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Low-Level Rifampin Resistance and *rpoB* Mutations in *Mycobacterium tuberculosis*: an Analysis of Whole-Genome Sequencing and Drug Susceptibility Test Data in New York

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ABSTRACT Rapid and reliable detection of rifampin (RIF) resistance is critical for the diagnosis and treatment of drug-resistant and multidrug-resistant (MDR) tuberculosis. Discordant RIF phenotype/genotype susceptibility results remain a challenge due to the presence of *rpoB* mutations that do not confer high levels of RIF resistance, as have been exhibited in strains with mutations such as Ser450Leu. These strains, termed low-level RIF resistant, exhibit elevated RIF MICs compared to fully susceptible strains but remain phenotypically susceptible by mycobacterial growth indicator tube (MGIT) testing and have been associated with poor patient outcomes. Here, we assess RIF resistance prediction by whole-genome sequencing (WGS) among a set of 1,779 prospectively tested strains by both prevalence of *rpoB* gene mutation and phenotype as part of routine clinical testing during a 2.5-year period. During this time, 139 strains were found to have nonsynonymous *rpoB* mutations, 53 of which were associated with RIF resistance, including both low-level and high-level resistance. Resistance to RIF (1.0 μg/ml in MGIT) was identified in 43 (81.1%) isolates. The remaining 10 (18.9%) strains were susceptible by MGIT but were confirmed to be low-level RIF resistant by MIC testing. Full *rpoB* gene sequencing overcame the limitations of critical concentration phenotyping, probe-based genotyping, and partial gene sequencing methods. Universal clinical WGS with concurrent phenotypic testing provided a more complete understanding of the prevalence and type of *rpoB* mutations and their association with RIF resistance in New York.

KEYWORDS *Mycobacterium tuberculosis*, WGS, *rpoB*, *rpoA*, *rpoC*, RIF, low-level resistance, MIC, rifampin, next-generation sequencing

Rifampin (RIF) is a critical component of drug regimens for treating tuberculosis (TB) infection, and detection of RIF resistance is crucial for the diagnosis and treatment of drug-resistant and multidrug-resistant (MDR) tuberculosis. RIF resistance is mainly caused by the presence of specific mutations in the *rpoB* gene, which encodes the RNA polymerase β subunit, an observation first described in *Escherichia coli* (1, 2). In 1993, Telenti et al. described the molecular basis for RIF resistance in 64 *Mycobacterium tuberculosis* clinical isolates and found resistance polymorphisms to be restricted to a highly conserved region of *rpoB*, a key finding that paved the way for the development of rapid genotypic methods for the determination of resistance (3, 4). This highly conserved region of *rpoB* would later be designated the 81-bp RIF resistance-determining region (RRDR) due to the frequency with which resistance polymorphisms were detected in this region (5, 6).

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As genotypic methods to detect RIF resistance became widely implemented, rare variants associated with resistance were described, including several located outside the RRDR (7, 8). Among these less common variants, a class of mutations emerged that confer a lower level of RIF resistance than typical variants (9, 10). These mutations, referred to as low-level resistant mutations in this paper, were found to be susceptible to rifampin by critical concentration phenotypic susceptibility testing. It was proposed that these mutations are still clinically relevant (9, 11). This group of mutations has garnered increased attention since being linked to poor clinical outcomes when RIF is included in the treatment regimen (10, 12–15). Data regarding the prevalence of low-level RIF resistance in *M. tuberculosis* are still lacking, particularly in settings with low incidences of tuberculosis. Current reports suggest that the prevalence of these mutations vary by setting (12, 16, 17). Furthermore, mutations located outside the RRDR and rare variants within the RRDR can be challenging to detect or interpret, which may contribute to underreporting of these mutations, especially when some commonly used methods are used.

RIF resistance is most commonly found in conjunction with isoniazid (INH) resistance, but a minority of strains are resistant to only RIF and not INH or other TB drugs (RIF monoresistant). Reliable identification of RIF monoresistance is critical, as these strains have been associated with lower rates of successful treatment outcomes and may lead to the development of MDR-TB if treated suboptimally (18). The World Health Organization (WHO) estimated 1.1% of patients worldwide were infected with RIF monoresistant TB in 2014, but regional reports have suggested that the rate varies significantly, surpassing 20% in some jurisdictions (19, 20). RIF monoresistance has been reported at higher rates among HIV-positive patients compared to HIV-negative patients, while rates of resistance to other drugs, such as INH, have not been found to vary by HIV status (19, 21).

