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Mutations outside RRDR lead to increased MICs for M. tuberculosis isolates with «borderline» rpoB mutations

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Abstract*— Rapid and accurate detection of rifampicin resistance in Mycobacterium tuberculosis is crucial for effective tuberculosis control. While molecular methods targeting the rifampicin resistance determining region (RRDR) of the rpoB gene have revolutionized resistance detection, challenges arise with mutations outside this region. This study investigates the correlation between the rpoB borderline mutations Asp435Tyr and His445Asn and phenotypic resistance in M. tuberculosis. The sequencing results revealed that 11 of the 20 isolates with the Asp435Tyr mutation also had the Asn487Ser mutation and one had the Gln429His mutation. Five of the 17 isolates with the His445Asn mutation also carried the Ile491Met mutation. The study found that the presence of additional mutations outside RRDR increased the degree of phenotypic resistance. These findings suggest that mutations outside the RRDR, that not detected widely used PCR-based tests, may play a role in the variable expression of resistance associated with the Asp435Tyr and His445Asn mutations. Understanding the impact of these mutations on rifampicin resistance is important for the development of more accurate diagnostic tests and effective treatment strategies for TB.

Index Terms—**Mycobacterium tuberculosis, rpoB, RRDR, resistance, mutations**

I. INTRODUCTION

In 2022, tuberculosis affected an estimated 10.6 million people worldwide, with an incidence rate that has risen by 3.9% from 2020 to 2022 The emergence of multidrug-resistant *Mycobacterium tuberculosis* strains poses a significant threat to public health. Among these, resistance to rifampicin - the marker of multidrug resistance and the most effective first-line drug - is particularly alarming. Russia remains one of the countries with the highest burden of multidrug-resistant tuberculosis. Accurate identification of rifampicin resistance is, therefore, essential for devising effective treatment regimens [1].

The primary mechanism of mycobacterial resistance to rifampicin involves mutations in the rifampicin resistance-determining region (81-base-pair central region of the *rpoB* gene, spanning codons 426 to 452 (*Escherichia coli* numbering 507-533), which encodes the mycobacterial RNA polymerase [2], [3]. Molecular genetic techniques allow for the identification of mutations within the *rpoB* gene that confer resistance of *M. tuberculosis* to rifampicin. These tools are not only less complex but also quicker than traditional phenotypic methods [4]–[7].

The World Health Organization (WHO) has established guidelines that classify mutations within the rifampicin resistance-determining region (RRDR) of the *rpoB* gene, excluding synonymous mutations, and *rpoB* I491F mutation as conferring resistance to rifampicin. This includes mutations that have not been previously described [8]. However, the landscape of *rpoB* mutations is complex, with some mutations classified as "borderline" or "disputed." These mutations do not always correlate with a high level of phenotypic resistance and their clinical significance remains a topic of debate [9]–[12]. The second edition of the WHO

catalog of *M. tuberculosis* mutations associated with drug resistance (2023) listed seven «borderline» mutations: Leu430Pro, Asp435Tyr, His445Leu, His445Asn, Leu452Pro, His445Ser and Ile491Phe [10].

To reduce the percentage of discrepancies, WHO in 2021 lowered the critical concentrations for rifampicin from 1 to 0.5 mg/L on Mycobacterial Growth Indicator Tube (MGIT) medium and on Middelbrook 7H10 medium. There is insufficient evidence to change the concentrations on Middelbrook 7H11 medium [9]. The changes were made with the understanding that they may not lead to complete match between the results of molecular-genetic and phenotypic methods, but they will increase the percentage of convergence. In the future, as more data is accumulated, a new revision of the values of rifampicin critical concentrations is possible.

This study focuses on investigating the correlation between the *rpoB* borderline mutations Asp435Tyr and His445Asn and the degree of phenotypic resistance in *M. tuberculosis* clinical isolates. Through the application of advanced molecular techniques and extensive drug sensitivity testing, we aim to unravel the mechanisms behind the variable expression of *M. tuberculosis* rifampicin resistance associated with these mutations.

