Infection and Drug Resistance

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ORIGINAL RESEARCH

Characterization of Genetic Variants Associated with Rifampicin Resistance Level in *Mycobacterium tuberculosis* Clinical Isolates Collected in Guangzhou Chest Hospital, China

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Objective: Rifampicin (RIF)-resistance, a surrogate marker for multidrug-resistant tuberculosis (TB), is mediated by mutations in the *rpoB* gene. We aimed to investigate the prevalence of mutations pattern in the entire *rpoB* gene of *Mycobacterium tuberculosis* clinical isolates and their association with resistance level to RIF.

Methods: Among 465 clinical isolates collected from the Guangzhou Chest Hospital, drug-susceptibility of 175 confirmed *Mtb* strains was performed via the proportion method and Bactec MGIT 960 system. GeneXpert MTB/RIF and sanger sequencing facilitated in genetic characterization, whereas the MICs of RIF were determined by Alamar blue assay.

Results: We found 150/175 (85.71%) RIF-resistant strains (MIC: 4 to >64 μg/mL) of which 57 were MDR and 81 pre-XDR TB. Genetic analysis identified 17 types of mutations 146/150 (97.33%) within RRDR (codons 426–452) of *rpoB*, mainly at L430 (P), D435 (V, E, G, N), H445 (N, D, Y, R, L), S450 (L, F) and L452 (P). D435V 12/146 (8.2%), H445N 16/146 (10.9%), and S450L 70/146 (47.94%) were the most frequently encountered mutations. Mutations Q432K, M434V, and N437D are rarely identified in RRDR. Deletions at (1284–1289 CCAGCT), (1295–1303 AATTCATGG), and insertion at (1300–1302 TTC) were detected within RRDR of three RIF^R strains for the first time. We detected 47 types of mutations and insertions/deletions (indels) outside the RRDR. Four RIF^R strains were detected with only novel mutations/indels outside the RRDR. Two of the four had (K274Q + C897 del + I491M) and (A286V + L494P), respectively. The other two had (G1687del + P454L) and (TT1835-6 ins + I491L) individually. Compared with phenotypic characterization, diagnostic sensitivities of GeneXpert MTB/RIF and sequencing analysis were 95.33% (143/150), and 100% (150/150) respectively.

Received: 24 May 2022
Accepted: 11 September 2022
Published: 27 September 2022

Conclusion: Our findings underscore the key role of RRDR mutations and the contribution of non-RRDR mutations in rapid molecular diagnosis of RIF^R clinical isolates. Such insights will support early detection of disease and recommend the appropriate anti-TB regimens in high-burden settings.

Keywords: rifampicin-resistance, *Mycobacterium tuberculosis*, *rpoB*, GeneXpert MTB/RIF, resistance-determining region

Introduction

Rifampicin (RIF), a semisynthetic derivative of rifamycin B, discovered in 1965 has a strong bactericidal activity against both active and latent bacilli.[1] Resistance to RIF generally co-occurs with isoniazid (INH) resistance and *Mycobacterium tuberculosis* (*Mtb*) strains resistant to both of these drugs are classified as multidrug-resistant (MDR) TB. RIF-resistant (RIF^R) TB is a proxy of MDR-TB and in 2018, WHO reported around 0.5 million RIF^R cases, of which 78% were MDR-TB.[2] After India, China has the second-highest burden of RIF^R TB and accounts for 14% of the global RIF^R TB cases.[2,3] About 68,200 RIF^R-TB cases were reported during 2015–2019, of which 48.1% were new cases.[4] The number and detection rate of RIF^R-TB incidences are increasing with each passing year, from (10,019 and 14.3%) in 2015 to (18,623 and 28.7%) in 2019.[3,4]

Among the confirmed TB cases reported in 2019, 81.9% of them were identified as resistant to RIF which was a considerable increase from 29.5% in 2015.[4] The epidemic situation of drug-resistant TB in Guangdong province is still a serious challenge. Recently, of the 30,362 strains, the total drug-resistant rate and the mono-RIF^R rate were 26.75% (8121/30,362) and 6.22% (1887/30,362) respectively.[5] Taking this into consideration, it has been observed that RIF^R/MDR-TB is one of the leading causes of morbidity and mortality among infectious diseases around the globe.[2]

RIF targets the DNA-dependent RNA polymerase (RNAP), impeding translocation followed by first phosphodiester bond formation, preventing RNA elongation, and thus inhibiting transcription.[6] RIF^R is mainly associated with mutations in the *rpoB* gene which encodes the β-subunit of RNA polymerase that is required for RNA transcription.[6] About 90–95% of the RIF^R strains harbor mutations within the 81-bp region of *rpoB*, known as RIF-resistance-determining region (RRDR) from codons 426 to 452 in *Mtb* or 507–533 (consensus numbering scheme of RNAP from *Escherichia coli*).[7,8] The remaining ~5–10% RIF^R strains contain mutations in the N-terminal or cluster II region of the *rpoB* or may have unexplored resistance mechanisms.[9]

Phenotypic susceptibility testing requires 2–8 weeks to collect the *Mtb* colonies, which not only causes a diagnostic delay but also hinders TB treatment.[2] WHO endorsed the Mycobacteria Growth Indicator Tube (MGIT) and Xpert MTB/RIF assay for rapid phenotypic and genetic characterizations, respectively.[10] The development of Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) is a breakthrough in molecular diagnosis as it is a fully automated and hemi-nested real-time nucleic acid amplification assay that provides rapid (2-hour) and reliable results using five overlapping molecular beacon probes (A–E) covering the RRDR to identify *Mtb* complex (MTBC) together with the mutations associated with resistance to RIF.[7,11] Xpert MTB/RIF Ultra is a newer version of the Xpert assay.[10] Commercial molecular assays are upgrading the landscape of diagnostic approaches, but these methods detect mutations only within the RRDR.[7,11] A recent survey in Eswatini (formerly known as Swaziland) revealed that up to 30% of MDR-TB had RIF-resistance-related mutation outside the RRDR.[12,13]

