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Rifabutin and Rifampin Resistance Levels and Associated *rpoB* Mutations in Clinical Isolates of *Mycobacterium tuberculosis* Complex

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Abstract

Cross-resistance in rifamycins has been observed in rifampin (RIF)-resistant *Mycobacterium tuberculosis* complex isolates; some *rpoB* mutations do not confer broad *in vitro* rifamycin resistance. We examined 164 isolates, of which 102 were RIF-resistant, for differential resistance between RIF and rifabutin (RFB). A total of 42 unique single mutations or combinations of mutations were detected. The number of unique mutations identified exceeded that reported in any previous study. RFB and RIF MICs up to 8 μg/ml by MGIT 960 were studied; the cut-off values for susceptibility to RIF and RFB were 1 μg/ml and 0.5 μg/ml, respectively. We identified 31 isolates resistant to RIF but susceptible to RFB with the mutations, D516V, D516F, 518 deletion, S522L, H526A, H526C, H526G, H526L and two dual mutations (S522L+K527R and H526S +K527R). Clinical investigations using RFB to treat MDR TB cases harboring those mutations are recommended.

INTRODUCTION

Multidrug-resistant tuberculosis (MDR-TB), defined as disease caused by strains of *Mycobacterium tuberculosis* complex (MTBC) that are resistant to isoniazid and rifampin (RIF), is a major obstacle to the treatment and control of tuberculosis (TB) globally (1). The concern over MDR-TB has necessitated not only initiatives to improve diagnostic testing capabilities and more efficient detection of drug resistance; it has also prompted a search for

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alternative drug options for treating MDR-TB. Rifamycin drugs are generally very effective against MTBC, with RIF serving as an important primary drug in the treatment arsenal for TB. Another drug in this class, rifabutin (RFB), has fewer interactions with protease inhibitor drugs compared to RIF, and is often used to treat *Mycobacterium avium* complex and MTBC in HIV-infected patients, but is not commonly used as a first-line treatment for TB. Although the effectiveness of RFB in treating patients with drug-susceptible TB has been demonstrated, documentation of successful treatment of MDR-TB patients with RFB, even in patients whose isolates are susceptible *in vitro* to RFB, is limited (2 – 5). This may partly be due to general concerns about potential cross-resistance among the rifamycins and the fact that clinical efficacy of RFB for treatment of RIF-resistant strains in MDR-TB patients has not yet been well-established (2, 6 – 10).

Certain mutations in the RIF resistance determining region (RRDR) of the *rpoB* gene of MTBC appear to confer cross-resistance to both RIF and RFB (11 – 21). The mutations S531L, H526Y and H526D are most common and are found in isolates from a majority of MDR-TB patients. However, RIF-resistant strains possessing certain mutations, mostly in codons 511, 516, 518, 522, 526, 533 may retain a level of *in vitro* susceptibility to RFB depending on the particular amino acid substitutions and have the potential to be clinically effective against RIF-resistant MTBC strains (11 – 21). Utilization of molecular assays capable of discriminating SNPs in the RRDR at the nucleotide level may allow for the prediction of culture-based drug susceptibility testing (CDST) results to RIF and RFB (14 – 15, 21 – 24). Owing to increased use of molecular diagnostics for detection of RIF resistance, an understanding of the relationship between different *rpoB* mutations and their association with differential resistance levels may be helpful to clinicians treating RIF-resistant TB or MDR-TB as a real-time complement to CDST which can take weeks to be completed.

While determination of RIF and RFB MICs in association with *rpoB* mutations has been investigated, most of those studies included either relatively low numbers of isolates or commonly encountered mutations (11 – 16, 18 – 21). California has the most TB and MDR-TB cases in the US. Our laboratory has used molecular methods to test clinical specimens or cultures for drug resistance mutations including those in RRDR since 2003. We have found that *rpoB* mutations are associated with a wide range of RIF MICs and that not all *rpoB* mutations confer RIF resistance as measured by a liquid culture-based standard phenotypic DST method, MGIT 960 (Becton Dickinson Diagnostic Systems, Sparks, MD). In this study, we determined RFB and RIF MICs using MGIT 960 on isolates with various *rpoB* mutations from our archived collection. All the MIC data were generated by the same CDST method and allowed a head-to-head comparison of the effect of a mutation on MIC changes for the two drugs. The goal of this study is to strengthen our understanding of and confidence in the role of *rpoB* mutations in differential resistance to RIF and RFB, and to lay a foundation for building a diverse collection of genotype/phenotype data to support the establishment of clinical trials to determine if the observed *in vitro* differential resistance can be used to guide treatment.