II. Material and Methods:

In this study, we obtained 20 *M. tuberculosis* isolates with the *rpoB* Asp435Tyr mutation and 17 isolates with the His445Asn mutation from the collection of several tuberculosis hospitals: Ural Research Institute for Phthisiopulmonology, a branch of the National Medical Research Center of Phthisiopulmonology and Infection Diseases (Yekaterinburg, Russia) - isolates with discrepancies and convergence data of molecular genetics and phenotypic tests; Regional Clinical Medical Center of Phthisiopulmonology and Infection Diseases (Yekaterinburg, Russia), Regional Clinical Phthisiopulmonology Center (Tyumen, Russia), Chelyabinsk Regional Clinical Phthisiopulmonology Hospital (Chelyabinsk, Russia) and Republic Phthisiopulmonology Hospital (Yoshkar-Ola, Russia) – only isolates with discrepancies data of molecular-genetic and phenotypic tests.

The initial detection of *M. tuberculosis rpoB* mutations in regional laboratories was carried out using one of the routinely used PCR-based tests in Russia: «TB-TEST» (BIOCHIP-IMB, Russia), «Amplitub-MDR RV» kit (Syntol, Russia) or «Xpert MTB/RIF» (Cepheid, USA).

The initial phenotypic drug susceptibility testing to rifampicin was performed using one of the methods: BACTEC MGIT 960 technology (rifampicin critical concentration was 0,5 or 1 mg/L), absolute concentration method on Lowenstein-Jensen medium (rifampicin concentration was 40 mg/L) or proportion method on Middlebrook 7H11 medium (rifampicin concentration was 1 mg/L). All isolates were collected during 2021– 2023 years.

In this work, all 37 *M. tuberculosis* isolates were sub-cultured on Lowenstein-Jensen medium. Phenotypic drug susceptibility testing to rifampicin was performed using BACTEC MGIT 960 technology (Becton Dickenson and Company, USA) with rifampicin concentrations of 0.25, 0.5 and 1 mg/L, and a critical concentration of 0.5 mg/L. The minimal inhibitory concentration (MIC) of rifampicin was determined by the serial dilutions' method on Middlebrook 7H11 medium (Becton Dickenson and Company, USA) with concentrations 0.25; 0.5; 1; 2; 4; 8; 16, 32 mg/L, and a critical concentration 1 mg/L. The results were considered after 28 days. The laboratory strain *M. tuberculosis* H37Rv was used as a control strain for phenotypic drug susceptibility testing.

Genomic DNA was extracted from *M. tuberculosis* suspension aliquots prepared for phenotypic drug susceptibility testing using the "Amplitub-RV" kit (Syntol, Russia) according to the manufacturer's instructions. The sample was further examined by the «TB-TEST» (BIOCHIP-IMB, Russia) method, according to the manufacturer's instructions. This method determines the genotypes of *M. tuberculosis* (LAM, Ural, Haarlem, Beijing and its clone Beijing B0) and 116 mutations associated with drug resistance in the *rpoB, katG, inhA, ahpC, gyrA, gyrB, rrs, eis,* and *embB* genes.

For amplification and Sanger sequencing of the *rpoB* gene fragment, including the rifampicin resistance-determining region and the areas above and below this site, primer pairs: rpoB-459-F GCTGATCCAAAACCAGATCC (position in the *rpoB* gene 1131-1150), rpoB-459-R TCCTCGTCGGCGGCGGTCAGGTA (position in the *rpoB* gene 1570-1589) were used. Amplification was performed on C1000 thermal cycler (BioRad, USA) according to the follow program: $94C^0$ - 3min.; 40 cycles: $94C^0$ - 30 sec., $58C^0$ – 30 sec., $72C^0$ - 1 min.; $72C^0$ – 5 min. Sequencing was performed on an ABI 3500 genetic analyzer using the manufacturer's protocol (Applied Biosystems, Foster City, CA). The results of *rpoB* gene sequencing were processed using the open-source software Unipro UGENE (ver.42.0), using the Sanger data analysis tool. The *rpoB* gene sequence of *M. tuberculosis* H37Rv was used as a reference.

