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# Comparative Study on Genotypic and Phenotypic Second-Line Drug Resistance Testing of *Mycobacterium tuberculosis* Complex Isolates[▽]

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**The mycobacterium growth indicator tube (MGIT960) automated liquid medium testing method is becoming the international gold standard for second-line drug susceptibility testing of multidrug- and extensively drug-resistant *Mycobacterium tuberculosis* complex isolates. We performed a comparative study of the current gold standard in the Netherlands, the Middlebrook 7H10 agar dilution method, the MGIT960 system, and the GenoType MTBDR*sl* genotypic method for rapid screening of aminoglycoside and fluoroquinolone resistance. We selected 28 clinical multidrug- and extensively drug-resistant *M. tuberculosis* complex strains and *M. tuberculosis* H37Rv. We included amikacin, capreomycin, moxifloxacin, prothionamide, clofazimine, linezolid, and rifabutin in the phenotypic test panels. For prothionamide and moxifloxacin, the various proposed breakpoint concentrations were tested by using the MGIT960 method. The MGIT960 method yielded results 10 days faster than the agar dilution method. For amikacin, capreomycin, linezolid, and rifabutin, results obtained by all methods were fully concordant. Applying a breakpoint of 0.5 μg/ml for moxifloxacin led to results concordant with those of both the agar dilution method and the genotypic method. For prothionamide, concordance was noted only at the lowest and highest MICs. The phenotypic methods yielded largely identical results, except for those for prothionamide. Our study supports the following breakpoints for the MGIT960 method: 1 μg/ml for amikacin, linezolid, and clofazimine, 0.5 μg/ml for moxifloxacin and rifabutin, and 2.5 μg/ml for capreomycin. No breakpoint was previously proposed for clofazimine. For prothionamide, a division into susceptible, intermediate, and resistant seems warranted, although the boundaries require additional study. The genotypic assay proved a reliable and rapid method for predicting aminoglycoside and fluoroquinolone resistance.**

The emergence of multidrug-resistant tuberculosis (MDR-TB) in the 1990s, and more recently, extensively drug resistant tuberculosis (XDR-TB), has revealed the need for new drugs and alternative, second-line treatment regimens. Many of these second-line drugs are either old drugs that had not been frequently used because of side effects or unproven efficacy or newer drugs intended primarily for treatment of other infections (3). Their use necessitated an evaluation of drug susceptibility testing (DST) and a determination of the critical concentrations of these alternative drugs.

A variety of techniques are now available for second-line DST, of which the mycobacterium growth indicator tube automated liquid culture system (MGIT960) is probably the most used and best validated at this moment (13). The latest addition to second-line DST are genotypic methods, which detect mutations in the *gyrA* gene of *Mycobacterium tuberculosis* that are associated with fluoroquinolone resistance and mutations in the *rrs* operon that are associated with resistance to capreomycin and the aminoglycosides (2–5).

Despite the arrival of these novel tools, many uncertainties remain. Not all methods have been evaluated in comparative studies. Moreover, the critical concentrations for resistance to several second- and third-line drugs, including moxifloxacin and prothionamide, remain the subject of debate (7, 11, 13).

In the Netherlands, the Middlebrook 7H10 agar dilution method has been used for second-line DST for 2 decades (18). In order to comply with international standardization requirements, a switch to the MGIT960 method has been initiated.

In this study, we have compared the results of the MGIT960 method and the GenoType MTBDR*sl* assay, a commercially available genotypic second-line DST method, to our reference method, the Middlebrook 7H10 agar dilution method. For the MGIT960 method, we tested the various critical concentrations published for prothionamide and moxifloxacin but also included clofazimine in our drug panel. For the latter drug, which has become increasingly important in the treatment of MDR and XDR-TB, no *in vitro* DST data for the MGIT960 method were available.

## MATERIALS AND METHODS

From our laboratory database, we selected 28 multidrug-resistant clinical isolates (26 *M. tuberculosis* and 2 *Mycobacterium bovis*) and the *M. tuberculosis* H37Rv reference strain. Isolates were nonrandomly selected to include those previously designated susceptible and resistant to each of the drugs included in the test panel, except linezolid, for which no resistant strains are available. All strains were identified by a GenoType MTBC assay (Hain Lifescience, Nehren, Germany).

