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Profiling of *rpoB* Mutations and MICs for Rifampin and Rifabutin in *Mycobacterium tuberculosis*

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Resistance to rifampin (RIF) and rifabutin (RFB) in *Mycobacterium tuberculosis* is associated with mutations within an 81-bp region of the *rpoB* gene (RIF resistance-determining region [RRDR]). Previous studies have shown that certain mutations in this region are more likely to confer high levels of RIF resistance, while others may be found in phenotypically susceptible isolates. In this study, we sought to determine the relationship between the MICs of RIF and RFB and *rpoB* RRDR mutations in 32 multidrug-resistant (MDR), 4 RIF-monoresistant, and 5 susceptible *M. tuberculosis* clinical isolates. The MICs were determined using the MGIT 960 system. Mutations in the *rpoB* RRDR were determined by Sanger sequencing. RpoB proteins with mutations S531L (a change of S to L at position 531), S531W, H526Y, and H526D and the double mutation D516A-R529Q were associated with high MICs for RIF and RFB. Five isolates carrying the mutations L511P, H526L, H526N, and D516G-S522L were found to be susceptible to RIF. Several mutations were associated with resistance to RIF and susceptibility to RFB (F514FF, D516V, and S522L). Whole-genome sequencing of two MDR isolates without *rpoB* RRDR mutations revealed a mutation outside the RRDR (V146F; RIF MIC of 50 μg/ml). The implications of the polymorphisms identified in the second of these isolates in RIF resistance need to be further explored. Our study further establishes a correlation between the mutations and the MICs of RIF and, also, RFB in *M. tuberculosis*. Several *rpoB* mutations were identified in RIF- and RFB-susceptible isolates. The clinical significance of these findings requires further exploration. Until then, a combination of phenotypic and molecular testing is advisable for drug susceptibility testing.

The incidence and mortality rates of tuberculosis (TB) are slowly declining. However, *Mycobacterium tuberculosis* still claims the lives of more than 1 million people worldwide and remains the second leading cause of death from an infectious disease (1).

In recent years, there has been an alarming increase in reports of multidrug- and extensively drug-resistant TB (MDR-TB and XDR-TB, respectively), both of which may hinder current advances in TB control. In 2013, there was a worldwide estimate of 450,000 new cases of MDR-TB, defined as resistance to both isoniazid (INH) and rifampin (RIF) (1). WHO estimates that 5% of MDR-TB cases may be XDR-TB, which is defined as MDR-TB with resistance to a fluoroquinolone and a second-line injectable drug (1). Accurate and rapid detection of drug resistance in TB patients is essential for the successful control of the disease.

RIF is one of the most potent sterilizing drugs available for TB treatment. RIF, in conjunction with INH, ethambutol (EMB), and pyrazinamide (PZA), make up the four agents most widely used as first-line drugs against TB (1).

Rifabutin (RFB) is a semisynthetic derivative of rifamycin S and, together with RIF, is part of the rifamycin family (2). Both RIF and RFB inhibit mycobacterial growth by blocking the DNA-dependent RNA polymerase subunit B (RpoB) (3, 4). RFB is recommended for TB treatment in HIV-coinfected patients because it has fewer drug-drug interactions than RIF in patients receiving antiretroviral therapy (5).

Resistance to rifamycins in *M. tuberculosis* is largely associated with mutations within an 81-bp RIF resistance-determining region (RRDR) in the *rpoB* gene, which corresponds to codons 507 to 533 of RpoB (3, 6, 7). However, the mechanism of resistance in about 5% of *M. tuberculosis* RIF-resistant isolates is unknown, suggesting alternative mechanisms, such as lowered cell wall permeability or efflux pump activity (8, 9).

Previous studies have shown that certain mutations in the *rpoB* RRDR are more likely to confer higher levels of RIF resistance (10–13). At the same time, certain *rpoB* mutations have been associated with RIF-susceptible phenotypes (6, 11, 14–18). Although the data on this particular issue are contradictory, it has been suggested that the critical concentration used for phenotypic drug susceptibility testing (DST) of both RIF and RFB should be reevaluated (7, 11, 16). Similarly, little is known with respect to RIF and RFB resistance levels in *M. tuberculosis* isolates without *rpoB* RRDR mutations (7).

Although cross-resistance to RIF and RFB is common, RIF-resistant/RFB-susceptible isolates have been reported and RFB has been suggested as a reasonable alternative to treat MDR-TB and XDR-TB associated with particular *rpoB* mutations (19). Given all of these points, characterization of *rpoB* mutations and their association with RIF and/or RFB resistance is needed in order to better interpret molecular and phenotypic results generated in the clinical laboratory.

In this study, we sought to characterize a subset of *M. tuberculosis* clinical isolates representing MDR, RIF monoresistant, and

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RIF-/RFB-susceptible isolates and to correlate their *rpoB* mutations with MIC values for both RIF and RFB.

Our study shows that high MICs for RIF and RFB are associated with specific mutations at codons 531 and 526, while mutations at other positions are generally associated with low or moderate MICs.

Although such isolates are rare, we also characterized 5 *M. tuberculosis* isolates that had *rpoB* RRDR mutations but were phenotypically susceptible to RIF by both the MGIT (automated mycobacteria growth indicator tube) 960 liquid system (Becton, Dickinson) and the agar proportion method.