Precise assessment of drug susceptibility based on the detection of resistance-conferring mutations in *Mtb* is essential for reliable early diagnosis and to determine effective treatment regimen(s). Yet there are limited data about the prevalence and the obscure role of various *rpoB* mutations leading to RIF resistance in MDR-TB patients in southern China. Using comprehensive phenotypic and genetic characterization, we investigated the distribution and frequency of RIF^R-associated mutations in- and outside the RRDR in clinical isolates collected from Guangzhou, the central city in southern China. This prospective study will be useful in molecular diagnosis to identify the potential role of the newly discovered *rpoB* mutations causing RIF resistance in *Mtb*.

Materials and Methods

Collection of *Mtb* Clinical Isolates

A total of 465 clinical isolates were collected randomly from the patients (age range, 15 to 89 years) registered from September 2019 to November 2021 at Guangzhou Chest Hospital, the biggest specialized TB hospital in southern China. Medical records were further reviewed to categorize the TB patients who experienced poor clinical response based on

previous treatment. Usually, the suspected cases are tested by sputum smear microscopy and culture testing, QuantiFERON–TB Gold In-Tube test (QFT–GIT), Tuberculin Skin Test (TST), and imaging evaluation. If the etiology is not in line with the clinical examination, the patients are given medications to treat common inflammation or lung infections. However, if the general anti-inflammatory drugs are found ineffective and the etiological tests support the diagnosis of TB, the patients are treated with anti-TB drugs while observing the treatment effects. The previously treated drug-resistant TB patients and the new cases with severe TB symptoms and positive etiological tests are admitted to this hospital. All admitted patients get TB culture testing and then drug susceptibility testing (DST) is performed only for those patients which are confirmed by positive TB cultures. *Mtb* species were also confirmed by Ziehl–Neelsen staining and commercial MPB64 monoclonal antibody assay (Genesis, Hangzhou, China).[14] 175 confirmed *Mtb* strains were selected to proceed this study.

Drug Susceptibility Testing of *Mtb* Strains

Phenotypic DST was determined using the proportion method on Löwenstein–Jensen (LJ) medium following previously recommended WHO-approved guidelines.[15–19] The following critical concentrations (CC: μg/mL) for anti-TB drugs were used: INH (0.2), RIF (40.0), ethambutol (2.0), streptomycin (4.0), levofloxacin (3.0), moxifloxacin (2.0), amikacin (40.0), rifabutin 20.0, and prothionamide (40.0). To carry out DST, diluted bacteria were cultured onto LJ medium with and without drugs and incubated at 37°C for 42 days. The critical concentrations were selected as the lowest concentrations of anti-TB drugs that inhibited the 99% in vitro growth of phenotypically susceptible *Mtb* strains. The strain was also considered as resistant to the tested drug when *Mtb* growth rate was ≥1% compared with the drug-free medium.[17–19] Resistance to RIF and pyrazinamide (at 1 and 100 μg/mL, respectively) was also confirmed by Bactec MGIT 960 liquid culture system (Becton Dickinson, Franklin Lake, NJ, USA) according to the manufacturer's instructions and in line with approved guidelines.[15,18] The wild-type *Mtb* H37Rv (ATCC27294^{T}) reference strain was used as a control.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined by microplate-based Alamar blue assay.[20] The bacterial strains were cultured in 7H9 medium supplemented with 10% oleic acid albumin dextrose catalase (OADC, Difco, VWR, Radnor, PA) up to 0.4–0.8 at OD_{600}. The cultures were then diluted to OD_{600}: 0.01 and 100 μL of culture was added per well. Twofold dilutions of the drug were prepared in 7H9 broth in 96-well plates and the gradient concentrations of RIF ranging from 0.5 to 64 μg/mL were used to access MIC. Seven days post-incubation at 37°C, Alamar blue solution (Alamar Bio-sciences/Accumed, Westlake, OH) 10% of the total volume of the well was added to each well, and the plates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of drug that prevented the change in color.

GeneXpert MTB/RIF Assay

To detect RIF^{R} *Mtb* due to mutations in RRDR, Xpert MTB/RIF assay was performed using the first/older version of GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) and results were read following the manufacturer's instructions.[10,21] Briefly, the sample reagent was mixed according to 3:1 to 0.5 mL of decontaminated specimen. The tube was manually stirred twice during incubation for 15 min at room temperature and then transferred 2 mL of the reagent-sample mixture to the Xpert-test cartridge. The cartridges containing this mixture were placed in the Xpert machine and the results readouts were obtained after 90 min of the fully automated process. The probes in the Xpert system hybridize to the sequence of RRDR of *rpoB*: A (codons 507–511), B (codons 512–518), C (codons 518–523), D (codons 523–529) and E (codons 529–533).[11] If at least two of the five probes exhibit a cycle threshold (Ct) ≤38, it indicates the presence of MTBC. Whereas the strain displaying no hybridization of one or more probes or when the variance between first and last Ct is >3.5 is recognized as RIF^{R} *Mtb*.[11,22]

