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Original Article

Rifabutin Cross-resistance and Associated *rpoB* Mutations in Rifampicin-resistant *Mycobacterium tuberculosis* Complex Isolates

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ABSTRACT

OBJECTIVE: This study aimed to phenotypically evaluate rifabutin susceptibility in rifampicin-resistant *Mycobacterium tuberculosis* complex isolates and to identify, through DNA sequence analysis of the rifampicin resistance-determining region (RRDR), specific *rpoB* mutations associated with rifabutin susceptibility.

MATERIAL AND METHODS: A total of 43 rifampicin-resistant and 23 rifampicin-susceptible (control) *Mycobacterium tuberculosis* complex isolates, collected between 2000 and 2020 at the Mycobacteriology Laboratories of Manisa Celal Bayar University and Ege University Faculty of Medicine, were included. Phenotypic drug susceptibility testing was performed using the MGIT 960 system. Mutations in a 682-bp fragment encompassing the 81-bp RRDR of the *rpoB* gene were investigated by Sanger sequencing.

RESULTS: Among the 43 rifampicin-resistant isolates, 39 (91%) were resistant to rifabutin, while four (9%) remained susceptible. All rifampicin-susceptible isolates were also susceptible to rifabutin. A total of 44 non-synonymous single nucleotide polymorphisms were detected in rifampicin-resistant isolates, whereas no mutations were observed in the control group. Seven distinct mutation patterns were identified, including one isolate with a double mutation. The most common mutations were S531L (76%), H526Y (12%), and D516Y (4%). The mutations D516Y, L511P, and L533P, as well as the M515I+D516Y double mutation, were associated with rifabutin susceptibility.

CONCLUSION: Early identification of *rpoB* mutations associated with rifabutin susceptibility may allow substitution of rifampicin with rifabutin in selected rifampicin-resistant cases, potentially improving treatment outcomes and limiting the emergence of resistance to second-line drugs. Further *in vitro* studies, including minimum inhibitory concentration testing, and additional clinical data on rifabutin-containing regimens are needed.

KEYWORDS: *Mycobacterium tuberculosis* complex, rifampicin resistance, rifabutin, *rpoB*, mutation

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INTRODUCTION

Tuberculosis (TB) remains one of the most significant infectious diseases threatening global public health. According to the World Health Organization (WHO) Global TB Report 2025, an estimated 10.7 million individuals developed TB in 2024, and there were 1.23 million TB-related deaths worldwide.[1] One of the major obstacles to successful TB control is drug resistance. While the successful treatment rate for drug-susceptible TB remained high at 88% in 2024, it was lower at 71% for rifampicin-resistant TB.[1]

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Rifampicin is a cornerstone of TB treatment due to its potent sterilizing activity, and rifampicin resistance is monitored by the WHO as a proxy for multidrug-resistant TB.[1] Rifabutin, a rifamycin derivative with similar antimycobacterial activity, induces cytochrome P450 enzymes to a lesser extent and is therefore preferred in patients co-infected with human immunodeficiency virus or receiving immunosuppressive therapy.[2,3]

In most rifampicin-resistant *Mycobacterium tuberculosis* complex isolates, resistance-conferring mutations occur within the rifampicin resistance-determining region (RRDR) of the *rpoB* gene.[4] Although these mutations frequently result in cross-resistance to rifabutin, certain substitutions—particularly those affecting codons 511, 516, and 533—have been associated with retained *in vitro* susceptibility to rifabutin.[5] Identifying such mutations in rifampicin-resistant isolates may enable the use of rifabutin-containing regimens without resorting to second-line drugs. The present study aimed to phenotypically assess rifabutin cross-resistance in rifampicin-resistant isolates and to characterize the accompanying *rpoB* mutations.

MATERIAL AND METHODS

Mycobacterium tuberculosis Complex Isolates

The study included 43 rifampicin-resistant and 23 rifampicin-susceptible *Mycobacterium tuberculosis* complex isolates collected between 2000 and 2020 at the Mycobacteriology Laboratories of Manisa Celal Bayar University and Ege University Faculty of Medicine. Only one isolate per patient was analyzed. *M. tuberculosis* H37Rv was used as the reference strain.