Studies have suggested that drug resistance in bacteria often results in a fitness cost, which may impact growth rate, virulence, and/or transmissibility (22, 23). In the case of *rpoB* mutations, compensatory mutations have been described in the *rpoA* and *rpoC* genes, which encode the α and β' subunits of the RNA polymerase and have been suggested to compensate for the fitness cost of resistance mutations in *rpoB* (24). It has also been proposed that additional mutations in *rpoB* itself compensate for the fitness cost of resistance mutations, although these mutations are still not well characterized, likely due to the challenge discerning which mutations play a role in resistance versus compensation (23, 25). Reports of compensatory mutations are currently limited, but increased surveillance may further our understanding of how RIF resistance is acquired and spread.

To address these gaps in our knowledge, between January 2016 and October 2018 we tested one isolate of *M. tuberculosis* complex (MTBC) from each newly diagnosed patient in New York, a low-incidence setting (3.8 cases per 100,000) (26). Each isolate was tested by whole-genome sequencing (WGS), phenotypic susceptibility testing (Bactec mycobacterial growth indicator tube [MGIT] 960 and agar proportion), and selective MIC testing (Sensititre MYCOTB MIC plate). This report describes the incidence of low-level RIF mutations in *M. tuberculosis* strains in a prospective analysis in a low-incidence setting. Furthermore, we report on all *rpoB* gene mutations identified, their corresponding phenotypic interpretation, compensatory mutations, strain lineage, and genomic clustering among these strains.

MATERIALS AND METHODS

Clinical isolates. A total of 1,779 MTBC strains from unique patients, received as isolates or cultured in-house from clinical specimens by the Mycobacteriology Laboratory at the Wadsworth Center, New York State Department of Health (NYSDOH), were included in this study. Prior to leaving the biosafety level 3 (BSL-3) laboratory for DNA extraction, liquid culture aliquots of clinical isolates were heat inactivated at 80°C for 60 min.

Phenotypic DST. Culture-based RIF drug susceptibility testing (DST) was performed using the liquid culture MGIT 960 system (Bactec MGIT 960 SIRE package insert; Becton, Dickinson) and solid 7H10 agar proportion method according to the Clinical and Laboratory Standards Institute's recommendations,

using a critical concentration of 1.0 μg/ml (27). Isolates received in the laboratory were subcultured in MGIT medium prior to first-line DST being set up. A subset of 67 isolates was selected for MIC testing based on mutation profile and MGIT DST result. This group contained 53 isolates identified with *rpoB* gene mutations and a group of 14 wild-type strains. MIC testing was performed on cultures initially grown on Middlebrook 7H10 plates using the Sensititre MYCOTB AST plate (Thermo Scientific), including a RIF concentration range of 0.12 to 16 μg/ml. MIC testing was performed in triplicate, with plates being read by one analyst. Repeat MIC results were within 1 dilution for all isolates tested.

Real-time PCR. An in-house-developed real-time PCR assay (28) was utilized to detect MTBC in all samples received. Prior to WGS, this assay was repeated as a quality control check on DNA extracts to assess purity and quantitation of MTBC DNA.

DNA extraction. DNA was extracted from 1 ml of heat-inactivated isolates using a modified version of the InstaGene/FastPrep (IG/FP) method described by Shea et al. (29). Specifically, the 56°C incubation was reduced from 30 min to 10 min, and the volume of InstaGene matrix added to the pellets was altered to be 130 to 200 μl, based on the size of the pellet observed. These changes were implemented to reduce extraction turnaround time and increase DNA yield. DNA yields were measured by Qubit fluorometry and compared to real-time PCR results to confirm purity.

WGS. Paired-end 250-bp DNA sequencing was carried out using the Illumina MiSeq platform following Nextera XT library preparation with a 15-cycle PCR indexing step (30). Sequencing runs were composed fully of 15 to 17 MTBC samples or of MTBC with other bacterial, viral, and/or parasitic samples.

Bioinformatic analysis. Sequence analysis was performed using the Wadsworth Center TB WGS bioinformatics pipeline as previously described (29). Specifically, mutations were determined by a minimum 10× depth of coverage, and mutations were detected with the GATK package, using the diploid mode to allow for detection of heteroresistance. Major lineage identification was based on the presence or absence of lineage-defining single-nucleotide polymorphisms (SNPs; see Table S1 in the supplemental material), with nomenclature according to Gagneux and Small (31). Genomic clusters were determined by an SNP distance of ≤5 SNPs to any sequence(s) in our database, independent of epidemiological data.

