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Predicting differential rifampicin resistance in clinical *M. tuberculosis* isolates by specific *rpoB* mutations

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Abstract

Setting—Rifampin (RIF)-resistant *Mycobacterium tuberculosis* (*Mtb*) is usually assumed to be resistant to all rifamycins. Increasing evidence indicates, however, that some *rpoB* mutations, detectable by rapid molecular diagnostics, confer resistance to RIF but not rifabutin (RFB), suggesting RFB may be effective for treatment of *Mtb* with these mutations.

Objective—To determine if specific *rpoB* mutations reliably predict differential phenotypic resistance to RIF and RFB.

Design—We selected 60 clinical *Mtb* isolates from a repository of multinational MDR-TB isolates and stratified them into two groups: 1) Those with *rpoB* mutations suspected to confer differential resistance to RIF and RFB and 2) Those expected to be cross-resistant to RIF and RFB. These assumptions were tested by comparing the phenotypic susceptibilities of RIF/RFB with those predicted by mutations in the *rpoB* gene.

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Results—Of 20 suspected RIF-resistant/RFB-susceptible isolates, 15 were RIF resistant but RFB susceptible, 3 were RIF and RFB sensitive, and 2 were cross-resistant to both RIF and RFB. In comparison, 40 of 40 suspected cross-resistant isolates were both RIF and RFB resistant.

Conclusion—Our data supports the association between specific *rpoB* mutations and differential resistance of *Mtb* to RIF and RFB. Clinical studies are required to investigate the efficacy of RFB for treatment of *Mtb* harboring these mutations.

Keywords

MDR-tuberculosis; rifampin; rifabutin; diagnostics

Introduction

Rifamycins are a cornerstone of tuberculosis (TB) treatment. With their introduction in the 1960's, the rifamycins allowed for the reduction of TB treatment duration from a minimum of 18 months to 9 months due to the sterilizing properties of the drugs.¹ Rifampin (RIF) is the rifamycin of choice in TB therapy.² However, a major obstacle to RIF use is the induction of hepatic enzymes altering the metabolism of a large number of medications.¹ It has therefore been recommended that HIV-TB co-infected individuals that receive concomitant therapy of both conditions should be treated with RFB as it has far less activity than RIF as an inducer of hepatic enzymes.^{3,4} This recommendation is supported by multiple studies demonstrating the equivalent efficacy of RFB and RIF in TB treatment.⁵ The other, less well-understood and rarely used application of RFB is in the treatment of infection with *Mycobacterium tuberculosis* (*Mtb*) isolates that are RIF resistant, but RFB susceptible.

The importance of exploring the use of RFB in RIF-resistant, RFB-susceptible *Mtb* treatment is highlighted by the threat RIF resistance poses to TB control worldwide.⁶ The World Health Organization (WHO) has identified the diagnosis and treatment of multi-drug resistant TB (MDR-TB)—TB resistant to RIF and isoniazid (INH)—a top priority. An estimated 480,000 people developed MDR-TB in 2013, resulting in an estimated 210,000 deaths.⁶ With the loss of efficacy of RIF in MDR-TB, treatment duration is increased up to 24 months. The available alternative medications are less effective and less well-tolerated by patients, resulting in extremely difficult and costly treatment courses.^{7,8} Developing a rapid, accessible method for identifying individuals who may benefit from RFB when RIF resistance is found could improve both treatment outcomes and treatment tolerability.

Determining RIF resistance by phenotypic methods is challenging, slow (a minimum of 4 weeks, usually longer) and does not distinguish between clinical *Mtb* isolates that are resistant to RIF, RFB, or both. On the contrary, molecular assays such as GeneXpert (Cepheid, Sunnyvale, CA), line probe assays⁹, and pyrosequencing¹⁰ have reduced the time to diagnosis of RIF resistance to a matter of hours. However, not all molecular methods identify the specific *rpoB* mutations present in the isolate, necessary for rapid differentiation of RIF and/or RFB resistance.