Together, our data suggest that although most of the mutations commonly present in the *rpoB* RRDR gene correlate with RIF and RFB resistance, caution must be used in interpreting phenotypic and molecular test results.

MATERIALS AND METHODS

***M. tuberculosis* clinical isolates.** Thirty-nine clinical isolates corresponding to 37 TB cases were selected for this study based on their availability from the clinical laboratory, their previously characterized *rpoB* mutations (20), and their phenotypic drug susceptibility pattern. Of the 37 cases, 8 corresponded to 4 paired clusters. In addition, 50 INH-resistant/RIF-susceptible isolates were screened for the presence of *rpoB* mutations. Two of them were found to have *rpoB* mutations and were included in this study. All isolates were obtained from specimens submitted to the Public Health Ontario Laboratories for routine clinical testing for tuberculosis. Frozen isolates were revived on Lowenstein-Jensen (LJ) slants and MGIT (Becton, Dickinson, Sparks, MD) liquid medium. Drug susceptibility patterns were available as part of the routine clinical testing using the Bactec MGIT 460 or 960 system (Becton, Dickinson, Sparks, MD). Thirty-two isolates were MDR, and 24 of these were also RFB resistant. Four isolates were monoresistant to RIF, and two of them were resistant to RFB as well. Five isolates were RIF and RFB susceptible. Two of these were identified during screening of INH-monoresistant isolates, while the remaining 3 were identified in a previous study (20).

MIC determination. MICs were determined using the Bactec MGIT 960 system. Inoculum preparation was performed according to the manufacturer's recommendations. The drug concentrations tested for RIF included 0.125, 0.25, 0.5, 1, 2, 4, 8, 20, 50, 100, and 160 μg/ml, and RFB concentrations of 0.25, 0.5, 1, and 5 μg/ml were tested. These drug concentrations were selected based on previous reports of clinical drug susceptibility testing routinely performed at critical concentrations of 1 μg/ml for RIF and 0.5 μg/ml for RFB. For example, RIF-resistant isolates were tested with concentrations starting at 2 μg/ml and susceptible isolates were tested with RIF concentrations below 1 μg/ml. A similar rationale was used for RFB MIC determination.

Four of the five RIF-susceptible isolates with *rpoB* RRDR mutations were also tested using the Middlebrook 7H10 agar proportion method (21). The RIF concentrations tested by this method were 0.06, 0.125, 0.25, 0.5, 1, 2, 40, and 80 μg/ml.

DNA extraction, PCR, and Sanger sequencing. *rpoB* RRDR mutations for the majority of isolates included in this study had been previously characterized (20). All susceptible isolates and the two resistant isolates with no identified *rpoB* RRDR mutations were subjected to PCR and Sanger sequencing to confirm their sequences. *M. tuberculosis* isolates with no previous information on *rpoB* mutations, including 50 INH-resistant/RIF-susceptible isolates, were also analyzed. These isolates were grown in MGIT tubes according to the manufacturer's recommendations. Cells were harvested and then inactivated by boiling at 95°C for 20 min in TE buffer (10 mM Tris, 1 mM EDTA). The lysates were the DNA source for *rpoB* RRDR PCR. The PCR primers used to amplify *rpoB* RRDR were *rpoB*-F (5′-GTACGGTCGGGCGAGCTGA-3′) and *rpoB*-R (5′-GTTGT CGTGCATCACAGTGA-3′). The cycling conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension of 72°C for 10 min.

Sanger sequencing of the amplicons was performed using the same *rpoB* RRDR primers and the BigDye 3.1 kit (Applied Biosciences, Life Technologies) as recommended by the manufacturer. Sequences were acquired on the ABI Prism 3730xl and analyzed using BioEdit (version 7.1.19) (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

WGS. RIF-resistant isolates with no *rpoB* RRDR mutations were analyzed by whole-genome sequencing (WGS). *M. tuberculosis* cells were revived from glycerol stocks on LJ slants. After incubation at 37°C for 2 to 4 weeks, *M. tuberculosis* colonies from each slant were harvested and DNA was extracted. DNA extraction was performed as previously described, with minor modifications (22). Briefly, colonies were resuspended in 0.4 ml TE buffer (10 mM Tris, 1 mM EDTA) and heat inactivated for 60 min at 85°C. Prior to lysozyme and protease treatment, samples were delipidated with an equal volume of chloroform-methanol (2:1, vol/vol). All DNA samples were treated with RNase following standard protocols and quantified using the Qubit system (Life Technologies). One nanogram of high-quality genomic DNA (gDNA) was prepared for sequencing using the Nextera XT sample preparation kit following the manufacturer's protocol (Illumina, San Diego, CA). Libraries were run on the Illumina MiSeq instrument according to the manufacturer's instructions for 150-bp paired-end reads. Illumina paired-end reads were trimmed using quality scores and then aligned to the *M. tuberculosis* H37Rv reference genome (GenBank accession number NC_000962.3) using the CLC Genomics workbench software (version 6.0.2) (Aarhus, Denmark). Single-nucleotide polymorphisms (SNPs) and small insertion-deletion (indel) events were identified using a probabilistic variant detection with a cutoff of a minimum read depth of 20× and were subsequently filtered to select variants with a read frequency of at least 75%.

