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Rifampin Resistance Missed in Automated Liquid Culture System for *Mycobacterium tuberculosis* Isolates with Specific *rpoB* Mutations

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WHO-endorsed phenotypic drug susceptibility testing (DST) methods for *Mycobacterium tuberculosis* are assumed to be the gold standard for identifying rifampin (RMP) resistance. However, previous results indicated that low-level, yet probably clinically relevant, RMP resistance linked to specific *rpoB* mutations is easily missed by some growth-based methods. We aimed to compare the level of resistance detected on Löwenstein-Jensen (LJ) medium with resistance detected by the Bactec MGIT 960 automated DST (MGIT-DST) system for various *rpoB* mutants. Full agreement between LJ and MGIT-DST was observed for mutations located at codons 513 (Lys or Pro) and 531 (Leu, Trp), which were always resistant by both methods. For mutations 511Pro, 516Tyr, 533Pro, 572Phe, and several 526 mutations, LJ and MGIT results were highly discordant, with MGIT-DST failing to give a result or declaring the strains susceptible. Our data show that phenotypic RMP resistance testing of *M. tuberculosis* is not a binary phenomenon for some *rpoB* mutations and that the widely used automated MGIT 960 system is prone to miss some RMP resistance-conferring mutations, while careful DST on LJ missed hardly any. Given the association of these mutations with poor clinical outcome, our findings suggest that the gold standard for rifampin resistance should be reconsidered, in order to address the present confusion caused by discrepancies between phenotypic and genotypic results. The impacts of these mutations will depend on the frequency of their occurrence, which may vary from one setting to another.

Drug-resistant tuberculosis (TB), specifically multidrug-resistant (MDR) and extremely drug-resistant (XDR) TB increasingly jeopardize TB treatment success worldwide (1). Rapid and accurate laboratory diagnosis is the first requirement to treat these patients correctly, which will in turn limit transmission of MDR and XDR TB. The World Health Organization (WHO) has endorsed phenotypic and genotypic assays for rapid detection of MDR TB.

The endorsed phenotypic assays comprise the solid medium-based proportion and absolute concentration methods, the commercial liquid medium-based Mycobacteria Growth Indicator Tube system (MGIT; Becton, Dickinson), the noncommercial colorimetric nitrate reductase assay (NRA) (2), the resazurin microtiter assay (REMA) (3), and microscopy-based (MODS) (4) assays (5). Recommended genotypic assays include line probe assays (LPAs) and the quantitiative PCR-based automated GeneXpert MTB/RIF (Cepheid). Phenotypic assays can provide data on all first- and second-line drugs, although reliability of results varies with the drug tested and method being used, whereas the endorsed genotypic assays are limited to rifampin (RMP; GeneXpert and INNO-LiPA Rif.TB; Innogenetics, Belgium) or RMP and isoniazid (GenotypeMTBDR*plus*; HainLifeSciences, Germany).

It is well known that these genotypic assays can produce fast and reliable results, even directly from clinical specimens, but also fail to detect 100% of clinically relevant resistance because of DNA mutations outside the targeted region and other mechanisms of resistance. The proportion of resistance missed by genotypic testing depends on the drug investigated (6, 7). Also, the level of phenotypic resistance may vary with the type of mutation, e.g., low-level isoniazid resistance for *inhA* mutants (8).

WHO-endorsed phenotypic drug susceptibility testing (DST) methods are assumed to correctly identify all clinically relevant resistant cases. A study among the Supranational Reference Laboratories (SRL), however, has shown that low-level but probably clinically relevant RMP resistance linked to specific *rpoB* mutations is easily missed by these standard growth-based methods, particularly the automated broth-based Bactec systems (9). However, these rounds of proficiency testing included only a few such strains, which might also not have been representative. The current study covers a large number of representative strains with a broad variety of *rpoB* mutations. We aimed at defining the level of resistance on Löwenstein-Jensen (LJ) medium, compared with resistance detected by the Bactec MGIT 960 automated system.