Phenotypic Drug Susceptibility Testing

Stock isolates were subcultured in MGIT liquid medium, and rifabutin susceptibility was determined using the MGIT 960 automated mycobacterial culture system (Becton Dickinson, USA). Rifabutin (CAS no 72559-06-9, Sigma-Aldrich, USA) was prepared in methanol and diluted to achieve a final critical concentration of 0.5 µg/mL, in accordance with Clinical and Laboratory Standards Institute recommendations.[6] Following completion of the drug susceptibility test, cultures were processed for DNA extraction.

Main Points

- A subset (9%) of rifampicin-resistant *Mycobacterium tuberculosis* isolates remained susceptible to rifabutin, demonstrating that rifampicin resistance alone should not preclude the use of rifabutin.
- Borderline rpoB mutations (D516Y, L511P, L533P) are often associated with an increased likelihood of rifabutin susceptibility, but they may cause phenotypic false susceptibility in rifampicin testing. Correction: In contrast, mutations associated with high-level rifampicin resistance (S531L, H526Y) consistently confer resistance to both rifamycins.
- Molecular identification of *rpoB* mutation profiles can enable personalized rifamycin selection, potentially improving treatment outcomes and preserving second-line drug options.

DNA Extraction, Polymerase Chain Reaction and Sequencing

Genomic DNA was extracted from bacterial suspensions using the GeneMark Bacteria DNA Purification Kit (DP025, GeneMark Technology Co. Ltd., Taiwan) and stored at –20 °C. The genome of *M. tuberculosis* H37Rv (NC_000962.3) was retrieved from the NCBI database and a 682-bp fragment encompassing the *rpoB* RRDR sequences was selected as the target region. Primers were designed in-house using the NCBI Primer-BLAST tool. The primer sequences were as follows: F: 5′-GTCAGACCACGATGACCGTT-3′ and R: 5′-TAGTCCACCTCAGACGAGGG-3′. Polymerase chain reaction (PCR) was performed with an initial denaturation at 94 °C for 2 min, followed by 30 cycles (94 °C for 30 s, 58 °C for 20 s, and 72 °C for 40 s) and a final extension at 72 °C for 5 min. Amplicon integrity was confirmed by agarose gel electrophoresis, and the amplicons were visualized under ultraviolet light before storage at –20 °C for sequencing.

PCR amplicons targeting the *Mycobacterium tuberculosis rpoB* RRDR were sequenced using the BigDye™ Terminator v3.1 kit. Reactions were performed using the original PCR primers and analyzed by capillary electrophoresis on an Applied Biosystems 3730 DNA Analyzer.

Raw chromatograms (AB1) were processed for base calling and quality trimming using the sangerseqR v1.40.0 package. Sequences were aligned to the H37Rv reference (NC_000962.3) using Biostrings and Pwalign packages, with results independently validated via MEGA software. Rifampicin resistance-associated mutations were identified through visual inspection of high-quality consensus sequences and their corresponding chromatograms using the *Mycobacterium tuberculosis* H37Rv (NC_000962.3) strain as the reference sequence.

This study was conducted with the approval of the Health Sciences Ethics Committee of Manisa Celal Bayar University Faculty of Medicine (ref: 1656, date: 01.02.2023).

Statistical Analysis

The frequency of mutations in the study was calculated using percentage values.

RESULTS

Phenotypic susceptibility results for the 43 rifampicin-resistant isolates showed that rifabutin resistance was detected in 39 (91%), while 4 isolates (9%) were found to be rifabutin-susceptible. All 23 rifampicin-susceptible control isolates were also found to be susceptible to rifabutin.

A total of 44 non-synonymous mutations were detected in the rifampicin-resistant isolates. No mutations were observed in the control isolates. The identified mutations resulted in seven distinct genetic variants, with a double mutation observed in one variant. The mutations observed in rifampicin-resistant cases, in order of frequency, were: S531L (76%), H526Y (12%),

D516Y (4%), H526D (2%), L511P (2%), M515I (2%), and L533P (2%).

