay.

Conclusions

Our study confirms that first-line treatment of tuberculosis with low-level rifampicin resistance, specifically *rpoB* Leu430Pro mutations, has an equally poor prognosis. In analyzing the 14 codons of the Rifampicin Resistance Determining Region (RRDR) of the *rpoB* gene, the Leu430Pro codon displayed the highest odds ratio of 2.98, with a p-value of 0.0591. Although this result is not statistically significant, it suggests a potential association with rifampicin resistance that warrants further investigation, especially in regions with a high prevalence of drug-resistant tuberculosis. Low-level RIF resistance poses significant diagnostic and treatment challenges in our population and may be underreported, especially in strains lacking other drug resistance. As a group, these

patients have a clinical impact similar to high-level rifampicin resistance and should be treated with a second-line regimen. These findings emphasize the urgent need for better detection methods for low-level resistance and development of personalized treatment strategies to enhance patient outcomes. Further research with larger sample sizes is warranted to confirm these findings and to investigate the underlying mechanisms driving the association between specific rpoB mutations and treatment failure.

Abbreviations

RR-TB	Rifampicin-resistant Tuberculosis
DR-TB	Drug-resistant tuberculosis
MDR TB	Multidrug-resistant tuberculosis
TB	Tuberculosis
MIC	Minimum inhibitory concentration
DST	Drug susceptibility test
CI	Confidence interval
RRDR	Rifampicin resistant determination region
HIV	Human immunodeficiency virus
WHO	World health organization
MGIT	Microbial growth indicator tube
PCR	Polymerase Chain reaction
BLAST	Basic local alignment search tool
PMDT	Programmatic management of drug-resistant tuberculosis
OR	Odds ratio

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Clinical trial
Not applicable.

Authors' contributions
All authors contributed to the conception and design of the study. MJV, VJN, UB, VR, SP, AM, RMB, SSR,GP,SS and GS all participated in data analysis and interpretation. MM drafted the manuscript, and all authors contributed to revisions and approved the final version.

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Data availability
All primary and secondary data are available with the corresponding author and in the Nikshay portal, Government of India. Permission is granted to the corresponding author to access the data through login credentials. The datasets generated and analyzed during the current study are part of the first author's Ph.D. thesis and are not publicly available. The datasets are available from the corresponding author upon reasonable request. Contact no: + 91 9,944,737,597 Email.ID:drmuthurajm@gmail.com.
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Declarations

Ethics approval and consent to participate
This retrospective study received ethical approval from the Ethics and Scientific Review Committee of the General Hospital Institute of the Directorate of Health and Family Welfare Services, Pondicherry (No. GHIEC/2020/244 ft. 08–06-2020), which granted a waiver for informed consent. The research was conducted in strict accordance with the ethical principles of the Helsinki Declaration as outlined by the World Medical Association, and all data were maintained with confidentiality.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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