MOLEKUL
eISSN: 2503-0310

Articles
https://doi.org/10.20884/1.jm.2025.20.1.13215

Regional Variations in rpoB Gene Mutations and Their Association with Rifampicin Resistance in *Mycobacterium tuberculosis*

Widodo[1,2]*, Daniel Joko Wahyono[3], Oedjijono[3]

[1]PhD Student in Biological Science PhD Programme, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto 53123, Indonesia
[2]Medical Laboratory Technology, Health Polytechnic of the Ministry of Health, Semarang, Indonesia
[3]Department of Molecular Biology, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto53123, Indonesia

*Corresponding author email: daniel.wahyono@unsoed.ac.id

Received November 22, 2024; **Accepted** February 20, 2025; **Available online** March 20, 2025

ABSTRACT. The increasing incidence of tuberculosis (TB) remains a global challenge, driven by the emergence of *Mycobacterium tuberculosis* resistance to antituberculosis drugs. This study investigated the relationship between rpoB gene mutations, rifampicin resistance levels, and the geographic origin of multidrug-resistant M. tuberculosis (MDR-TB) isolates. A total of 30 MDR-TB isolates were collected from the Central Java Provincial Health and Calibration Testing Laboratory between January and December 2023. Rifampicin resistance levels ranged from 5 to 80 μg/mL and were assessed using the microculture method, while DNA sequencing identified rpoB mutations. The results showed that rifampicin exposure significantly influenced rpoB mutation frequencies, with Ser531Leu (50%), His526 (16.7%), Leu511 (6%), Leu533 (6%), and Gln513 (6%) being the most common. Geographic variations were observed, with isolates from the ex-residencies Pekalongan, Pati, and Banyumas Residencies harboring seven, six, and four distinct mutations, respectively. Mutations such as Leu511Arg+His526Ser and Asp516Val were detected at low rifampicin concentrations (5–10 μg/mL), whereas Ser531Leu mutations dominated at moderate levels (20–80 μg/mL). These findings confirmed that rpoB mutations were influenced by both the region of origin and rifampicin resistance levels, providing critical insights for improving TB diagnosis and optimizing MDR-TB treatment strategies.

Keywords: *Mycobacterium tuberculosis*, rifampicin resistance, rpoB gene mutation

INTRODUCTION

Tuberculosis (TB) remains one of the leading causes of infectious disease-related deaths worldwide, with approximately 10 million people infected annually. In 2021, TB claimed 1.4 million lives, and 465,000 cases of drug-resistant TB were reported globally (WHO, 2021; Parums, 2021; Dheda et al., 2022; Hatami et al., 2022). The burden of TB is disproportionately concentrated in 16 countries, with India, Indonesia, and the Philippines contributing the highest number of cases.

In Indonesia, the Ministry of Health has set ambitious targets to control TB, including identifying 73,856 cases in Central Java Province, with an achievement goal of 69,823 cases (95%) by 2023 (Ministry of Health of the Republic of Indonesia, 2023). Among the regions in Central Java, the highest TB incidence rates are observed in Tegal City (762.10 cases per 100,000 population), Magelang City (507.30 cases per 100,000 population), and Banyumas Regency (205.90 cases per 100,000 population) (Central Java BPS, 2021).

The global increase in TB cases presents significant challenges for disease control, particularly due to the emergence of *M. tuberculosis* strains resistant to antituberculosis drugs. Resistance to these drugs complicates treatment regimens and contributes to delayed detection, incomplete characterization of resistant strains, and increased TB morbidity, mortality, and transmission rates (Dlamini et al., 2019; Goossens et al., 2022; Mirzayev et al., 2021; Vos et al., 2021).

The phenotypic determination of drug resistance in *M. tuberculosis* has undergone significant advancements. Historically, the conventional Lowenstein-Jensen (LJ) method, which uses duck eggs as a nutritional source, required an extended incubation period of 6 to 8 weeks for resistance

diagnosis (Muluwork et al., 2022; Myo & Lin, 2018). More recent approaches, such as the Mycobacteria Growth Indicator Tube (MGIT) method, have significantly reduced this timeframe to 7–21 days. In this study, we utilized a modified microplate method incorporating MGIT reagents with the alamarBlue indicator. This innovative technique offers a promising alternative for phenotypic resistance testing and assessing the resistance level of *M. tuberculosis* to first-line antituberculosis drugs (Abdel-Aziz et al., 2009; Lin et al., 2009; Longhin et al., 2022; WHO, 2022).