Genetic Analysis of *rpoB* Gene

Genomic DNA was extracted from *Mtb* cultures using MagMAX Total Nucleic Acid Isolation Kit (Ambion, Life Technologies, NY, USA). The purified DNA was quantified with the NanoDropND-1000 spectrophotometer (NanoDrop Technologies). The complete 3519-bp long *rpoB* gene was amplified in RIF^{S} and RIF^{R} *Mtb* strains using

the two sets of primers designed for this study (*rpoB* F1: 5'–ACTTGACACCGTGGTCTTAG–3', *rpoB* R1: 5'–CGAGACGTCCATGTAGTCCAC–3'), covering 1098-bp (including 180-bp of the upstream region) and (*rpoB* F2: 5'–ATCGAAACGCCGTACCGCAAG–3', *rpoB B* R2: 5'–GACCGATGCGGAGTTCATCG– 3') 2088-bp (including 213-bp of the first part of the gene and 84-bp of the downstream region to avoid the omission during sequencing analysis), to detect the presence of resistance-associated mutations in- and outside the RRDR. PCR products were examined on 1.0% agarose gels, purified by PCR purification kit (Qiagen, Hilden, Germany), and sequences were determined via standard Sanger DNA sequencing on a ABI3730XL genetic analyzer (BGI, Guangzhou, China). Sequencing data were aligned with the reference sequence of wild-type *Mtb* H37Rv strain (GenBank accession number: NC_000962.3) using the software BioEdit version 7.2.6.1. The codon numbers were reported according to the *Mtb rpoB* gene numbering system (TubercuList: http://genolist.pasteur.fr/TubercuList/).

Statistical Analysis

The sensitivity, specificity and their 95% confidence intervals (CIs) were calculated, and the data were treated statistically by using MEDCALC statistical software (https:/www.medcalc.org/calc/diagnostic_test.php).

Results

Demographic Features

Among the 175 *Mtb* clinical isolates in this study, the proportion of TB cases was higher in male patients 115/175 (65.71%), while 60/175 (34.28%) were in female patients. The maximum number of positive *Mtb* cases 87/175 (49.71%) were from the age group of 45–65-year-old patients[2,7,23] with the highest number 74/150 (49.33%) of RIF^R TB patients. Moreover, most of the *Mtb* isolates 105/175 (60.00%) analyzed in this study were from retreated patients of TB, whereas 70/175 (40.00%) were listed as new TB cases (Table 1).

Drug Susceptibility Profile of *Mtb* Isolates

Of the 175 confirmed TB strains, 150/175 (85.71%) were identified as resistant and 25/175 (14.28%) were susceptible to RIF. Among them, 57 MDR and 81 pre-XDR *Mtb* strains were detected cumulatively covering 138/175 (78.85%) of the isolates. Four strains were mono-resistant to RIF, while the remaining 8/150 (5.33%) RIF^R strains possessed different

Table 1 Demographic Features of 175 *Mtb* Clinical Isolates

Characteristics	No. of Clinical Isolates (%)		OR (95% CI)	*P*-value
	RIF^R (n= 150)	RIF^S (n= 25)		
Gender				
Male	98 (65.33)	17 (68.00)	0.88 (0.35–2.19)	0.79
Female	52 (34.66)	8 (32.00)	1.00 (Ref.)	–
Age group				
<25	7 (4.66)	2 (8.00)	0.90 (0.15–5.21)	0.90
25–45	31 (20.66)	8 (32.00)	1.00 (Ref.)	–
45–65	74 (49.33)	13 (52.00)	1.46 (0.55–3.89)	0.43
>65	38 (25.33)	2 (8.00)	4.90 (0.96–24.78)	0.05
Treatment history				
New Case	59 (39.33)	11 (44.00)	1.00 (Ref.)	–
Retreated	91 (60.66)	14 (56.00)	1.21 (0.51–2.84)	0.65

Abbreviations: RIF^R, rifampicin-resistant; RIF^S, rifampicin-susceptible; OR, odds ratio; CI, confidence intervals.

patterns of drug resistance. In this study, the number of previously treated RIF^R *Mtb* patients 91/150 (60.66%) was considerably higher compared to the treatment-naive cases 59/150 (39.33%).

Genetic Characterization of *rpoB* in *Mtb*

The genetic testing of *Mtb* strains was determined by the detection of resistance-conferring mutations in *rpoB* gene. Besides the mutations in RRDR, we also identified the nonsynonymous mutations outside the RRDR which remain undetected by GeneXpert MTB/RIF assay. 150/150 (100%) RIF^R strains contained resistance-conferring mutations or indels in *rpoB* gene. Sixty-seven different types of mutations were detected in the entire region of *rpoB*, comprising 46 nonsynonymous mutations, 13 synonymous mutations, 4 deletions, and 4 insertions.

RRDR of *rpoB*

Interestingly, 146/150 (97.33%) RIF^R isolates harbored single or multiple mutations/indels at codons from 426 to 452 of the *rpoB* in RIF^R strains, of which 143/150 (95.33%) were covered by Xpert probes while 3/150 (2%) were only detected by sequencing of whole *rpoB* gene. Seventeen different types of nonsynonymous mutations were identified within the RRDR in RIF^R *Mtb* strains (Figure 1). Most of the mutations were detected at codons L430 (P), D435 (V, E, G, N), N437 (D), H445 (N, D, Y, R, L), and S450 (L, F). The most frequently encountered mutations were D435V 12/146 (8.2%), H445N 16/146 (10.9%), and S450L 70/146 (47.94%) respectively. Compared to these substitutions, other RIF^R-conferring *rpoB* mutations were present at lower frequencies. Amino acid substitutions Q432K (3/146; 2.05%), M434V (2/146; 1.36%), N437D (5/146; 3.42%), R448L (3/146; 2.05%) and L452P (3/146; 2.05%) are very rarely

