RMP discordant strains

*Mycobacterium tuberculosis* strains with highly discordant rifampin susceptibility test results

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Abstract

Setting: the tuberculosis Supra-National Reference Laboratory (SRL) Network

Objectives: to investigate the origin of highly discordant rifampin (RMP) drug susceptibility test results obtained for Mycobacterium tuberculosis strains during proficiency testing

Design: nine SRLs tested RMP susceptibility of 19 selected Mycobacterium tuberculosis strains, using standard culture-based methods. Strains were classified as definitely resistant (R, n=6) or susceptible (S, n=2), and probably resistant (PR, n=8) or susceptible (PS, n=3), based on rpoB mutations and treatment outcome.

Results: all methods yielded a susceptible result for the two S and three PS strains lacking a rpoB mutation, and a resistant result for one R strain with a Ser531Leu and one PR strain with a double mutation. Although the remaining twelve R and PR strains had rpoB mutations (four Asp516Tyr, three Leu511Pro, two Leu533Pro, one each His526Leu/Ser, and one Ile572Phe), they were all susceptible by the radiometric BACTEC™ 460TB or BACTEC™ 960 MGIT methods. In contrast, only one was susceptible by the proportion method on Löwenstein-Jensen, and two on Middlebrook 7H10 agar.

Conclusions: low-level but probably clinically relevant RMP resistance linked to specific rpoB mutations is easily missed by standard growth-based methods, particularly the automated broth-based systems. Further studies are required to confirm these findings, to determine the frequency of these low-level resistant isolates, and to identify technical improvements that may identify such strains.
**Introduction**

The prevalence of multidrug-resistant TB (MDRTB) is rising globally, posing a serious threat to tuberculosis (TB) control. MDRTB does not respond to treatment with first-line drugs, and its management using second-line drugs has not yet been properly organized by most control programmes. Although MDRTB is defined as resistance to at least isoniazid and rifampin (RMP), the key determinant for treatment failure is RMP resistance. Detection of RMP resistance has thus been proposed as a proxy for MDRTB diagnosis as well as for epidemiological monitoring. (14,20,24) RMP drug susceptibility testing (DST) is generally considered as most reliable, by conventional methods based on growth as well as by newer genetic techniques. (1,8) Highly consistent results were obtained during the early proficiency testing (PT) rounds among the Supra-National TB Reference Laboratories (SRL) of the World Health Organization (WHO) / International Union against Tuberculosis and Lung Disease network. Consequently, Laszlo et al proposed a 99% efficiency target for RMP DST by the SRLs. (8) However, 15 of 240 quality control strains (6.2%) distributed from 1999 to 2007 yielded less than 80% agreement for RMP resistance among the SRLs, insufficient for a judicial result. The panels were designed to contain approximately 50% resistance to all first-line TB drugs in various combinations. This pre-condition resulted in over-representation of rare profiles. The SRLs employed one of the four recognized standard culture-based DST methods and the discordant results were not clearly correlated with a particular method or systematic technical errors. DNA-sequencing of the PT rounds’ problem strains invariably showed some rpoB gene mutation. All the mutations encountered had been described previously and were generally considered to confer RMP-resistance, though sometimes at a low level, (12) and available clinical data were usually suggestive of RMP resistance.
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We report here the results of an SRL investigation into the cause of this RMP resistance testing problem.
Materials and methods

The coordinating SRL in Antwerp, Belgium, constituted a panel of 19 *M. tuberculosis* strains isolated from retreatment cases (Table 1), selected either on the basis of discordant results in earlier PT, or because of a RMP minimal inhibitory concentration (MIC) close to the breakpoint at pre-testing on Löwenstein-Jensen (LJ). The strains were further characterised by *rpoB* sequencing, covering all regions of the gene with known resistance conferring mutations, including those outside Cluster I of the core region, (15) supplemented with information on the final outcome of standard treatment with first-line drugs, when available. Strains were classified as resistant (R), probably resistant (PR), susceptible (S) or probably susceptible (PS) to RMP applying the following criteria:

- **R**: mutation present and clinical failure on a RMP-containing treatment
- **PR**: mutation present and treatment outcome either unknown, or “cure” (usually with subsequent bacteriologically-proven relapse) on the standard retreatment regimen, WHO Category 2 (23)
- **PS**: no mutation but subsequent failure or relapse of Category 2 treatment
- **S**: no mutation, cured without registered relapse

