J Antimicrob Chemother 2013; **68**: 2915 – 2920
doi:10.1093/jac/dkt284 Advance Access publication 9 July 2013

Journal of Antimicrobial Chemotherapy

Phenotypically occult multidrug-resistant *Mycobacterium tuberculosis*: dilemmas in diagnosis and treatment

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Received 22 March 2013; returned 6 June 2013; revised 7 June 2013; accepted 13 June 2013

Objectives: The clinical significance of the emergence of *Mycobacterium tuberculosis* (MTB) isolates that contain *rpoB* mutations (genotypic resistance), but are phenotypically susceptible to rifampicin (RIF G^R P^S), remains uncertain. The aim of this study was to determine the prevalence of MTB cases that demonstrate this discordant rifampicin resistance pattern and to establish whether these patients have poorer treatment outcomes with rifampicin-based regimens.

Methods: *rpoB* sequencing was performed on all MTB isolates demonstrating phenotypic resistance to one or more first-line antituberculosis agents (excluding rifampicin). Rifampicin MICs were determined for *rpoB* mutation-positive isolates and clinical case notes were reviewed to identify treatment outcomes in these patients.

Results: Of the 214 phenotypically drug (excluding rifampicin)-resistant isolates tested, 5 contained *rpoB* mutations (4 isoniazid resistant and 1 pyrazinamide resistant). These isolates demonstrated elevated rifampicin MICs (low-level resistance), despite testing susceptible using phenotypic broth-based methods. One patient experienced a relapse of tuberculosis (TB) 2 years after completion of a rifampicin-containing regimen. These findings are consistent with a recent study that reported treatment failure with rifampicin-based regimens in patients with isoniazid-resistant MTB and genotypic rifampicin resistance.

Conclusions: While MTB RIF G^R P^S strains remain relatively uncommon, they can be associated with low-level rifampicin resistance and poorer treatment outcomes with rifampicin-based regimens. This recently recognized form of multidrug-resistant TB should be adequately detected and managed.

Keywords: rifampicin, *rpoB* mutation, low-level resistance, treatment failure, high-dose rifampicin

Introduction

Rifampicin interferes with the transcription of *Mycobacterium tuberculosis* (MTB) by binding to the β subunit of the RNA polymerase encoded by the *rpoB* gene.[1] Mutations within an 81 bp region of the *rpoB* gene account for ∼96% of all rifampicin-resistant MTB isolates and the detection of rifampicin resistance is used as a surrogate marker for multidrug-resistant (MDR) disease, i.e. resistance to both isoniazid and rifampicin.[1] Phenotypically occult MDR tuberculosis (TB) refers to MTB strains that are isoniazid resistant and contain *rpoB* mutations, but test susceptible to rifampicin using standard phenotypic drug susceptibility methods. There is limited knowledge about the prevalence and clinical significance of MTB cases with this discordant genotypic – phenotypic rifampicin resistance pattern; however, clinical failures whilst on treatment with rifampicin-based regimens have been reported.[2] In this study, we determined the prevalence of MTB that are genotypically resistant but phenotypically susceptible to rifampicin (RIF G^R P^S) and describe the microbiological and clinical outcomes of these cases. We discuss the current diagnostic dilemmas and treatment options that pertain to RIF G^R P^S disease and propose some preliminary alternatives to current practice.

Materials and methods

MTB isolates

All MTB isolates demonstrating resistance to one or more first-line anti-TB drugs (excluding rifampicin), using the BACTEC Mycobacterial Growth Indicator Tube (MGIT) 960 system or BACTEC 460 TB radiometric system (BD,

Sparks, MD, USA), between 2004 and 2012 from New South Wales, Australia, were identified by searching the NSW Mycobacterium Reference Laboratory database, together with a control group of an equal number of fully susceptible MTB isolates matched by year. Drug susceptibility testing (DST) for these isolates had previously been performed at the recommended critical concentrations of rifampicin (1.0 and 2.0 mg/L), isoniazid (0.1 + 0.4 mg/L and 0.1, 0.4 + 2.5 mg/L) and ethambutol (5.0 and 7.5 mg/L) using BACTEC MGIT 960 and BACTEC 460, respectively. Pyrazinamide testing was performed at a concentration of 100 mg/L for BACTEC MGIT 960 or by using the pyrazinamidase test.[3] DNA extracts, stored at −70°C, were retrieved for these isolates. Where DNA extracts were unavailable, MTB isolates stored at −70°C were cultured on Middlebrook 7H10 agar and DNA extraction was performed using Bio-Rad InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA).