RESULTS

Correlation of RIF MICs and *rpoB* RRDR mutations. Of the 41 isolates tested, 32 (78%) were MDR, 24 of which were also resistant to RFB; 2 (5%) were resistant to RIF and RFB; 2 (5%) were resistant to only RIF, and 5 (12%) were RIF and RFB susceptible. All isolates, except for two MDR, contained mutations in the *rpoB* RRDR. All results were in agreement with the phenotypic DST pattern reported by the clinical laboratory during routine testing.

In order to facilitate interpretation, we selected MIC ranges arbitrarily to catalogue the resistance to RIF as high (MIC ≥100 μg/ml), moderate (≥20 to <100 μg/ml), or low (≥1 to <20 μg/ml). Of the 36 RIF-resistant isolates, 26 (72.2%), 9 (25%), and 1 (2.8%) had high, moderate, and low levels of resistance, respectively (Table 1).

The most frequent *rpoB* RRDR mutation was a change of S to L at position 531 (S531L) ($n = 19$, 46.3%), followed by S531W, D516V, and S522L ($n = 3$ isolates each, 7.3% each). Mutations H526Y, H526L, and L511P were present in 2 isolates each (4.9% each). The remaining mutations were only observed in single isolates (i.e., mutations V146F, H526D, D516G-S522L, D516A-R529Q, and H526N and a duplicated phenylalanine residue, F514FF), with a distribution of 2.4% each (Table 1 and Fig. 1).

All isolates bearing mutations at position 531 (S531L or S531W) had very high levels of resistance to RIF. The levels of RIF resistance in isolates with mutations at position 526 were found to be dependent on the amino acid change. H526Y and H526D were associated with high levels of resistance, while H526L was associated with either low levels of resistance (MIC = 2 μg/ml) or susceptibility to RIF (MIC of 0.5 μg/ml). H526N was not associated with RIF resistance (MIC of 0.125 μg/ml).

The substitution of a valine residue for an aspartic acid at position 516 (D516V) was associated with moderate resistance levels.

TABLE 1 Distribution of *rpoB* mutations and MICs of rifampin and rifabutin

Mutation(s)	MIC (μg/ml) of[a]: RIF	RFB	No. of isolates with mutation(s)	DST pattern[b]
V146F	50	1	1	MDR
L511P	**≤0.125**	**≤0.25**	1	INH-R
L511P	**≤0.125**	**≤0.25**	1	Pan-S
F514FF	20	**≤0.25**	1	MDR
D516A, R529Q	160	5	1	MDR
D516G, S522L	**0.5**	**≤0.25**	1	INH-R
D516V	20	**≤0.25**	1	MDR
D516V	50	**0.5**[c]	2	MDR
H526D	160	5	1	MDR
H526L	2	**0.5**[c]	1	MDR
H526L	**0.5**	**0.5**[c]	1	INH-R
H526N	**0.25**	**≤0.25**	1	INH-R
H526Y	100	≥5	1	MDR
H526Y	≥160	≥5	1	MDR
S522L	20	**0.5**[c]	1	MDR
S522L	20	**0.5**[c]	2	RIF-R
S531L	100	5	6	MDR
S531L	160	5	10	MDR
S531L	≥160	5	2	MDR
S531L	160	**≤0.25**	1	MDR
S531W	≥160	≥5	1	MDR
S531W	≥160	≥5	2	RIF-R
None	20	**0.5**[c]	1	MDR

[a] RIF, rifampin; RFB, rifabutin. Values highlighted in boldface correspond to MICs below the critical concentration, and isolates with these values are therefore classified as susceptible.

[b] Drug susceptibility test (DST) patterns correspond to the clinical diagnosis performed using the Bactec MGIT 960 and the critical concentration (RIF, 1 μg/ml; RFB, 0.5 μg/ml). MDR, multidrug resistant; INH-R, isoniazid resistant; Pan-S, pan-susceptible; RIF-R, RIF resistant.

[c] Value based on DST performed during routine clinical diagnosis procedures. When tested at 1 μg/ml of RFB, growth for these isolates was inhibited.

Substitutions at position 516 were also observed in the two double mutants, one of which was susceptible (D516G-S522L, MIC of 0.5 μg/ml) and the other highly resistant (D516A-R529Q, MIC of 160 μg/ml).

Isolates with the L511P mutation were found to be RIF susceptible. The RIF susceptibility of isolates containing the mutations L511P, H526L, and H526N, with MICs of <1 μg/ml obtained in the Bactec MGIT 960 system, was also confirmed by the agar proportion method (data not shown).

Correlation between RFB MICs and *rpoB* RRDR mutations. Twenty-six of the RIF-resistant isolates were also resistant to RFB (26/36, 72.2%). All *rpoB* mutants that were RIF susceptible were also susceptible to RFB.

Five isolates had RFB MIC levels of ≥5 μg/ml. These isolates were characterized by mutations S531W ($n = 3$) and H526Y ($n = 2$).

RFB-resistant isolates characterized by mutations S531L ($n = 18$), H526D ($n = 1$), and the double mutation D516A-R529G ($n = 1$) presented MICs of 5 μg/ml. A single MDR isolate with the mutation S531L that was highly resistant to RIF (MIC = 160 μg/ml) was found to be susceptible to RFB during routine DST. Susceptibility to RFB in this isolate was confirmed by the determination of a MIC of ≤0.25 μg/ml.