Mutation classification. Mutations in *rpoB* were sorted into three categories of RIF resistance, i.e., no resistance, low-level resistance, and high-level resistance, based on culture-based MGIT and MIC results. Strains with high-level resistance had clearly elevated RIF MIC (≥16 μg/ml) and were phenotypically resistant at 1.0 μg/ml in MGIT. Strains with low-level resistance had RIF MIC ranging from 0.25 to 1.0 μg/ml and tested phenotypically susceptible at 1.0 μg/ml in MGIT. Remaining mutations with no evidence of RIF resistance (susceptible at 1.0 μg/ml in MGIT and RIF MIC of ≤0.25 μg/ml) were determined not to be associated with RIF resistance. As the MIC ranges overlap at 0.25 μg/ml for low-level resistant and nonresistant strains, reports of mutations in the literature were used to finalize classification.

Data availability. Sequences analyzed in this article have been provided to the Centers for Disease Control and Prevention on a monthly basis to contribute to the National TB Genotyping program (https://www.cdc.gov/tb/programs/genotyping/default.htm) as well as the Relational Sequencing TB Data Platform (ReSeqTB) (https://platform.reseqtb.org/), which catalogs a vast amount of genotypic, phenotypic, and related metadata from *M. tuberculosis* strains to enable the development of clinically useful, WHO-endorsed *in vitro* diagnostic assays for rapid drug susceptibility testing.

RESULTS

***rpoB* mutation identification and classification.** Among 1,779 isolates tested, 139 were found to have one or more nonsynonymous mutation(s) in the *rpoB* gene. A total of 119 had a single mutation, while the remaining 20 had two or three *rpoB* mutations each. Of the 139 strains, 53 (38%) were found to have mutations associated with phenotypic RIF resistance (both high- and low-level resistance) based on MGIT DST and MIC testing. These RIF-resistant isolates contained 17 specific mutations across 11 codons in *rpoB* (Fig. 1), including 42 (79.2%) MDR, 2 (3.8%) extensively drug-resistant (XDR), and 9 (17%) RIF monoresistant strains (Table 1). Nearly all RIF-resistant strains harbored mutations within the RRDR, 49/53 (92.5%), compared to 4/53 (7.5%) strains with single *rpoB* mutations outside the RRDR. Among the remaining 86 RIF-susceptible strains, four other mutations, including one nonsynonymous mutation (Thr427Ala) and three silent mutations (1 of Thr427Thr and 2 of Arg447Arg), were identified within the RRDR with no effect on resistance.

MGIT DST, low-level resistance mutations, and RIF monoresistance. Of the 53 strains with *rpoB* resistance mutations, 43 (81.9%) were resistant to RIF in MGIT at 1 μg/ml. The remaining 10 (18.9%) strains were susceptible in MGIT at 1 μg/ml but were determined to be low-level RIF resistant by MIC testing. Three of the mutations detected in these strains were located outside the RRDR (Ile59Thr [1], Ile491Phe [2]), and seven were within the RRDR (Leu430Pro [3], Asp435Tyr [1], Ser441Gln [1], His445Gln [1], Leu452Pro [1]). Two other strains were found to have mutations associated with low-level resistance, but each contained an additional resistance-associated