The study was approved by the Local Ethics Committee of the National Medical Research Center for Phthisiopulmonology and Infection Disease, Ekaterinburg, Russia (approval protocol № 102, 24.11.2021). Clinical isolates included in this study were obtained from collections routinely assembled as a part of the practices of the microbiology laboratory. No personal patients' data, or experiments on humans, or human tissues, were utilized in this study.

III. Results:

The sequencing results confirmed the *rpoB* Asp435Tyr mutation in 20 isolates, 11 of which also had the Asn487Ser mutation (outside RRDR) and one had the Gln429His mutation (within RRDR). Another 17 isolates had the His445Asn mutation, with five of them also carrying the Ile491Met mutation (outside RRDR).

According to the «TB- TEST» data, all 11 isolates with combination of Asp435Tyr and Asn487Ser mutations had additional mutations: *katG* S315T (isoniazid resistance), *embB* M306V (ethambutol resistance), *eis* c14t (aminoglycosides resistance) and the Beijing genotype. Ten of them had six different types of *gyrA* mutation (fluoroquinolone resistance). One isolate with *rpoB* Asp435Tyr and Gln429His mutation had the LAM genotype. Of the nine isolates with a single *rpoB* Asp435Tyr mutation seven belong to the Beijing genotype and other two to the LAM and Ural genotypes. They had different combinations of mutation in *katG, embB, eis* and *gyrA* genes or none in these genes at all.

Isolates with His445Asn and Ile491Met mutations belong to the Beijing genotype and had *katG* Ser315Tyr mutation. Nine isolates with single His445Asn mutation belonged to the Beijing B0 genotype the other three to the Ural and Beijing genotypes.

Phenotypic testing revealed an increased MIC values in isolates with an additional *rpoB* mutation. On BACTEC MGIT 960, all eight isolates with the single Asp435Tyr mutation were sensitive to 1 mg/L, and six of them were also sensitive to 0.5 mg/L of rifampicin. In the case of the single His445Asn mutation, all 12 isolates were sensitive to 0.5 mg/L, and 11 of them were sensitive to 0.25 mg/L. On Middlebrook 7H11 medium, the MICs ranged from 1-8 mg/L for isolates with the single Asp435Tyr mutation and were 0.5-1 mg/L (sensitive) for isolates with the single His445Asn mutation (Table 1). The median MICs for H37Rv strain was 0.25-0.5 mg/L.

Table 1. The genetic and phenotypic drug susceptibility testing data of *M. tuberculosis* clinical isolates with the *rpoB* borderline mutations D453T and H345N.