The remaining RFB-resistant isolate, with a mutation outside the *rpoB* RRDR (V146F), had a MIC of 1 μg/ml. Isolates with the mutation S522L (1 MDR and two RIF-monoresistant isolates) and MDR isolates with mutations H526L, F514FF, and D516V were also susceptible to RFB (Table 1, Fig. 1).

WGS of RIF-resistant isolates with no *rpoB* RRDR mutations. Whole-genome sequencing (WGS) of two MDR isolates without *rpoB* RRDR mutations was performed in order to identify potential targets of drug resistance to RIF. After resequencing analysis using the *M. tuberculosis* H37Rv reference genome, the average read coverages were 70× and 80× for isolates A (MDR-A) and B (MDR-B), respectively. Variant analysis revealed 919 polymorphic sites in MDR-A, including 862 SNPs and 57 small indels, compared to the H37Rv reference genome. Polymorphisms associated with the PPE and PE-PGRS gene family were not included in the analysis due to the high frequency of sequencing error reported in these sites (23). In order to select potential polymorphisms associated with drug resistance, the SNPs and indels were filtered against a list of variants from pan-susceptible strains sequenced in our laboratory, as well as from variants available at the TB database (www.tbdb.org). In total, 394 variants were found to be unique to MDR-A. These included 211 nonsynonymous SNPs, 112 synonymous SNPs, 48 noncoding SNPs, and 22 indels (see Table S1 in the supplemental material). A nonsynonymous mutation in the *rpoB* gene, resulting in mutation V170F (V146F in *Escherichia coli* nomenclature), was identified as the most likely cause for RIF resistance in this isolate. This isolate had a RIF MIC of 50 μg/ml and an RFB MIC of 1 μg/ml.

For strain MDR-B, variant analysis revealed 1,846 SNPs and 123 indels compared to the H37Rv reference genome. Following the same variant filtering described above, a total of 218 variants were found to be unique to MDR-B. These included 83 nonsynonymous SNPs, 45 synonymous SNPs, 22 noncoding SNPs, and 15 indels (see Table S2 in the supplemental material). Although one of these variants could be responsible for the RIF resistance phenotype observed in this isolate, preliminary analysis of the data did not reveal obvious genetic targets potentially responsible for RIF resistance. This isolate had a RIF MIC of 20 μg/ml and was susceptible to RFB.

DISCUSSION

Rapid and accurate laboratory detection of drug resistance in *M. tuberculosis* is essential for TB control at the population level, as well as for individual patients, such that quick detection of resistance allows for early treatment modification and greatly improves treatment success. In the past few decades, several molecular diagnostics methods that allow the rapid detection of *M. tuberculosis* drug resistance directly from specimens have been developed. The available commercial molecular assays include the INNO-LiPA (Innogenetic, Belgium), GenoType MTBDRplus (Hain LifeScience, Germany), and GeneXpert MTB/RIF (Cepheid, USA). These genotypic methods produce fast and accurate results but can miss resistant isolates with mutations outside the target region or with alternative mechanisms of drug resistance (11). In addition, commercial assays may yield false-positive results for isolates with silent mutations in *rpoB*. The level of resistance is not determined by these and other molecular methods. Culture-based phenotypic DST remains the gold standard, as it is expected to detect all clinically relevant resistant cases. However, recent reports suggest that *M. tuberculosis* isolates with *rpoB* mutations but resistance levels below the critical concentration used