Rifampicin (RIF), a cornerstone in TB treatment for over 50 years, has derivatives including rifapentine, rifalazil, rifabutin, and rifacinna. Rifampicin works by binding to RNA polymerase (RNAP) in *M. tuberculosis*, thereby disrupting protein synthesis and exerting bactericidal effects (Jankute et al*.,* 2015; Ma et al*.,* 2021; Zaw & Lin, 2018). Resistance to rifampicin is primarily associated with mutations in the rpoB gene, particularly within the 81-base-pair Rifampicin Resistance Determining Region (RRDR), which accounts for approximately 95% of resistance cases. Codons 531, 526, and 516 are the most frequently mutated sites (Jankute et al., 2015; Ma et al., 2021; Zaw & Lin, 2018).

Understanding rpoB gene mutations is crucial for assessing phenotypic resistance and its implications for treatment outcomes (Mani et al., 2003). This study hypothesizes that the distribution of rpoB gene mutations varies among the Ex-Residencies (*Ex-Karesidenan*) in Central Java, potentially contributing to regional differences in rifampicin resistance patterns. The RRDR, which spans nucleotides 760,932 to 761,335 and corresponds to codons 507–570, serves as a key marker for rifampicin resistance. A common mutation within this region is the substitution of cytosine (C) with thymine (T) at codon 531, leading to an amino acid change from serine (S) to leucine (L) rimfampicin (Hamze et al., 2015; Mboowa, 2016; Ullah et al., 2016; Unissa et al., 2016).

The rpoB gene encodes the RNAP β-subunit, the primary target of rifampicin. Although conserved across bacterial species, its length and sequence vary. To enable cross-species comparison of rifampicin resistance mutations, a standardized numbering system for rpoB mutations was established in 1993, based on the annotation of the *Escherichia coli* (*E. coli*) sequence (Andre et al., 2017). In this system, mutations at codons 435, 445, and 450 in *E. coli* correspond to codons 516, 526, and 531 in *M. tuberculosis*, facilitating a standardized framework for understanding rifampicin resistance mechanisms (Andre et al., 2017).

Despite extensive research on rpoB mutations, studies focusing on the regional distribution of these mutations in Indonesia remain limited. The majority of global studies have concentrated on broader epidemiological trends, leaving gaps in understanding how geographic factors influence mutation patterns at a local level. Moreover, previous studies have primarily relied on whole-genome sequencing (WGS), while alternative methods such as the microculture system combined with DNA sequencing remain underexplored. This study sought to fill these gaps by analyzing the correlation between rpoB mutations, rifampicin resistance levels, and regional variations in Central Java. The findings contributed to a more targeted approach in TB diagnostics and treatment, particularly in regions with high multidrug-resistant TB (MDR-TB) prevalence.

EXPERIMENTAL SECTION

Bacterial Isolates

Determination of Isolate Count

This study employed a descriptive cross-sectional design. MDR-TB isolates were collected using a purposive sampling method from the Central Java Provincial Health and Calibration Testing Laboratory (Balapkes Provinsi Jawa Tengah). Sample collection was conducted between January and December 2023, resulting in 700 isolates. These isolates were first tested for resistance to isoniazid, after which isoniazid-resistant *M. tuberculosis* isolates were further tested for rifampicin resistance. Isolates resistant to both rifampicin and isoniazid were classified as MDR-TB. The required sample size for this study was determined using the formula by Lemeshow et al. (1990) and Lachenbruch et al. (1991):

Sample Size Estimation:

$$N = \frac{(1.96)^2 \text{ x } (1 - 0.05)^2/2}{\left(2 \text{ x } 0.0800\left(1 - 0.0800\right)\right) / 0.26)^2}$$

$$N = 28 \text{ Isolates}$$

Parameters:

Alpha (α)	: 0.0500
Power	: 0.8000
Delta (Δ)	: 0.2600
p_0	: 0.3700
p_a	: 0.6300
Z-score	: 1.96

Thus, the minimum number of isolates required was 28. However, 30 MDR-TB isolates that tested positive for rifampicin and isoniazid resistance were included in the study.

Sample Criteria

Samples included in this study met the following criteria:

- The isolates were confirmed as *M. tuberculosis* using the TB MPT64 Ag kit.
- The isolates were identified as MDR-TB, exhibiting resistance to both isoniazid and rifampicin.
- The isolates demonstrated good growth on Lowenstein-Jensen (LJ) medium and were able to form bacterial suspensions with a density of 1 McFarland (McF), equivalent to 3×10^8 cells/mL, with a growth age of 3–4 weeks.