DNA sequencing

A segment of the *rpoB* gene containing codons 462–591, including 509–533 of the rifampicin resistance-determining region, was amplified by PCR using primers *rpoB*-F (5′-GACGACATCGACCACTTCGGCAAC-3′) and *rpoB*-R (5′-GAACGGGTTGACCCGCGCGTACA-3′)[4] in a reaction mixture containing the following: 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.2 mM $MgCl_2$; 0.01% gelatin; 0.01% Tween 20; 0.01% NP-40; 200 μM dNTPs; 250 nM *rpoB*-F and *rpoB*-R primers; 1.0 U of HotStar*Taq* DNA polymerase (QIAGEN, Hilden, Germany); and 10 μL of chromosomal DNA. The following thermocycling parameters were applied: initial denaturation at 95°C for 15 min; denaturation, primer annealing and extension at 94°C for 1 min, 64°C for 1 min and 72°C for 15 s for 4 cycles, then 94°C for 30 s, 64°C for 15 s and 72°C for 15 s for 30 cycles; and then a final extension at 72°C for 5 min. Sequencing of the purified DNA product was performed in forward and reverse directions using an automated sequencer (AB 3730*xl* DNA analyser) with 3 pmol of the sequencing primers *rpoB*-SF (5′-AAACCAGATCCGGGT CGGCATGT-3′) and *rpoB*-SR (5′-GCGTACACCGACAGCGAGCCGA-3′).[4] DNA sequences were prepared using Chromas Pro version 1.33 and compared with the MTB reference strain H37Rv using BLAST (http://blast.ncbi.nlm.nih.gov).

MICs

Stock drug solutions of rifampicin were prepared in DMSO at the recommended critical concentrations and as serial 2-fold dilutions to achieve the following rifampicin concentrations: 1.0, 0.5, 0.25 and 0.12 mg/L. Stored MTB isolates were retrieved from cases with *rpoB* mutations and a control group of randomly selected, fully susceptible isolates with no *rpoB* mutations, and cultured on Middlebrook 7H10 agar for DST. The MICs were determined using MGIT 960[5] at the above concentrations of rifampicin. Culture contamination was excluded by performing a Gram and Ziehl–Neelsen stain from MGIT 960 cultures and the inclusion of a *p*-nitrobenzoic acid-containing MGIT 960 culture tube for each isolate tested.[6] Statistical analysis was performed using SPSS version 21. The MICs with < or > values were rounded to the nearest dilution (e.g. MIC <0.12 rounded to 0.06). Two-sample *t*-tests were performed on log-transformed MIC values and the average differences were back-transformed and expressed as ratios of MICs together with a 95% CI, to determine the difference in the MIC values between *rpoB*-containing MTB isolates and the control group.

Xpert MTB/RIF and GenoType MTBDR*plus*

Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) testing was conducted on isolates with *rpoB* mutations, using 0.5 mL of MGIT 960 culture solution added to 1.5 mL of sample reagent, with the remainder of the test performed as previous described.[7] GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) testing was performed on these isolates as detailed previously.[8]

Genotyping

Molecular typing of isolates was determined by the analysis of 24 loci variable number tandem repeats of mycobacterial interspersed repetitive units (MIRU-24)[9] and MTB lineage was assigned using the MIRU-VNTR web application (http://www.miru-vntrplus.org/MIRU).

Patient treatment outcomes

Clinical case notes of patients with *rpoB* mutations were reviewed to determine patient demographics, site of infection, drug regimen received and treatment outcome. Ethics approval was granted by the Western Sydney Local Health District Human Research Ethics Committee (HREC2013/2/6.6(3661) AU RED LNR/13/WMEAD/24).

