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Evaluation of MGIT 960-Based Antimicrobial Testing and Determination of Critical Concentrations of First- and Second-Line Antimicrobial Drugs with Drug-Resistant Clinical Strains of *Mycobacterium tuberculosis*

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The objectives of this study were to (i) compare agreement of the MGIT 960 system for first-line drugs with a methodology (the resistance ratio method [RRM]) that had been used in clinical trials, relating drug susceptibility to clinical outcome; (ii) compare the performance of the MGIT 960, RRM, and microtiter plate assay (MPA) methodologies for second-line drug testing; and (iii) define critical concentrations for ciprofloxacin and moxifloxacin for liquid-culture-based testing. The large collection of clinical isolates of *Mycobacterium tuberculosis* (*n* = 247) used included 176 (71%) multidrug-resistant isolates. The results for MGIT 960 and the RRM for rifampin and isoniazid (*n* = 200) were in excellent (99 to 100%) agreement for all strains. For streptomycin, 97% of the results at the critical concentration and 92% at high concentration, and for pyrazinamide 92% of results overall, were concordant, but for ethambutol, fewer than 85% (65% for the critical concentration and 84% for the high concentration) of the MGIT-based results were concordant with those for the RRM. The MGIT 960, RRM, and MPA assays (*n* = 133) correlated well for most second-line drugs tested. For susceptibility to ofloxacin, the MGIT 960 and MPA results were in full agreement. The amikacin and rifabutin results obtained by MGIT 960 agreed with the RRM results in 131 (99%) cases, and for capreomycin, they agreed for 129 of 133 isolates tested (97%). For prothionamide testing, only a limited number of drug-resistant isolates were available for testing and drawing definitive conclusions. We propose critical concentrations of 1.0 μg/ml and 0.125 μg/ml for ciprofloxacin and moxifloxacin, respectively, for liquid-culture-based testing.

For the first-line antituberculosis drugs, there is a clear correlation between the drug susceptibility testing (DST) results assayed in vitro and the clinical usefulness of the drug (25). Moreover, for many years there has been a generally accepted consensus on how laboratory testing of the drug susceptibility of *Mycobacterium tuberculosis* should be performed (5, 6, 14, 24, 27). For most second-line drugs and new alternative compounds for treatment of drug-resistant tuberculosis (TB), there is a lack of accepted standard techniques for drug testing as well as full understanding of the clinical interpretation of test results.

Much recent attention has focused on assessing the global burden of multidrug-resistant (MDR) TB and predicting the future threat of the pathogen. At present, MDR TB continues to be a serious problem, particularly in developing countries in Asia (11, 12), but also in the Baltic region (9, 22) and in other parts of the former Soviet Union (3, 10, 26, 29, 33).

The increase in MDR TB rates has led to pressing demands for appropriate treatment with second-line antituberculosis drugs, and accurate and reliable drug susceptibility testing, not only for individual case management, but also for drug resis-

tance surveillance (13). Consequently, laboratories are challenged to provide reliable (and ideally rapid) drug susceptibility testing for first- and second-line drugs to ensure effective treatment of tuberculosis worldwide.

The reliability of the Becton Dickinson MGIT 960 system for rapid testing of *Mycobacterium tuberculosis* susceptibility to front-line drugs has been evaluated in several single- and multicenter studies (1, 2, 4, 5, 19, 21, 31, 32). However, in most of these studies, only critical concentrations of drugs were tested (1, 19, 32) and/or limited numbers of drug-resistant and MDR *M. tuberculosis* strains were included in the analysis (2, 4, 18, 19, 21, 32). Drug-resistant isolates are likely to exhibit physiological variations in parameters such as growth compared to drug-sensitive strains.

In seven out of the eight studies described above, the MGIT 960 system was evaluated against the BACTEC 460TB system, confirming good reproducibility between liquid-medium-based systems made by the same company. There is no published analysis available for the MGIT 960 system for second-line DST including significant numbers of drug-resistant isolates.

In light of this, the primary aim of our study was to test a large number of *M. tuberculosis* isolates (132 MDR, 40 drug-resistant, and 28 drug-sensitive *M. tuberculosis* isolates) against the critical and high concentrations of first-line drugs using a solid-medium-based resistance ratio method (RRM) as a reference method for comparison with the MGIT 960 system.

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Similarly, our study contributes to the development of standards for second-line drug susceptibility testing, as we analyzed 133 MDR *M. tuberculosis* isolates (arguably the most relevant group for analysis), comparing drug susceptibly analyses using the new MGIT 960 system and the resistance ratio reference solid-culture-medium method against amikacin (AMI), capreomycin (CAP), ofloxacin (OFL), prothionamide (PRO), and rifabutin (RIFB). In addition, critical concentrations for ciprofloxacin (CIP) and moxifloxacin (MOX) were established for the MGIT 960 system.