Sample Grouping

Samples were grouped based on their origin within the Ex-Residencies (*Ex-Karesidenan*) of Central Java Province, as shown in **Table 1**. The purpose of this grouping was to facilitate mapping the distribution of *M. tuberculosis* mutations in the rpoB, katG, and inhA-mabA genes.

Resistance Level Testing Procedure

Resistance testing was conducted using 96-well microplates. The procedure was as follows:

1. Wells A1–A12 were filled with 300 μL of working reagent.
2. Wells B1–H12 were filled with 150 μL of MGIT medium without isoniazid or rifampicin.
3. Serial dilutions were performed starting from well A1, transferring 150 μL to well B1, followed by homogenization. This was repeated sequentially until reaching G1, ensuring thorough homogenization at each step.
4. Growth control wells (H1–H12) were left untreated, without isoniazid or rifampicin.

Rifampicin and isoniazid concentrations were prepared as follows:

- Rifampicin : 160, 80, 40, 20, 10, 5, and 2.5 μg/mL
- Isoniazid : 4, 2, 1, 0.5, 0.25, 0.125, and 0.065 μg/mL

5. Each well was inoculated with 10 μL of *M. tuberculosis* suspension at a density of 1 McFarland.
6. The microplates were incubated at 37°C for 2 weeks.
7. Following incubation, 10 μL of alamarBlue was added, and the plates were incubated for an additional 24 hours.
8. Color changes were observed:
   - Blue: Negative culture (no bacterial growth).
   - Purple: Positive culture (bacterial growth).

Modifications to the standard protocol included:

- Replacing the factory-provided plate cover with qPCR Plate Seals.
- Adding 10 mL of sterile aquabidest between wells to reduce evaporation rates.

The classification of resistance levels based on Minimum Inhibitory Concentration (MIC) is presented in **Table 2**.

Table 1. Isolate Grouping Based on the Residency System

No.	Ex-Residency	Regency Groups
1	Banyumas	Banyumas, Banjarnegara, Cilacap, Purbalingga
2	Kedu	Purworejo, Temanggung, Wonosobo, Kebumen, Magelang Regency, Magelang City
3	Pati	Pati, Kudus, Jepara, Blora, Rembang
4	Pekalongan	Pekalongan, Batang, Tegal, Tegal City, Brebes, Pemalang
5	Semarang	Semarang City, Semarang Regency, Salatiga City, Kendal, Demak, Grobogan
6	Surakarta	Klaten, Boyolali, Wonogiri, Sukoharjo, Sragen, Surakarta City, Karanganyar

Table 2. Classification of Resistance Levels

No.	Resistance Level	Isoniazid (μg/mL)	Rifampicin (μg/mL)
1	Low	MIC ≤ 0.25	MIC ≤ 10
2	Moderate	0.5 ≤ MIC < 2.0	10 < MIC ≤ 80
3	High	MIC ≥ 2.0	MIC ≥ 80

Note: The classification of resistance levels follows the criteria established by Zhang et al. (2015).

Mutation Testing

MDR-TB Isolate Subculturing

A single loopful of the MDR-TB isolate was retrieved from a −70°C cryovial storage tube and transferred into a 1 mL sterile distilled water tube containing sterile glass beads to separate *M. tuberculosis* colonies. The tube was vortexed to homogenize the suspension and left to settle for 15 minutes. A 100 µL aliquot of the homogenized suspension was inoculated into a McCartney bottle containing Lowenstein-Jensen (LJ) medium. The bottle was incubated at 35–37°C for 24 hours at a 30-degree angle. After incubation, the bottle was positioned upright, sealed tightly, and incubated for 2–3 weeks. Bacterial growth was observed weekly, and the resulting colonies were used for resistance testing and DNA extraction. The required bacterial density for resistance testing was 1 McFarland (3×10^8 cells/mL), while DNA extraction required 10^9 cells/mL in Phosphate-Buffered Saline (PBS) (Ministry of Health of Indonesia, 2012; WHO, 2022).

DNA Extraction

DNA extraction was performed using the Zymo Research D3024 kit. A 200 µL bacterial suspension (10^9 cells/mL) was added to a ZR Bashing Bead Lysis Tube (0.1 mm and 0.5 mm beads), followed by the addition of 750 µL Bashing Bead solution. The ZR Bashing Bead process consisted of three steps:

- Homogenization,
- High-speed shaking,
- Centrifugation at 13,000 RPM, repeated three times for 20 minutes each (total 1 hour).