Results

Between 2004 and 2012, 3147 MTB isolates from individual patients were identified; 51 were phenotypically rifampicin resistant [45 MDR (1.4% of all MTB), 5 rifampicin monoresistant and 1 rifampicin and ethambutol resistant]. Two hundred and four were isoniazid monoresistant (6.5% of all MTB) and 12 had other resistance patterns. *rpoB* sequencing was performed on 202 isoniazid-resistant isolates (2 isoniazid-resistant isolates could not be located) and the 12 isolates with other resistance patterns. *rpoB* mutations were identified in five isolates, of which four were isoniazid resistant (two high level at 0.4 mg/L and two low level at 0.1 mg/L), accounting for 2.0% of all isoniazid-resistant isolates, and one was pyrazinamide monoresistant. *rpoB* sequencing of 202 fully susceptible MTB isolates revealed no *rpoB* mutations. RIF G^R P^S MTB isolates accounted for 9% of all MTB that had either phenotypic or genotypic rifampicin resistance.

There was a significant difference in the rifampicin MICs for the *rpoB* mutation-containing isolates compared with a control group of 12 *rpoB* mutation-negative, fully susceptible MTB isolates ($P < 0.001$). On average, rifampicin MICs were 7.3 times higher (95% CI 3.9–13.7 times) in the *rpoB*-positive isolates compared with the control group. Four of the five MTB isolates were of the Beijing lineage and had significantly higher rifampicin MICs compared with the one East African Indian strain. Xpert MTB/RIF and GenoType MTBDR*plus* assays correlated with *rpoB* sequencing results, demonstrating all isolates to be rifampicin resistant (Table 1).

Drug treatment regimens in these cases consisted of four first-line TB drugs, continued for the duration of therapy in the majority of isoniazid-resistant cases. Treatment duration varied between 6 and 12 months (Table 1). All patients received directly observed therapy and were confirmed by clinic nurses to be compliant with the prescribed treatment. Case 4, with low-level isoniazid-resistant non-cavitary pulmonary TB, received treatment with four first-line drugs for 6 months (Table 1). Two years after completion of therapy, this patient experienced recurrent pulmonary TB with an MTB strain that had an identical MIRU-24, *rpoB* genotype and phenotypic DST profile to the initial isolate.

Discussion

Out of the first-line TB agents, rifampicin has the most potent sterilizing activity, including activity against semi-dormant organisms,[10] and plays a key role in early bacterial killing[10,11] and in the continuation phase against persisting organisms.[12] The

Table 1. Clinical and microbiological characteristics of first-line drug (excluding rifampicin)-resistant MTB strains containing *rpoB* mutations

	Patient characteristics				Microbiological results of MTB strains					
	age (years), gender, country of birth, site of disease	previous TB treatment	TB regimen received[a]	treatment outcome	phenotypic drug resistance[b]	*rpoB* mutation	MTB lineage	rifampicin MIC (mg/L)[c]	Xpert MTB/RIF	Genotype MTBDR*plus*
1	62, F, Philippines, pulmonary TB (cavitary)	yes; >20 years prior in the Philippines; 3 drugs for 3 months	RHEZ daily for 1.5 months and RHEZ 3×/week for 7.5 months	lost to follow-up after completion of treatment	HL INH monoresistant	511 CTG→CCG (Leu→Pro)	East African Indian	0.25	RIF resistant	MDR-TB
2	28, M, China, pleural TB	no	RHEZ 3×/week for 12 months	successful; 1.5 years of follow-up	LL INH monoresistant	533 CTG→CCG (Leu→Pro)	Beijing	1.0	RIF resistant	MDR-TB
3	29, M, China, pleural TB	no	RHEZ daily for 1 month, RHEZ 3×/week for 5 months and REZ 3×/week for 3 months	successful; 2 years of follow-up	HL INH monoresistant	516 GAC→GGC (Asp→Gly); 518 AAC→GAC (Asn→Asp)	Beijing	0.5	RIF resistant	MDR-TB
4	44, F, China, pulmonary TB (non-cavitary)	no	RHEZ daily for 1 month and RHEZ 3×/week for 5 months	recurrence of TB 2 years post-treatment completion	LL INH monoresistant	511 CTG→CCG (Leu→Pro)	Beijing	0.5	RIF resistant	MDR-TB
5	26, F, Vietnam, pulmonary TB (cavitary)	no	RHEZ daily for 2 months, RHEZ 3×/week for 2 months and RH 3×/week for 5 months	successful; 0.5 years of follow-up	PYZ monoresistant	511 CTG→CCG (Leu→Pro)	Beijing	1.0	RIF resistant	RIF resistant