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TABLE 1. Drug concentrations and breakpoints evaluated in this study

| Drug | Concn(s) evaluated (μg/ml)[a] | |
|---|---|---|
| | 7H10 | MGIT960 |
| Amikacin | 1, 2, **5**, 10, 20 | **1** |
| Capreomycin | 2, 5, **10**, 20, 40 | **2.5** |
| Prothionamide | 1, 2, **5**, 10, 20 | **2.5**, **5** |
| Moxifloxacin | 0.125, 0.25, 0.5, **1**, 2 | **0.125**, 0.25, 0.5, **1** |
| Linezolid | 0.125, 0.25, 0.5, **1**, 2 | 0.25, 0.5, **1** |
| Rifabutin | 0.2, **0.5**, 01, 2, 5 | **0.5** |
| Clofazimine | 0.5, 1, **2**, 5 | 0.125, 0.25, 0.5, 1, 2 |

[a] Boldface type indicates the breakpoint concentration (cutoff point for resistance) (7, 11-13, 18). 7H10, Middlebrook 7H10 agar dilution; MGIT960, mycobacterium growth indicator tube.

We performed DST using the 25-well Middlebrook 7H10 agar dilution method as previously described (18), the automated MGIT960 method with the TBeXist application and EpiCenter software package (BD Bioscience, Erebodegem, Belgium) (16), and the genotypic GenoType MTBDR*sl* reverse line blot methodology (Hain Lifescience, Nehren, Germany) (4, 5). This molecular test detects mutations in the *rrs* (16S) gene that give rise to amikacin, kanamycin, and capreomycin resistance, as well as *gyrA* gene mutations that lead to fluoroquinolone resistance. The agar dilution method was considered the gold standard, and the selection of strains was based on previous results obtained by this method.

The applied drug concentrations and breakpoints, based on previous publications (7, 11–13, 18), are depicted in Table 1. For the agar dilution method, a MIC equal to the breakpoint concentration is reported as susceptible; plates were read after 4, 7, 10, and 14 days of incubation and on each working day thereafter. For the MGIT960 method, growth at the breakpoint concentration is considered resistant; drug-containing tubes were read at the moment the growth control was signaled positive on the MGIT960 system. Research was conducted in a blinded manner with respect to previous test results and to results obtained by the other methods in this study.

## RESULTS

The turnaround time of the MGIT960 method is substantially shorter than that of the agar dilution method; after a mean of 8 days, the growth control tubes were positive (187.8 h; standard deviation [SD], 58.3 h) versus a mean duration of 18 days (SD, 3 days) for the agar dilution method ($P < 0.01$).

The DST results for amikacin, capreomycin, linezolid, and rifabutin are presented in Table 2. Phenotypic DST results for rifabutin, linezolid, and amikacin were fully concordant between the two methods. For capreomycin, 97% concordance was noted; one strain was borderline susceptible (MIC, 10 μg/ml) when tested by the agar dilution method, but resistant (MIC, >2.5 μg/ml) when tested by the MGIT960 method (Table 2) (sensitivity, 95%; specificity, 100%); the genotypic assay detected an *rrs* gene mutation, which resolves this issue in favor of the MIT960 result. Complete cross-resistance between amikacin and capreomycin was noted when the MGIT method was used; there was 97% cross-resistance when the agar dilution method was used.

The results for moxifloxacin are presented in Table 3. For moxifloxacin, the use of the 0.125-μg/ml breakpoint concentration for the MGIT960 method led to one false-resistance result, yielding a 97% concordance (sensitivity, 95%; specificity, 100%); the use of the 1.0-μg/ml breakpoint led to two false-susceptibility results (93% concordance; sensitivity, 100%; specificity, 71%). Applying the 0.5-μg/ml breakpoint, complete agreement was noted between results obtained by the MGIT960 and agar dilution methods.

TABLE 2. Phenotypical drug susceptibility testing results for amikacin, capreomycin, rifabutin, and linezolid[a]

| Drug | 7H10 result (no. of isolates) | No. of isolates with indicated MGIT960 result | | Concordance (%) |
|---|---|---|---|---|
| | | S | R | |
| Amikacin | S (21) | 21 | 0 | 100 |
| | R (8) | 0 | 8 | |
| Capreomycin | S (22) | 21 | 1[b] | 97 |
| | R (7) | 0 | 7 | |
| Rifabutin | S (14) | 14 | 0 | 100 |
| | R (15) | 0 | 15 | |
| Linezolid | S (29) | 29 | 0 | 100 |

[a] S, susceptible; R, resistant; 7H10, Middlebrook 7H10 agar dilution method; MGIT960, mycobacterium growth indicator tube.
[b] This strain harbored the A1401G *rrs* mutation, favoring the MGIT960 result and a "resistant" designation.