MATERIALS AND METHODS

Bacterial isolates

A total of 164 MTBC isolates (102 RIF-resistant and 62 RIF-susceptible) were included in the study. Of the 102 RIF-resistant isolates, 71 were from TB patients from California (including many global immigrants) and 31 were contributed by India (Mumbai), Philippines (Manila) and across South Africa and Moldova through the Global Consortium for Drug-resistant TB Diagnostics (GCDD) (25). RIF-resistant isolates were selected to include a wide variety of *rpoB* mutations as determined by a well-established pyrosequencing assay (23); 41 of them with known RFB results previously determined by the agar proportion. We randomly selected 42 susceptible strains that had wild-type *rpoB* sequences. We also included 20 strains which had *rpoB* mutations but tested RIF-susceptible by MGIT 960.

Drug solutions

RFB was obtained from Sigma-Aldrich (St. Louis, MO) and US Pharmacopeia (Rockville, MD), and RIF from US Pharmacopeia. The drugs were dissolved in HPLC-grade absolute methanol into 16 μg/ml or 32 μg/ml stock solutions for RFB and RIF, respectively, and then filter-sterilized using 0.22 μm filters (VWR). The stock solutions were aliquoted in small volumes and stored at −70°C. The 8 μg/ml concentration was diluted in methanol from the stock solution, and the lower concentrations were made in sterile, deionized water for both drugs. These dilutions were frozen at −20°C in small aliquots for future use, and they were limited to three freeze-thaw cycles before being discarded. Note: all the concentrations were expressed as the test concentrations with MGIT 960.

Testing for MIC

MICs of RIF and RFB were determined by MGIT 960. Isolates were sub-cultured onto Löwenstein-Jensen (LJ) slants (Becton Dickinson, Sparks, MD) and used for testing within 4 weeks of inoculation. The manufacturer's instructions for testing primary drugs of isolates growing on solid media were followed with a modification. Instead of a 1:5 dilution, a 1:3 dilution of the cell suspension equivalent to the 0.5 McFarland standard was used to inoculate the drug-containing MGIT. This modification yielded a 100% correlation with a 15 to 24 h shortened turnaround time when compared with the unmodified procedure (unpublished data). All 164 isolates were tested for RFB MICs from 0.0625 to 8 μg/ml. We also tested RIF MIC from 0.125 to 8 μg/ml on a subset of 91 isolates from the 164 isolates; 87 isolates comprised of strains with representative *rpoB* mutations and 4 isolates were of wildtype *rpoB* sequence. As there is no published WHO critical concentration for determining whether an isolate is "resistant" to RFB, we used a breakpoint concentration of 0.5 μg/ml, as suggested in multiple previous studies (13, 26 – 30). The critical concentration for RIF was 1.0 μg/ml, as per manufacturer's protocol and the WHO published standard (26, 31).

Pyrosequencing

The *rpoB* sequence from codons 507 to 533 was determined for each isolate by pyrosequencing. It was performed as described previously (23) using the Qiagen PyroMark Q96 ID system (Qiagen, Valenicia, CA).

Quality control (QC)

For the QC strain of *M. tuberculosis*, H37Rv (ATCC 27294), MIC of RIF was tested from 0.0625 to 0.5 μg/ml and that of RFB was tested from 0.0312 to 0.125 μg/ml for each batch of CDST or once a week if more than one run was performed. This was to assess drug performance with each drug dilution lot and to demonstrate that the potency of RIF and RFB was maintained properly during the study. For the QC of pyrosequencing, the QC reference strain and PCR-grade water were included in each run. If the expected values for the controls were not obtained, the test run was repeated.