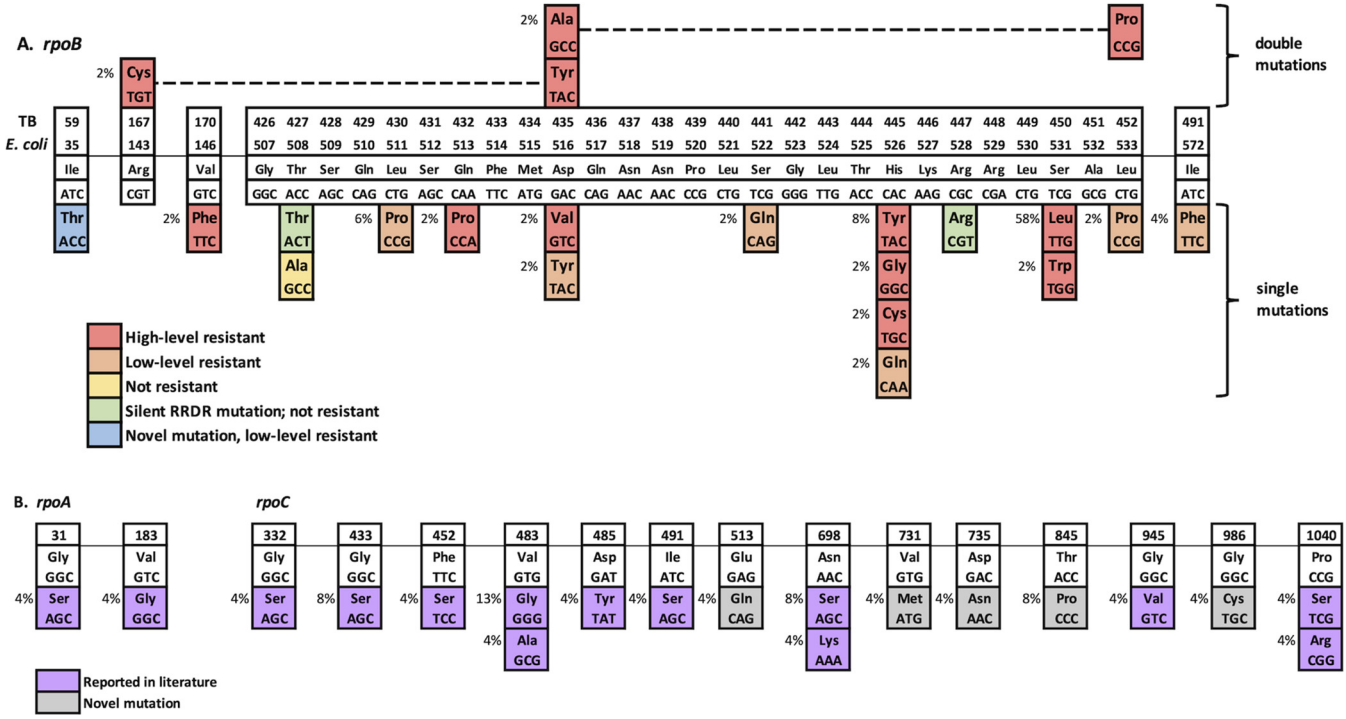

FIG 1 Schematic representations of mutations in *rpoB*, *rpoA*, and *rpoC* genes. (A) *rpoB*. Cell color indicates corresponding phenotypic MGIT result. (B) *rpoA* and *rpoC*. Compensatory mutations among strains with *rpoB* gene resistance mutations. Cell color indicates previous literature reports. Lineage-specific mutations are not shown.

mutation in *rpoB*, and both were phenotypically resistant to RIF in MGIT (Table 1). RIF monoresistance was more prevalent among low-level resistant strains (7/10, 70%) than high-level resistant strains (2/43, 4.6%). The 7 patients with low-level RIF monoresistant strains were HIV negative, while one of the two patients infected with high-level RIF monoresistant strains was HIV positive. HIV status for the remaining patient with high-level RIF monoresistant TB was unavailable.

MIC ranges. MIC results were performed for a total of 67 strains, including 53 with *rpoB* mutations and 14 wild-type strains. The results were grouped by *rpoB* mutation type: *rpoB* wild-type strains and *rpoB* mutations not associated with resistance were combined with a MIC range of $<$0.12 to 0.5 μg/ml ($n = 40$), *rpoB* mutation associated with low-level RIF resistance with a MIC range of 0.25 to 1.0 μg/ml ($n = 9$), and *rpoB* mutation associated with high-level RIF resistance with a MIC of $>$16 μg/ml ($n = 18$) (Fig. 2). Strains with high-level RIF resistance mutations had distinct MIC results compared to all other strains. The MIC ranges of *rpoB* wild-type and low-level *rpoB* mutated strains overlapped at 0.25 μg/ml and 0.5 μg/ml; one strain with *rpoB* His445Gln had a MIC of 0.25 μg/ml, while two *rpoB* wild-type strains had a MIC of 0.5 μg/ml.

Compensatory mutations. Resistant strains were screened for the presence of potential compensatory mutations in the *rpoA*, *rpoC*, and *rpoB* genes. Thirty-two of 53 (60.4%) RIF-resistant strains harbored one of these mutations, 22 in *rpoC*, 8 in *rpoB*, and 2 in *rpoA*. Both *rpoA* mutations and 16/22 (73%) of the *rpoC* mutations were reported previously in RIF-resistant clinical isolates (Fig. 1). Mutations in compensatory genes were associated mainly with high-level RIF resistance; 30/43 (70%) of high-level RIF-resistant strains had a compensatory mutation compared to just 2/10 (20%) of low-level RIF-resistant strains. To our knowledge, we are the first to report *rpoC* mutations Glu513Gln, Val731Met, Asp735Asn, Thr845Pro, and Gly986Cys. We also considered all nonresistance SNPs in *rpoB* as potential compensatory mutations and found eight such mutations, not previously reported, in seven unique isolates with *rpoB*

