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Mycobacterium tuberculosis Strains with Highly Discordant Rifampin Susceptibility Test Results▽

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The objectives of this study were to investigate the origin of highly discordant rifampin (rifampicin) (RMP) drug susceptibility test results obtained for *Mycobacterium tuberculosis* strains during proficiency testing. Nine Supra-National Tuberculosis Reference Laboratories tested the RMP susceptibilities of 19 selected *M. tuberculosis* strains, using standard culture-based methods. The strains were classified as definitely resistant (R) ($n = 6$) or susceptible (S) ($n = 2$) or probably resistant (PR) ($n = 8$) or susceptible (PS) ($n = 3$) based on *rpoB* mutations and treatment outcome. All methods yielded a susceptible result for the two S and three PS strains lacking an *rpoB* mutation and a resistant result for one R strain with a Ser531Leu mutation and one PR strain with a double mutation. Although the remaining 12 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 medium and two on Middlebrook 7H10 agar. 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.

The prevalence of multidrug-resistant (MDR) tuberculosis (TB) is rising globally, posing a serious threat to TB control. (25) MDR TB does not respond to treatment with first-line drugs, (3), and its management using second-line drugs has not yet been properly organized by most control programs (25). Although MDR TB is defined as resistance to at least isoniazid and rifampin (rifampicin) (RMP), the key determinant for treatment failure is RMP resistance. Detection of RMP resistance has thus been proposed as a proxy for MDR TB diagnosis, as well as for epidemiological monitoring (14, 20, 24). RMP drug susceptibility testing (DST), by conventional methods based on growth as well as by newer genetic techniques, is generally considered the most reliable (1, 8). Highly consistent results were obtained during the early proficiency testing (PT) rounds among the Supra-National TB Reference Laboratories (SRLS) 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 precondition resulted in overrepresentation 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 of the mutations encountered had been described previously and were generally considered to confer RMP resistance, though sometimes at a low level (11), and available clinical data were usually suggestive of RMP resistance.

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 an RMP MIC close to the breakpoint at pretesting on Löwenstein-Jensen (LJ) medium. The strains were further characterized 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 by applying the following criteria: R, mutation present and clinical failure on an RMP-containing treatment; PR, mutation present and treatment

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TABLE 1. Panel strain classification and characteristics

Classification and code no.	Resistance to H, E, and S[a]	*rpoB* mutation	Treatment			Country of origin
			Regimen[c]	Outcome	Relapse	
R1	None	Ser531Leu	Cat. 1	Failure	NA[d]	Bangladesh
R2	HE	Leu511Pro	Cat. 2	Failure	NA	Bangladesh
R3	HES	Leu511Pro	Cat. 2	Failure	NA	Bangladesh
R4	H	Asp516Tyr	Cat. 2	Failure	NA	Bangladesh
R5	HES	Asp516Tyr	Cat. 2	Failure	NA	Bangladesh
R6	HS	Asp516Tyr	Cat. 2	Failure	NA	Bangladesh
PR1	HES	Asp516Tyr	Unknown	Unknown	Unknown	DR Congo
PR2	HES	Leu533Pro	Unknown	Unknown	Unknown	Azerbaijan
PR3	None	Leu533Pro	Cat. 2	Cure	Yes	Bangladesh
PR4	H	His526Leu	Cat. 2	Cure	Yes	Bangladesh
PR5	None	His526Ser	Cat. 2	Cure	Yes	Bangladesh
PR6	HE	Leu511Pro	Cat. 2	Cure	Yes	Bangladesh
PR7	HE	Met515Ile Asp516Tyr	Cat. 2	Cure	Yes	Bangladesh
PR8	None	Ile572Phe	Cat. 2	Cure	No	Bangladesh
PS1	H	WT[b]	Cat. 2	Cure	Yes	Bangladesh
PS2	H	WT	Cat. 2	Failure	NA	Bangladesh
PS3	HES	WT	Cat. 2	Failure	NA	Bangladesh
S1	None	WT	Cat. 2	Cure	No	Bangladesh
S2	None	WT	Cat. 1	Cure	No	Bangladesh

[a] H, isoniazid; E, ethambutol; S, streptomycin.
[b] WT, wild type, no mutation found.
[c] Cat. 1, Cat. 2, WHO standard first-line treatment regimens: category 1 for new cases and category 2 for retreatment cases (see the text).
[d] NA: not applicable.