RESULTS

Determination of the RRDR sequences

The *rpoB* gene of all isolates was successfully sequenced from codon 507 through 533 using pyrosequencing. We identified a total of 42 mutations including 24 unique single nucleotide substitutions, three deletions involving single or multiple codons, 14 multiple mutations and one synonymous mutations (Table 1). The 31 isolates from global sources contributed 13 unique mutations; five of them were new to our laboratory including the H526C mutation and 4 other mutations involving multiple codons. Of the 42 mutations, 24 (57%) were not found in the TB Drug Resistance Mutation Database (www.tbdreamdb.com) (32), although we were unable to verify if all of them were novel mutations. In addition, there were 42 RIF-susceptible isolates with wildtype sequences that were exact matches to the QC strain.

RIF and RFB MICs of the QC strain

We tested RIF MIC for the QC strain with the same cell suspension in parallel using RIF from the MGIT 960 SIRE kit (Becton Dickinson Diagnostic Systems, Sparks, MD) and from US Pharmacopeia to ensure the RIF powder from US Pharmacopeia would perform equally with the RIF from the MGIT 960 SIRE kit, which is routinely used with MGIT 960. We found RIF MIC consistently tested at 0.125 μg/ml for the QC strain using RIF from either source. We also performed RFB MIC for the QC strain using RFB from two different sources and found the RFB MIC tested consistently at 0.0312 to 0.0625 μg/ml.

RIF and RFB MICs and the association with RRDR mutations

Table 1 shows the results of MIC tested by MGIT 960 for RIF and RFB. Overall the observed MICs were considered narrow. We noticed RIF MIC range appeared to be wider for isolates with 526C and 526L mutations; similarly RFB MICs range was wider with isolates having 516V and 531L mutations. However, the variability of these MICs was still within plus/minus one 2-fold dilution of the mean MIC. We categorized isolates into 3 groups. Group 1 isolates were susceptible to both RIF and RFB, group 2 isolates were resistant to RIF but susceptible to RFB, and group 3 isolates were resistant to both RIF and

RFB. For group 1, RIF MICs were 0.125 to 1 μg/ml and RFB MICs were ≤ 0.0625 μg/ml to 0.25 μg/ml. Besides the 42 isolates with wildtype *rpoB* sequence, we found 20 isolates harboring 10 unique mutations were also in this group: a synonymous mutation, 7 mis-sense mutations [L511P, D516Y, H526N, H526S (nucleotide changes of AGC and TCC), S531C, L533P], a deletion of codons 508–509 and a dual mutation of M515I and H526N. For group 2, the RIF MICs were 2 to ≥8 μg/ml and RFB MICs were 0.0625 to 0.5 μg/ml. The most frequently detected mutation in this group was D516V; nine other mutations detected were D516F, S522L, H526A, H526C, H526G, H526L, codon 518 deletion and two dual mutations (S522L plus L527R, and H526S plus L527R). For group 3, the RIF and RFB MICs were >8 μg/ml and 1 to >8 μg/ml, respectively. The most common mutation found in MDR-TB cases was S531L. Additional 23 mutations detected were Q513E, Q513K, Q513L, Q513P, N519K, H526D, H526R, H526Y, S531F, S531W, 2 multiple codon deletion and 11 multiple mutations (Table 1).

DISCUSSION

We detected 42 different *rpoB* mutations from a total of 164 strains studied, representing a wide variety of mutations including 24 infrequently encountered single or multiple mutations that have not been listed in the TB Drug Resistance Mutation database to date. Our study demonstrated the association of various RRDR mutations with the differential expression of phenotypic resistance or susceptibility to RIF and RFB as measured by MGIT 960. These data may serve as a starting point to establish a database containing MIC values of RFB and RIF associated with specific *rpoB* mutations, which will evolve as new mutations are detected and new MIC values added. The need for establishing such a database connecting results of molecular drug susceptibility (MDST) and CDST has been suggested in recent publications (33 – 34) and is critical to the formulation of trial treatment regimens based on rapidly detectable genotypes and predicted phenotypes. The inclusion of a relatively large number of RIF-resistant strains and the diversity of mutations we observed is a significant contribution to the growing body of knowledge suggesting certain *rpoB* mutations are associated with differential RIF/RFB resistance.