The tubes were centrifuged at 10,000 × g for 1 minute, and 400 µL of the supernatant was transferred into a Zymo-Spin III-F filter column, placed in a 1.5 mL microcentrifuge tube, and centrifuged at 8,000 × g for 1 minute. The filtrate was mixed with 1.200 µL Genomic Lysis Buffer, and 800 µL of this mixture was loaded into a Zymo-Spin II-CR Column with a collection tube, then centrifuged at 10.000 × g for 1 minute. The filtration step was repeated twice until the solution was fully processed.

DNA Washing Steps:

- 200 µL of Pre-Wash Buffer was added to the Zymo-Spin II-CR column and centrifuged at 10,000 × g for 1 minute.
- 500 µL of DNA Wash Buffer was added and centrifuged at 10,000 × g for 1 minute.

The column was then transferred to a new 1.5 mL microcentrifuge tube, 50 µL of DNA Elution Buffer was added, incubated for 1 minute, and centrifuged at 10,000 × g for 30 seconds. The extracted DNA was stored at −20 °C. The concentration and purity of the extracted DNA were analyzed using a UV-Vis spectrophotometer.

DNA Analysis Using qPCR

DNA amplification was performed using the BIONER Excycler 96 quantitative polymerase chain reaction (qPCR) system with BIO 86005 SensiFast reagents. The qPCR mixture was prepared as follows:

- 20 µL of mastermix,
- 1.6 µL each of forward and reverse primers (10 µM),
- 16.4 µL of DNA template (10 µg).

PCR cycling conditions were optimized as follows:

- Initial denaturation : 95 °C for 3 minutes,
- Denaturation: 95 °C for 5 seconds,
- Annealing : 54.5 °C for 10 seconds,
- Elongation : 72 °C for 15 seconds,
- 40 cycles.

Fluorescence signals appeared within 20 cycles. The annealing temperatures were:

- katG : 54.5 °C
- rpoB : 53.4 °C
- inhA-mabA : 52.5 °C

The PCR products were sent to PT Genetika Science for sequencing. The sequencing results were analyzed using BioEdit and MEGA 10 software to identify mutations causing amino acid changes. Mutation points were determined using the RRDR for rpoB, while katG and inhA-mabA were directly compared to the wild-type *M. tuberculosis* genome.

Gene Primers

The primer sequences were as follows:

Nucleotide sequence	760932																											761012
H37RV Nc-000962.3	GGCACCAGCCAGCTGAGCCAATTCATGGACCAGAACAACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTG																											
81-bp mutation region	GGCACCAGCCAGCTGAGCCAATTCATGGACCAGAACAACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTG																											
Sample Number 1 rpoB	GGCACCAGCCAGCTGAGCCAATTCATGGACCAGAACAACCCGCTGTCGGGGTTGACCTACAAGCGCCGACTGTCGGCGCTG																											
Amino acid H37Rv	G	T	S	Q	L	S	Q	F	M	D	Q	N	N	P	L	S	G	L	T	H	K	R	R	L	S	A	L
Codon number	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	
Amino acid change																				Y								

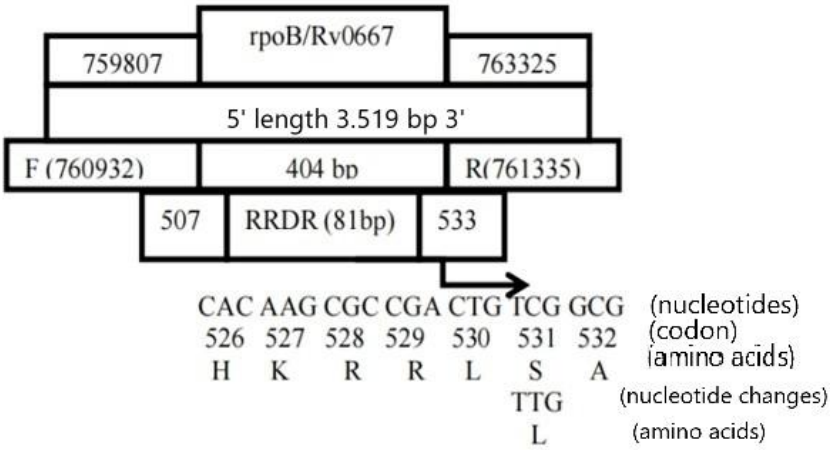

Figure 1. Mutation Location in the rpoB Gene and Primer Positions

Mutations in *M. tuberculosis* isolates were detected using specific primers targeting the rpoB gene to amplify the 81-base-pair (bp) RRDR region (**Figure 1**). The primers were designed based on previous studies rimfampicin (Hamze et al., 2015; Mboowa, 2016; Ullah et al., 2016; Unissa et al., 2016). The rpoB gene primers were designed as follows:

- Forward (F): 5′-GCGAGCTGATCCAAAACCAG-3′
- Reverse (R): 5′-GTACGGCGTTTCGATGAACC-3′

These primers amplified a 288-bp fragment of the *M. tuberculosis* H37Rv genome, designed using Primer3Plus software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and validated using in silico PCR analysis (http://insilico.ehu.es/PCR/index.php?mo=Mycobacterium).