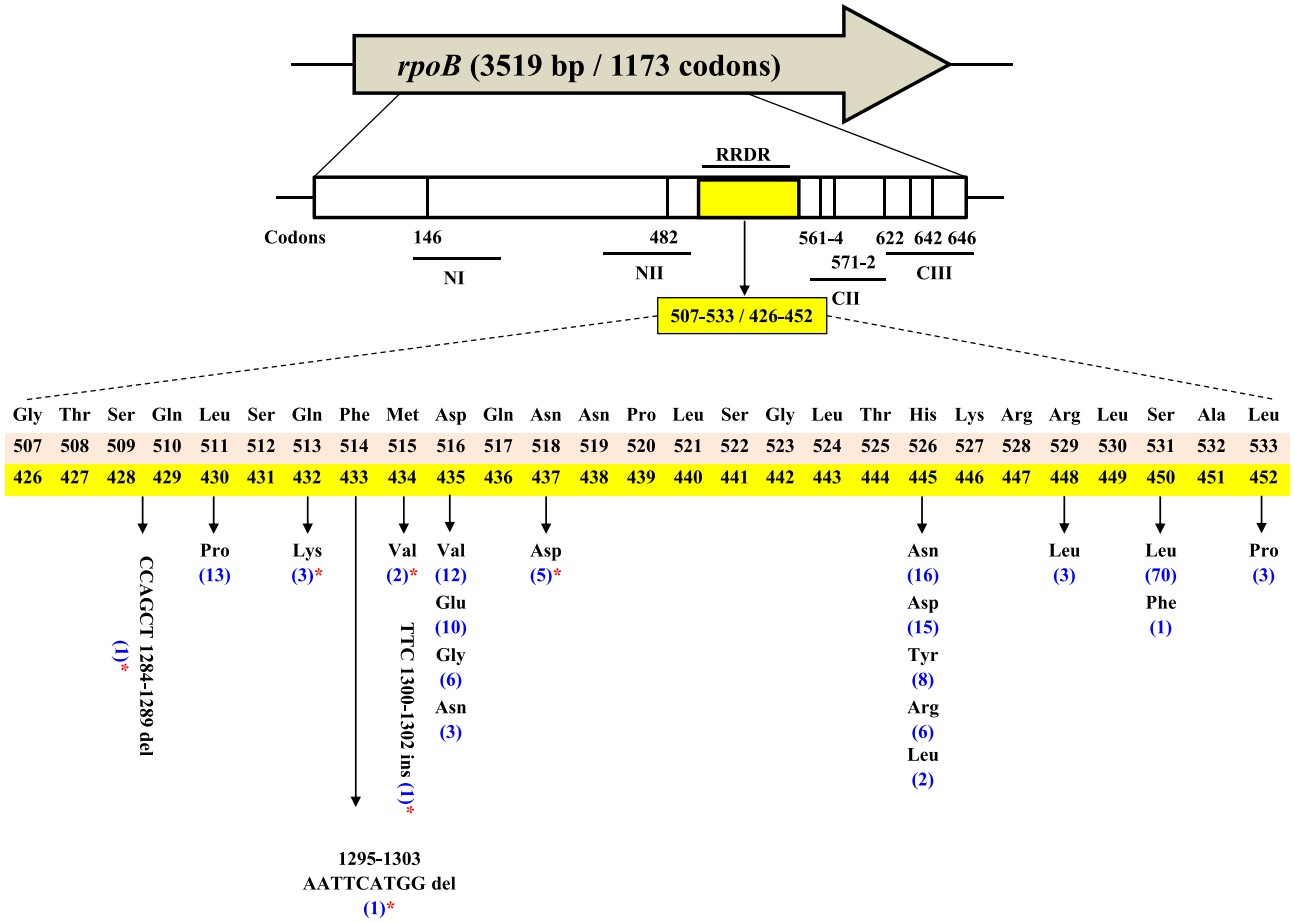

Figure 1 Mutations and indels in the RRDR of *rpoB* (507–533) *E. coli* numbering system; (426–452) *Mtb* numbering system; *Novel and rarely detected mutations and indels in RIF^R strains in this study; Number of *Mtb* strains containing *rpoB* mutations are shown in brackets.

detected in RRDR but found in our study in 16 RIF^R strains. Whereas 9/150 (6%) strains were identified with indels along with the mutations at codons 435, 445, or 450. By sequencing of whole *rpoB* gene, the insertion of an amino acid TTC at 1300–1302 (F434) without any other amino acid substitution is identified the first time in this study. Similarly, two phenotypically RIF^R strains which also remained obscure by Xpert probes were found with the deletion of CCAGCT at 1284–1289 and AATTCATGG at 1295–1303 nucleotide positions for the first time in RRDR of *rpoB*.

Non-RRDR of *rpoB*

The sequencing results of the complete *rpoB* gene also showed 47 different types of non-RRDR mutations/indels (including 13 synonymous mutations) in *rpoB* (Figure 2). Among them, 27 nonsynonymous mutations/indels were observed only in resistant strains, two (R1162H, A1166T) were present in both resistant and susceptible strains, and five in only susceptible strains. Whereas I491 (L, M), P541 (T, K, Q), N673 (S), and A1166 (T) were frequently encountered non-RRDR mutations. Nonsynonymous mutations outside the RRDR of RIF^R isolates were mostly found with mutations inside the RRDR which might be the reason for the increased level of RIF resistance in these strains.