TABLE 1 Compensatory mutations, susceptibility, and major lineage of strains with *rpoB* resistance mutations[e]

Mutation	Description			No. resistant	No. sensitive	MIC	Resistance type	No. in major lineage: 1	2	3	4
rpoB RRDR nonresistance SNPs	*rpoB* compensatory	*rpoA*	*rpoC*								
Thr427Thr[a]				0	1	NA	Pan-susceptible				1
Thr427Ala				0	1	≤0.12	Pan-susceptible			1	
Arg447Arg[a]				0	2	NA	Pan-susceptible				2
rpoB low-level resistance mutations	*rpoB* compensatory	*rpoA*	*rpoC*								
Ile59Thr[d]				0	1	0.5	Mono		1		
Leu430Pro				0	3	0.5–1	1 MDR, 2 mono	1	1		1
Asp435Tyr				0	1	0.5	MDR		1		
Ser441Gln				0	1	NA	MDR				1
His445Gln				0	1	0.25	Mono				1
Leu452Pro				0	1	1–2	Mono		1		
Ile491Phe[d]			Thr845Pro	0	2	1–2	Mono				2
rpoB high-level resistance mutations	*rpoB* compensatory	*rpoA*	*rpoC*								
Val170Phe[d]		Val183Gly		1	0	N/A[b]	MDR				1
Gln432Pro		Gly31Ser		1	0	>16	MDR			1	
Asp435Val				1	0	NA[b]	MDR				1
His445Cys				1	0	>16	MDR				1
His445Gly	His674Arg			1	0	>16	MDR				1
His445Tyr			Val731Met[c]	1	0	>16	MDR				1
His445Tyr			Glu513Gln	1	0		MDR		1		
His445Tyr	Glu563Ala			2	0		MDR		2		
Ser450Trp				1	0	>16	MDR		1		
Ser450Leu			Gly332Ser	1	0	>16	MDR		1		
Ser450Leu			Gly433Ser	2	0		MDR		2		
Ser450Leu			Phe452Ser	1	0		MDR			1	
Ser450Leu			Val483Ala	1	0		MDR	1			
Ser450Leu			Val483Gly	3	0		MDR		1		2
Ser450Leu			Asp485Tyr	1	0		MDR		1		
Ser450Leu			Ile491Ser	1	0		MDR				1
Ser450Leu			Asn698Lys	1	0		MDR		1		
Ser450Leu			Asn698Ser	2	0		MDR		1	1	
Ser450Leu			Asp735Asn	1	0		MDR		1		
Ser450Leu			Gly945Val	1	0		MDR		1		
Ser450Leu			Gly986Cys	1	0		MDR	1			
Ser450Leu			Pro1040Ser	1	0		MDR				1
Ser450Leu			Pro1040Arg	1	0		MDR		1		
Ser450Leu				9	0		7 MDR, 2 Mono		6	1	2
Ser450Leu	Arg552His			1	0		MDR		1		
Ser450Leu	Asp574Glu			1	0		MDR				1
Ser450Leu	Val534Ala			1	0		XDR				1
Ser450Leu	Lys258Thr, Ser1039Ala			1	0		MDR		1		
Double *rpoB* mutations	*rpoB* compensatory	*rpoA*	*rpoC*								
Arg167Cys, Asp435Tyr				1	0	>16	XDR				1
Asp435Ala, Leu452Pro	Asp265Gly			1	0	>16	MDR		1		

[a]Silent mutation.
[b]Sample exhausted; no MIC result.
[c]Heterozygous mutation.
[d]Mutation located outside RRDR.
[e]TB numbering system used for *rpoB* gene. NA, not applicable.

resistance mutations: Asp265Gly, Val534Ala, Arg552His, Glu563Ala, Asp574Glu, His674Arg, and Lys258Thr plus Ser1039Ala.

