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Rapid Detection of *Mycobacterium tuberculosis* Resistance to Second-Line Drugs by Use of the Manual Mycobacterium Growth Indicator Tube System[▽]

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The objective of this study was to evaluate the manual mycobacterium growth indicator tube (MGIT) system for the testing of *Mycobacterium tuberculosis* susceptibility to second-line drugs compared to the proportion method. One hundred eighty-eight *M. tuberculosis* isolates were tested for susceptibility to ofloxacin, kanamycin, ethionamide, and capreomycin by the manual MGIT, and results were compared to those obtained with the proportion method on 7H11 agar, considered a reference method. Results for ofloxacin and capreomycin were excellent, with 100% accuracy, and a result of 99.4% accuracy was achieved for kanamycin. For ethionamide, accuracy was lower, with a result of 86.7% compared to that of the proportion method. We proposed the following critical concentrations for the drugs: for ofloxacin, 2.0 μg/ml; for kanamycin, 2.5 μg/ml; for ethionamide, 5 μg/ml; and for capreomycin, 2.5 μg/ml. The time required to obtain results was an average of 8 days by the manual MGIT and 3 weeks by the reference method. Our results show that the manual MGIT is an accurate method for the rapid susceptibility testing of *M. tuberculosis* to second-line drugs. There is no need for a machine when using the manual MGIT, and results can be read with a simple UV lamp or with a semiquantitative reader, which considerably reduces the cost of the method.

The emergence of multidrug-resistant tuberculosis (MDR-TB) caused by *Mycobacterium tuberculosis* and, recently, extensively drug-resistant tuberculosis caused by an MDR strain that is also resistant to any fluoroquinolone and at least one of the three injectable second-line drugs (kanamycin [KAN], amikacin [AK], and/or capreomycin [CM]) is a real threat for TB control programs (28). It is obvious that there is a great necessity for rapid, reliable, and economical methods for testing the susceptibility of *M. tuberculosis* not only to first-line drugs but also to second-line drugs. Access to drug susceptibility testing (DST) is a priority, and TB culture is an essential component of TB management. Using the standardized conventional DST methods, it takes a minimum of 3 to 8 weeks to identify resistant or susceptible strains on solid media (6, 7). The introduction of liquid culture media such as the manual mycobacterium growth indicator tube (MGIT) reduces the turnaround time compared to that of solid media, taking an average of 15 days to get results (1, 5, 19, 23, 25). In June 2007, the World Health Organization issued a recommendation for the use of liquid media for culture and DST in middle- and low-income countries to address challenges due to the epidemic of human immunodeficiency virus-associated TB and drug-resistant TB, especially in resource-limited settings (29). Fully automated commercial systems such as the BACTEC MGIT 960 (Becton Dickinson) have shown their usefulness for the rapid detection of resistance to second-line drugs (12, 24); however, they require heavy equipment and are still constrained by the cost of the machines. The manual MGIT system has been reported to be a sensitive and rapid method for DST to first-line drugs (19, 23), but unfortunately it has not been fully standardized for testing second-line drugs. The objective of this study was to establish critical concentrations for the major second-line anti-TB drugs ofloxacin (OFX), KAN, ethionamide (ETH), and CM with clinical isolates of *M. tuberculosis*. Results were compared with those obtained with the gold standard proportion method (PM) on 7H11 agar. An interpretation of discordant results was attempted by determining the MIC, using the colorimetric resazurin microtiter assay (REMA) plate (14).

MATERIALS AND METHODS

Strains. One hundred eighty-eight clinical isolates of *M. tuberculosis* were studied. These isolates originated from the collection of the Institute of Tropical Medicine in Antwerp, Belgium. One hundred fifty-three isolates were MDR, and 35 were susceptible. The reference strain H37Rv (ATCC 27294), from the American Type Culture Collection, was used as the reference susceptible control. All isolates were freshly subcultured on Löwenstein-Jensen medium before being used and tested by the different methods.

Antimicrobial agents. All drugs were in chemically pure powder. ETH, OFX, and CM sulfate were obtained from Sigma-Aldrich (St. Louis, MO), and KAN monosulfate was obtained from ICN Biomedicals, Inc. (OH). KAN and CM were dissolved in sterile distilled water. OFX was dissolved in 0.1 N NaOH solution, and ETH was dissolved in dimethyl sulfoxide. All drugs were filter sterilized. Stock solutions were made at 1 mg/ml and stored at −20°C for no more than 6 months.