It was interesting that the association of four *Mtb* strains with RIF resistance was not established when evaluated by Xpert MTB/RIF assay, though these strains were phenotypically resistant to RIF, but sequencing analysis of the complete region of *rpoB* revealed that missense mutations/indels were located only outside the RRDR which cannot be covered by Xpert probes (Figure 2). Two of them were detected with the novel nonsynonymous mutations (K274Q + C897 del + I491M + A1075A) and (A286V + L494P + A1075A), respectively. Of the remaining two strains, one showed deletion of G nucleotide at position 1687 along with P454L mutation and another showed the insertion of TT nucleotides at 1835–6 position + I491L + A1075A. The

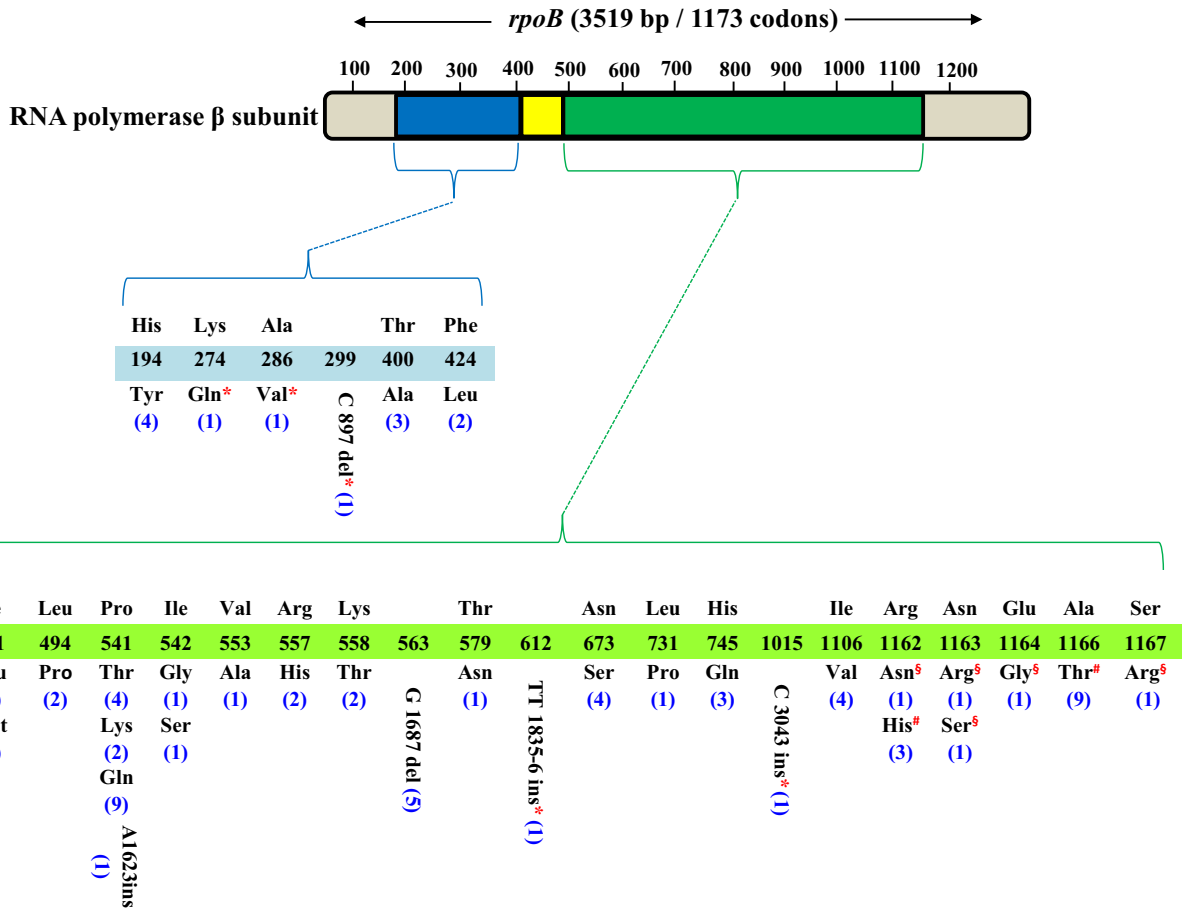

Figure 2 Mutations and indels outside the RRDR of *rpoB*. *Novel and rarely detected mutations and indels in RIF^R strains in this study; #Nonsynonymous mutations in both RIF^R and RIF^S strains; §Nonsynonymous mutations only in RIF^S strains; Number of *Mtb* strains containing *rpoB* mutations are shown in brackets.

synonymous mutations A1075A and L1160L were present in most of the RIF^R and RIF^S strains. Besides, 25/175 (14.28%) RIF^S strains presented synonymous mutations, ineffectual substitutions or wild-type *rpoB* gene.

Association Between *rpoB* Mutations and RIF Resistance Level

Minimum inhibitory concentration (MIC) testing indicated that mutations/indels in the RRDR of *rpoB* were mainly associated with resistance to RIF (CC > 1 µg/mL), with MICs ranging from 4 to >64 µg/mL. Interestingly, RIF^R strains having double RRDR mutations or both RRDR and non-RRDR mutations together showed higher MIC levels compared to the strains with only a single mutation in RRDR (Table S1). Mainly, RIF^R strains having mutations at codons 445 and S450L mutation developed a higher level of resistance. All RIF^S isolates exhibited MICs of <0.5 µg/mL. Generally, there is a direct connotation between the distance of mutated amino acids at the drug-binding site and the level of drug resistance. The mutations mainly responsible for higher levels of RIF resistance in *Mtb* are found at exactly or near to RIF-binding pocket (RBP) (Figure 3). However, some frequently detected non-RRDR mutations are also marked in Figure 3. The presence of these mutations in variant distant regions facilitates to understand their effect on RIF binding and the resistance level.