MATERIALS AND METHODS

***Mycobacterium tuberculosis* strains.** From our research collection, we selected a total of 129 *Mycobacterium tuberculosis* isolates from Bangladesh ($n = 76$) and Kinshasa, Democratic Republic of Congo ($n = 53$), representing all *rpoB* mutations encountered at least twice among sputum specimens from the same study population and for which a viable culture isolate was available. Sequencing of the *rpoB* gene and routine DST on LJ medium were performed independently. For each mutation type that was encountered at least twice in our study population, all available strains, to a maximum of 15 strains isolated from different patients, were included. Only strains showing a single mutation were included; those with double mutations or heteroresistant sequence profiles for the *rpoB* gene (wild type and mutant) were excluded from this study. Additional MIC testing with the MGIT 960 system was performed for a subset of 24 strains representing 11 single-nucleotide mutations as well as three wild-type strains;

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TABLE 1 Summary of MICs determined on LJ or by DST in the automated Bactec MGIT 960 system, stratified by *rpoB* mutation type for 129 *M. tuberculosis* isolates

rpoB mutation type	No. of strains	LJ result (MIC_{99} [μg/ml])	No. (%) of isolates determined to be resistant via:			No. (%) of isolates with invalid MGIT result
			LJ (at MIC_{99})	MGIT (at 1 μg/ml)	MGIT (at 0.5 μg/ml)	
511Pro	6	80–640	6 (100)	0	1	0
513Lys	2	>640	2 (100)	2 (100)	2 (100)	0
513Pro	3	>640	3 (100)	3 (100)	3 (100)	0
516Phe	4	>640	4 (100)	4 (100)	4 (100)	0
516Val	15	320–>640	15 (100)	15 (100)	15 (100)	0
516Tyr	6	320–>640	6 (100)	0	1 (20)	1 (20)
522Gln	11	320–>640	11 (100)	8 (73)	8 (73)	2 (18)
522Leu	2	>640	2 (100)	2 (100)	2 (100)	0
526Asp	11	≥640	11 (100)	8 (73)	8 (73)	3 (27)
526Arg	5	>640	5 (100)	5 (100)	5 (100)	0
526Leu	10	≥640	10 (100)	6 (60)	9 (90)	0
526Tyr	14	≥640	14 (100)	10 (71)	12 (86)	2 (14)
526Asn	5	80–>640	5 (100)	1(20)	1(20)	1 (20)
531Leu	10	≥640	10 (100)	10 (100)	10 (100)	0
531Trp	4	≥640	4 (100)	4 (100)	4 (100)	0
533Pro	14	160–>640	14 (100)	0	0	2 (14)
572Phe	7	40–>640	6 (86)	0	6 (86)	0

15 of these strains were part of the 129 strains presented here, whereas 9 were additional strains from previous experiments.

Strains were grown from our −80°C culture collection on LJ medium and further processed for phenotypic resistance testing.

***rpoB* sequencing.** Detection of *rpoB* mutations, targeting a 1,674-bp region from codon 176 to 672, from which RMP resistance-conferring mutations have been described, was performed as described before (10). DNA extracts from clinical specimens were prepared using an adapted Maxwell extraction method (Promega). Thermolysates from grown cultures were prepared by transferring a loop full of bacilli into 1× Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA; pH 7.5) and subsequent boiling for 5 min at 100°C.

Phenotypic RMP resistance testing. We determined the RMP MIC on LJ using the following drug concentrations: 20, 40, 80, 160, 320, and 640 μg/ml. Pure RMP powder was dissolved in dimethyl sulfoxide (both from Sigma-Aldrich, St. Louis, MO) at a concentration of 100 mg/ml with subsequent dilutions in sterile distilled water.

Bacterial suspensions were prepared in sterile 0.01% Tween 80 and adjusted to an opacity equal to McFarland standard 1. This bacterial suspension was used for the inoculation on LJ and for the primary culture required for further testing in the Bactec MGIT 960 system. For LJ, drug-containing slant and a control slant with plain medium were inoculated using a 10^{-2} dilution of the bacterial suspension. A second control was inoculated with a 10^{-4} dilution. Tubes were incubated at 35 to 38°C and read after 4 and 6 weeks of incubation. Interpretation of the MIC inhibiting growth of 99% of the bacilli (MIC_{99}) was done using the 10^{-4} control slant with a countable number of colonies, as per the proportion method (11).