F, female; M, male; INH, isoniazid; PYZ, pyrazinamide; RIF, rifampicin.
[a]R, rifampicin; H, isoniazid; E, ethambutol; Z, pyrazinamide.
[b]Drug resistance testing performed using MGIT. High-level (HL) isoniazid resistant = MIC ≥0.4 mg/L. Low-level (LL) isoniazid resistant = MIC 0.1–0.4 mg/L.
[c]MTB isolates with MICs >1.0 mg/L were considered to be phenotypically resistant; MICs for the control group of 12 fully susceptible MTB isolates with no *rpoB* mutations: <0.12 mg/L (median); <0.12–0.25 mg/L (range).

appropriate use of this drug is dependent on informative laboratory susceptibility testing and reporting. We present here five cases of RIF G^R P^S TB that had coexisting resistance to another first-line agent (isoniazid in four cases and pyrazinamide in one case). Recurrent disease occurred in one case, 2 years after completion of a rifampicin-based regimen. These RIF G^R P^S MTB isolates had significantly elevated rifampicin MICs, associated with *rpoB* mutations that have been described previously, with varying degrees of rifampicin resistance.[13–15] Our findings are consistent with a recent study by Williamson *et al*.,[2] where four patients with RIF G^R P^S, isoniazid-resistant MTB (4.3% of all isoniazid-resistant MTB) also had elevated rifampicin MICs. In three of these patients (clinical data were missing in the fourth case), treatment failure in the form of recurrent, persistent or progression to culture-positive disease occurred during treatment on a rifampicin-based regimen.[2] In addition, a study describing an outbreak of low-level rifampicin-resistant disease reported treatment failure on standard first-line therapy.[16]

These findings challenge several aspects of our current diagnostic and treatment practices. First, the established gold standard DST method is to employ phenotypic, most commonly broth-based, assays at a recommended critical drug concentration (e.g. 1.0 mg/L rifampicin for MGIT 960). This form of testing will miss both genotypic resistance and low-level phenotypic rifampicin resistance. This is particularly apparent with the use of automated broth-based assays, which although they offer more rapid DST results are less sensitive at detecting lower-level drug resistance compared with solid agar-based assays.[17] This issue is supported by a recent pharmacokinetic/pharmacodynamic (PK/PD) study suggesting the need for a 4-fold reduction in the current critical concentration of rifampicin in DST to most optimally reflect rifampicin bactericidal activity.[18] This would consequently result in a dramatic increase in the number of MDR-TB cases reported. Whilst our study and that by Williamson *et al*.[2] have shown that RIF G^R P^S MTB associated with low-level rifampicin resistance are clinically important and are more likely to result in treatment failure or recurrent disease, such a large reduction in the critical concentration of rifampicin would be premature in the absence of additional supportive clinical data. A more pragmatic approach could involve the use of two rifampicin concentrations in DST (e.g. 1.0 and 0.5 mg/L for MGIT 960) to reflect higher- and lower-level resistance, analogous to the current isoniazid DST recommendations. An alternative strategy, depending on laboratory capacity, could be to screen all phenotypically drug (excluding rifampicin)-resistant MTB for *rpoB* mutations using e.g. DNA sequencing or Xpert MTB/RIF.

Based on a similar rationale to lowering critical concentrations of rifampicin in DST, the use of higher doses of rifampicin (e.g. 900 or 1200 mg) should be re-explored in the context of low-level rifampicin resistance. The antibacterial effect of rifampicin against MTB is concentration dependent and best reflected by the area under the curve (AUC)/MIC and the maximum concentration of drug (C_{max})/MIC ratio.[19] The current standard 600 mg (10 mg/kg) dose of rifampicin has been consistently shown in PK/PD studies to be suboptimal at preventing the emergence of resistance and inducing sufficient bactericidal effects at the site of infection.[19–21] Animal models have shown that higher doses of rifampicin improve the sterilizing activity and survival.[22–24] Two recent reviews provide an excellent summary of the existing evidence for the use of higher-dose rifampicin and conclude this to be an extremely promising option to improve outcomes and allow shortening of TB drug regimens.[25,26] Toxicities in the form of a 'flu-like' syndrome have been associated with high-dose intermittent rifampicin in studies from the 1970s;[27,28] however, there is good evidence from the use of daily high-dose rifampicin in other conditions[29–31] that this form of rifampicin dosing in TB is likely to be tolerated. A clinical trial of 46 patients in Indonesia, randomized to standard- (10 mg/kg) versus higher-dose (13.3 mg/kg) rifampicin showed a significantly greater proportion of patients with adequate rifampicin plasma concentrations in the higher-dose rifampicin group.[32] We await with interest the results of current Phase II high-dose rifampicin studies on drug tolerability and treatment outcomes.