RIF resistance is often associated with the presence of mutations in the beta subunit of the *rpoB* gene, but the effect of these *rpoB* mutations on RFB resistance is less well understood.^{11,12} Recent data indicate that mutation-specific phenotypic sensitivity to RFB is maintained in a moderate proportion of RIF-resistant *Mtb* isolates depending on their *rpoB* mutation. These specific mutations can be detected by some of the existing and next generation rapid diagnostic platforms.^{10,13–19} The ability to distinguish RIF resistant isolates that retain RFB sensitivity at point-of-treatment could have important implications in improving MDR-TB treatment efficacy, duration, toxicity, and cost.

In this study we evaluated the quantitative, phenotypic RIF and RFB resistance in a multinational sample of MDR-TB isolates with a diverse set of *rpoB* mutations. Our primary aim was to test the hypothesis that specific *rpoB* mutations reliably predicted differential phenotypic resistance to RIF and RFB.

Materials and methods

Source and selection of *Mtb* isolates

A total of 60 *Mtb* isolates were selected from a repository of 416 MDR-TB and Extensively Drug-resistant TB (XDR-TB) isolates collected by the Global Consortium for Drug-resistant TB Diagnostics (GCDD). The GCDD aims to understand the genotypic and phenotypic basis of drug-resistance from regions of high M/XDR-TB burden in India, Moldova, Philippines and South Africa. Details of the collection and processing of the parent set of GCDD *Mtb* isolates, including standardized reference phenotypic DST, DNA extraction, PCR and Sanger sequencing, are described in detail by Hillery et al. and Rodwell et al.^{11,20} We used existing GCDD Sanger sequence data to identify isolates with *rpoB* mutations known to confer resistance to RIF.¹¹ A subset of all the RIF resistant isolates with *rpoB* mutations was then stratified into two groups: (1) isolates with *rpoB* mutations previously documented to confer differential phenotypic resistance to RIF and RFB (RIF-resistant/RFB-susceptible), and (2) a random sample of isolates with *rpoB* mutations documented to confer cross-resistance to RIF and RFB (RIF-resistant/RFB-resistant).^{13–19} This study was approved by the institutional review board of the University of California San Diego (UCSD).

Quantitative Phenotypic Drug Susceptibility Testing

We used quantitative drug susceptibility testing (DST) to determine RIF MICs using the Mycobacterial Growth Indicator Tube (MGIT) 960 platform, with EpiCenter software (BD Diagnostic Systems, Franklin Lakes, NJ, USA), and standard critical concentration DST for determining RFB resistance. The RIF drug concentrations for determining RIF MICs were 1.0mg/L, 2.0mg/L, and 4.0mg/L. If RIF MICs on MGIT 960 were ≥ 1 mg/L, the isolate was considered susceptible according to WHO-recommendations.²¹ A single critical concentration of RFB (0.5mg/L) was used to determine if an isolate was resistant to RFB as per clinical recommendations by the Clinical and Laboratory Standards Institute (CLSI).²²

Results

As shown in Table 1, 20 isolates were analyzed in the group of isolates hypothesized to be RIF-resistant/RFB-susceptible based on their *rpoB* mutations: GAC516GTC (n=15), GAC516TAC (n=2), CAC526CTC (n=2), and TCG522TTG (n=1). Forty isolates with a variety of other *rpoB* mutations were analyzed in the suspected RIF-resistant/RFB-resistant group (Table 1). Of the 20 suspected RIF-resistant/RFB-susceptible group, 15 were RIF resistant (>1.0mg/L) and RFB susceptible (<0.5mg/L) as hypothesized, 2 were resistant to both RIF and RFB, and 3 were susceptible to both RIF and RFB. The two isolates expected to be differentially resistant that were actually cross-resistant contained the same *rpoB* mutation (GAC516GTC) as the majority of the differentially resistant group. Cross-susceptibility was found in two of two isolates harboring the mutation GAC516TAC and one of two isolates harboring the mutation CAC526CTC. Of the suspected RIF-resistant/RFB-resistant group, 40 of 40 (100%) were RIF and RFB resistant.

Low-level resistance to RIF (MIC=2.0 mg/L) was also found in one of two isolates in the differentially resistant group harboring the CAC526CTC mutation (Table 1). This isolate was RIF resistant at the critical concentration of 1.0mg/L, but susceptible at a RIF concentration of 2.0mg/L. In contrast, all of the 40 isolates in the suspected RIF-resistant/RFB-resistant group had a RIF MIC>4.0mg/L.