The rpoB gene (Rv0667) consists of 3,519 bp, spanning nucleotides 759.807–763.325 of the H37Rv genome (NC_000962.3, total length: 4,411,532 bp) (https://www.ncbi.nlm.nih.gov/nuccore/ 448814763). The targeted mutation region spans nucleotides 760.932–761.335 (codons 507–570). A common example is the C-to-T substitution at codon 531, resulting in an amino acid change from serine (S) to leucine (L). The RRDR region has been widely established as a key marker of rifampicin resistance (Hamze et al., 2015; Mboowa, 2016; Ullah et al., 2016; Unissa et al., 2016).

Sequential Examination Procedure

The samples used in this study were previously identified as *M. tuberculosis* isolates. These isolates were subcultured to assess their resistance to rifampicin and isoniazid. Isolates meeting the criteria were subjected to rifampicin and isoniazid resistance testing using the 96-well microculture method.

Following resistance testing, DNA extraction was performed using the Zymo Research D3024 kit. The extracted DNA was analyzed using qPCR (BIONER Excycler 96) with BIO 86005 SensiFast reagents. The PCR products were sent to PT Genetika Science for sequencing. The sequencing results were analyzed using BioEdit and MEGA 10 software to determine whether mutations caused amino acid changes.

Mutation points were identified using the RRDR for rpoB, while katG mutations were mapped to predefined regions, and inhA-mabA mutations were directly compared to the wild-type *M. tuberculosis* genome.

RESULTS AND DISCUSSION

MDR-TB Sample Distribution

A total of 30 samples meeting the MDR-TB criteria were collected from the Central Java Provincial Health and Calibration Testing Laboratory between January 2023 and December 2023. **Table 3** shows the distribution of MDR-TB cases across the ex-residencies in Central Java. The highest number of cases was observed in the ex-residency Pekalongan (9 isolates, 30%), followed by the ex-residency Banyumas (7 isolates, 23%), the ex-residency Pati (6 isolates, 20%), and the ex-residency Kedu (3 isolates, 10%).

The results of this study revealed the frequency of rpoB gene mutation variations as follows: Ser531Leu (50%), His526 (16.7%), Leu511 (6%), Leu533 (6%), and Gln513 (6%). The distribution of rpoB mutations by regional groupings showed the highest frequency in the ex-residency Pekalongan (7 mutations), followed by the ex-residency Pati (6 mutations), and the ex-residency Banyumas (4 mutations). These findings align with previous studies using whole-genome sequencing (WGS), which identified Ser450Leu (equivalent to Ser531Leu in *E. coli* numbering) as the dominant rpoB mutation (Andre et al., 2017; Tania et al., 2020).

Table 3. Mutations in the rpoB Gene Based on Sample Origin

No.	Sample Origin	rpoB Gene Mutation	Total Samples
1	Ex-residency Banyumas	Leu511Pro (1), Leu533Pro, Ser571Tyr (1), Ser531Leu (4), His526Tyr (1)	7
2	Ex-residency Kedu	Ser531Le (3)	3
3	Ex-residency Pati	His526Tyr, Phe573Ser, Gly574Val (1), Leu511Arg, His526Ser (1), Leu533Pro (1), Gln513Lys, Phe537Ser, Gly574Val (1), Ser531Leu (1), Ser531Trp (1)	6
4	Ex-residency Pekalongan	Phe573Ser, Gly574Val (1), His526Gly (1), His526Tyr (1), Gln513Pro(1), Ser531Leu(3), Ser531Leu, Asp597Met, Val598Leu (1)	9
5	Ex-residency Semarang	Asp516Val(1), Ser531Leu(1)	2
6	Ex-residency Surakarta	Gln513Lys,His526Leu(1), Ser531Leu(2)	3

This study also revealed that the mutations in the rpoB gene alter the structure of the β-subunit of RNA polymerase (RNAP), reducing rifampicin binding affinity and thereby conferring resistance. This aligned with the studies on *M. tuberculosis* resistance isolates which indicate that 90–100% of rifampicin-resistant strains harbor mutations in the rpoB gene (Siddiqi et al., 2002; Dookie et al., 2018). These mutations can include insertions, deletions, and single-base substitutions, with single-base mutations being the most common (Casali et al., 2014; Coll et al., 2018; Farhat et al., 2019).