Assessment of Xpert and DNA Sequencing Results

To evaluate the performance of molecular methods in the prediction of RIF resistance, the phenotypic and genotypic data for 175 *Mtb* isolates were compared statistically. Considering the phenotypic outcomes as a reference, detection of RIF

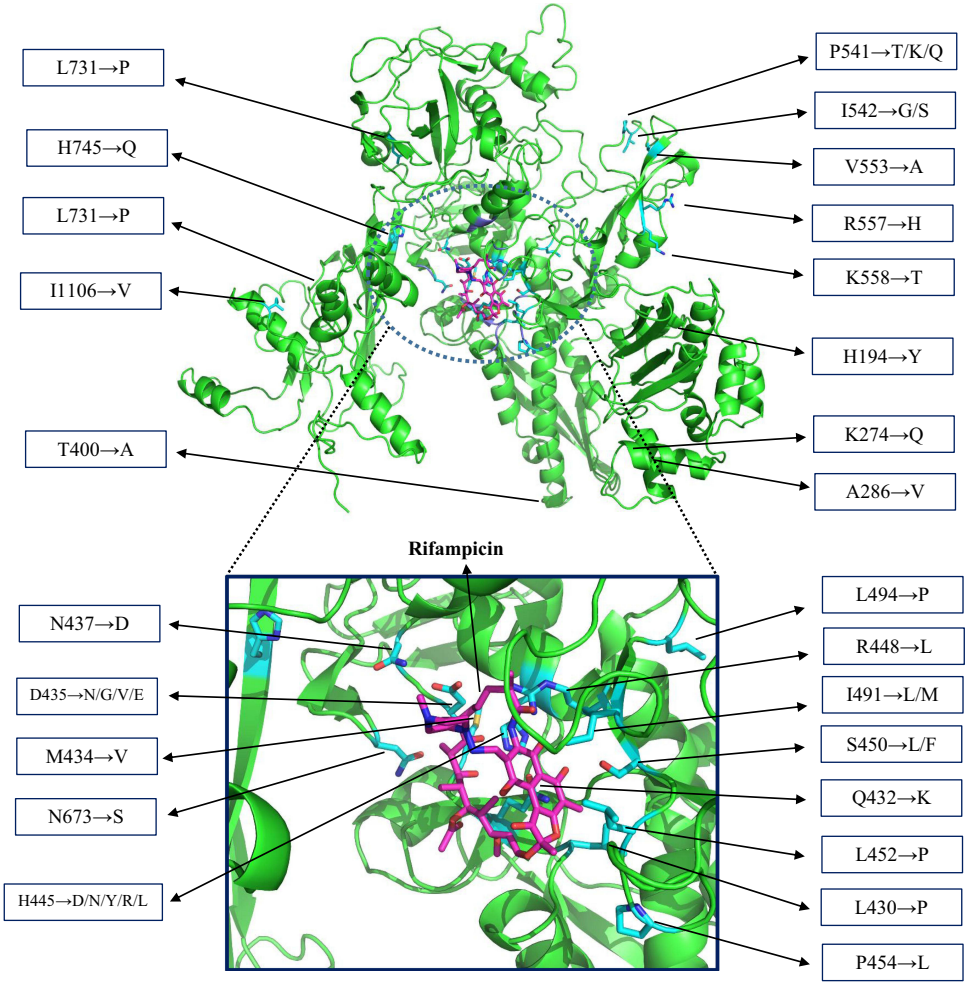

Figure 3 Locations of mutations and binding site residues in RpoB.

Table 2 Evaluation of Molecular Susceptibility Testing Methods

Methods	RIF^R Isolates n = 150		RIF^S Isolates n = 25		Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% CI)
	Resistance-Associated Nonsynonymous Mutations (%)	Wild-Type/ Synonymous Mutations (%)	Resistance-Associated Nonsynonymous Mutations (%)	Wild-Type/ Non-Resistant /Synonymous Mutations (%)			
Xpert probes	143 (95.33)	7 (4.66)	0 (0.0)	25 (100)	95.33 (90.62–98.10)	100 (86.28–100)	96.00 (91.93–98.38)
rpoB^E	150 (100.00)	0 (0.0)	0 (0.0)	25 (100)	100 (97.57–100)	100 (86.28–100)	100 (97.91–100)

Notes: Xpert probes: RIF^R *Mtb* strains detected by Xpert probes of GeneXpert MTB/RIF system covering the RRDR of *rpoB*; *rpoB*^{E}: RIF^R *Mtb* strains detected by resistance-associated mutations via sequencing analysis of the entire region of the *rpoB* gene.
Abbreviations: RIF^R, rifampicin-resistant; RIF^S, rifampicin-susceptible.

resistance by Xpert assay showed a sensitivity of 95.33% (95% CI: 90.62–98.10%) and a specificity of 100% (95% CI: 86.28–100.00%) with the accuracy of 96.0% (95% CI: 91.93–98.38%). Whereas the sequencing analysis of the entire region of *rpoB* to detect resistance-conferring mutations exhibited a sensitivity of 100% (95% CI: 97.57–100%) and the specificity of 100% (95% CI: 86.28–100.00%) showing the accuracy of 100% (95% CI: 97.91–100%) (Table 2). Our analysis showed that covering the RRDR of *rpoB* by the Xpert system provides sensitivity (95.33%) almost similar to the entire *rpoB* gene sequencing (100%) with maximum accuracy of the tests regarding phenotypic resistance. However, Xpert system remained unresponsive to detect the RIF resistance of seven *Mtb* strains which later were clarified by sequencing of the whole *rpoB* gene. Of these, 3/7 were found with indels within RRDR and 4/7 had only non-RRDR mutations.