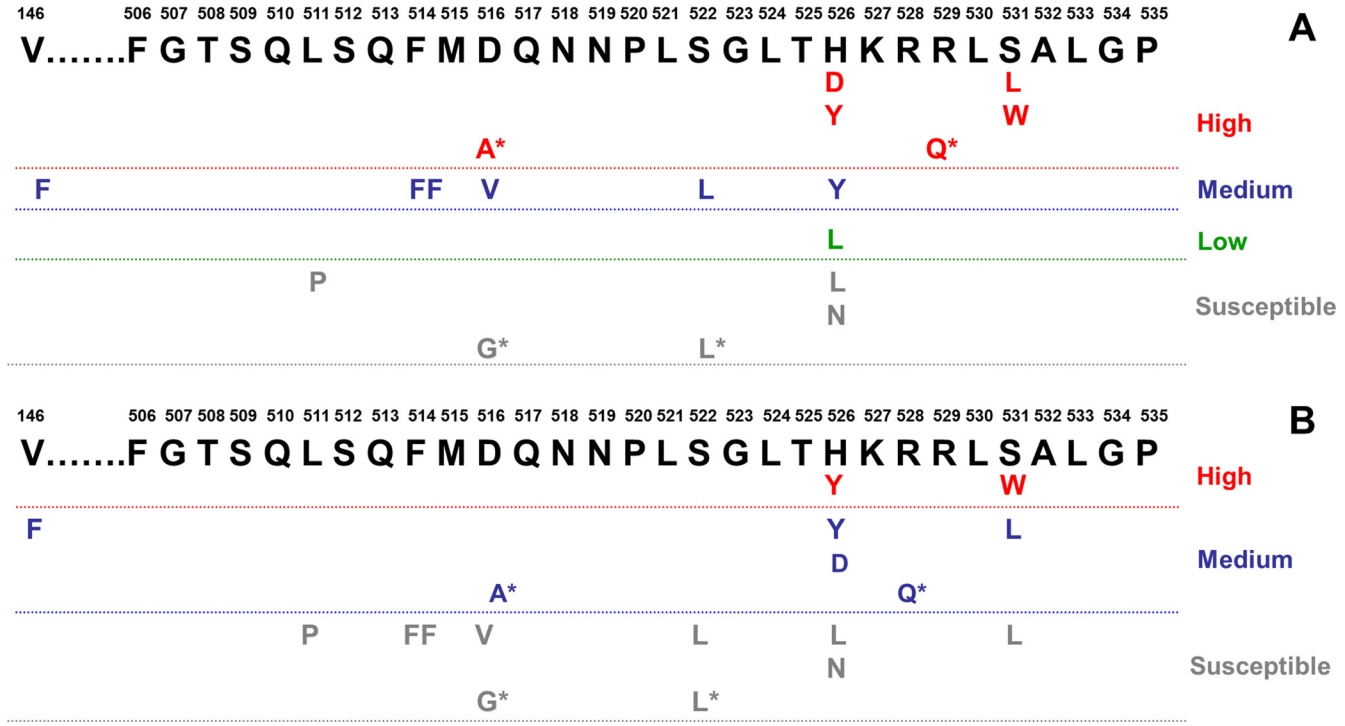

FIG 1 *rpoB* mutations and their correlation to RIF (A) and RFB (B) resistance levels. Asterisks indicate *rpoB* RDRR double mutants. Arbitrarily selected MIC ranges were used to catalogue resistance to RIF as high (MIC ≥100 μg/ml), medium (≥20 to <100 μg/ml), or low (≥1 to <20 μg/ml). Arbitrarily selected MIC ranges were used to catalogue resistance to RFB as high (MIC ≥5 μg/ml) or medium (≥0.5 to <5 μg/ml).

for phenotypic DST exist and may indeed be clinically relevant (7, 11, 14).

Similarly, RFB-susceptible isolates with known *rpoB* mutations have also been reported. For both RIF and RFB, these cases have been linked to a suboptimal critical concentration used for DST (7).

RIF resistance is an indication of MDR, and therefore, its accurate and rapid detection is imperative. Similarly, accurate characterization of RFB resistance in MDR- and XDR-TB cases is also important. The replacement of RIF by RFB can positively affect the treatment outcome in these hard-to-treat patients (19).

For all of these reasons, it is crucial to understand the association of specific *rpoB* mutations with RIF and RFB resistance levels and use this information to provide rapid feedback to physicians and their TB patients.

In our study, we included a variety of *M. tuberculosis* clinical isolates representing different drug susceptibility patterns. The majority of RIF resistance in isolates included in this study was associated with *rpoB* RRDR mutations at codons 531 and 526 (Fig. 1). This observation is similar to those of previous reports elsewhere (7, 11, 12, 24–26). Accordingly, mutations at position 531 were also associated with high levels of resistance to RIF in the data from these reports.

The levels of resistance to RIF in isolates with *rpoB* RRDR mutations at codon 526 were found to be residue dependent (Fig. 1). The replacement of histidine with tyrosine or aspartic acid correlated with high levels of RIF resistance, while the replacement of the histidine residue with leucine was characterized by low levels of RIF resistance or susceptibility. Similarly, H526N was not related to RIF resistance.

We also identified two RIF-susceptible isolates with the *rpoB* RRDR mutation L511P. Both H526N and L511P have previously been associated with RIF resistance (10, 25). However, these mutations are also part of a group of known disputed RIF-causing mutations, found in both susceptible and resistant isolates (10, 14, 16, 27). In this study, we identified these mutations in two INH-resistant/RIF-susceptible isolates and one pan-susceptible isolate (according to the results of the phenotypic DST). The MICs for these three isolates were confirmed to be almost 4 to 10 times below the critical breakpoint (≤0.125 μg/ml to 0.25 μg/ml). Some studies have indicated that these mutations may yield contradictory DST results depending on the method used (11, 14), and a recent study suggested that genotypic results for these disputed mutations should be confirmed using the proportion method in solid medium (16). However, these three isolates were consistently susceptible by both the MGIT 960 and agar proportion method.

In addition to the H526N isolate, the H526L isolate, and the two L511P isolates, we identified a fifth RIF-susceptible isolate with *rpoB* RRDR mutations, the D516G-S522L double mutant. It is possible that the isolate with mutation H526L may indeed be borderline resistant (MIC of >0.5 but <1 μg/ml). This isolate presented 214 growth units (GU) at the time of reading (compared to 400 GU for the control), which indicates slower but observable growth at 1 μg/ml. Unfortunately, access to clinical information and treatment outcomes for these five cases was not available and the clinical relevance of these mutations in this particular setting remains unknown.