The agreement between both phenotypical DST methods was low in prothionamide testing (Table 4). Applying the 2.5-μg/ml breakpoint for the MGIT960 method led to six false-resistance results and one false-susceptibility result (76% concordance; sensitivity, 73%; specificity, 86%; positive predictive value [PPV], 94%; negative predictive value [NPV], 50%). When applying the 5-μg/ml breakpoint, three false resistances and one false susceptibility were noted (86% concordance; sensitivity, 86%; specificity, 86%; PPV, 95%; NPV, 67%). From these results, it is clear that at the lowest MICs (≤2 [7H10]/≤2.5 μg/ml [MGIT960]) and highest MICs (>10 [7H10]/>5 μg/ml [MGIT960]), the concordance is the highest for the two phenotypic methods (Table 4).

All 29 strains proved susceptible to clofazimine by the 7H10 agar dilution method. By the MGIT960 method, two strains were found to be resistant to 0.25 μg/ml of clofazimine (i.e., a MIC of 0.5 μg/ml); all others proved susceptible to this concentration or the lower concentrations (Table 5). From these data, a $MIC_{90}$ of 0.5 μg/ml for the agar dilution method can be calculated; for the MGIT960 method, the $MIC_{90}$ is 0.25 μg/ml.

The MTBDR*sl* assay revealed *rrs* mutations in eight strains

TABLE 3. Distribution of moxifloxacin MICs determined by two phenotypic methods[a]

| 7H10 MIC of moxifloxacin (μg/ml) | No. of isolates with indicated MGIT960 MIC (μg/ml) | | | | |
|---|---|---|---|---|---|
| | *≤0.125* | 0.25 | ***0.5*** | *1* | >1 |
| ≤0.125 | 14 | 1 | 0 | 0 | 0 |
| 0.25 | 4 | 0 | 0 | 0 | 0 |
| 0.5 | 3 | 0 | 0 | 0 | 0 |
| ***1*** | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 1 | 2 |
| >2 | 0 | 0 | 0 | 1 | 3 |

[a] Twenty-nine isolates were tested. Italics indicate the previously published breakpoint concentration and cutoff for resistance. Boldface indicates the proposed breakpoint stemming from the current study, 0.5 μg/ml for moxifloxacin (MGIT960). 7H10, Middlebrook 7H10 agar dilution method; MGIT960, mycobacterium growth indicator tube.

TABLE 4. Distribution of prothionamide MICs determined by two phenotypic methods

| 7H10 MIC of prothionamide (μg/ml) (no. of strains) | No. of isolates with indicated MGIT960 MIC (μg/ml)[a] | | |
|---|---|---|---|
| | ***≤2.5*** | >2.5 and ≤5 | ***>5*** |
| <1 (11) | 11 | 0 | 0 |
| **2** (3) | 2 | 1 | 0 |
| *5* (8) | 3 | 2 | 3 |
| **10** (4) | 1 | 0 | 3 |
| >20 (3) | 0 | 0 | 3 |

[a] Twenty-nine isolates were tested. Italics indicate the previously published breakpoint concentration and cutoff for resistance. Boldface indicates proposed breakpoints stemming from the current study; MICs of ≤2 (7H10) or ≤2.5 (MGIT960) μg/ml indicate prothionamide susceptibility, MICs of >10 (7H10) or >5 (MGIT960) μg/ml indicate resistance, and those in between indicate intermediate susceptibility. 7H10, Middlebrook 7H10 agar dilution method; MGIT960, mycobacterium growth indicator tube.

TABLE 5. Distribution of clofazimine MICs determined by two phenotypic methods

| 7H10 MIC of clofazimine (μg/ml) | No. of isolates with indicated MGIT960 MIC (μg/ml)[a] | | | | |
|---|---|---|---|---|---|
| | ≤0.125 | 0.25 | 0.5 | **1** | 2 |
| ≤0.5 | 13 | 11 | 2 | 0 | 0 |
| 1 | 0 | 3 | 0 | 0 | 0 |
| *2* | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 |

[a] Twenty-nine isolates were tested. Italics indicate the previously published breakpoint concentration and cutoff for resistance. Boldface indicates the proposed breakpoint stemming from the current study, 1 μg/ml for clofazimine (MGIT960). 7H10, Middlebrook 7H10 agar dilution method; MGIT960, mycobacterium growth indicator tube.

(6 *M. tuberculosis*, 2 *M. bovis*) and *gyrA* mutations in seven strains (5 *M. tuberculosis*, 2 *M. bovis*). Five strains harbored the A1401G *rrs* (16S rRNA) gene mutation, and three harbored a C1402T mutation (1 *M. tuberculosis*, 2 *M. bovis*). Hybridization with the probe for the A1401G mutation was generally weak. All isolates with *rrs* mutations proved amikacin and capreomycin resistant, except for one strain with an A1401G *rrs* mutation that proved amikacin resistant by 7H10 agar dilution and MGIT960 testing but had a MIC of 10 μg/ml for capreomycin, i.e., at the breakpoint, by the 7H10 agar dilution method. No false-positive or false-negative results were recorded.