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 after category 2 treatment; and S, no mutation and cured without registered relapse.

The presence of a mutation described as conferring resistance to RMP thus took precedence over the 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 TB patients seem to be cured spontaneously, independent of drug resistance (3, 6). Table 1 shows details of the panel strains. Of the 14 strains with *rpoB* mutations, 6 (R1 to R6) were classified as resistant to RMP (mutation plus treatment failure) and 8 (PR1 to PR8) as probably resistant, 5 of which were isolated from relapse cases after category 2 treatment. The mutations identified from the panel strains were Asp516Tyr (n = 4), Leu511Pro (n = 3), Leu533Pro (n = 2), His526Leu, His526Ser, Ser531Leu, and Ile572Phe (n = 1 each), by the *Escherichia coli* codon numbering system. One strain had the double mutation Met515Ile and 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 to PS3) were considered probably susceptible to RMP (no mutation but category 2 treatment failure or relapse). The two strains called susceptible (S1 and S2) showed a wild-type *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 medium or Middlebrook 7H10 agar) or on the manufacturer's instructions (Bactec 460 TB radiometric and Bactec 960 MGIT) (2). Six of the participating SRLs performed DST using the LJ proportion method, two reported results by the Middlebrook 7H10 agar proportion method, and two by Bactec 460 radiometric and two by Bactec 960 MGIT DST. Three SRLs reported results with the proportion method, as well as one of the Bactec methods, and some reported incomplete sets of results. To provide more detailed information, the MIC was determined by each method, using RMP at 10, 20, 30, 40, and 80 μg/ml in LJ medium or at 0.25, 0.5, 1, 2, and 4 μg/ml in agar and Bactec medium, but maintaining the interpretation criteria recommended for each method. The ratio of the MICs to the standard critical concentration for the medium used (40 μg/ml for LJ medium, 2 μg/ml for radiometric Bactec medium, 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 MIC/critical concentration ratio of >1 was interpreted as resistant.

RESULTS

Figure 1 shows summary DST results by strain as the average MIC/critical concentration ratio for each method. All methods were able to detect resistance for strains R1 (Ser531Leu) and PR7 (Met515Ile Asp516Tyr double mutation), 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), which tested susceptible by the agar proportion method.

Individual results on LJ medium are shown in Fig. 2 for five SRLs. The ratio of the MIC to the 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 1 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 from 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