Discussion

Our study demonstrates a strong association between specific *rpoB* mutations and differential resistance to RIF and RFB. Specifically, we found, with a few exceptions, that *rpoB* mutations GAC516GTC, CAC526CTC, and TCG522TTG were associated with phenotypic resistance to RIF and susceptibility to RFB in a diverse selection of MDR-TB isolates from a multinational sample. Conversely, in isolates with a variety of *rpoB* mutations suspected to confer cross-resistance to RIF and RFB resistance, all were found to be phenotypically resistant to both drugs with high RIF MICs (>4.0mg/L). Our findings are consistent with prior studies indicating that the same specific *rpoB* mutations (GAC516GTC, CAC526CTC, and TCG522TTG) are associated with differential resistance to RIF and RFB, while most other resistance-conferring *rpoB* mutations are associated with cross-resistance. It is important to note that there are other *rpoB* mutations associated with RIF/RFB differential resistance and many other mutations associated with cross-resistance, for example at codons 506, 508, 511, 512, 514, 518, 529, and 533 that were not represented in our isolates.^{13–19,23–25} Given that globally, only a few hundred RIF resistant *Mtb* isolates have been evaluated for RIF/RFB differential resistance to date, it is important to build confidence in these associations, even if incrementally.

Two of the 15 isolates with the GAC516GTC mutation that we predicted would be RIF resistant/RFB susceptible were found to be phenotypically resistant to both drugs. This finding reflects the probabilistic nature and complexity of using mutations to predict resistant phenotypes. Most of the *Mtb* genes that confer phenotypic resistance do not have 100% accuracy, likely resulting from natural phenotypic variation in *Mtb* isolates with MICs near the critical concentration.¹¹ The small proportion of isolates deviating from the

predicted susceptibility pattern also emphasizes the fact that the RFB critical concentration we used to determine resistance (0.5mg/L), used by many clinical laboratories but not yet approved by the WHO, might be slightly low. A low critical concentration could result in a small proportion of isolates with the GAC516GTC mutation to have variably resistant phenotypes. Detailed RFB MIC studies on a large number of wild type and *rpoB* mutants may help clarify this issue.

Three isolates in the group we expected to be RIF resistant and RFB susceptible were susceptible to both RIF and RFB. While this does not affect the prediction of RFB susceptibility in isolates with these mutations, it is another demonstration of the phenomenon described above. It has been well documented that *Mtb* isolates with GAC516TAC and CAC526CTC mutations in *rpoB* have variable low-level RIF resistance or “borderline resistance”.^{26–28} Isolates with these mutations have RIF MICs near the critical concentration used as a threshold to determine if an isolate is resistant or susceptible. Grown in the presence of RIF on solid media, isolates with these mutations most often survive and are determined to be resistant. However, the DST result can change based on probabilistic growth dynamics that are different every time the DST is run. In contrast, in liquid media (e.g. MGIT960 that was used in this study), isolates with these mutations are more likely to be determined to be “susceptible” due to the growth dynamics and critical concentration of MGIT 960 DST. As with solid media DST though, liquid media DST results can change on repeat runs.^{26–28} This highlights the importance of a nuanced interpretation of the different *rpoB* mutations and emphasizes the need for global molecular surveillance of the phenotypic and genotypic nature of RFB and RIF resistant strains in order to develop reliable molecular diagnostic interpretation algorithms.

In the context of the other studies showing similar findings, our results indicate that to optimally utilize molecular diagnostics for clinical decision-making in patients with RIF-resistant TB, a deeper understanding of *rpoB* mutations is needed. Beyond identifying the presence of an *rpoB* mutation, we need to identify (1) which *rpoB* mutation is present and (2) what the likely drug-specific MIC consequences of that mutation are likely to be. Only then are we going to be able to design rational clinical studies to test these hypotheses clinically.