Among these, the mutations D516Y, L511P, and L533P were identified as associated with rifabutin susceptibility. In addition, one rifabutin-susceptible isolate harbored the M515I+D516Y double mutation. In the four isolates found to be rifampicin-resistant but rifabutin-susceptible, the mutations GAC516TAC, CTG511CCG, ATG515ATC, and CTG533CCG were present; one strain harbored a double mutation of ATG515ATC and GAC516TAC. While the most frequently observed mutation in the 39 isolates phenotypically resistant to both drugs was TCG531TTG (85%, 33/39), the most common mutation in the four rifabutin-susceptible isolates was GAC516TAC (50%, 2/4) (Table 1).

DISCUSSION

Cross-resistance between rifampicin and rifabutin is a well-recognized phenomenon in *Mycobacterium tuberculosis* complex isolates and is largely driven by mutations in the *rpoB* RRDR. However, increasing evidence indicates that not all rifampicin resistance-conferring mutations result in uniform cross-resistance to rifabutin, highlighting a clinically relevant heterogeneity within rifampicin-resistant isolates. In the present study, susceptibility to rifabutin was observed in 9% of phenotypically rifampicin-resistant isolates. Although this proportion is lower than the 13–28% range reported in previous studies, this finding should not be interpreted solely as a limitation.[7-9] Rather, it underscores the population-specific distribution of *rpoB* mutations and reinforces the concept that rifabutin susceptibility is restricted to a molecularly defined subset of rifampicin-resistant isolates. From a clinical perspective, even a relatively small proportion of rifabutin-susceptible isolates may represent a meaningful therapeutic opportunity, particularly in settings where preserving first-line drug backbones is desirable. However, the presence of borderline *rpoB* mutations (often located outside the core 81-bp hot spot) presents a diagnostic challenge, as they frequently confer low-level rifampicin resistance that may lead to phenotypic false-susceptibility in conventional tests.[10]

The WHO's 2023 "Catalogue of Mutations in *Mycobacterium tuberculosis* Complex and Their Association with Drug Resistance" provides a robust framework for interpreting the clinical relevance of *rpoB* mutations by stratifying them according to the strength of evidence linking genotype to phenotypic resistance.[10] Within this framework, the majority of mutations identified in our study—namely S531L, H526Y, H526D, D516Y, L511P, and L533P—are classified as Group 1 mutations associated with rifampicin resistance. The predominance of these mutations among rifabutin-resistant isolates in our cohort is therefore concordant with both molecular expectations and prior epidemiological data. Studies covering the Aegean, Marmara, and Central Anatolia regions of Türkiye indicate that mutations responsible for rifampicin

Table 1. Observed mutations and phenotypic resistance patterns in *Mycobacterium tuberculosis* complex isolates

Codon*	Nucleotide substitution	Amino acid change		Number (%)**	Phenotypic resistance
531	TCG→TTG	S531L	Ser - Leu	33 (76%)	R^R/RFB^R
526	CAC→TAC	H526Y	His-Tyr	5 (12%)	R^R/RFB^R
526	CAC→GAC	H526D	His-Asp	1 (2%)	R^R/RFB^R
516	GAC→TAC	D516Y	Asp-Tyr	2 (4%)	R^R/RFB^s
511	CTG→CCG	L511P	Leu-Pro	1 (2%)	R^R/RFB^s
533	CTG→CCG	L533P	Leu-Pro	1 (2%)	R^R/RFB^s
515+516	*ATG→ATC*	M515I	Met-Ile	1 (2%)	R^R/RFB^s
	GAC→TAC	D516Y	Asp-Tyr		
Wild type				23	R^S/RFB^s

*Based on *Escherichia coli* numbering system[10], **Number (%) indicates the number and percentage of mutation events (n = 44), not the number of isolates
RFB: Rifabutin, ^{R}: Rifampicin, ^{r}: Resistant, ^{s}: Susceptible