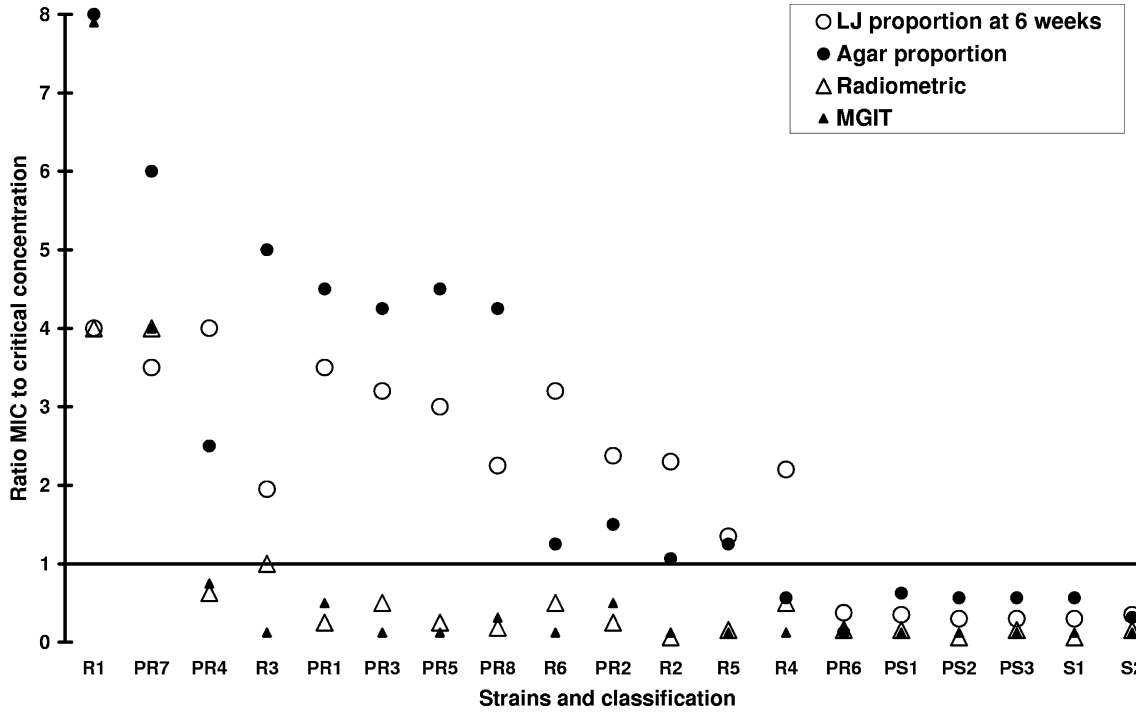

FIG. 1. Average results of RMP susceptibility tests by method and strain. The ratio of the MIC to the critical concentration is shown, with resistance defined as a ratio of >1. LJ, LJ medium; Radiometric, Bactec 460 radiometric method; MGIT, Bactec 960 MGIT system; R1 to R6, PR1 to PR8, PS1 to PS3, S1, and S2, individual strain codes, based on the presumptive RMP resistance classification.

PR7 strains were found to be resistant, while all others were consistently declared susceptible. Overall, 40/47 (85%) of the R and PR Bactec test results were susceptible.

DISCUSSION

Our study shows that RMP DST can yield highly discordant results, even among proficient laboratories, due to the existence of *M. tuberculosis* strains with borderline susceptibility. Alternative explanations, such as mixtures consisting of susceptible and resistant strains, (22) or heteroresistance 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 wild-type nucleotide. Second, for nine strains with highly discordant results in the PT rounds, IS*6110* fingerprinting had systematically shown identical patterns for all 10 clones tested per strain (data not shown).

It was obvious that particular DST methods are more prone to missing low-level RMP resistance. Four SRLs using the Bactec radiometric or MGIT 960 method declared all borderline strains susceptible, yielding a resistant result only when the average ratio of the MIC to the critical concentration was at least 4. The Centers for Disease Control and Prevention (CDC) DST performance evaluation program found in their 2008 round that only 19% of laboratories using the MGIT and 42% of those using the Bactec radiometric method reported such an RMP borderline strain as resistant versus 70% of agar proportion method users (CDC Atlanta, GA, unpublished data). Susceptible Bactec results from genotypically RMP-resistant strains have been reported occasionally in the literature. Traore and coworkers found that 4/39 (10%) RMP-resistant isolates from Uganda, with mutations in codon 511, 516, or 533 and resistant by phage and colorimetric DST, were missed by the Bactec radiometric method (21).