An important finding of this study was the identification of *rpoB* RRDR mutations in MDR isolates that are not associated

with RFB resistance. Previous studies have shown a proportion of RIF-resistant isolates with susceptibility to RFB ranging from 13 to 26% (7, 28, 29). In these studies, the most common mutations associated with RIF resistance/RFB susceptibility were associated with residues 516, 529, and 533. In our study, the proportion of RIF-resistant isolates that were RFB susceptible was only slightly higher than the proportions reported elsewhere (28%) (7, 28, 29). Mutations H526L, F514FF, D516V, and S522L were all associated with RIF resistance/RFB susceptibility patterns. In these cases, early inclusion of RFB in the first few months of treatment may be advisable. Additional studies with a higher proportion of strains and follow-up of treatment outcomes are needed.

A single MDR isolate with the S531L mutation and high resistance to RIF was found to be susceptible to RFB. This finding is puzzling, as S531L has been shown to significantly reduce rifamycins' affinity to RNA polymerase in both *M. tuberculosis* and *E. coli* (30) and all other isolates with the S531L mutation in this study were RFB resistant. The reason for the RFB susceptibility in this isolate is unknown. Given the rarity of this finding, identification of S531L should generally be an indication of RIF and RFB cross-resistance. This isolate is currently undergoing additional investigation.

The majority of the RIF resistance isolates are linked to *rpoB* RRDR mutations (3). Here, we report two isolates with no mutations in this region. WGS analysis revealed mutation V146F as the most likely cause for RIF resistance in isolate MDR-A. This mutation has been previously described and associated with resistance to both RIF and RFB, but it appears to be rare (31, 32). Other variants of interest that could potentially be related to drug resistance in this strain were also identified and corresponded to two SNPs in the efflux pump-encoding genes Rv2994 (T326A) and Rv0783c (G406V). Based on homology analyses, both SNPs are located in conserved positions within the substrate-binding translocation pore. Rv2994 and Rv0783 have previously been reported to be overexpressed in MDR isolates (33) and RIF monoresistant isolates (13), respectively. WGS of isolate MDR-A also revealed two nonsynonymous mutations in the *rpoC* gene (Gly594Glu and Pro1040Ala). Compensatory mutations in *rpoC* and *rpoA* have been previously identified in RIF-resistant isolates (34, 35). Although these compensatory mutations seem to be associated mostly with *rpoB* S531L, a small percentage of *rpoB* mutants harboring other mutations also present *rpoC* compensatory mutations. However, this is the first report of *rpoC* mutations in a V146F *rpoB* mutant. Further studies are needed to prove that this mutation is an actual compensatory change conferring RIF resistance. Whole-genome analysis of strain MDR-B revealed several hundred polymorphic sites. The association of these SNPs and RIF resistance remains unknown, but this information opens the field for future studies on alternative mechanisms of resistance to RIF.

Conclusion. Our findings support previous reports highlighting that certain mutations in *rpoB* RRDR are more likely to confer higher levels of RIF resistance (10–13). Similarly, our findings confirm that current DST methods using MGIT 960 and the agar proportion method may miss isolates with *rpoB* RRDR mutations and borderline RIF resistance, such as the isolate with mutation H526L. *rpoB* RRDR sequencing should be strongly considered in all patients with RIF treatment failure or relapse patients, as the present conventional phenotypic test methods may fail to detect borderline resistant isolates with certain *rpoB* mutations.

Conversely, isolates with the previously identified disputed mutations L511P and H526N appear to be truly susceptible, as demonstrated by very low RIF MICs (<0.125 μg/ml) in the MGIT 960 and confirmation of susceptibility in the agar proportion method, but the clinical relevance of these mutations in our particular study setting remains unknown.

Although cross-resistance to RIF and RFB is common, RIF-resistant/RFB-susceptible isolates have been reported, and RFB has been suggested as a good alternative to treat MDR-TB and XDR-TB associated with particular *rpoB* RRDR mutations (19). We identified additional *rpoB* mutations associated with RIF-resistant and RFB-susceptible patterns, particularly in MDR isolates. Our findings need to be validated in larger studies, and if they are confirmed, it may be advisable to perform *rpoB* RRDR sequencing in all suspected MDR specimens/isolates in order to rapidly determine whether RFB is a possible treatment option for these patients.

In summary, we performed a comprehensive characterization of several *rpoB* RRDR mutations and their correlation with RIF and RFB resistance levels in a variety of *M. tuberculosis* clinical isolates. We suggest that a combination of molecular and phenotypic methods may be needed until more information regarding the clinical relevance of phenotypically susceptible isolates with *rpoB* RRDR mutations is generated.