MATERIALS AND METHODS

Study site and strains. A master panel of 247 isolates, including 176 MDR *M. tuberculosis* isolates, was created from the United Kingdom Health Protection Agency Mycobacterium Reference Unit (MRU) strain collection, and two subpanels were formed for the different phases described below (Fig. 1).

(i) Phase I. A selection of 200 *M. tuberculosis* clinical isolates from the master panel with a variety of resistance patterns were tested. Of these, 179 isolates were cultured originally from the sputa of individual patients from the Samara Region in the Russian Federation and 21 isolates were obtained from TB patients registered in the United Kingdom.

(ii) Phase II. Clinical isolates ($n = 133$) which had been identified initially as MDR *M. tuberculosis* either by the national United Kingdom or Estonian Reference Laboratory using the resistance ratio method on Löwenstein-Jensen medium and the BACTEC 460TB system, respectively, were included for analysis. In all cases, the reference method was repeated in parallel with the MGIT 960 analysis. Out of 133 MDR *M. tuberculosis* clinical isolates, 85, 46, and 2 isolates originated in Russian, Estonian, and United Kingdom patients.

(iii) Phase III. The same panel was used as for phase II.

Identification of strains. All *M. tuberculosis* strains included in this study were identified using molecular (Accuprobe [GenProbe, San Diego, CA] and Innolipa [Innogenetics, Ghent, Belgium]) or classical biochemical criteria (e.g., microscopic appearance, growth, and pigmentation).

Preparation of inocula. Inocula were prepared following the manufacturer's instructions. All cultures were inoculated/subcultured onto Löwenstein-Jensen medium and were used for antimicrobial susceptibility testing no later than 14 days after the first appearance of colonies on the slant. All colonies were transferred into a sterile tube containing 4 ml of Middlebrook 7H9 medium with 8 to 10 sterile glass beads. The suspension was vortexed for 3 min and left standing undisturbed for 20 min. The supernatant was transferred into a sterile tube and left standing undisturbed for 15 min. Finally, the supernatant was transferred into a third sterile tube, and the turbidity was adjusted to 0.5 McFarland standard with sterile saline. A 1:5 dilution of this suspension in sterile saline was used for inoculating 0.5-ml volumes into the MGIT streptomycin (STR), isoniazid (INH), rifampin (RIF), and ethambutol (EMB) (SIRE) and pyrazinamide (PZA) sets. All inoculated sets were loaded into the BACTEC MGIT 960 instrument within 2 h of inoculation. For the RRM, 10 μl of suspension adjusted to 0.5 McFarland standard was inoculated onto each drug-containing slope and the drug-free control. For the microtiter plate assay (MPA), the suspension was adjusted to 1 to 1.5 McFarland standards, and 10 μl of cell suspension was added to each well of the prepared microtiter plate.

Reagents and drug concentrations. (i) MGIT 960. Lyophilized drugs (BACTEC MGIT 960 SIRE kit, MGIT 960 STR4.0 kit, MGIT 960 INH0.4 kit, MGIT 960 EMB7.5 kit, and MGIT 960 PZA kit; Becton Dickinson, Baltimore, MD) were dissolved in diluent according to the manufacturer's instructions. From the dissolved drug solutions, 100 μl was pipetted into a 7-ml MGIT 960 tube. All drugs used in phase II were obtained from the manufacturers in a chemically pure form. The drugs used were AMI from Bristol-Myers Squibb, Syracuse, N.Y., dissolved in deionized (DI) water; CAP sulfate from Sigma, St. Louis, Mo., dissolved in DI water; OFL from Ortho/R. W. Johnson Pharmaceutical, Raritan, N.J., dissolved in 1/10 N NaOH solution; CIP from Miles, West Haven, Conn., dissolved in 1/10 N NaOH solution; MOX, generously provided by Bayer HealthCare AG, Leverkusen, Germany, dissolved in 1/10 N NaOH solution; PRO, a gift from Fatol Arzneimittel GmbH, Schiffweiler, Germany, dissolved in ethylene glycol; and RIFB from Pharmacia, Spa, Italy, dissolved in methanol. The subsequent dilutions for all drugs were made in DI water, and aliquots of the stock solution were stored at −70°C for 6 months. The final drug concentrations used were 1.0 and 4.0 μg/ml for STR; 0.1 and 0.4 μg/ml for INH; 5.0 and 7.5 μg/ml for EMB; 1.0 μg/ml for RIF; 100 μg/ml for PZA; 1.0 μg/ml for AMI; 1.25 μg/ml for