The bacteriologically unfavorable 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 encountered 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 represented 18% (40/221) (data not shown). Population studies based on molecular screening without culture-based DST preselection are thus required, particularly among early MDR TB suspects (late converters, failures of WHO category 1 treatment, and 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 the least impairing, while very rare mutations or those known only from in vitro experiments show severe growth inhibition with some assays

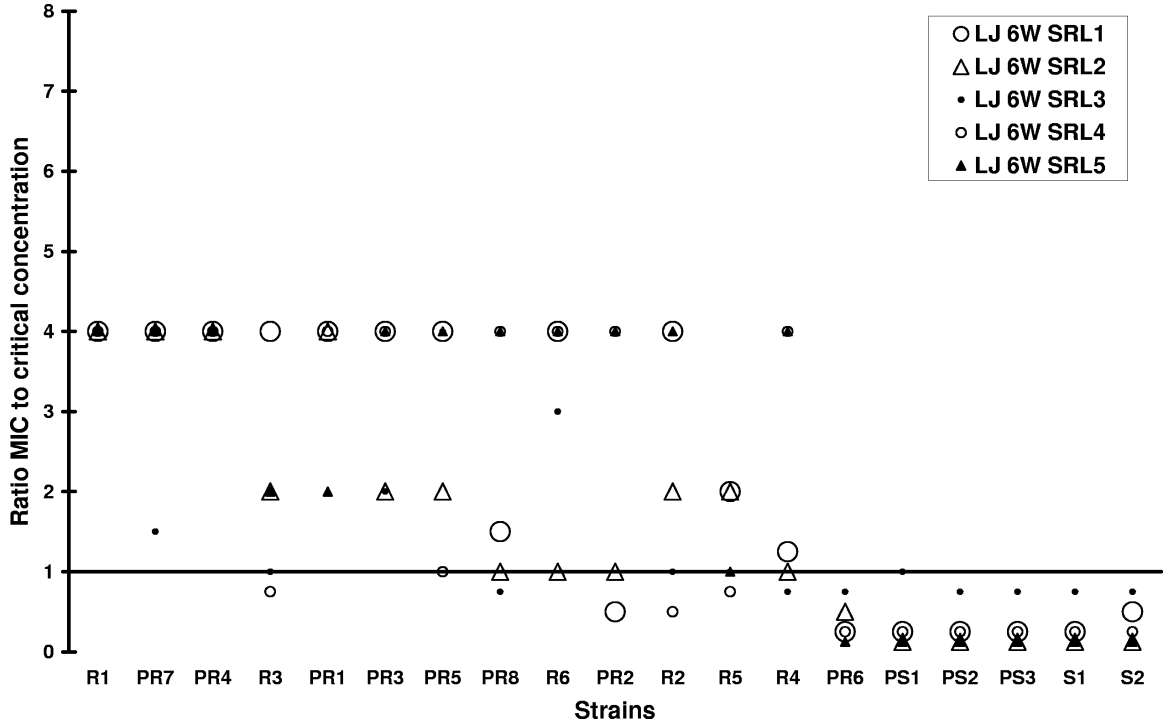

FIG. 2. RMP MIC ratios by the proportion method on LJ medium, read after 6 weeks of incubation, by SRL and strain. The ratio of the MIC to the critical concentration is shown, with resistance defined as a ratio of >1. LJ 6W, LJ medium, read after 6 weeks of incubation; R1 to R6, PR1 to PR8, PS1 to PS3, S1, and S2, individual strain codes, based on the presumptive RMP resistance classification.

(9, 12). 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 (11), although a very high MIC has occasionally been reported, as well (10). The strain with the highest MIC, diagnosed as resistant by all methods and SRLs, had the Ser531Leu mutation. The only other (probably) resistant strain consistently detected, albeit with lower MICs, had the double mutation Met515Ile Asp516Tyr. Both are known to confer low-level resistance (11), but together they resulted in a MIC higher than those of 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 bacillus survival mechanism, an argument for considering these low-resistance mutations 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 pretesting by the coordinating SRL (our 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/ International Union against Tuberculosis and Lung Disease PT rounds 6 to 14, only 27% of 106 Bactec results from seven SRLs indicated RMP resistance, although all of these strains had an *rpoB* mutation. In order to avoid calling such strains RMP susceptible, our methods may thus need modification. Prolonged incubation and a larger inoculum size may be necessary to disclose the resistance of poorly growing strains, and the 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 the 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 of 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).