Discussion

RIF resistance is a surrogate marker for MDR-TB[2,7,23] and the emergence of RIF^R/MDR-TB is a serious public health problem. The lack of rapid TB diagnostic facilities and inappropriate treatment regimens for TB therapy are the key reasons for the emergence of RIF^R/ MDR-TB. The exploration of molecular-level performance is essential to identify the RIF^R-related *rpoB* mutations for better interpretation of phenotypic results and to continue the development of improved diagnostic assays. To investigate the factors associated with resistance to RIF among sputum smear and culture-positive new and previously treated drug-resistant TB patients have been scrutinized in various studies through genetic testing by commercially available molecular diagnostic approaches and sequencing analysis.[24–27] Based on phenotypic and genetic characterization, the identification of 85.71% RIF^R strains in the current study is higher than the study from Zhejiang (77.7%),[25] and Vietnam (44.57%)[26] but lower than that from Wuhan (96.71%)[27] and South Korea (98.15%).[20] The early diagnosis of TB and the rapid detection of resistance to anti-TB drugs are indispensable for the effective treatment and constrain the emergence of MDR-TB. Herein, we provide the detailed insights to comprehend the role of nonsynonymous mutations in- and outside of the RRDR of *rpoB* on RIF susceptibility.

In our study, 71/146 (48.63%) of RIF^R RRDR mutants harbored mutations at codon 450 alone and with other mutations within the *rpoB* gene, whereas mutation S450L ranked first (70/146; 47.94%) in the data associated with high RIF resistance level. This endorsed an earlier report that codon 450 (531 in the *E. coli* numbering system) is the most frequently mutated in the *rpoB* gene.[28] However, the frequency of S450L mutation can be different in various settings around the world, such as, Kazakhstan (80.9%),[29] Bangladesh (69.4%),[22] Taiwan (66.7%),[30] China (60.9%),[31] India (57.81%),[32] South Africa (56%),[33] South Korea (53.1%),[24] Brazil (52.4%),[34] Russia (47%)[35] and Vietnam (37.8%).[26]

Besides, the next most abundant RRDR mutations occurred in the present study at codons positions 430 (13/146; 8.90%), 435 (31/146; 21.23%), and 445 (47/146; 32.19%) respectively in RIF^R strains. The mutation frequencies at these codons have been reported within the ranges of 1.1–20.4%, 6.8–32%, and 0–8%, respectively.[24,29,36–38] Our study elucidated that S450L, followed by H445N and D435V were the most common mutations for both RIF mono-resistant and MDR strains. The variances in the *rpoB* codons substitution and the frequency of *rpoB* mutations can have adverse

effects on RNAP functions and DNA transcription. Therefore, the mutations at codons 430, 435, 445, and 452 have been associated with relapse or treatment failure in clinical settings.[37]

Genetic analysis in our study revealed 17 different types of mutations within RRDR in 146/150 (97.33%) RIF^R *Mtb* strains which have been shown to confer resistance to RIF.[24,37,39] Interestingly, the rarely detected RRDR mutations Q432K, M434V, and N437D in RIF^R strains can also act as potential RIF resistance determinants consistent with an earlier report where mutations at codon 432 occurred exclusively in high-level RIF^R strains.[40] Moreover, the deletions at (1284–1289 CCAGCT), (1295–1303 AATTCATGG), and insertion at (1300–1302 TTC) were also detected within RRDR in three different RIF^R strains first time in this study. These deletions and insertion were possibly involved in the impairment of RBP and therefore played a key role in the development of RIF resistance, as no other mutation was located in these resistant strains. Forty-seven different types of mutations were observed outside the RRDR, 13 of these mutations were synonymous, two of which D103D and A1075A were commonly documented in prior studies.[38] Synonymous mutation A1075A has been associated with the Beijing genotype,[41] and earlier reports confirmed the abundance of Beijing genotype in this region[15,42]; therefore, A1075A was observed in both RIF^S and RIF^R isolates.

Four RIF^R strains remained obscure by Xpert MTB/RIF assay as it covers only RRDR of the *rpoB*. However, sequencing analysis of the entire *rpoB* gene revealed new mutations outside the RRDR. Among these four strains, two resistant strains conserved the nonsynonymous mutations and the other two strains, one had G1687 del along with (P454L + F248F + I271I + F294F) mutations, and the second was found with TT ins at 1835–6 position + I491L + A1075A. The role of *rpoB* mutations at codon 491 in developing RIF resistance has been investigated,[12,40] and we found two different types of amino acid substitutions at this position, I491L in four and I491M in two MDR strains respectively. These four RIF^R strains without RRDR mutations were initially classified as RIF^R at CC (1 µg/mL) which were later confirmed by MIC determination assay where they were still able to grow at 16 µg/mL of RIF. Though, similar to earlier reports,[40] the other nonsynonymous mutations outside the RRDR in RIF^R strains co-existed with RRDR mutations; however, an increased level of RIF MIC was observed in these strains in comparison with those RIF^R strains that possessed mutations only at codons 435, 445 and 450. Thus, the presence of these non-RRDR mutations only in RIF^R strains and their influence on RIF MIC throws light on the potential role of non-RRDR mutations in conferring RIF resistance in *Mtb* strains.