Rapidly identifying *rpoB* mutations associated with cross-resistance, differential resistance and different levels of resistance to RIF and RFB presents an intriguing potential means of identifying RIF-resistant isolates (including MDR-TB). Patients with these strains could possibly be treated with a regimen including RFB or even increased RIF dosing, considerably improving the current treatment options. However, the clinical implications of treating RIF-resistant isolates that retain phenotypic RFB susceptibility are not currently well understood. While evidence supports the equivalent efficacy of RFB and RIF in pansusceptible TB⁵, the clinical use of RFB in RFB-susceptible MDR-TB has been evaluated in very few patients.^{29,30} One recent study found higher treatment success rates in RFB-susceptible MDR-TB patients receiving an RFB-containing regimen as compared to RFB-resistant MDR-TB patients receiving a DST-guided regimen (85.7% vs. 52.4% success rates). Although this was a small retrospective study, the findings are encouraging given

their support of the clinical relevance of differential RFB/RIF resistance.³¹ More clinical trials are needed to confirm the effectiveness of RFB in RFB-susceptible RIF-resistant TB.

A limitation of our study was that RFB susceptibility was determined based only on the CLSI-recommended concentration of 0.5mg/L. A finer-grained estimation of the RFB MICs might have helped us understand the clinical relevance of these mutations better,²⁴ since any mutations in *rpoB* appear to cause higher RFB MICs than wild type *rpoB* genotypes.³² However, the objective of this study was not to test whether the recognized RFB interpretive concentration (0.5mg/L) is clinically relevant, but rather, to test whether specific *rpoB* mutations are associated with RFB susceptibility relative to an RFB concentration currently recognized as the critical concentration for making clinical decisions to use RFB in treatment.

Conclusion

Our study adds further evidence to the body of accumulating data that suggests detection of the *rpoB* mutations GAC516GTC, CAC526CTC, and TCG522TTG could be used to rapidly and reliably identify RFB-susceptible MDR-TB isolates that might benefit from RFB treatment. Previous studies have reported that up to 10 to 30 percent of MDR-TB isolates might contain these and other *rpoB* mutations associated with RFB susceptibility.^{17,19,23,25,33–35} If RFB proves to be clinically effective in RFB-susceptible MDR-TB, ignoring these mutations misses a potential opportunity to shorten the duration and limit the toxicities of MDR-TB treatment in an estimated 48,000 to 144,000 patients worldwide.⁶ Prospective clinical trials are urgently needed to determine if the *in vitro* promise of these rapidly detectable mutations can be translated into improved treatment outcomes.

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Table 1

MICs for RIF and RFB with associated *rpoB* sequences stratified by DST results*

Study group	Nucleotide changes	Number of isolates		MIC (mg/L)		DST results
		Rifampin	Rifabutin	Rifampin	Rifabutin	
1: <i>rpoB</i> mutations suspected to confer differential resistance to RIF and RFB	GAC516TAC	2	0.5	1.0	0.5	RIF-S/RFB-S
	CAC526CTC	1	0.5	1.0	0.5	
	GAC516GTC	13	0.5	>4.0	0.5	RIF-R/RFB-R
	TCG522TTG	1	0.5	>4.0	0.5	
	CAC526CTC	1	0.5	2.0	0.5	
	GAC516GTC**	2	>0.5	>4.0	>0.5	RIF-R/RFB-S
	AGC509CGC	1	>0.5	>4.0	>0.5	
	CAA513AAA	4	>0.5	>4.0	>0.5	
	CAG517CAA	2	>0.5	>4.0	>0.5	
	CAC526CGC	2	>0.5	>4.0	>0.5	
2: <i>rpoB</i> mutations suspected to confer cross-resistance to RIF and RFB	CAC526GAC	2	>0.5	>4.0	>0.5	
	CAC526TAC	3	>0.5	>4.0	>0.5	
	TCG531TTG	14	>0.5	>4.0	>0.5	
	TCG531TGG	5	>0.5	>4.0	>0.5	
	GAC516GGC/CTG533CCG	6	>0.5	>4.0	>0.5	
	CTG511CCG/AGC512ACC/GAC516TAC	1	>0.5	>4.0	>0.5	

* MIC, minimum inhibitory concentration; RIF, rifampin; RFB, rifabutin; DST, drug susceptibility testing;

* RIF-S, rifampin susceptible; RIF-R, rifampin resistant; RFB-S, rifabutin susceptible; RFB-R, rifabutin resistant

** Two of fifteen isolates with the GAC516GTC mutation (from Group one, suspected to be RIF-R/RFB-S) tested RIF-R/RFB-R and thirteen of fifteen isolates tested RIF-R/RFB-S