Guidelines for using MGIT 960 to test second-line antituberculous drugs are available (26, 31). Testing RFB by MGIT 960 using 0.5 μg/ml as a breakpoint concentration for interpreting RFB susceptibility results has been demonstrated in multiple studies (28, 30) to perform comparably to the BACTEC 460 (29) and agar proportion methods. In this study, we demonstrated that the breakpoint of 0.5 μg/ml was at least 4-fold higher than MICs for wild-type isolates (RIF-susceptible with no *rpoB* mutations). These data are consistent with previous studies and support use of this breakpoint with MGIT 960 to detect *in vitro* RFB-resistance. While this breakpoint sufficiently distinguishes wild-type isolates from those with *rpoB* mutations that confer RIF and RFB cross-resistance *in vitro*, establishment of a critical concentration that can reliably guide effective therapy, especially in instances where some level of RIF resistance is determined, should ideally incorporate additional information obtained through clinical and pharmacological studies (35).

From the RFB and RIF MIC data generated in this study, we observed 42 distinct *rpoB* mutations. Twenty (48%) of these 42 *rpoB* mutations (Group 1 and Group 2 combined) were

associated with *in vitro* RFB susceptibility. This suggests that RFB may have clinical efficacy in patients with isolates harboring these differential resistance mutations (36), although the frequency of isolates possessing these mutations was far lower than those, such as S531L, found in most MDR-TB patients,. These findings and similar findings in other studies demonstrate SNP-specific differential phenotypic resistance to the various drugs within a class of drugs such as rifamycins and fluoroquinolones (37). They also highlight the advantage of using sequence-based molecular methods, which provide the mutation identity when a mutation is detected, over probe-based methods, which either don't identify individual SNPs (such as GeneXpert's MTB/RIF assay) or only identify few common mutations (such as HAIN or INNO-LIPA line-probe assays). As evidence accumulates for the added value of rapid SNP-specific phenotypic predictions, sequence-based MDST methods may have significant clinical advantages (33).

We also tested isolates with so called "disputed" mutations, such as L511P, D516Y, H526N, H526S, S531C, L533P, S522TTG and H526L, which have been reported to confer highly discordant RIF results by CDST (38 – 40). Due to concerns over failure to detect phenotypic RIF-resistance by MGIT 960 in isolates harboring these disputed mutations, a consideration of lowering the critical concentration of RIF to bring *rpoB* genotype and phenotype into concordance has been discussed. However, as shown in our study, RIF MICs associated with those disputed mutations, except for S522L and H526L, either overlapped with that of the wildtype strains or were only slightly increased. Lowering the critical concentration of RIF to 0.5 μg/ml will improve our detection of these strains, but will not allow us to detect all of them. Yet the risk of generating false RIF-resistance may be increased because the critical concentration is close to the MIC of wildtype strains. False RIF-resistance may cause removal of RIF from regimens and adverse impacts on TB patient management may ensue.

In conclusion, our study demonstrated that various *rpoB* mutations were associated with differential RIF and RFB susceptibility or resistance. The accumulated information on RRDR mutations and the associated RIF and RFB MICs deepens our confidence in the potential for using MDST to rapidly predict CDST results several weeks before those results are typically available. However, while our data suggest the clinical potential of using RFB for treating patients whose isolates contain *rpoB* mutations associated with *in vitro* RFB susceptibility, clinical investigations are required to verify that RFB can be used effectively in these patients to improve outcomes.

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Highlights

This study includes the following core findings:

- Differential resistance between RIF and RFB was studied in 164 clinical isolates.
- 122 isolates had *rpoB* mutations; 42 were unique.
- RFB and RIF MIC's were determined for isolates with or without rpoB mutations.
- We identified 10 *rpoB* mutations conferring RIF-resistance but RFB-susceptibility.
- Clinical studies to assess RFB treatment in certain MDR-TB cases are recommended.