The mechanism of rifampicin resistance is predominantly associated with mutations in the Rifampicin Resistance Determining Region (RRDR), an 81-base-pair (bp) segment of the rpoB gene. Common mutations within this region include Ser531Leu, His526Tyr, His526Asp, and Asp516Val, with Ser531Leu being the most prevalent, accounting for 41–74% of cases (Nguyen et al., 2018). The Ser531Leu mutation is particularly common in strains from the Beijing lineage in Germany (Hillemann, et. al., 2005), while the Asp516Val mutation is more frequently observed in strains from Russia (Lipin et al., 2007; Xu et al., 2021).

rpoB Gene Mutations Based on Rifampicin Concentration

Table 4 summarizes the rpoB gene mutations identified at various rifampicin concentrations. Mutations were categorized into two groups based on rifampicin levels: low (5–10 µg/mL) and moderate (20–80 µg/mL). The highest number of rpoB mutations was observed in the low resistance group, with 20 isolates, where Ser531Leu was the most frequent mutation. Similarly, in the moderate resistance group (10 isolates), Ser531Leu remained the dominant mutation (**Table 4**). Further classification was conducted for inhA-mabA mutations based on isoniazid concentration.

At low rifampicin levels, the observed mutations included Leu511Arg + His526Ser (1), Asp516Val (1), Phe573Ser + Gly574Val (1), His526Gly (1), His526Tyr (1), Leu533Pro + Ser571Tyr (1), and Ser531Leu (2). At moderate levels, mutations included Gln513Pro (1), Ser531Leu (6), Leu533Pro (1), His526Leu (1), His526Tyr (1), His526Tyr + Phe573Ser + Gly574Val (1), Gln513Lys + Phe537Ser + Gly574Val (1), Ser531Trp (1), Leu511Pro (1), Gln513Lys + His526Leu (1), Ser531Leu (3), and Ser531Leu + Asp597Met + Val598Leu (1).

Interestingly, some mutation points, such as codons 511 and 526, were observed at both low and moderate levels. However, the amino acid changes differed: Leu511Arg was associated with low rifampicin levels, while Leu511Pro was identified at moderate levels. These differences suggest that amino acid changes contribute to increased rifampicin resistance. The Ser531Leu mutation was the most frequent at moderate levels, indicating that codon 531 is a dominant mutation site in rifampicin resistance cases. These findings partially align with Zhang et al. (2022), who categorized rifampicin resistance into three MIC-based groups: low (1–8 µg/mL), medium (16–64 µg/mL), and high (≥128 µg/mL). Zhang's study linked moderate resistance to the D435V mutation and high resistance to S450L, H445D, H445Y, and H445R. In contrast, the present study observed the dominance of Ser531Leu at moderate levels (20–80 µg/mL), differing from Zhang's finding that S450L is associated with high rifampicin resistance (MIC ≥128 µg/mL).

Table 4. rpoB Gene Mutations Based on Rifampicin Concentration

No.	Rifampicin Concentration (μg/mL)	rpoB Gene Mutations	Total Isolates	Resistance Level
1	5	Leu511Arg + His526Ser (1)	1	Low
2	10	Asp516Val (1), Phe573Ser + Gly574Val (1), His526Gly (1), His526Tyr (1), Leu533Pro + Ser571Tyr (1), Ser531Leu (2)	7	Low
3	20	Gln513Pro (1), Ser531Leu (6), Leu533Pro (1), His526Leu (1), His526Tyr (1), His526Tyr + Phe573Ser + Gly574Val (1), Gln513Lys + Phe537Ser + Gly574Val (1)	12	Moderate
4	40	Ser531Leu (3), Ser531Trp (1)	4	Moderate
5	80	Leu511Pro (1), Gln513Lys + His526Leu (1), Ser531Leu (3), Ser531Leu + Asp597Met + Val598Leu (1)	6	Moderate

Mutations in the *M. tuberculosis* RRDR enhance the bacterium's resistance to first-line antituberculosis drugs (OAT) (Fernando et al., 2019; Ma et al., 2021). Common rpoB mutations include Asp441Val, Ser456Trp, Ser456Gln, Arg454Gln, His451Gly, and His451Pro (Amusengeri, Khan, & Bishop, 2022). Mutations such as Ser450Leu, His445Asp, His445Tyr, and His445Arg are strongly associated with high rifampicin resistance (MIC ≥128 μg/mL), whereas Asp435Val is linked to moderate resistance (Li et al., 2022).