Compared to other studies, we have detected a wide range of different types of mutations associated with the diverse range of resistance. In particular, mutations at codons 445 and 450 have consistently been associated with high levels of RIF resistance.[30] Also, the MIC values for other RRDR mutants were somewhat lower than those having S450L mutations, but, higher than the previous reports of low levels of RIF resistance due to mutations at codons 430 and 435, particularly D435Y.[43] The type and frequency of RIF^R-related *rpoB* mutations may vary by settings and generally H445N and L430P mutations are associated with low-level RIF resistance. The mutations detected alone have been linked with low RIF MICs but showed higher levels of RIF resistance when multiple types of mutations were detected.[44] Likewise, H445N and L430P mutants in the current study showed lower level of RIF resistance compared to those strains containing these mutations accompanied by high RIF^R associated mutation (eg, S450L). A similar phenomenon was observed in another study where L430P RIF^R mutant showed the MIC of 4 µg/mL but the MIC of a RIF^R strain increased up to 32 µg/mL containing L430P mutation along with D435G.[40] This indicates that H445N and L430P mutations alone are involved in low-level RIF resistance and only influence the MIC when co-exist with additional mutations responsible for high RIF resistance. Low-level RIF resistance causes diagnostic and treatment challenges and the data related to the low-level RIF^R TB incidences are still lacking, so more comprehensive studies with a wider pool of isolates will facilitate to overcome such challenges.

Considering the structural relatedness and similar modes of action, it is assumed that all rifamycins hold common resistance mechanisms, causing a cross-resistance among all drugs of this group. This cross-resistance occurs, but strains resistant to RIF and susceptible to other rifamycins have been previously explained.[45,46] In the current study, ~74.66% of RIF^R strains were cross-resistant to rifabutin comparable to the other reports, where cross-resistance between the two drugs was observed from 72.2% to 85.4% of RIF^R strains.[45,46] Notably, the pattern of RIF^R/rifabutin-susceptible has been associated with certain *rpoB* mutations, including M434I, D435Y, D435V, or H445L[45,46] which was consistent with our findings, thus the presence of these mutations in clinical isolates may confirm not only RIF resistance but also indicates the susceptibility to rifabutin.

Resistance to RIF is generally caused by mutations in the RBP of RNAP leading to loss in drug affinity, and there is a strong association between the resistance level of RIF and the distance of the mutated residues to the drug-binding site in the RpoB.[47] Therefore, majority (97.33%) of our detected mutations in the RRDR of *rpoB* have an obvious effect on RIF resistance, because most of the affected residues are located in the RBP site. Interestingly, similar to previous studies[40,48] some mutations (R1162H, A1166T) were observed in both RIF^S and RIF^R isolates and R1162N, N1163R, N1163S, E1164G, S1167R substitutions only in RIF^S isolates. These mutations were considered ineffectual on the interaction between RIF and RBP, therefore not involved in the development of RIF resistance. Considering previous observations, it was anticipated that such mutations may act as compensatory mutations to alleviate fitness impairment acquired by other mutations directly associated with drug resistance.[40,48] However, the number of isolates harboring certain types of *rpoB* mutations was limited. Further investigation through mechanistic approaches will clarify the effects of non-RRDR mutations on the interaction between RpoB and RIF in susceptible and resistant strains, particularly when the patient's diagnostic or treatment outcomes are not as expected. The RIF resistance level is important as some drugs may convert the low-level RIF-resistance into RIF-susceptible and the patients could be cured more easily.

Conclusions

In conclusion, this study revealed comprehensive phenotypic and genetic profiles of RIF^R *Mtb* clinical isolates. However, evaluation of non-RRDR of *rpoB* reveals the unnoticed resistance-associated nonsynonymous mutations for the detection of false-susceptible strains and the patients with false-negative results most likely receive the treatment therapy for drug-susceptible TB and tend to have poor treatment outcome. However, we observed that assessing mutations outside the RDRR contributed only ~5% increase in the diagnostic sensitivity because Xpert probes covering the RRDR showed ~95% diagnostic sensitivity. Hence, improving the access of WHO-endorsed GeneXpert MTB/RIF could be useful for rapid molecular diagnosis of RIF resistance in *Mtb*. Lastly, resistance-associated mutations in- and outside the RRDR of *rpoB* highlight their role as potential diagnostic resistance markers and possible target sites for RIF and other rifamycins in *Mtb*. These findings may facilitate in designing the new probes for various alleles associated with RIF resistance to increase the sensitivity of molecular diagnostic methods for *Mtb* isolates in different geographic settings and also to understand RIF resistance for developing precise TB therapy for MDR-TB patients.

Ethical Approval

This study followed WHO guidelines and was approved by the ethics committee of Guangzhou Chest Hospital (GZXK-2019–21).

Funding

This work was supported by the National Key R&D Program of China (2021YFA1300904), partially by the National Natural Science Foundation of China (NSFC 81973372), Joint Research the Russian Science Foundation (RSF)-NSFC Collaboration grant numbers 21-45-00018 (to D.M.), 82061138019 (to T.Z.), the Joint Research Health Research Council of New Zealand (HRC)-NSFC Collaboration grant numbers 20/1211 (to G.C.), 82061128001 (to T.Z.), supported by China-New Zealand Joint Laboratory on Biomedicine and Health, Department of Science and Technology of Guangdong Province (2019B110233003), the Chinese Academy of Sciences (154144KYSB20190005, YJKYYQ20210026), State Key Laboratory of Respiratory Disease (SKLRD-OP-202324 to Tianyu Zhang), and China Postdoctoral Fellowship, Guangdong province, Huangpu, GIBH-University of Chinese Academy of Sciences, President's International Fellowship Initiative-CAS and State Key Laboratory of Respiratory Disease(SKLRD-Z-202301 to H.M. Adnan Hameed). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure

Dr Dmitry A Maslov reports grants from Russian Science Foundation, during the conduct of the study. All authors approved the study to publish in your esteemed journal and declare no competing interests.

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