Table 1

Observed MIC for RFB and RIF and associated *rpoB* mutations

Amino acid change (nucleotide changes)	Observed MIC (μg/ml)	
	RIF MIC (Isolates tested)	**RFB MIC (Isolates tested)**
Group I, RIF-S and RFB-S		
Wildtype	≤ 0.125 (2), 0.125 (2)	≤ 0.0625 (37), 0.125 (5)
L511P(CCG)	≤ 0.25 (2), 0.25 (1)	≤ 0.0625 (2), 0.0625 (1)
F514F(TTT)	0.125 (2)	≤ 0.0625 (2)
D516Y(TAC)	0.25 (2), 0.5 (2)	0.0625 (4)
H526N(AAC)	0.125 (2), 0.25 (1)	≤ 0.0625 (2), 0.125 (1)
H526S(AGC)	0.5 (1), 1 (1)	≤ 0.0625 (1), 0.125 (1)
H526S(TCC)	0.25 (1)	≤ 0.0625 (1)
S531C(TGT)	≤ 0.125 (1)	0.0625 (1)
L533P(CCG)	0.5 (2)	0.125 (1), 0.25 (1)
T508 to S509 deletion	0.5 (1)	≤ 0.0625 (1)
M515I(ATA) + H526N(AAC)	1 (1)	0.125 (1)
Group II, RIF-R and RFB-S		
D516V(GTC)	8 (3), > 8 (15)	0.125 (2), 0.25 (6), 0.5 (10)
D516F(TTC)	2 (1)	0.0625 (1)
S522L (TTG)	2 (1)	0.0625 (1)
H526A(GCC)	2 (1)	0.125 (1)
H526C(TGC)	2 (1), 8 (1)	0.125 (2)
H526G(GGC)	2 (1)	0.125 (1)
H526L(CTC)	2 (2), 4 (1), 8 (1)	0.125 (2), 0.25 (1), 0.5 (1)
N518 deletion	4 (1)	0.125 (1)
S522L(TTG) + K527R(AGG)	8 (1)	0.5 (1)
H526S(TCC) + K527R(CGG)	4 (1)	0.25 (1)
Group III, RIF-R AND RFB-R		
Q513E(GAA)	> 8 (2)	1 (2)
Q513K(AAA)	> 8 (3)	> 8 (3)
Q513L(CTA)	> 8 (1)	> 8 (1)
Q513P(CCA)	> 8 (2)	1 (1), 2 (1)
H526D(GAC)	> 8 (3)	8 (2), > 8 (1)
G526R(CGC)	> 8 (2)[a]	8 (2), > 8 (3)
H526Y(TAC)	> 8 (1)[a]	8 (2), > 8 (2)
S531F(TTC)	> 8 (1)	> 8 (1)
S531W(TGG)	> 8 (3)[a]	4 (1), 8 (2), >8 (1)
S531L(TTG)	> 8 (5)[a]	2 (7), 4 (25), 8 (1)

Amino acid change (nucleotide changes)	Observed MIC (μg/ml)	
	RIF MIC (Isolates tested)	RFB MIC (Isolates tested)
S509R(CGC) + H526Y(TAC)	> 8 (1)	8 (1)
S509R(AGG) + H526L(CTC)	> 8 (1)	8 (1)
Q510L(CTG) + D516V(GTC)	> 8 (1)	1 (1)
L511P(CCG) + S512T(ACC) + D516Y(TAC)	> 8 (1)	8 (1)
L511P(CCG + D516Y(TAC)	> 8 (2)	2 (2)
Q513L(CTA) + H526N(AAC)	> 8 (1)	> 8 (1)
F514F(TTT) + S531L(TTG)	> 8 (1)	8 (1)
515 to 521 deletion	> 8 (1)	8 (1)
D516E(GAG) + S522L(TTG)	> 8 (1)	1 (1)
D516V(GTC) + S531L(TTG)	> 8 (1)	4 (1)
D516G(GGC) + L533P(CCG)	> 8 (1)	4 (1)
H526Q(CAG) + L533P(CCG)	> 8 (1)	1 (1)

[a]These mutations are commonly found in isolates from MDR TB patients and they are known to confer high level resistance to RIF; RIF MIC was performed for limited numbers of those strains. RFB MIC was performed for all strains included in this study.