Suo et al. 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 medium, applying a

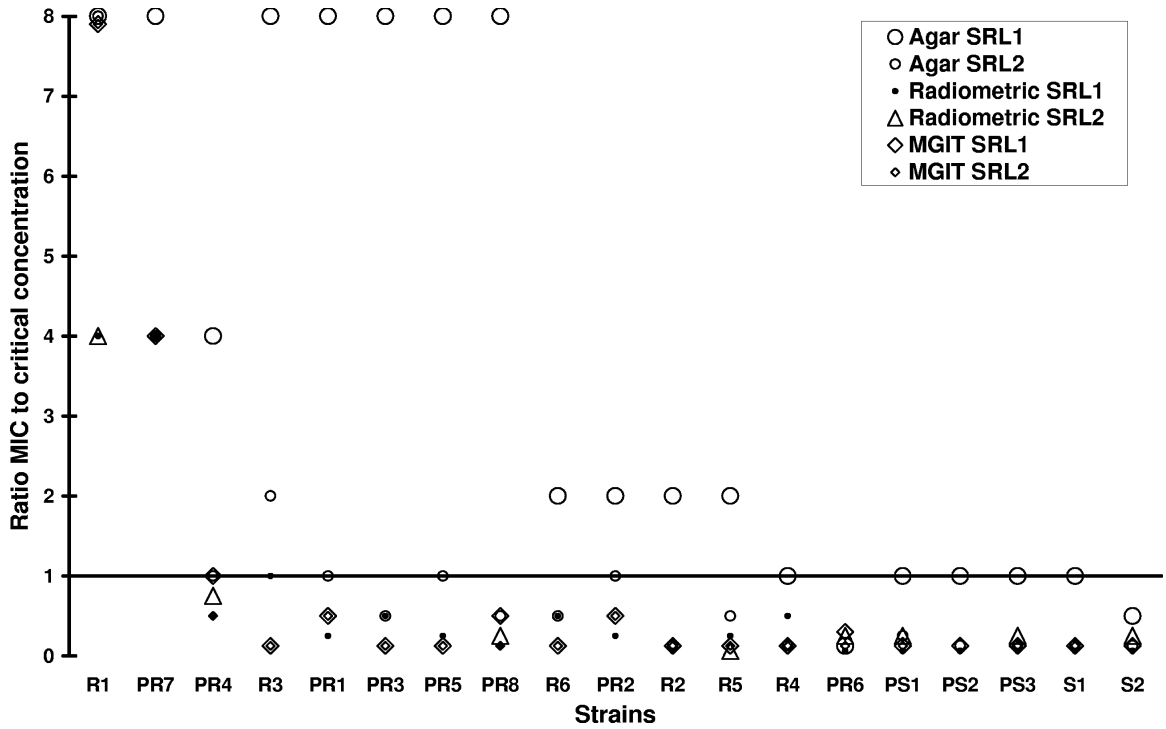

FIG. 3. RMP MIC ratios with the agar proportion and Bactec radiometric or Bactec MGIT method, by SRL and strain. The ratio of the MIC to the critical concentration is shown, with resistance defined as a ratio of >1. Radiometric, Bactec 460 radiometric method; MGIT, Bactec 960 MGIT system; R1 to R6, PR1 to PR8, PS1 to PS3, S1, and S2, individual strain codes, based on the presumptive RMP resistance classification.

10% criterion for the lower concentration) was originally suggested by Canetti et al. as a more accurate variant of the proportion method (2).

Under TB control program conditions, a very high sensitivity is more important than a few days less turnover time for RMP DST, which may represent only a minor fraction of the total delay before the start of MDR TB treatment (26). Missing early RMP resistance has serious consequences because of the highly standardized care in high-prevalence, low-income countries, resulting in death or default from treatment and continued transmission of RMP-resistant TB. Moreover, human immunodeficiency virus-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.

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