REFERENCES

1. **World Health Organization.** 2014. Global tuberculosis report 2013. World Health Organization, Geneva, Switzerland. www.who.int/tb/publications/global_report/en/.
2. **Marsili L, Pasqualucci CR, Vigevani A, Gioia B, Schioppacassi G, Oronzo G.** 1981. New rifamycins modified at positions 3 and 4. Synthesis, structure and biological evaluation. J. Antibiot. **34:**1033–1038.
3. **Ramaswamy S, Musser JM.** 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber. Lung Dis. **79:**3–29. http://dx.doi.org/10.1054/tuld.1998.0002.
4. **Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T.** 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet **341:**647–650. http://dx.doi.org/10.1016/0140-6736(93)90417-F.
5. **Lee SS, Meintjes G, Kamarulzaman A, Leung CC.** 2013. Management of tuberculosis and latent tuberculosis infection in human immunodeficiency virus-infected persons. Respirology **18:**912–922. http://dx.doi.org/10.1111/resp.12120.
6. **Yang B, Koga H, Ohno H, Ogawa K, Fukuda M, Hirakata Y, Maesaki S, Tomono K, Tashiro T, Kohno S.** 1998. Relationship between antimycobacterial activities of rifampicin, rifabutin and KRM-1648 and rpoB mutations of *Mycobacterium tuberculosis*. J. Antimicrob. Chemother. **42:**621–628. http://dx.doi.org/10.1093/jac/42.5.621.
7. **Schön T, Juréen P, Chryssanthou E, Giske CG, Kahlmeter G, Hoffner S, Angeby K.** 2013. Rifampicin-resistant and rifabutin-susceptible *Mycobacterium tuberculosis* strains: a breakpoint artefact? J. Antimicrob. Chemother. **68:**2074–2077. http://dx.doi.org/10.1093/jac/dkt150.
8. **Musser JM.** 1995. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin. Microbiol. Rev. **8:**496–514.
9. **Louw GE, Warren RM, Gey van Pittius NC, McEvoy CRE, Van Helden PD, Victor TC.** 2009. A balancing act: efflux/influx in mycobacterial drug resistance. Antimicrob. Agents Chemother. **53:**3181–3189. http://dx.doi.org/10.1128/AAC.01577-08.
10. **Ohno H, Koga H, Kohno S, Tashiro T, Hara K.** 1996. Relationship between rifampin MICs for and rpoB mutations of *Mycobacterium tuberculosis* strains isolated in Japan. Antimicrob. Agents Chemother. **40:**1053–1056.
11. **Rigouts L, Gumusboga M, de Rijk WB, Nduwamahoro E, Uwizeye C, de Jong B, Van Deun A.** 2013. Rifampin resistance missed in automated liquid culture system for *Mycobacterium tuberculosis* isolates with specific *rpoB* mutations. J. Clin. Microbiol. **51:**2641–2645. http://dx.doi.org/10.1128/JCM.02741-12.