RIF resistance and mutation prevalence by major lineage. All four major tuberculosis lineages (lineages 1 to 4) were represented in our collection (14.6%, 21.6%,

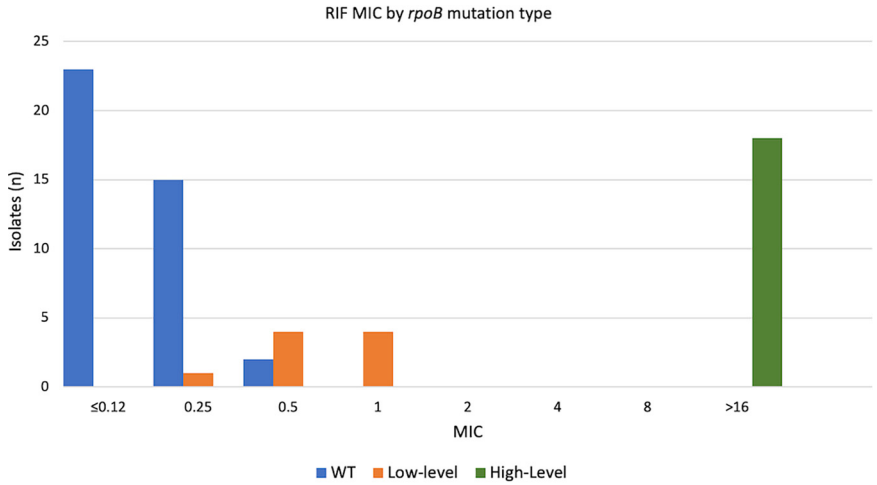

FIG 2 Rifampin MIC by *rpoB* gene mutation type: 67 strains, including 40 with no resistance mutations, 9 with low-level RIF resistance mutations, and 18 with high-level RIF resistance mutations.

7.5%, and 51.7% of strains, respectively). Eighty-one strains were determined to be non-*M. tuberculosis* species in the MTBC (32 *M. bovis* BCG, 26 *M. bovis*, 18 *M. africanum*, 4 *M. orygis*, and 1 *M. caprae*), but none of these strains exhibited any RIF resistance. The distribution of strains with resistance-conferring *rpoB* mutations across the four major lineages of *M. tuberculosis* varied drastically, ranging from 5.7% in lineage 1 to 50.9% in lineage 2. In particular, resistance mutations in *rpoB* were overrepresented in lineage 2 strains, given the prevalence of these strains in our population (Table 2). Compensatory mutations in strains with *rpoB* resistance mutations were found at similar rates across each major lineage.

Strain clustering. In our study population, a SNP distance of ≤5 SNPs across the entire genome is suggestive of recent transmission based on follow-up epidemiological investigations (data not shown). WGS analysis identified three clusters among strains with *rpoB* resistance mutations. Cluster 1 was comprised of two strains with *rpoB* Ser450Leu and were genetically identical (0 SNPs). Clusters 2 and 3 included strains with *rpoB* Ile491Phe and His445Tyr, respectively. Each pair of strains in clusters 2 and 3 was separated by 3 SNPs. The remaining strains with *rpoB* resistance mutations did not belong to any genomic clusters. SNP-based estimates of recent transmission for strains with *rpoB* resistance mutations (5.7%) were lower than those for strains without *rpoB* resistance mutations (14.3%).

DISCUSSION

Since implementation in January 2016, routine, universal WGS in our laboratory has improved surveillance and detection of RIF resistance while providing insights into the background and characteristics of RIF-resistant strains in New York. In this study, we analyzed a prospective set of 1,779 MTBC isolates from unique patients over a 2.5-year

TABLE 2 Major lineage and RIF resistance type summary

Lineage	No. (%) of strains	No. (%) of strains with RIF resistance[a]	No. MDR/DR	No. RIF monoresistant	No. (%) of RIF-resistant strains with compensatory mutations
Lineage 1 (Indo-Oceanic)	260 (14.6)	3 (5.7)	3	0	2 (67)
Lineage 2 (Beijing)	384 (21.6)	27 (50.9)	23	4	16 (59)
Lineage 3 (Central Asian)	134 (7.5)	4 (7.6)	4	0	3 (75)
Lineage 4 (Euro-American)	920 (51.7)	19 (35.8)	14	5	11 (58)
Other lineages[b]	81 (4.6)	NA	NA	0	NA
Total	**1,779**	**53**	**44**	**9**	**32**

[a]Includes low-level and high-level RIF resistance.
[b]Includes *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. orygis*, and *M. caprae*.

period to determine the frequency and type of *rpoB* resistance mutations and their association with phenotypic resistance, including MIC testing. Additional data were collected to assess the relationship of compensatory mutations and strain lineage with RIF resistance.