DST. The PM was performed on 7H11 agar for all drugs according to the standard procedure with the following recommended critical concentrations: 10 μg/ml of ETH and CM, 2 μg/ml of OFX, and 6 μg/ml of KAN (17). Results were read 21 days after incubation at 37°C in 5% CO_2 atmosphere.

MGIT. The standard protocol for DST in the MGIT was followed as recommended for first-line drugs according to the manufacturer's instructions (Becton

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TABLE 1. Results of 188 *M. tuberculosis* isolates tested by the PM and the manual MGIT for second-line drugs[a]

Drug (concn, μg/ml)	PM result on 7H11 agar	No. of isolates with indicated MGIT result and drug (concn, μg/ml)		Manual MGIT		
		S	R	% Sensitivity	% Specificity	% Accuracy
		OFX (2)				
OFX (2)	S	147	0	100	100	100
	R	0	41			
		KAN (2.5)				
KAN (6)	S	116	0	98.6	100	99.4
	R	1	71			
		ETH (2.5)				
ETH (10)	S	125	36	96.2	77.6	80.3
	R	1	26			
		ETH (5)				
ETH (10)	S	137	25	100	84.5	86.7
	R	0	26			
		CM (2.5)				
CM (10)	S	157	0	100	100	100
	R	0	31			

[a] R, resistant; S, sensitive.

Dickinson, Sparks, MD). Briefly, MGITs were supplemented with 0.5 ml of oleic acid-albumin-dextrose-catalase. Culture suspension for inoculation was adjusted to a McFarland standard of 0.5 and further diluted 1:5 with sterile saline. Drugs were added to the MGIT to have final concentrations of 2.5 μg/ml and 5 μg/ml of ETH, 2.5 μg/ml of CM, 2 μg/ml of OFX, and 2.5 μg/ml of KAN. A growth control (GC) tube was prepared without antibiotic. The tubes were inoculated with 0.5 ml of the inoculum diluted 1:5. A positive control tube was prepared by adding 5 ml of a 0.4% sodium sulfite solution to an empty MGIT, and an uninoculated MGIT was used as negative control. After 3 days of incubation at 37°C, the fluorescence of the MGITs was read with a 365-nm UV transilluminator and with the BD MicroMGIT fluorescence reader. A strain was considered suseeptible if the drug tube did not show fluorescence within 2 days of the positivity of the GC tube and was considered resistant if the drug tube was positive within 2 days of the positive result of the GC tube. In this study, weekend reading was not possible, so when the GC tube was positive on the weekend, resistant results were reported on Monday if the drug-containing tube and the GC tube were both fluorescent. If the GC tube was positive when examined on Monday and the drug-containing tube was negative, then Monday was considered day 1 of GC tube fluorescenee, and the drug-containing tube was considered sensitive if it remained negative through Wednesday.

REMA. The MICs of discordant second-line drugs were determined as described by Martin et al. (14). The concentration ranges for each drug used in this test were 0.62 to 20 μg/ml of ETH and KAN, 0.3 to 10 μg/ml of CM, and 0.25 to 8 μg/ml of OFX.

Analysis of data. The sensitivity was calculated as the true positive (TP) rate expressed as a pereentage (TP/TP + false negative), and the specificity was calculated as the true negative (TN) rate expressed as a percentage (TN/TN + false positive) for each drug compared to the PM on 7H11 agar. We have also calculated the accuracy of the MGIT manual that represents all TP and TN results among all results of the test.

RESULTS

One hundred eighty-eight isolates of *M. tuberculosis* were tested for susceptibilities to OFX, KAN, CM, and ETH.

With the manual MGIT, results were available after an average of 8 days of incubation compared to 3 weeks with the PM 7H11 agar. Table 1 shows the results of DST obtained by the PM and the manual MGIT. Among the 188 isolates, 41 were resistant to OFX, 71 were resistant to KAN, 26 were resistant to ETH, and 31 were resistant to CM according to the PM. Complete agreement was found between results obtained for the detection of OFX resistance by the PM on 7H11 agar and by the manual MGIT using 2 μg/ml of OFX as the critical concentration. Complete agreement was also found for the detection of CM resistance between both methods using a concentration of 2.5 μg/ml of CM for the manual MGIT.