Among the seven strains with *gyrA* mutations, one harbored an A90V mutation, three harbored an S91P mutation (including the 2 *M. bovis* strains), and one had the D94G mutation. Two strains were found to have multiple mutations; one had A90V and D94A mutations, and the other had A90V, S91P, and D94N mutations. For both, one mutant probe hybridized well and yielded a high-intensity band, whereas the others were weak. In 3 out of 8 strains (38%) with mutations in *gyrA*, wild-type sequences were also detected by the MTBDR*sl* assay. All seven isolates with mutations in *gyrA* proved moxifloxacin resistant by the 7H10 agar dilution method; no false-positive or false-negative results were recorded. All seven isolates with *gyrA* gene mutations had MICs above 0.5 μg/ml for moxifloxacin when tested by the MGIT960 method.

## DISCUSSION

The results of second-line DST for *M. tuberculosis* complex bacteria obtained by the 7H10 agar dilution method and MGIT960 method are largely similar. The much shorter turnaround time for the MGIT960 method (mean, 8 versus 18 days) is a major advantage over that of the previous standard, the 7H10 agar dilution method. Moreover, the reading in the MGIT system is done automatically, which is preferred over the reading by eye in the classical 7H10 agar method. Fast yet robust laboratory results are of paramount importance to guide the choice of drugs in MDR- and XDR-TB treatment. Before confidently switching to MGIT960 as the gold standard for second-line DST, the uncertainties involving moxifloxacin and prothionamide breakpoints must be resolved.

Of all quinolones, moxifloxacin is considered the most promising antituberculosis drug (3, 9, 14). As a result, *in vitro* DST for this drug has become an important issue. However, the breakpoint concentration for the MGIT method is still a subject of debate, with published breakpoints ranging from 0.125 μg/ml (7) to 0.5 μg/ml (11, 17) and 1.0 μg/ml (12). Our comparison of the two phenotypic methods and the genotypic method supports the choice of 0.5 μg/ml as the breakpoint. One strain without detectable *gyrA* mutations proved resistant to 0.125 μg/ml of moxifloxacin, a previously proposed breakpoint (7); false-resistance results are major errors and could lead to unwarranted diagnoses of XDR-TB and unnecessary restrictions in the selection of active drugs for individual treatment regimens. Two strains with *gyrA* mutations grew at 0.125, 0.25, and 0.5 μg/ml but not at 1 μg/ml. Here, the 1.0-μg/ml breakpoint (12) would have led to false-susceptibility results; this can lead to the inclusion of presumably inactive drugs in treatment regimens, with serious consequences, and counts as a very major error (7).

For moxifloxacin, the 0.5-μg/ml MGIT960 breakpoint concentration seems to correlate well with the drug's bioavailability. In a recent study, the regular 400-mg once-daily dosage of moxifloxacin led to maximum serum concentrations of 4.7 μg/ml, an area under the curve (AUC) of 48.2 mg · h/liter, and trough concentrations (24 h after intake) of 0.78 μg/ml (9). The AUC/MIC ratio best describes the activity of moxifloxacin against *M. tuberculosis* (2, 15) and should be >100 for optimal bactericidal activity, although this is rarely reached in TB treatment (10). Applying the 1.0-μg/ml (7H10) and 0.5-μg/ml breakpoints (MGIT) to designate susceptible isolates, the $MIC_{90}$ was 0.5 (7H10) or 0.25 (MGIT) μg/ml, leading to an $AUC/MIC_{90}$ of 96.4 or 192.8, both compatible with substantial clinical activity.

Based on the suggested breakpoint concentrations for prothionamide, MGIT960 test results compare poorly with those of the agar dilution method, at 76 to 86% concordance. This discordance is not a novel observation, although its extent is larger than that seen in previous studies. Two previous extensive studies noted more major and very major errors in prothionamide testing than for any other second-line drug (7, 13). Owing to the inclusion of many susceptible strains, both studies still showed 96% and 97% percent agreement when applying the 2.5- and 5.0-μg/ml breakpoints for the MGIT960 method, compared to results for the resistance ratio method (7) or BACTEC460 radiometric method (13). In our study, the concordance was highest at the lowest and highest MICs, which

suggests that one breakpoint defining susceptibility and resistance may not be adequate for prothionamide. A division into susceptible, intermediate, and resistant may be preferable, at least if there are pharmacokinetic and clinical consequences. The existence of low-level resistance has also been suggested by previous investigators, on the basis of discrepancies in drug susceptibility testing (13, 16). Larger studies are needed to define the suitability of this division and its MIC breakpoints as well as the genomic mutations underlying the different degrees of resistance (16). On the basis of our data, isolates with MICs of ≤2 (7H10) or ≤2.5 (MGIT) μg/ml should be considered susceptible, those with MICs of >10 (7H10) or >5 (MGIT) μg/ml should be considered resistant, and all those in between should be considered intermediate susceptible. For the intermediate group, pharmacokinetic and pharmacodynamic studies would be needed to determine the possible efficacy of prothionamide treatment.