Resistance mutations detected in *rpoB* mutations were diverse, located both within and outside the RRDR, and had various impacts on the level of RIF resistance. Among mutations not associated with RIF resistance, four were notable for being located within the RRDR (Thr427Ala [1], Thr427Thr [1], and Arg447Arg [2]). These SNPs, which may result in false-positive predictions of resistance when tested by probe-based methods, such as Hain LPA or GeneXpert MTB/RIF, were detected at an exceedingly low rate (4/1,779 [0.22%]) in our strain population. There have been reports of silent RRDR mutations in the past, typically identified at rates below 1% in each setting for which data are available (10, 32–35).

Resistance-conferring *rpoB* mutations were detected most commonly in MDR and XDR strains; however, a significant portion (9/53 [17%]) of RIF-resistant strains were found to be RIF monoresistant. The incidence of RIF monoresistance varies considerably by setting, ranging from 0% in some settings to as high as 21.4% in South Africa (19, 36–39). Reported incidences of RIF monoresistance may be underestimated if low-level resistant strains are undetected or were excluded from analysis as a result of using critical-concentration MGIT DST. It is noteworthy that other DST systems, such as the Löwenstein-Jensen (LJ) proportion method, reportedly have fewer discrepancies when it comes to these mutations (40). In the present study, 70% of low-level RIF-resistant strains were also RIF monoresistant. Detecting and counting these strains may drastically affect reported rates of RIF resistance, particularly RIF monoresistance. A clear link between HIV positivity and RIF monoresistance is not supported by our data, although this may be a result of the relatively high rates of low-level RIF monoresistance in this study. Previous reports linking HIV infection with RIF monoresistance almost exclusively describe high-level RIF resistance (21). It is notable that the single patient with high-level RIF monoresistance for which HIV status was available was HIV positive. The conclusions we can draw from these data are limited by our small sample size. Although *rpoB* mutation has been used as a presumptive positive identification of MDR-TB in some settings, our findings suggest that this approach overestimates the rates of additional/multidrug resistance. Therefore, it may be prudent, at least in low-incidence settings, to be cautious of using *rpoB* mutation as a proxy for MDR-TB. Resistance to isoniazid and to other first-line drugs should be confirmed before switching to a regimen with more serious side effects and potential drug toxicity (41, 42).

WGS identified several *rpoB* mutations outside the RRDR, most of which were determined to be low-level RIF resistant by follow-up DST. Three strains, none of which were found to exhibit resistance to any other drugs, contained this type of mutation (Ile59Thr [1] and Ile491Phe [2]), and in each case WGS was the first and only indication that a standard therapy was not appropriate. Strains harboring such mutations are at particular risk for treatment failure, as they may go undetected by both conventional targeted genotypic and phenotypic DST (7, 9, 10). In the absence of WGS or MIC testing, these may have gone completely undetected, potentially leading to treatment failure and the acquisition of further resistance. The remaining low-level resistance mutations detected were located within the RRDR and were found in strains with no other resistance ($n = 4$) and INH-resistant strains ($n = 3$). Resistance in strains with mutations within the RRDR may be detected more readily than in cases with mutations located outside the RRDR, yet RIF resistance still will be detected only if a sequencing method that evaluates a large portion of the *rpoB* gene or MIC testing is performed.

MIC testing was performed on a subset of the test strains, including *rpoB* wild-type strains and strains with *rpoB* resistance mutations (associated with both low- and high-level RIF resistance). MICs for strains with high-level resistance and double mutations were $>16\ \mu$g/ml for all isolates tested. The mutations found in double *rpoB* mutated strains often have low RIF MICs when found alone, but studies utilizing the Bactec

MGIT 960 culture system and the agar proportion method have independently reported finding higher levels of RIF resistance when more than one of these mutations is detected (42–47). Low-level resistance mutations were found to have a much lower range of MICs, 0.25 to 1.0 μg/ml, while the MIC range of *rpoB* wild-type strains was $\leq$0.12 to 0.5 μg/ml. The MIC range for high-level RIF-resistant strains was clearly distinct compared to the ranges of low-level resistant and susceptible strains; whether using MIC or genotyping, RIF resistance would be readily identified in all strains with high-level resistance.