It is clear that further treatment outcome data are required to guide the management of RIF G^R P^S and/or low-level rifampicin-resistant disease. Retrospective cohort studies, similar to our study, in larger multicentre settings, that employ primary phenotypic DST, would be the most ideal methodology. This will minimize bias and avoid the ethical dilemma of withholding potentially important clinical information that may be problematic in a prospective study. Whilst awaiting these studies and the trial results of higher-dose rifampicin, a 'more conservative' treatment regimen should be considered in RIF G^R P^S MTB and/or low-level rifampicin-resistant disease, in the form of a longer treatment duration and/or the use of additional anti-TB drugs.

There are several limitations to our study. The conclusions from our findings are limited by the relatively small sample size and the number of RIF G^R P^S cases observed. In three out of the five cases reported, the follow-up period was <2 years; therefore, recurrent disease outside of this time frame has not yet been determined. Less than 10% of fully drug-susceptible isolates were sequenced for *rpoB* mutations; hence, although no mutations were detected in this group, these cannot be excluded in the whole drug-susceptible MTB cohort. In addition, the number of wild-type MTB isolates we used for comparing MIC values with *rpoB*-containing strains was small; however, we believe the magnitude of the difference we detected in these two groups is likely to reflect a reliable and significant difference. To our knowledge, wild-type MIC distributions in MTB have not previously been performed using MGIT 960.

Although 25% (one out of four) of the isoniazid-resistant RIF G^R P^S TB cases in this study experienced treatment failure whilst receiving a rifampicin-based regimen, the rate of treatment failure associated with the entire isoniazid-resistant TB cohort is unknown. It is known, however, that the relapse rate for all TB cases in Australia (drug susceptible and drug resistant) is extremely low (0.9%–1.3%).[33] Of note, the one patient who experienced relapsed disease in this study received four first-line drugs for 6 months, whereas the remaining patients received 9–12 months of therapy, a considerably longer course than that recommended by the WHO[34] and administered to patients in most TB endemic countries for isoniazid-resistant pulmonary disease. Hence, the clinical significance of RIF G^R P^S MTB may be even more relevant in these high-burden settings, although further studies are required to confirm this.

On a global scale, the significance of MTB that is RIF G^R P^S may be overshadowed by the enormity of the MDR-TB epidemic. However, we believe that if global targets for TB control[35] are to be achieved, all TB diagnostic and treatment avenues must be explored and should begin in regions that can afford to do this.

In summary, we have found that MTB isolates that are RIF G^R P^S are associated with low-level rifampicin resistance and poorer treatment outcomes with standard-dosing rifampicin-based regimens. This resistance pattern is not detected by current phenotypic methods; thus, a lower concentration of rifampicin in DST or screening all phenotypically drug (excluding rifampicin)-resistant MTB for *rpoB* mutations is required. Further studies are needed to determine the prevalence of RIF G^R P^S TB and treatment outcomes in this condition. Previous and emerging evidence suggests that a higher dose of rifampicin should be considered in TB with low-level rifampicin resistance and in TB treatment in general, to ensure the most optimal efficacy of this valuable drug.

Acknowledgements

We are grateful to Karen Byth for statistical assistance, Hazel Goldberg for assistance with data collection, Alex Outhred for manuscript review and staff of the Mycobacterium Reference Laboratory of Westmead Hospital (Basel Suliman and Andrea Bustamante) for technical assistance.

Funding

This work was supported by a grant provided by the Westmead Research Network.

Transparency declarations

None to declare.

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