Simultaneously, from the same bacterial suspension, a subculture and subsequent RMP resistance to 0.5 μg/ml and 1 μg/ml were determined with the automated Bactec MGIT 960 system by using the standard procedure recommended by the manufacturer. All new batches of medium and supplements were checked for their quality upon arrival and prior to use, as per the manufacturer's recommendations.

Strains presenting growth problems with controls not reaching the minimum growth cutoff required for the automated MGIT were retested once from a freshly grown subculture. MIC testing in the MGIT 960 system was done using the following concentrations: 0.25, 0.5, 1.0, 2.0, and 4.0 μg/ml.

Spoligotyping. Spoligotyping was performed using primers (DRa and DRb) corresponding to the direct repeat (DR) region of the genome of *Mycobacterium tuberculosis* and an in-house membrane according to the procedure described elsewhere (12).

RESULTS

Results are presented in Table 1, based on the *Escherichia coli rpoB* codon numbering system. Overall, high MICs on LJ were found, even for most of the strains with supposed low-level resistance mutations, but many of those were found susceptible by simultaneous testing in the MGIT system. Only one strain (the 572Phe mutant) remained just below the breakpoint of an 80-μg/ml MIC on LJ and were interpreted as susceptible. In contrast, by MGIT testing, 11 strains failed to yield a valid result, while 40 were considered susceptible to RMP at 1.0 μg/ml, and 27 strains were considered susceptible at 0.5 μg/ml.

Full agreement between LJ and MGIT DST was observed for mutations located at positions 513 (Lys or Pro) and 531 (Leu, Trp), which were always resistant by both methods.

For positions 511, 533, and 572, LJ and MGIT results were largely discordant. Six strains showing a 511Pro mutation had MICs ranging from 80 to 640 μg/ml on LJ medium, while they were identified as susceptible by MGIT at an RMP concentration of 1.0 μg/ml, and one of these was identified as resistant in the MGIT at an RMP concentration of 0.5 μg/ml. Similarly, all strains with a 572Phe mutation ($n = 7$) were systematically identified as RMP susceptible by MGIT at a RMP concentration of 1 μg/ml, and six of them were also susceptible at 0.5 μg/ml, whereas the MICs ranged from 80 to 640 μg/ml LJ for six strains. The LJ-susceptible strain had a MIC of 40 μg/ml. All 14 533Pro mutated strains were resistant on LJ, with MICs ranging from 160 to >640 μg/ml, but none was classified as resistant by MGIT, irrespective of the RMP critical concentration; two of those yielded repeatedly invalid results.

For positions 516, 522, and 526, the rate of agreement depended on the amino acid substitution. All strains with a 516Val or Phe mutation were found resistant in the MGIT, but only 1 of 6 with a 516Tyr mutation were resistant, and only at 0.5 μg/ml. For the 522 position, all Leu mutations were resistant in the MGIT,

TABLE 2 Summary of spoligotyping results, stratified by *rpoB* mutation type

rpoB mutation type	No. of strains	Observed spoligotype(s) (no. of strains, if >1)[a]
511Pro	6	1 (2), 26, 138, 458, NT
513Lys	2	1404, NT
523Pro	3	1 (2), LAM
516Phe	4	52, 144, 471, Cameroon
516Tyr	6	1, 73 (2), 1435, West Africa (2)
516Val	15	52, 53, 96 (2), 123, 1491, Ghana, LAM (2), Uganda (1, 2=), orphan (2), NT
522Gln	11	292, 535, LAM (4=, 2), Uganda, orphan, NT
522Leu	2	1420, 1434
526Arg	5	1, 1567 (2), Uganda, NT
526Asn	5	48, 1423, LAM, Uganda, africanum
526Asp	11	1, 125, 535 (2), 1422, 1425, 1910 (2), EAI (2), NT
526Leu	10	52, 61, 373, 403, 1141, 1391, 1424, 1430, Uganda, LAM
526Tyr	14	1 (5), 292, 358, 1390, 1404, 1423, 1580, CAS, Haarlem, NT
531Leu	10	1 (3), 26, 292, 535 (2), Ghana (2=), Haarlem
531Trp	4	48, LAM, NT (2)
533Pro	14	1 (3), 48 (2), 52, 126, 292, 317, 882, 1396, CAS, Cameroon, orphan
572Phe	7	48, 411 (2), 882, 1389, LAM, EAI
Total	129	

[a] Spoligotypes were assigned according to the MIRU-NVTR*plus* database (http://www.miru-vntrplus.org/MIRU/index.faces); "=" indicates identical profiles. NT, not tested.

but only 73% of the Gln substitutes were. The 526 mutations presented the largest variation, ranging from 1 of 5 (20%) Asn mutations to all 5 Arg mutations classified as resistant by the MGIT, compared to 71 to 90% of the other mutations (Asp, Leu, or Tyr). Except for a few with the 526Asn mutation, all these strains showed high MICs (320 to >640 μg/ml) in LJ.