Rifampicin is bactericidal (rifampicin is well known as bactericidal of M. tuberculosis) agent against *M. tuberculosis* in both log and stationary growth phases, functioning by inhibiting RNA polymerase (RNAP). It blocks RNA elongation during transcription at the 5' end or reduces RNAP's affinity for short RNA transcripts. Mutations in the rpoB gene disrupt rifampicin binding to the RNAP β-subunit, thereby reducing the drug's efficacy.

The rpoB gene, which encodes the RNAP β-subunit, is a conserved rifampicin target across bacterial species, though its length and sequence vary. In 1993, a standardized numbering system was established based on the *E. coli* sequence annotation to compare rpoB mutations in *M. tuberculosis*. Using this system, amino acid codons 435, 445, and 450 in *E. coli* correspond to codons 516, 526, and 531 in *M. tuberculosis* (Andre et al., 2017). Mutations in the RRDR region significantly increase *M. tuberculosis* resistance to OATs, including rifampicin, isoniazid, and ethambutol (Fernando et al., 2019; Ma et al., 2021).

CONCLUSIONS

This study highlights the significant role of rpoB gene mutations in rifampicin resistance among *M. tuberculosis* isolates. The findings demonstrate that the region of origin of the isolate and the rifampicin concentration are key factors influencing the frequency and variation of rpoB mutations. The most prevalent mutation observed was Ser531Leu (50%), followed by mutations at codons 526 (16.7%), 511 (6%), 533 (6%), and 513 (6%).

The Ser531Leu mutation, located within the Rifampicin Resistance Determining Region (RRDR), emerged as the dominant marker of rifampicin resistance, consistent with its established role in conferring high resistance. Variations in amino acid substitutions at low and moderate rifampicin levels suggest that rifampicin concentration influences the selection pressure driving specific mutations.

These results emphasize the importance of region-specific surveillance to monitor mutation patterns and improve diagnostic accuracy. Understanding the mutation landscape of the rpoB gene provides valuable insights for tailoring rifampicin-based therapies and combating multidrug-resistant tuberculosis (MDR-TB). Further studies, including whole-genome sequencing and phenotypic resistance testing, are recommended to elucidate the mechanisms underlying resistance and explore strategies to mitigate the spread of MDR-TB.

ACKNOWLEDGMENTS

We extend our deepest gratitude to Mrs. Tri Sugiyatmini from Balepkes for her invaluable guidance and expertise, which greatly contributed to the success of this study. We also sincerely thank Mr. Hendro Pramono and Ms. Dini Riyandini for their generous support, insightful discussions, and dedication of time to this research.

REFERENCES

BPS. (2021). Number of disease cases by regency/city and type of disease in Central Java Province in 2021. Retrieved from https://jateng.bps.go.id/statictable/2022/03/21/2584/jumlah-kasus-penyakit-menurut-kabupaten-kota-dan-jenis-penyakit-di-provinsi-jawa

de Vos, M., Scott, L., David, A., Trollip, A., Hoffmann, H., Georghiou, S., Carmona, S., Ruhwald, M., Stevens, W., Denkinger, C. M., & Schumacher, S. G. (2021). Comparative analytical evaluation of four centralized platforms for the detection of *Mycobacterium tuberculosis* complex and resistance to rifampicin and isoniazid. *Journal of Clinical Microbiology, 59*.

https://doi.org/10.1128/JCM.02168-20

Dheda, K., Perumal, T., Moultrie, H., Perumal, R., Esmail, A., Scott, A. J., Udwadia, Z., Chang, K. C., Peter, J., Pooran, A., von Delft, A., von Delft, D., Martinson, N., Loveday, M., Charalambous, S., Kachingwe, E., Jassat, W., Cohen, C., Tempia, S., Fennelly, K., & Pai, M. (2022). The intersecting pandemics of tuberculosis and COVID-19: Population-level and patient-level impact, clinical presentation, and corrective interventions. *The Lancet Respiratory Medicine, 2600*, 1–20. https://doi.org/10.1016/s2213-2600(22)00092-3

Mboowa, G., & Sserwadda, W. (2016). Rifampicin resistance mutations in the 81 bp RRDR of *rpoB* gene in *Mycobacterium tuberculosis* clinical isolates using Xpert MTB/RIF in Khyber Pakhtunkhwa, Pakistan: A retrospective study. *BMC Infectious Diseases, 16*, 1–5. https://doi.org/10.1186/s12879-016-1745-2