For all *M. tuberculosis* isolates determined to be susceptible to KAN by the PM on 7H11 agar, the specificity was 100% for the manual MGIT using a concentration of 2.5 μg/ml of KAN. For the resistant strains, one discordant result was found to be resistant by the PM but susceptible by the manual MGIT, giving a sensitivity of 98.6%. This isolate was repeated by both methods, giving the same results. A third method, the REMA plate, was then tested for this isolate, and the MIC result for KAN obtained by the REMA method was 2.5 μg/ml, which corresponds to a borderline strain since the cutoff for KAN in this system has been determined to be 2.5 μg/ml (14). This same strain gave a reading of 11 using the semiquantitative MGIT reader, which also corresponds to a borderline strain.

For ETH, we evaluated two different critical concentrations in the MGIT manual system. Using a concentration of 2.5 μg/ml of ETH, 36 isolates with discordant results were found to be false resistant, giving a specificity of 77.6%. One discordant result was classified as false susceptible, giving a sensitivity of 96.2%. When using a critical concentration of 5 μg/ml of ETH, 25 discordant isolates were classified as false resistant and no false susceptible results were found, giving a specificity and a sensitivity of 84.5% and 100%, respectively. We have repeated testing of these 25 discordant isolates by both methods using a critical concentration of 5 μg/ml of ETH in the manual MGIT. The same results were obtained by the PM and by the manual MGIT. We then used the REMA to determine the MICs of these isolates,

TABLE 2. Comparison of critical concentration ranges for second-line drugs in *M. tuberculosis* obtained by different studies using liquid medium

Study, publication yr (reference)	Method	ETH	KAN	AK	OFX	CM
Chen et al., 1989 (8)	BACTEC TB-460	0.3–1.25			0.5–2.0	1.5–3.0
Heifets et al., 1987 (9) and 1991 (10)	BACTEC TB-460	0.3–1.25			0.5–2.0	1.5–3.0
Rastogi et al., 1996 (22)	BACTEC TB-460	0.25–5.0	2.0–4.0	0.5–1.0	0.5–1.0	1.0–2.0
Pfyffer et al., 1999 (20)	BACTEC TB-460	1.25–2.5	2.5–5.0	0.5–1.0	1.0–2.0	1.25–2.5
Pracharktam et al., 2001 (21)	alamarBlue				1.0–2.0	
Bastian et al., 2001 (4)	alamarBlue		2.5			
Bastian et al., 2001 (4)	Manual MGIT		5.0			
Martin et al., 2003 (14)	REMA plate	2.5	2.5		2.0	2.5
Barreto et al., 2003 (2)	MB/BacT	1.25		2.0	2.0	
Morcillo et al., 2004 (16)	MTT plate	2.0	4.0			
Martin et al., 2005 (13)	Manual MGIT				2.0	
Palaci et al., 2006 (18)	Manual MGIT				2.0	
Rüsch-Gerdes et al., 2006 (24)	BACTEC MGIT 960	5.0		1.0	2.0	2.5
Krüüner et al., 2006 (12)	BACTEC MGIT 960	5.0		1.25	1.0	1.0
Umubyeyi et al., 2006 (26)	REMA plate				2.0	

and out of the 25 false-resistant isolates, 3 borderline isolates had an intermediate MIC, 2 isolates were found to be susceptible by the REMA, and the other remaining strains were found to be resistant. No difference was found when the reading was made using the semiquantitative reader or the UV lamp. The time to obtain results was 8.6 days when results were read with the semiquantitative reader and 8.8 days when results were read using the UV lamp.

DISCUSSION

In vitro DST to second-line drugs is recommended for high MDR prevalence settings, and it plays a key role in deciding individualized treatment for MDR-TB patients (30). Two multicenter studies using the BACTEC MGIT 960 automated system for validation of DST of *M. tuberculosis* to second-line drugs have been described in the literature. Also, only three studies have been reported in the literature using the manual MGIT for second-line drugs, and they focused on only two drugs (3, 13, 18).

The aim of this study was to develop a basic protocol to establish critical concentrations for the major second-line anti-TB drugs in the manual MGIT. In our study, critical concentrations of OFX, KAN, CM, and ETH were chosen based on the two existing multicenter studies using the BACTEC MGIT 960 but also based on critical concentrations of second-line drugs established in liquid systems such as the BACTEC 460TB and the colorimetric redox-indicator assays (Table 2).