Additional MIC testing in the MGIT 960 system for a subset of 24 strains in our study confirmed the initial findings and failed to classify all discordant mutants as resistant: one 531Leu mutant showed an elevated MIC of >4.0 μg/ml and two strains had a MIC of 1.0 μg/ml (one 526Leu and one 516Tyr), whereas all other mutants showed a low MIC (3 with 0.5 μg/ml and 16 with <0.25 μg/ml), as did 3 wild-type strains (<0.25 μg/ml).

We did not observe a significant difference in time to positivity for primary cultures versus DST among discordant and concordant mutation types. Nevertheless, 11 strains failed to provide valid MGIT-DST results, even after repeat testing, with all of them showing a mutation of the discordant or variable group (516Tyr, 522Gln, 526Asp, 526Asn, 526Tyr, or 533Pro).

To ensure our observations were not biased by the clonal spread of a single strains for each mutation type, spoligotyping profiles were determined for all but 9 isolates (Table 2). For 11 of 17 mutation types, some degree of clustering by spoligotyping was observed, mostly grouping only one or three isolates. For mutations 526Tyr and 522Gln, 5 of 14 and 4 of 11 isolates were grouped in the Beijing and LAM clades, respectively.

DISCUSSION

Our data show that phenotypic RMP resistance testing of *M. tuberculosis* is not a black-and-white story for some types of *rpoB* mutations and that the widely used automated MGIT 960 system is prone to miss RMP resistance, while DST on LJ missed hardly any, although we cannot exclude that strains with certain mutations failed to grow *in vitro* at all and were therefore not included in our sample.

Our sample covered a good number of randomly selected different strains, representing all common and less common mutations from our study population. The mutations systematically missed by MGIT-DST were either located at the extreme ends of the *rpoB* hot spot for resistance (511Pro and 533Pro) or outside this core region (572Phe). Discordance was limited and less pronounced within the 81-bp core region, except for mutation 516Tyr, which was also missed systematically by standard MGIT-DST. The MIC_{99} on LJ medium was high to very high, except for a few mutations (3/6 of the 511, 2/7 of the 572, and 1/5 of the 526Asn mutations). The mutations with the highest MICs (320 to ≥640 μg/ml) were consistently found resistant by MGIT also.

Our observations are supported by data reported by other laboratories on two occasions. A study conducted by 9 supranational TB reference laboratories (SRLs) around the world provided evidence that our observation is a method/technique-related problem rather than being explicit to our laboratory (9). In that study, 12 of the difficult strains (mutations 511Pro, 516Tyr, 526Leu, 526Ser, 533Pro, and 572Phe) were tested together with some others, using their preferred WHO-endorsed method(s). All methods declared some of these mutated strains susceptible, but the 2 Bactec 460- and the 2 MGIT 960-using laboratories found them all susceptible. On average, the LJ proportion missed the least, followed by the agar proportion DST method. All methods/laboratories declared the strain with the universally most common 531Leu mutation as well as one strain with a double mutation to be resistant. A second study was recently that entailed investigation of 10 partly different SRLs, using a panel comprising 9 of the strains we report here (unpublished data). The 5 SRLs using MGIT as the primary method reported 1 to 7 of these 9 strains as susceptible.

Our observation of MDR strains reaching very high RMP MICs on solid medium (16-fold times the critical concentration in LJ in our study) is supported by data for South African clinical isolates, with MICs of 100 μg/ml or higher on Middlebrook 7H10 agar, which is 100-fold times the critical concentration (13). Ohno and colleagues documented MICs of ≥64 μg/ml up to 512 μg/ml for mutations in codon 531 and a variable level of RMP susceptibility among strains containing amino acid substitutes in either codon 516 or 526 when they used the broth dilution method (14).