Presence of a mutation described as conferring resistance to RMP thus took precedence over treatment outcome, since it is known that patients may fail or relapse from treatment due to other reasons than (RMP) drug resistance, while conversely a low proportion of tuberculosis patients seem to cure spontaneously, independent of drug resistance.(3,6) Table 1 shows details of the panel strains. Of the 14 strains with *rpoB* mutations, six (R1-6) were classified as resistant to RMP (mutation plus treatment failure), and eight as probably resistant (PR1-8), of which five were isolated from relapse cases after Category 2 treatment. Mutations identified from the panel...
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strains were Asp516Tyr (n=4); Leu511Pro (n=3); Leu533Pro (n=2), His526Leu, His526Ser, Ser531Leu and Ile572Phe (n= each 1), with the E. coli codon numbering system. One strain had the double mutation Met515Ile & Asp516Tyr. Ten clones of this strain were tested and showed identical nucleotide changes, thus ruling out a possible mixture of strains. None of the rpoB sequencing patterns showed simultaneously a wild type and a mutation peak, also suggesting the absence of strain mixtures. Three strains (PS1-3) were considered as probably susceptible to RMP (no mutation but Category 2 treatment-failure or relapse). The two strains called susceptible (S1-2) showed a wildtype rpoB sequence without any clinical suspicion of RMP resistance. Most R, PR and PS strains were resistant to one or more of the other first-line TB drugs. All strains except two originated from long-term monitoring of drug resistance among retreatment cases in Bangladesh.

This panel was sent to nine volunteer SRLs for blinded RMP DST. Each SRL used its standard RMP susceptibility testing method(s), based on the original publication of the proportion method (performed on LJ or Middlebrook 7H10 agar), or on the manufacturer’s instructions (BACTEC™ 460 TB radiometric and BACTEC™ 960 MGIT). Two of the participating SRLs performed DST using the LJ proportion method, two reported results by the Middlebrook 7H10 agar proportion method, two by BACTEC 460 radiometric and two by BACTEC 960 MGIT DST. Three SRLs reported results with the proportion as well as one of the BACTEC methods, and some reported incomplete sets of results. To provide more detailed information, the MIC was determined with each method, using RMP at 10, 20, 30, 40 and 80 µg/ml in LJ, or at 0.25, 0.5, 1, 2 and 4 µg/ml in agar and BACTEC, but maintaining the interpretation criteria recommended for each method. The ratio of the MICs to the standard critical concentration for the medium used...
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(40 µg/ml for LJ, 2 µg/ml for radiometric BACTEC, and 1 µg/ml for agar and MGIT) was calculated to allow comparison of MICs obtained with the different methods used. MICs out of the range of RMP concentrations tested were arbitrarily assigned a value corresponding to the next higher or lower dilution. A ratio MIC/critical concentration > 1 was interpreted as resistant.
Results

Figure 1 shows summary DST results by strain as the average MIC to critical concentration ratio for each method. All methods were able to detect resistance for strains R1 (Ser531Leu) and PR7 (double mutation Met515Ile&Asp516Tyr), both yielding the highest ratios, but they all indicated strain PR6 (Leu511Pro) as susceptible with ratios ranging from 0.13 (agar proportion and BACTEC) to 0.38 (LJ proportion method). All S and PS strains tested susceptible by all methods, on liquid or solid media. All other R and PR strains were considered susceptible with liquid but resistant with solid media, except strain R4 (Asp516Tyr), testing susceptible by the agar proportion method.

Individual results on LJ are shown in Figure 2 for five SRLs. The ratio of MIC to critical concentration never exceeded 4, since the highest concentration used (80 µg/ml) was only twice the critical concentration. Most results confirmed the presumptive resistance classification, but 8/14 R and PR strains resulted in an occasional discordant result, and one strain (PR6) was consistently declared susceptible. Overall, 21/67 (31%) MICs for these strains remained below the resistance breakpoint.

Figure 3 shows individual results with the agar proportion, BACTEC radiometric and MGIT methods (each two SRLs). On agar, only the R4 and PR6 strains were consistently susceptible, but 9/14 R and PR strains showed discordant results due to a large difference in MICs between the two SRLs, and 12/27 (44%) MICs for these strains remained below the resistance breakpoint. With both radiometric BACTEC 460TB and BACTEC 960 MGIT and at all four SRLs, only the R1 and PR7 strains were found resistant, while all others were consistently declared susceptible. Overall, 40/47 (85%) of R and PR BACTEC tests were susceptible.
Discussion

Our study shows that RMP DST can yield highly discordant results, even among proficient laboratories, due to the existence of \textit{M. tuberculosis} strains with borderline susceptibility. Alternative explanations, such as mixtures consisting of susceptible and resistant strains, (22) or hetero-resistance with simultaneous presence of susceptible and resistant clones of the same strain, (16) are unlikely. First, none of the DNA sequencing patterns showed an overlapping mutation and wildtype nucleotide. Second, for nine strains with highly discordant results in the PT rounds, IS\textit{6110} fingerprinting had systematically shown identical patterns for all ten clones tested per strain (data not shown).