Based on the $MIC_{90}$ of 0.25 μg/ml for clofazimine (Table 5), we propose a breakpoint concentration of 1 μg/ml for the MGIT960 method, although the stability of clofazimine in 7H9 medium warrants separate investigation. However, the mechanism of action of clofazimine against *M. tuberculosis* remains uncertain (3), and the difficulties in isolating clofazimine-resistant strains (1) raise a question about the existence of clofazimine resistance. In fact, the only isolate we previously found to be clofazimine resistant (18) was intentionally included in this study and now proved susceptible by both phenotypic methods, rendering the previous observation most likely a laboratory mistake. The mutations needed to acquire clofazimine resistance may have an impact on the viability of *M. tuberculosis* (1). For nontuberculous mycobacteria, clofazimine resistance exists, mainly among rapid growers, though its underlying mechanism is not known (19).

The molecular assay proved highly reliable in detecting amikacin, capreomycin, and moxifloxacin resistance. All *rrs* mutations predicted amikacin and/or capreomycin resistance. Therefore, the positive predictive value of the genotypic tests may be considered very high. One strain with a "MUT1" (A1401G) *rrs* mutation still tested capreomycin susceptible, albeit borderline, by the 7H10 agar dilution method. However, according to a previous study, this mutation induces capreomycin resistance (8). In contrast, for three strains, no hybridization with the wild-type probe was noted in the absence of MUT1 (i.e., the A1401G mutation) probe hybridization. This specific result indicates the presence of the C1402T mutation (4), which is supposed to result in amikacin susceptibility and capreomycin resistance (8). Our three strains all proved to be cross-resistant to both drugs; Hillemann et al. also noted this phenomenon for one strain in their study of the MTBDR*sl* assay's performance (5). Since the hybridization of the MUT1 probe was generally weak, A1401G mutations may have potentially been misinterpreted as C1402T mutations.

For moxifloxacin resistance, the obtained MTBDR*sl* results were fully concordant with the MGIT960 results if the 0.5-μg/ml breakpoint was applied. In fact, the molecular analysis provides further support for this breakpoint. Complete concordance with the 7H10 agar dilution was noted. Multiple mutations were found for two strains. Interestingly, in three out of eight strains with mutations in *gyrA*, wild-type sequences were also detected. This suggests that in these isolates, both wild-type (susceptible) and mutant (resistant) bacterial populations are present, in a ratio sufficient to allow hybridization of the wild-type and mutant probes. These results were also frequent (21.9%) in the study by Hillemann and coworkers (5). This heteroresistance may result from the emergence of resistant subpopulations or infection by multiple strains, as has been noted for isoniazid and rifampin (6).

While the MTBDR*sl* assay includes probes for *embB* gene mutation analysis, we decided not to compare these results with ethambutol results for the phenotypic methods, as low sensitivity of the molecular system has already been demonstrated (5) and ethambutol is not a second-line drug.

The intricacies of second-line DST by phenotypic methods and their importance in clinical care underline the need for quality control efforts, both internal and external. The use of control strains may be preferable to minimize intertest variance; external quality control schemes are already in existence for second-line DST to ensure interlaboratory reproducibility.

In summary, the 7H10 agar dilution and MGIT960 phenotypical second-line DST methods for *M. tuberculosis* yielded largely identical results, except for those for prothionamide. For moxifloxacin and clofazimine, we propose 0.5 μg/ml and 1 μg/ml, respectively, as breakpoint concentrations for the MGIT960 method. For amikacin, capreomycin, rifabutin, and linezolid, we support the 1.0-, 2.5-, 0.5-, and 1-μg/ml breakpoint concentrations suggested previously (7, 13). The MGIT960 method has a much shorter turnaround time than that of the conventional 7H10 agar method, which is essential for the timely optimization of patient treatment regimens. Here, the MTBDR*sl* molecular assay proved to be a reliable method for predicting aminoglycoside and fluoroquinolone resistance and thus for the rapid screening of (MDR-)TB strains for possible extensive drug resistance.

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