Finally, routine drug susceptibility testing on LJ medium, applying the proportion method with a 40-μg/ml critical concentration and 1% critical proportion, is an ISO15189-accredited test in our laboratory. Quality has been ensured by internal quality control of each batch of homemade medium, participation in external quality assessment panel testing provided by the CDC (until 2008), and the WHO/International Union Against Tuberculosis and Lung Disease since 1994, showing almost always 100% accuracy for rifampin and documented training records for qualified staff.

Similarly, *rpoB* sequencing and standard MGIT-DST are ISO15189-accredited assays in our laboratory. We previously re-

ported that the outcome of standardized first-line treatment of TB is very poor and virtually the same, irrespective of *rpoB* mutation. A comprehensive analysis of the clinical significance of prevailing *rpoB* mutants has been completed recently (9, 15). Missed RMP resistance will cause delays in timely and correct TB treatment adaptation or initiation, particularly under field program conditions in low-income settings. where treatment is standardized according to rigid guidelines that are applied by paramedics. Laboratory error will then usually result in treatment failure or multiple relapses in cases of low-level resistance and all too often to death. That this is not only a problem in low-income countries was recently reported from New Zealand, where MGIT-DST regularly missed clinically important resistance, with the same dire consequences (16). Moreover, with the current WHO emphasis on rapid genetic DST (17), discordant phenotypic/genotypic DST results are extremely confusing and complicate patient management.

Loss of relative fitness has been associated with specific mutations in the *rpoB* gene (18–20) and might explain why MGIT failed to detect RMP resistance in our study. The inability to obtain valid MGIT-DST results for some strains with the discordant mutations lends support to this hypothesis, pointing to slow growth as a factor responsible for the problematic drug susceptibility testing in rapid assays. Moreover, MGIT-DST at a lower critical concentration did not improve the results except for a few mutations, mainly, the 526 mutations, with a minor proportion of missed resistance. Thus, location of the mutation does not only seem to have an impact on the RMP binding site but also on the fitness of the strain, probably through a reduced functioning of the gene's product, the RNA polymerase.

However, it is not only the location of the mutation that plays a role, but also the resulting amino acid substitution. Part of the 516Tyr mutations were missed by MGIT, whereas 516Phe/Val showed fully concordant results.

Although spoligotyping did not identify high clonality among our isolates, our data need to be corroborated by increasing the number of isolates with discordant mutations, preferably from different geographic regions.

Future evaluations should include comparison of slow solid medium growth-based versus diverse rapid growth-based techniques, including NRA, MODS, and REMA, which might also be prone to miss such discordant strains, as well as sequencing versus LPA and GeneXpertMTB/RIF.

In our study, we applied an in-house sequence-based method to identify the mutations. Theoretically, all these strains with discordant mutations located within the core region will show an LPA profile lacking one or more wild-type band without showing specific mutation bands (ΔWT strains). There have been only a few reports on the GenotypeMTBDR*plus* system missing the 533Pro mutation, possibly because of its location at the very end of the segment amplified in this assay (21, 22). By extrapolation, this might also apply to the GeneXpertMTB/RIF system, which targets the same fragment, but we are not aware of any such reports.

The clinical and epidemiological significance of these difficult mutations is often contested, and still too little is documented. All mutation types investigated in this study have been reported before (www.TBDreamDB.org; update October 2011). The three most discordant mutation types (511Pro, 516Tyr, and 533Pro) have even been classified as highly confident mutations in this database, together with most of the concordant mutations; the former two classifications were based on radiometric Bactec data, and the latter were based on results from the proportion method.

Furthermore, reported mutation frequencies from different geographic regions in the world range from 0 to 9.4% for codon 511 and from 0 to 6.9% for codon 533 (23). The importance of these easily missed mutations thus needs to be further investigated, both in terms of frequency as well as determinations for first-line treatment outcomes in multiple geographical settings. The impacts of these mutations will depend on the frequency of their occurrence, which may vary from one setting to another.

Given the association of these mutations with poor clinical outcome, our findings suggest that the gold standard for rifampin resistance should be reconsidered, in order to address the present confusion caused by discrepancies between phenotypic and genotypic results.

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