Table 2. Grading of identified mutations according to the WHO catalogue

Variant	*E. coli* numbering	Reliability grading	Additional criteria*	Group
rpoB_S450L	S531L	Associated with resistance		1
rpoB_H445D	H526D	Associated with resistance		1
rpoB_H445Y	H526Y	Associated with resistance		1
rpoB_D435Y	D516Y	Associated with resistance	Borderline resistance	1
rpoB_L452P	L533P	Associated with resistance	Borderline resistance	1
rpoB_L430P	L511P	Associated with resistance	Borderline resistance	1
rpoB_M434I	M515I	Associated with resistance-interim		2

*Additional criteria were evaluated in accordance with the Catalogue of Mutations in *Mycobacterium tuberculosis* Complex and Their Association with Drug Resistance, second edition
WHO: World Health Organization

resistance are primarily concentrated in codons 531, 526, 516, and 513, although their frequency distribution varies by region. Among the specific alterations identified in these regions, the Ser531Leu mutation has been reported as the most prevalent variant.[11-14]

In contrast, mutations such as D516Y, L511P, and L533P occupy distinct positions within the resistance spectrum. These substitutions are also categorized as group 1 mutations, but are widely recognized as "borderline" or "disputed" mutations and are often associated with minimum inhibitory concentrations close to the critical threshold.[10] Importantly, multiple *in vitro* and clinical studies have demonstrated that isolates harboring these mutations may retain susceptibility to rifabutin despite being classified as rifampicin-resistant by standard phenotypic methods.[10,15-17] The observation that all isolates carrying these mutations in our study were susceptible to rifabutin provides further support for this genotype-phenotype dissociation and reinforces the biological plausibility of the use of rifabutin in this subset.

In this study, a double mutation (M515I and D516Y) was detected in one isolate, and this variant was found to be susceptible to rifabutin. In contrast, a study conducted by Jing et al.[18] reported that while single mutations at codons 511, 516, 526, and 533 were associated with rifabutin susceptibility, isolates harboring double mutations at these positions exhibited a rifabutin-resistant phenotype. This finding suggests that rifabutin susceptibility or resistance is determined not by the number of mutations but rather by the specific mutation pattern present. Table 2 presents the reliability grading of the mutations identified in this study according to the WHO mutation catalogue. In line with recent advancements, next-generation sequencing technologies are increasingly utilized for TB diagnosis and comprehensive drug resistance profiling, as outlined in current WHO guidelines.[19] Although next-generation sequencing is primarily employed for research purposes, it is anticipated to transition into routine commercial *in vitro* diagnostic use in the near future, offering a higher-throughput approach to identifying complex mutation patterns.

Study Limitations

The most important limitations of this study are the absence of minimum inhibitory concentration analysis and the limited number of isolates included. In addition, the recent WHO recommendation to lower the critical concentration for rifampicin to 0.5 µg/mL may alter phenotypic resistance profiles and increase the rate of false-positive rifampicin resistance. Therefore, further studies incorporating minimum inhibitory concentration testing, multiple culture systems, and sequencing of *rpoB* regions outside the RRDR are required.

CONCLUSION

Nine percent of phenotypically rifampicin-resistant isolates in this study were susceptible to rifabutin. High-level resistance mutations (S531L, H526Y, H526D) were associated with resistance to both rifampicin and rifabutin, whereas borderline resistance mutations (D516Y, L533P, L511P) were associated with susceptibility to rifabutin. The inclusion of rifabutin in treatment regimens for such cases may improve treatment outcomes and help prevent the rapid emergence of resistance to second-line drugs. Larger *in vitro* studies incorporating minimum inhibitory concentration data, together with additional clinical evidence on rifabutin-containing regimens, are required.

Ethics

Ethics Committee Approval: This study was conducted with the approval of the Health Sciences Ethics Committee of Manisa Celal Bayar University Faculty of Medicine (ref: 1656, date: 01.02.2023).

Informed Consent: Patient informed consent was not obtained because the research was retroactively performed on stored laboratory bacterial strains, ensuring complete patient anonymity.

Footnotes

Authorship Contributions

Concept: N.S., S.S., N.Ö., Design: N.S., S.S., Data Collection or Processing: N.S., Analysis or Interpretation: S.S., N.Ö., Literature Search: S.S., Writing: N.S.

Conflict of Interest: No conflict of interest was declared by the authors.

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