It was obvious that particular DST methods are more prone to missing RMP low-level resistance. Four SRLs using the BACTEC radiometric or MGIT 960 methods declared all borderline strains susceptible, yielding a resistant result only when the average ratio of the MIC over the critical concentration was at least four on average. The Centers for Disease Control and Prevention (CDC) drug susceptibility testing performance evaluation programme found in their 2008 round that only 19\% of laboratories using the MGIT and 42\% of those using the BACTEC radiometric method reported such a RMP borderline strain as resistant, against 70\% of agar proportion method users (CDC Atlanta, Georgia, unpublished report). Susceptible BACTEC results from genotypically RMP resistant strains have been reported occasionally in the literature. Traore and co-workers found that 4/39 (10\%) RMP resistant isolates from Uganda, with mutations in codons 511, 516 or 533 and resistant by phage and colorimetric DST, were missed by the radiometric BACTEC method. (21)
The bacteriologically unfavourable treatment outcomes for most of the borderline resistant strains from our panel suggest that these specific mutations may have clinical significance. Another question is how frequently they are met in clinical practice. Their reported rarity may be misleading, since virtually all publications describe the frequency of rpoB mutations starting from phenotypically RMP resistant isolates, while our study shows that they are easily missed by routine phenotypic DST. In a systematic sample of Hong Kong strains investigated independently of phenotypic DST results, Leu511Pro, Leu533Pro and His526Leu represented 22% (19/85) of all the mutations, compared to less than 10% among all phenotypically RMP resistant strains of previous years. (27) The distribution of rpoB mutations may differ with geographic origin and treatment history. However, among strains recovered from Bangladesh retreatment cases these three mutations also represent 18% (40 of 221; own data, not shown). Population studies based on molecular screening without culture-based DST pre-selection are thus required, particularly among early MDR-TB suspects (late converters, failures of WHO Category 1 treatment, first-line treatment relapses). Acquisition of RMP resistance may reduce the fitness of TB bacilli, depending on the type of mutation. The most prevalent Ser531Leu mutation has been shown to be least impairing, while very rare mutations or those only known from in-vitro experiments show a severe growth inhibition with some assays. (9,11) The fitness deficit may diminish or disappear due to compensation mechanisms with prolonged patient treatment. (4,5) Moreover, the Ser531Leu mutation and some mutations in codons 513 and 526 have generally been reported as conferring high level resistance, and they comprise 90% or more of those found among phenotypically RMP-resistant isolates. A large variety of other mutations have been occasionally or consistently
associated with low-level RMP resistance. (7,13,17,19) Those resulting in the lowest MICs and
most frequently missed in this study, i.e. Leu511Pro and Leu533Pro, have been considered
susceptible by some authors, (12) although a very high MIC has occasionally been reported as
well. (10) The strain with the highest MIC and diagnosed as resistant by all methods and SRLs
had the Ser531Leu mutation. The only other (probably) resistant strain consistently detected,
albeit with lower MIC values, had the double mutation Met515Ile&Asp516Tyr. Both are known
to confer low-level resistance, (12) but together they resulted in a MIC higher than the four single
Asp516Tyr mutated isolates in our panel. Of the double mutations reported in the literature,
usually at least one confers low-level resistance, and mutations such as Leu511Pro occurred
exclusively in combination in some series. (17) Acquisition and selection of additional mutations
under treatment pressure might be another bacilli survival mechanism, an argument to consider
these low-resistance mutations as clinically relevant. The Ile572Phe mutant from our panel, for
which we could find only one report, has not been associated with borderline resistance. (28)
However, four of our five strains with this mutation showed a low MIC at pre-testing by the
coordinating SRL (own unpublished data).

In our study using selected difficult strains, low level resistance was easily missed with the
current standard DST methods, and systematically with the rapid, automated BACTEC systems.
Considering all strains yielding discordant results in the WHO/IUATLD proficiency testing
rounds 6-14, only 27% of 106 BACTEC results from seven SRL indicated rifampin resistance,
although all these strains had a \textit{rpoB} mutation. In order to avoid calling such strains RMP
susceptible, our methods may thus need modifications. Prolonged incubation and a larger
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inoculum size may be necessary to disclose resistance of poorly growing strains, and RMP critical concentration used with the proportion and BACTEC methods may be too high.