In our data set, a MIC cutoff for RIF resistance of 0.5 μg/ml provides better performance, 96.2% sensitivity and 95% specificity, than *rpoB* resistance mutation detection. Using this cutoff, three strains would be misclassified: one strain with *rpoB* His445Gln would be considered susceptible, and two *rpoB* wild-type strains would be considered resistant. Previous reports have described the His445Gln mutation, although it has always been reported in strains with a secondary *rpoB* mutation (43, 44, 48–51). Based on the present study, the increase in MIC with this mutation alone appears to be quite small, but acquisition of a secondary *rpoB* mutation may result in a substantial increase in MIC. The two strains with MICs of 0.5 μg/ml and wild-type *rpoB* sequence would not be classified as resistant by genotype, but they exhibit a MIC that is higher than those of all other *rpoB* wild-type strains and overlaps the MICs of some low-level RIF-resistant strains. The absence of mutation in *rpoB* does not rule out the possibility of RIF resistance by another mechanism, but no other loci involved with RIF resistance have been well characterized thus far. These results may also represent the limitations of MIC testing, as interpreting MICs can be subjective and no cutoff perfectly delineates low-level RIF-resistant strains from RIF-susceptible strains.

Low-level resistance mutations in *rpoB* underscore the value of genotypic methods for diagnosing RIF resistance, particularly methods that interrogate the full-length *rpoB* gene. Testing algorithms may benefit from the inclusion of a genotypic method or a phenotypic method that detects resistance below the critical concentration of 1 μg/ml to detect all clinically relevant RIF resistance.

In addition to *rpoB*, whole-genome sequencing provided the ability to screen any other genes of interest for mutations possibly related to RIF resistance, including compensatory mutations in *rpoA* and *rpoC*. Excluding phylogenetic SNPs found in both resistant and susceptible isolates, we identified potential compensatory mutations in *rpoA*, *rpoB*, and *rpoC* in strains with *rpoB* resistance mutations. None of these putative compensatory mutations were detected in RIF-susceptible strains.

Each *rpoA* mutation detected in RIF-resistant strains has been previously reported to be associated with RIF resistance (52–56). Compensatory mutations in *rpoC* were found most frequently in strains with *rpoB* Ser450Leu. This link between compensatory mutations and strains with *rpoB* Ser450Leu has been well established (23, 25, 54, 57–60). A majority of *rpoC* mutations identified in our study have been described in previous reports (54, 57–66). Many of these mutations are located between codons 431 and 527, a particular region identified as harboring mutations that may compensate for the fitness cost of *rpoB* resistance mutations (53).

Major lineages were considered when analyzing the type and prevalence of resistance and compensatory mutations. While all four major lineages are represented in the strains isolated from New York State and New York City TB patients, the respective rates of these lineages vary. Moreover, the rates of RIF resistance among these lineages vary, significantly in some cases. RIF resistance was most prevalent among lineage 2 strains and especially low in lineage 1 strains. The association between lineage 2 and higher rates of drug resistance compared to other lineages found in this study has been previously documented (66–68). Other studies, however, have challenged this finding, which suggests that factors other than genetic background play a more critical role in the acquisition of resistance (69, 70).

RIF-resistant strains, and MDR strains in particular, are reportedly less transmissible than susceptible strains in studies done in both low- and high-incidence settings

(71–74). In our population, RIF-resistant strains were less likely than susceptible strains to be assigned to a genomic cluster, using a five-SNP threshold. While notable, this finding is limited by the relatively small number of RIF-resistant cases in New York State. Three strains with RIF resistance each matched one previous strain in our database, with zero, three, and three SNPs (clusters 1, 2, and 3). All of these strains had compensatory mutations in *rpoB* or *rpoC*, but a direct link between compensatory mutation and increased transmissibility has been rejected in previous studies with larger sample sizes (55, 65, 74). Investigations into clusters 1 and 2 revealed epidemiological links between the patients, further supporting recent transmission. No direct links between the patients in cluster 3 were established, but both of these patients were linked to an MDR outbreak in New York City. The strain implicated in this outbreak, referred to as strain W, was documented to exhibit high rates of transmission during the 1990s (75). These patients may have been independently infected with this strain during this time of high transmission and subsequently became sick after a period of latency.

In conclusion, routine whole-genome sequencing in a clinical setting is a powerful tool for understanding the prevalence and types of RIF resistance in a population while offering insights into strain background, compensation, and strain relatedness. Diagnosing all types of RIF resistance can be challenging, particularly when genotypic and phenotypic results are discordant. MIC testing is a useful tool for determining the significance of rare or novel *rpoB* mutations, supplementing routine phenotypic testing. Low-level RIF resistance, which causes diagnostic and treatment challenges, is significant in our population and may be underreported, particularly in strains with no other drug resistance. The present study begins to fill in the gaps of our knowledge regarding the prevalence and spread of RIF-resistant TB in New York.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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