Isolate number	The region	Mutation of drug resistance (TBTEST assey)					Genotype	Additional mutation in rpoB	Middle brook 7H11 MIC mg/L	Bactec MGIT (mg/L)*		
		Rif (*rpoB*)	H (*katG/inhA*)	Fq (*gyrA*)	Ag (eis)	Emb (*embB*)				0.25	0.5	1.0
469	Mari El	D435T	S315T/T8	wt	wt	M306I2	LAM	Q429H	4	R	R	R
13123	Khanty-Mansi	D435T	S315T	wt	c14t	M306V	Beijing	N487S	8	R	R	R(261)
28320	Sverdlovsk	D435T	S315T	A90V	c14t	M306V	Beijing	N487S	8	R	R	R
16023	Sverdlovsk	D435T	S315T	S91P	c14t	M306V	Beijing	N487S	8	R	R	S
2898	Yamalia	D435T	S315T	D94A S95T	c14t	M306V	Beijing	N487S	8	R	R	S
11622	Sverdlovsk	D435T	S315T	D94A S95T	c14t	M306V	Beijing	N487S	32	R	R	R
2097	Tyumen	D435T	S315T	D94N S95T	c14t	M306V	Beijing	N487S	8	R	R	R
22123	Sverdlovsk	D435T	S315T	D94N S95T	c14t	M306V	Beijing	N487S	8	R	R	R(258)
12821	Tyumen	D435T	S315T	D94N S95T	c14t	M306V	Beijing	N487S	8	R	R	R
2122	Sverdlovsk	D435T	S315T	D94N	c14t	M306V	Beijing	N487S	4	R	R	R
2623	Sverdlovsk	D435T	S315T	D94G S95T	c14t	M306V	Beijing	N487S	32	R	R	R
24221	Sverdlovsk	D435T	S315T	D94HS95T	c14t	M306V	Beijing	N487S	8	R	R	R
55	Mari El	D435T	S315T/T8	wt	wt	M306I2	LAM	no	2	R	S	S
30671	Chelyabinsk	D435T	S315T	D94G	wt	M306I1	URAL	no	1	R	S	S
2982	Sverdlovsk	D435T	S315T	wt	wt	wt	Beijing	no	2	R	R	S
6121	Sverdlovsk	D435T	S315T	wt	wt	wt	Beijing	no	4	R	S	S
21029	Sverdlovsk	D435T	S315T/T15	wt	wt	wt	Beijing	no	1	R	S	S
334	Tyumen	D435T	S315T/T15	D94G S95T	wt	wt	Beijing	no	2	R	S(92)	S
19983	Sverdlovsk	D435T	S315T	D94G S95T	wt	wt	Beijing	no	8	R	R	S(85)
3320	Orenburg	D435T	S315T	D94G S95T	wt	wt	Beijing	no	4	R	R	S(91)
19046	Sverdlovsk	D435T	S315T	D94A S95T	g37t	M306I3	Beijing	no	4	R	S	S
21022	Sverdlovsk	H526N	S315T	wt	wt	wt	Beijing	I491M	32	R	R	R
20222	Sverdlovsk	H526N	S315T	wt	wt	wt	Beijing	I491M	32	R	R	R
21223	Sverdlovsk	H526N	S315T	wt	wt	M306I1	Beijing	I491M	32	R	R	R
26919	Kurgan	H526N	S315T	wt	wt	M306V	Beijing	I491M	32	R	R	R
28721	Sverdlovsk	H526N	S315T	wt	wt	M306V	Beijing	I491M	32	R	R	R
38028	Sverdlovsk	H526N	S315T	wt	wt	wt	Ural	no	1	S	S	S
41013	Sverdlovsk	H526N	S315T	wt	wt	wt	Ural	no	1	S	S	S
31435	Chelyabinsk	H526N	wt	D94N S95T	wt	wt	Beijing	no	1	S	S	S
5983	Sverdlovsk	H526N	S315T	wt	wt	wt	BeijingB0	no	0,5	S	S	S
24889	Sverdlovsk	H526N	S315T	wt	wt	wt	Beijing B0	no	0,5	S	S	S
17421	Sverdlovsk	H526N	S315T	wt	wt	wt	BeijingB0	no	0,5	S	S	S
18204	Sverdlovsk	H526N	S315T	wt	g10a	wt	BeijingB0	no	1	S	S	S
19021	Sverdlovsk	H526N	S315T	wt	wt	M306I1	BeijingB0	no	0,5	R	S	S
15750	Sverdlovsk	H526N	S315T	A90V	g10a	G406A	BeijingB0	no	0,5	S	S	S
25941	Sverdlovsk	H526N	S315T	A90V	g10a	wt	BeijingB0	no	0,5	S	S	S
22038	Sverdlovsk	H526N	S315T	D94H S95T	wt	wt	BeijingB0	no	0,5	S	S	S
2224	Sverdlovsk	H526N	S315T	D94N S95T	c14t	Q497K	BeijingB0	no	1	S	S	S

Footnotes: * in the number of growth units is indicated in parentheses if it differed from 400

The presence of additional mutations in 12 isolates with Asp435Tyr significantly increased the rifampicin MIC value. On BACTEC MGIT 960, all of these isolates were resistant to 0.5 mg/L, and 10 of them were resistant to 1 mg/L. On Middlebrook 7H11 medium, the MICs ranged from 4-32 mg/L, indicating resistance. All five isolates with both the His445Asn and Ile491Met mutations were resistant to 1 mg/L on BACTEC MGIT 960 and had MICs >32 mg/L (resistant) on Middlebrook 7H11 medium.