12. **Clemente WT, Soares Lima SS, Palaci M, Silva MSN, Sumnienski Rodrigues VF, Dalla Costa ER, Possuelo L, Cafrune PI, Ribeiro FK, Gomes HM, Serufo JC.** 2008. Phenotypic and genotypic characterization of drug-resistant *Mycobacterium tuberculosis* strains. Diagn. Microbiol. Infect. Dis. **62:**199–204. http://dx.doi.org/10.1016/j.diagmicrobio.2008.06.013.
13. **Pang Y, Lu J, Wang Y, Song Y, Wang S, Zhao Y.** 2013. Study of the rifampin monoresistance mechanism in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **57:**893–900. http://dx.doi.org/10.1128/AAC.01024-12.
14. **Van Deun A, Barrera L, Bastian I, Fattorini L, Hoffmann H, Kam KM, Rigouts L, Rüsch-Gerdes S, Wright A.** 2009. *Mycobacterium tuberculosis* strains with highly discordant rifampin susceptibility test results. J. Clin. Microbiol. **47:**3501–3506. http://dx.doi.org/10.1128/JCM.01209-09.
15. **Van Deun A, Wright A, Zignol M, Weyer K, Rieder HL.** 2011. Drug susceptibility testing proficiency in the network of supranational tuberculosis reference laboratories. Int. J. Tuberc. Lung Dis. **15:**116–124.
16. **Van Deun A, Aung KJM, Bola V, Lebeke R, Hossain MA, de Rijk WB, Rigouts L, Gumusboga A, Torrea G, de Jong BC.** 2013. Rifampin drug resistance tests for tuberculosis: challenging the gold standard. J. Clin. Microbiol. **51:**2633–2640. http://dx.doi.org/10.1128/JCM.00553-13.
17. **Taniguchi H, Aramaki H, Nikaido Y, Mizuguchi Y, Nakamura M, Koga T, Yoshida S.** 1996. Rifampicin resistance and mutation of the *rpoB* gene in *Mycobacterium tuberculosis*. FEMS Microbiol. Lett. **144:**103–108. http://dx.doi.org/10.1111/j.1574-6968.1996.tb08515.x.
18. **Somoskovi A, Dormandy J, Mitsani D, Rivenburg J, Salfinger M.** 2006. Use of smear-positive samples to assess the PCR-based genotype MTBDR assay for rapid, direct detection of the *Mycobacterium tuberculosis* complex as well as its resistance to isoniazid and rifampin. J. Clin. Microbiol. **44:**4459–4463. http://dx.doi.org/10.1128/JCM.01506-06.
19. **Sirgel FA, Warren RM, Böttger EC, Klopper M, Victor TC, van Helden PD.** 2013. The rationale for using rifabutin in the treatment of MDR and XDR tuberculosis outbreaks. PLoS One **8:**e59414. http://dx.doi.org/10.1371/journal.pone.0059414.
20. **Bolotin S, Alexander DC, Chedore P, Drews SJ, Jamieson F.** 2009. Molecular characterization of drug-resistant *Mycobacterium tuberculosis* isolates from Ontario, Canada. J. Antimicrob. Chemother. **64:**263–266. http://dx.doi.org/10.1093/jac/dkp183.
21. **Pfyffer GE, Bonato DA, Ebrahimzadeh A, Gross W, Hotaling J, Kornblum J, Laszlo A, Roberts G, Salfinger M, Wittwer F, Siddiqi S.** 1999. Multicenter laboratory validation of susceptibility testing of *Mycobacterium tuberculosis* against classical second-line and newer antimicrobial drugs by using the radiometric BACTEC 460 technique and the proportion method with solid media. J. Clin. Microbiol. **37:**3179–3186.
22. **van Soolingen D, Hermans PW, de Haas PE, Soll DR, van Embden JD.** 1991. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J. Clin. Microbiol. **29:**2578–2586.
23. **Roetzer A, Diel R, Kohl TA, Rückert C, Nübel U, Blom J, Wirth T, Jaenicke S, Schuback S, Rüsch-Gerdes S, Supply P, Kalinowski J, Niemann S.** 2013. Whole genome sequencing versus traditional genotyping for investigation of a *Mycobacterium tuberculosis* outbreak: a longitudinal molecular epidemiological study. PLoS Med. **10:**e1001387. http://dx.doi.org/10.1371/journal.pmed.1001387.
24. **Williams DL, Waguespack C, Eisenach K, Crawford JT, Portaels F, Salfinger M, Nolan CM, Abe C, Sticht-Groh V, Gillis TP.** 1994. Characterization of rifampin-resistance in pathogenic mycobacteria. Antimicrob. Agents Chemother. **38:**2380–2386. http://dx.doi.org/10.1128/AAC.38.10.2380.
25. **Tang K, Sun H, Zhao Y, Guo J, Zhang C, Feng Q, He Y, Luo M, Li Y, Sun Q.** 2013. Characterization of rifampin-resistant isolates of *Mycobacterium tuberculosis* from Sichuan in China. Tuberculosis (Edinb.) **93:**89–95. http://dx.doi.org/10.1016/j.tube.2012.10.009.
26. **Bártfai Z, Somosköví A, Ködmön C, Szabó N, Puskás E, Kosztolányi L, Faragó E, Mester J, Parsons LM, Salfinger M.** 2001. Molecular characterization of rifampin-resistant isolates of *Mycobacterium tuberculosis* from Hungary by DNA sequencing and the line probe assay. J. Clin. Microbiol. **39:**3736–3739. http://dx.doi.org/10.1128/JCM.39.10.3736-3739.2001.
27. **Ramaswamy SV, Reich R, Dou S-J, Jasperse L, Pan X, Wanger A, Quitugua T, Graviss EA.** 2003. Single nucleotide polymorphisms in genes associated with isoniazid resistance in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **47:**1241–1250. http://dx.doi.org/10.1128/AAC.47.4.1241-1250.2003.
28. **Chen H-Y, Yu M-C, Huang W-L, Wu M-H, Chang Y-L, Che C-R, Jou R.** 2012. Molecular detection of rifabutin-susceptible *Mycobacterium tuberculosis*. J. Clin. Microbiol. **50:**2085–2088. http://dx.doi.org/10.1128/JCM.00652-12.
29. **Cavusoglu C, Karaca-Derici Y, Bilgic A.** 2004. In-vitro activity of rifabutin against rifampicin-resistant *Mycobacterium tuberculosis* isolates with known rpoB mutations. Clin. Microbiol. Infect. **10:**662–665. http://dx.doi.org/10.1111/j.1469-0691.2004.00917.x.
30. **Gill SK, Garcia GA.** 2011. Rifamycin inhibition of WT and Rif-resistant *Mycobacterium tuberculosis* and *Escherichia coli* RNA polymerases in vitro. Tuberculosis (Edinb.) **91:**361–369. http://dx.doi.org/10.1016/j.tube.2011.05.002.
31. **Heep M, Rieger U, Beck D, Lehn N.** 2000. Mutations in the beginning of the *rpoB* gene can induce resistance to rifamycins in both *Helicobacter pylori* and *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **44:**1075–1077. http://dx.doi.org/10.1128/AAC.44.4.1075-1077.2000.
32. **Siu GKH, Zhang Y, Lau TCK, Lau RWT, Ho P-L, Yew W-W, Tsui SKW, Cheng VCC, Yuen K-Y, Yam W-C.** 2011. Mutations outside the rifampicin resistance-determining region associated with rifampicin resistance in *Mycobacterium tuberculosis*. J. Antimicrob. Chemother. **66:**730–733. http://dx.doi.org/10.1093/jac/dkq519.
33. **Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, Venkatesan K, Sharma VD.** 2010. Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs. Microb. Drug Resist. **16:**21–28. http://dx.doi.org/10.1089/mdr.2009.0054.
34. **Brandis G, Wrande M, Liljas L, Hughes D.** 2012. Fitness-compensatory mutations in rifampicin-resistant RNA polymerase. Mol. Microbiol. **85:**142–151. http://dx.doi.org/10.1111/j.1365-2958.2012.08099.x.
35. **De Vos M, Müller B, Borrell S, Black PA, van Helden PD, Warren RM, Gagneux S, Victor TC.** 2013. Putative compensatory mutations in the *rpoC* gene of rifampin-resistant *Mycobacterium tuberculosis* are associated with ongoing transmission. Antimicrob. Agents Chemother. **57:**827–832. http://dx.doi.org/10.1128/AAC.01541-12.