Aminoglycosides and fluoroquinolones are the most potent second-line drugs for the treatment of TB, and in this study, results obtained for OFX and KAN showed a high degree of accuracy compared to that shown by the reference method. The critical concentration for OFX was the same as that recommended by the other two studies (13, 18) using the manual MGIT but also by Rüsch-Gerdes et al. (24) using the automated MGIT 960 system. On the contrary, in the study of Krüüner et al. (12), the authors recommend the use of a critical concentration of 1 μg/ml of OFX for the MGIT 960. In the present study, using a critical concentration of 2 μg/ml, no discordance was found between the PM and the manual MGIT (Table 3). For KAN, only one study using the manual MGIT to test this drug is reported in the literature, and the recommended critical concentration was 5 μg/ml (3). Comparing other studies that have tested KAN using the BACTEC 460TB or the colorimetric redox indicator tests, the critical concentration for KAN ranges between 2 and 5 μg/ml. An intermediate critical concentration of 2.5 μg/ml was therefore chosen as the breakpoint for evaluating KAN by the manual MGIT system with no discordant results.

Accurate ETH DST has always been difficult to obtain because the change in MICs associated with resistance is small and also because the drug is thermolabile (15). In the two multicenter studies using the MGIT 960 (12, 24), the highest discordant results were found with this drug. For this reason, we decided to test this drug using two critical concentrations of ETH, 2.5 and 5 μg/ml. According to the results obtained in this study with the manual MGIT, the critical concentration of ETH, 5 μg/ml, yielded the most concordant results between the PM on 7H11 and the manual MGIT. These results are in agreement with the recommended critical concentration for the MGIT 960 (12, 24). ETH is an important drug for the treatment of MDR-TB and is structurally analogous to isoniazid (INH). Despite this evidence for a common site of action, resistances to INH and ETH are based on different mechanisms, as resistance to INH does not confer resistance to ETH

TABLE 3. Final critical concentration established for the manual MGIT compared to that for BACTEC MGIT 960

Study, publication yr (reference)	Method	Critical concn (μg/ml)				
		ETH	KAN	AK	OFX	CM
Rüsch-Gerdes et al., 2006 (24)	BACTEC MGIT 960	5.0		1.0	2.0	2.5
Krüüner et al., 2006 (12)	BACTEC MGIT 960	5.0		1.25	1.0	1.0
Bastian et al., 2001 (4)	Manual MGIT		5.0			
Martin et al., 2005 (13)	Manual MGIT				2.0	
Palaci et al., 2006 (18)	Manual MGIT				2.0	
This study	Manual MGIT	5.0	2.5		2.0	2.5

(27). In our study, no cross-resistance between INH and ETH has been observed.

For CM, the manual MGIT has never been used before to define a critical concentration; therefore, we decided to use the recommended critical concentration of 2.5 μg/ml that is used for the MGIT 960 (24). Using this concentration, no discordance was found between both the manual MGIT and the PM.

Some discordant results between the PM and the manual MGIT have been attributed to the presence of borderline-resistant strains. These strains have been tested by the REMA plate. The REMA plate proved to be a very useful research tool in quickly defining the MIC.

DST of second-line drugs is not as simple as DST for first-line drugs, especially for the most accurate drugs, rifampin and INH (11). Critical concentrations of some second-line drugs are very close to the MIC, and the calibration of DST methods is in progress. Despite the publication of guidelines for second-line DST, we believe that this study, including a large number of *M. tuberculosis* isolates causing MDR-TB, contributes to the standardization of the critical concentration for second-line drugs for *M. tuberculosis*, especially for the use of the manual MGIT.

There are no commercial second-line drug kits including lyophilized vials available as exists for first-line drugs, known as the "SIRE kit." This is inconvenient because the working solutions of each drug should be prepared by the users and should be very precise. Quality control using the reference strain H37RV or well-known susceptible and resistant strains is also necessary.

Our results demonstrate that DST of second-line drugs with the manual MGIT is an accurate method for rapid susceptibility testing of *M. tuberculosis* as has already been shown using the MGIT 960 system. The great advantage of the manual MGIT is that there is no need for a machine, which represents a heavy investment of cost. Tubes can be read under a simple UV lamp or with the semiautomated reader system. No difference in result was found between the two reading systems.

Because of the increased importance of *M. tuberculosis* causing MDR-TB and extensively drug-resistant TB in the world, the manual MGIT for second-line drugs applied directly to sputum samples would save time by obtaining faster results of DST by omitting the preisolation step.

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