IV. Discussion:

Quick and accurate identification of drug resistance in *M. tuberculosis* is essential for improving treatment outcomes and reducing the spread of the disease. In recent years, several molecular diagnostic methods have been developed to enable the rapid detection of rifampicin resistance in *M. tuberculosis* directly from clinical specimens. However, these molecular tests may not detect strains with mutations outside the targeted area (RRDR) and cannot determine the level of rifampicin resistance. Additionally, some studies suggest that strains with *rpoB* mutations may still be susceptible to rifampicin, as confirmed by phenotypic drug susceptibility testing.

In this study, we investigated discrepancies between genotypic and phenotypic rifampicin resistance testing results in *M. tuberculosis* isolates with two borderline mutations. Mutations in the *rpoB* 435 and 445 codons were classified as variable resistance-associated variants as opposite to high resistance-associated variants (Ser450) and low-level resistance mutations (Leu430, Leu452, Ile491). Borderline mutations are often missed by phenotypic methods like BACTEC MGIT, possibly due to slower growth rates [13]–[15]. Our findings indicate that the single Asp435Tyr mutation exhibited a higher level of phenotype resistance compared to His445Asn. Lowering the critical concentration values for MGIT medium to 0,5 mg/L could enhance the alignment of phenotypic and molecular-genetic test results but may not be adequate for accurately distinguishing between sensitive and resistant isolates. However, isolates with additional mutations outside the RRDR region will be consistently identified as resistant.

For isolates harboring the single Asp435Tyr mutation, detection of drug resistance was more accurate on the Middlebrook 7H11 medium than on the BACTEC MGIT 960. This could potentially be attributed to the extended incubation period on Middlebrook media, allowing isolates with a reduced growth rate to manifest their resistant characteristics over a longer period of time.

Isolates carrying the single His445Asn mutation displayed sensitivity on both the Middlebrook 7H11 and BACTEC MGIT 960 media. Decreasing the rifampicin critical concentration on BACTEC MGIT 960 to 0.5 mg/L did not increase the accuracy of determination of phenotypic resistance. Similarly, further reduction of the critical concentration to 0.25 mg/L would not improve result consistency.

Given the low rifampicin MIC values for isolates with the single His445Asn mutation, we suggest considering the potential of rifampicin therapy for patients whose tuberculosis is caused by such *M. tuberculosis*. Successful treatment outcomes with high-dose rifampicin-based regimens in cases of pulmonary tuberculosis involving *M.tuberculosis* with borderline mutation Leu430Pro have been documented[16].

According to WHO catalogue, mutations *rpoB* Asn487Ser and Ile491Met have uncertain significance. However, our findings underscore their role in elevating rifampicin MICs in conjunction with borderline *rpoB* mutations.

Considering the similar mutation profiles and genotypes of isolates with the combination of Asp435Tyr and Asn487Ser, it is plausible that these isolates belong to the same genetic clone, widespread within the Ural region of Russia. A combination of mutations *katG* S315T, *embB* M306V, *eis* c14t and the Beijing genotype can serve as a marker for this variant when conducting studies utilizing the TB-TEST assay.

V. Conclusion:

Our findings highlight the importance of identifying mutations in *M. tuberculosis* isolates outside the RRDR for accurate prediction of phenotypic susceptibility to rifampicin. Understanding the impact of these mutations on Mtb drug resistance can inform the development of more accurate diagnostic tests and effective treatment strategies for tuberculosis.

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