One of the reasons for the BACTEC failures may be too early endpoint readings. Traore et al. reported a growth index below the resistance criterion for his strains, which might eventually have been reached after extended incubation. Extending incubation of borderline strains is usual in many laboratories using solid media, but this is not possible with the standard BACTEC MGIT automated system. That a sufficiently long incubation time is important to disclose drug resistance is common knowledge for the LJ proportion method. With only about 30% susceptible results for R and PR strains, in our study LJ proportion was the most sensitive method reading tests at the standard 6 weeks, but this proportion doubled for interim readings at 4 weeks reported additionally by four of the SRLs (details not shown).

Heifets recommended lowering the RMP breakpoint to 0.5 µg/ml with the radiometric method. (18) Screening at two concentrations (40 and 20 µg/ml in LJ, applying a 10% criterion for the lower concentration) has originally been suggested by Canetti as the more accurate variant of the proportion method. (2)

Under TB control programme conditions, a very high sensitivity is more important than a few days less turn-over time for RMP DST, which may represent only a minor fraction of the total delay before start of MDR-TB treatment. (26) Missing early RMP resistance will have more serious consequences because of the highly standardised care in high-prevalence, low-income countries, resulting in death or default from treatment besides continued transmission of RMP-resistant TB. Moreover, HIV-related immune deficiency and drug malabsorption might compensate for the fitness loss of these strains, with high rates of successful transmission.
Conclusions

Low-level but clinically probable *M. tuberculosis* RMP resistance, linked to specific *rpoB* mutations, is easily missed by standard growth-based methods, particularly the rapid, automated broth-based systems (BACTEC 460 and MGIT 960). Its true frequency remains unknown and should be investigated, but it might be considerable among patients with clinical suspicion of drug resistance. If this hypothesis is confirmed, adaptation of the standard DST methods will be needed.
Acknowledgements

We gratefully acknowledge the participation of the Barcelona, Brisbane and Lisbon SRLs in this study.
Reference List


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Table  Panel strain classification and characteristics

<table>
<thead>
<tr>
<th>Classification and code number</th>
<th>Resistance to H, E and S</th>
<th>rpoB mutation</th>
<th>Treatment</th>
<th>Outcome</th>
<th>Relapse?</th>
<th>Country of origin</th>
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Legend to the Table

- H, E, S: isoniazid, ethambutol, streptomycin
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- R, PR, PS, S: “resistant”, “probably resistant”, “probably susceptible” or “susceptible”, respectively
- WT: wildtype, no mutation found
- Cat. 1, Cat. 2: WHO standard first-line treatment regimens, Category 1 for new cases and Category 2 for retreatment cases (referenced in the text)
- NA: not applicable
**Figure 1** Average results of rifampin susceptibility tests by method and strain

![Graph showing rifampin susceptibility results](image-url)
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Figure 2  Rifampin MIC ratios with the proportion method on LJ, reading after 6 weeks incubation, by SRL and strain
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Figure 3  Rifampin MIC ratios with the agar proportion and BACTEC radiometric or BACTEC MGIT methods, by SRL and strain
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Legend to Figure 1
- MIC: minimal inhibitory concentration; the ratio MIC to critical concentration is shown, with resistance defined as ratio >1
- LJ: Löwenstein-Jensen medium
- Radiometric: BACTEC 460 radiometric™ method
- MGIT: BACTEC 960 MGIT™ system
- R1 to R6, PR1 to PR8, PS1 to PS3, S1 and S2: individual strain codes, based on presumptive rifampin resistance classification

Legend to Figure 2
- MIC: minimal inhibitory concentration; the ratio MIC to critical concentration is shown, with resistance defined as ratio >1
- LJ 6W: Löwenstein-Jensen medium, reading after 6 weeks incubation
- SRL1 to SRL5: supra-national tuberculosis reference laboratories 1 to 5
- R1 to R6, PR1 to PR8, PS1 to PS3, S1 and S2: individual strain codes, based on presumptive rifampin resistance classification

Legend to Figure 3
- MIC: minimal inhibitory concentration; the ratio MIC to critical concentration is shown, with resistance defined as ratio >1
- Radiometric: BACTEC 460 radiometric method
- MGIT: BACTEC 960 MGIT system
- SRL, SRL1, SRL2: supra-national tuberculosis reference laboratory (1 and 2)
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- R1 to R6, PR1 to PR8, PS1 to PS3, S1 and S2: individual strain codes, based on presumptive rifampin resistance classification