Goossens, S. N., Heupink, T. H., De Vos, E., Dippenaar, A., De Vos, M., Warren, R., & Van Rie, A. (2022). Detection of minor variants in *Mycobacterium tuberculosis* whole genome sequencing data. *Briefings in Bioinformatics, 23*, 1–9. https://doi.org/10.1093/bib/ bbab541

Hamze, M., Ismail, M. B., Rahmo, A. K., & Dabboussi, F. (2015). Pyrosequencing for rapid detection of tuberculosis resistance to rifampicin and isoniazid in Syrian and Lebanese clinical isolates. *International Journal of Mycobacteriology, 4*, 228–232. https://doi.org /10.1016/j.ijmyco.2015.05.007

Hatami, H., Sotgiu, G., Bostanghadiri, N., Abadi, S. S. D., Mesgarpour, B., Goudarzi, H., Migliori, G. B., & Nasiri, M. J. (2022). Bedaquiline-containing regimens and multidrug-resistant tuberculosis: A systematic review and meta-analysis. *Jornal Brasileiro de Pneumologia, 48*, e20210384. https://doi.org/10.36416/1806-3756/e20210384

Jankute, M., Cox, J. A. G., Harrison, J., & Besra, G. S. (2015). Assembly of the mycobacterial cell wall. *Annual Review of Microbiology, 69*, 405–423. https://doi.org/ 10.1146/annurev-micro-091014-104121

Ministry of Health of the Republic of Indonesia. (2023). Directorate General of P2P performance report for the first semester of 2023 (pp. 1–134).

Lachenbruch, P. A., Lwanga, S. K., & Lemeshow, S. (1991). Sample size determination in health studies: A practical manual. *Journal of the American Statistical Association, 86*, 1149. https://doi.org/10.2307/2290547

Lemeshow, S., Hosmer, D. W. Jr., Klar, J., & Lwanga, S. K. (1990). *Adequacy of sample size in health studies.* World Health Organization.

Ma, P., Luo, T., Ge, L., Chen, Z., Wang, X., Zhao, R., Liao, W., & Bao, L. (2021). Compensatory effects of *M. tuberculosis rpoB* mutations outside the rifampicin resistance-determining region. *Emerging Microbes & Infections, 10*, 743–752. https://doi.org/10.1080/22221751.2021.1908096

Mani, C., Selvakumar, N., Kumar, V., Narayanan, S., & Narayanan, P. R. (2003). Comparison of DNA sequencing, PCR-SSCP, and PhaB assays with indirect sensitivity testing for detection of rifampicin resistance in *Mycobacterium tuberculosis*. *International Journal of Tuberculosis and Lung Disease, 7*, 652–659.

Mirzayev, F., Viney, K., Linh, N. N., Gonzalez-Angulo, L., Gegia, M., Jaramillo, E., Zignol, M., & Kasaeva, T. (2021). World Health Organization recommendations on the treatment of drug-resistant tuberculosis, 2020 update. *European Respiratory Journal, 57*. https://doi.org/ 10.1183/13993003.03300-2020

Parums, D. V. (2021). Editorial: Updates from the World Health Organization (WHO) on global treatment recommendations for drug-susceptible and multidrug-resistant tuberculosis. *Medical Science Monitor, 27*, 1–3. https://doi.org/10.12659/MSM.934292

Ullah, I., Shah, A. A., Basit, A., Ali, M., Khan, A., Ullah, U., Ihtesham, M., Mehreen, S., Mughal, A., & Javaid, A. (2016). Rifampicin resistance mutations in the 81 bp RRDR of *rpoB* gene in *Mycobacterium tuberculosis* clinical isolates using Xpert MTB/RIF in Khyber Pakhtunkhwa, Pakistan: A retrospective study. *BMC Infectious Diseases, 16*, 4–9. https://doi.org/ 10.1186/s12879-016-1745-2

Unissa, A. N., Subbian, S., Hanna, L. E., & Selvakumar, N. (2016). Overview on mechanisms of isoniazid action and resistance in *Mycobacterium tuberculosis*. *Infection, Genetics and Evolution, 45*, 474–492. https://doi.org/10.1016/j.meegid.2016.09.004

World Health Organization. (2021). Treatment of drug-susceptible tuberculosis: Rapid communication (pp. 1–4).

Zaw, M. T., Emran, N. A., & Lin, Z. (2018). Mutations inside the rifampicin-resistance determining region of *rpoB* gene associated with rifampicin resistance in *Mycobacterium tuberculosis*. *Journal of Infection and Public Health, 11*, 605–610. https://doi.org/10.1016/j.jiph.2018.04.005