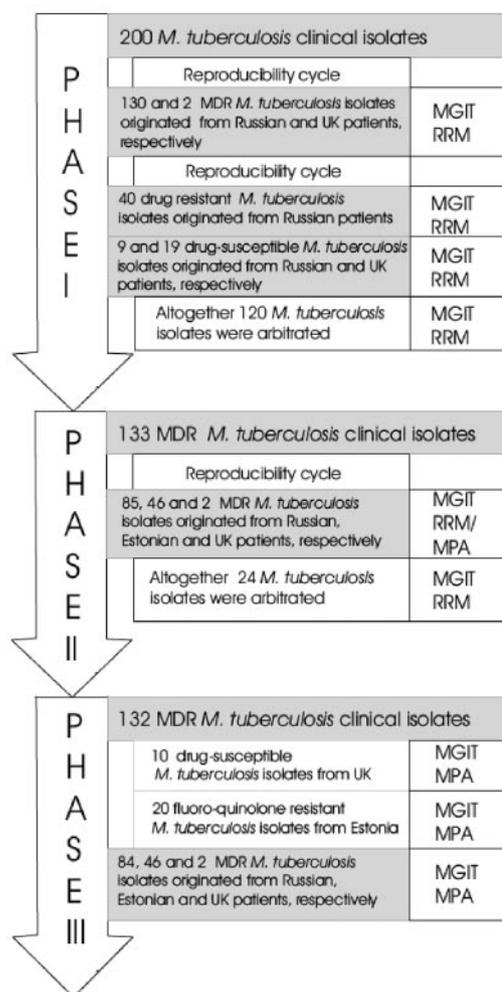FIG. 1. Flow diagram of the study.

CAP; 1.0 μg/ml for OFL; 1, 2, and 4 μg/ml for CIP; 0.125, 0.5, and 1 μg/ml for MOX; 2.5 and 5.0 μg/ml for PRO; and 0.5 μg/ml for RIFB.

(ii) RRM. The RRM is described for use in Löwenstein-Jensen medium without potato starch. To determine the strain MICs, various solutions of each drug were incorporated into the medium before inspissation to achieve the relevant final concentrations: 10, 20, and 40 μg/ml for STR; 0.05, 0.1, and 0.2 μg/ml for INH; 5, 10, and 20 μg/ml for RIF; 0.8, 1.6, and 3.2 μg/ml for EMB; 10, 20, and 40 μg/ml for AMI; 40, 80, and 160 μg/ml for CAP; 20, 40, and 80 μg/ml for PRO; and 5, 10, and 20 μg/ml for RIFB (8, 14, 16).

PZA was tested at a critical concentration of 66 μg/ml using a semisolid method as described previously (8, 16).

(iii) MPA. Antibiotic dilutions and 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, N.J.) were prepared as described by Caviedes et al. (7). The final drug concentration ranges were as follows: 0.25 to 16 μg/ml for OFL and CIP; 0.6 to 4 μg/ml for MOX.

Interpretation of susceptibility results. (i) MGIT 960 system. The predefined algorithms designed for the trial were followed throughout. For SIRE (low and high concentrations) and PZA, readings were automatically interpreted by the BACTEC MGIT 960 instrument and reported as either susceptible or resistant. For second-line drugs, readings were manually interpreted using the same algorithm as for first-line drugs. The "unloaded set report" listed growth units; time to results; and susceptible, resistant, or invalid results as described in the manufacturer's instructions.

(ii) RRM. The resistance ratio is defined as the ratio of the MIC for the test strain to the modal MIC for the drug-susceptible panel of five pansensitive clinical isolates tested in the same experiment. When read after 4 weeks of incubation, "growth" on any slope was defined as the presence of 20 or more

TABLE 1. Test results for clinical strains of *M. tuberculosis* ($n = 200$) for susceptibility to SIRE and PZA

Antibiotic (concn [µg/ml])	MGIT 960 result[a]	RRM result[a]		Overall agreement (%)	Sensitivity[b] (%)	Specificity[c] (%)	PPV[d] (%)	NPV[e] (%)
		S	R					
STR (1.0)	S	37	6	97	96	100	100	86
	R		157					
STR (4.0)	S	82	16	92	86	100	100	84
	R		102					
INH (0.1)	S	30	3[f]	99	98	100	100	91
	R		167					
INH (0.4)	S	31	3[f]	99	98	100	100	91
	R		166					
RIF (1.0)	S	68		100	100	100	100	100
	R		132					
EMB (5.0)	S	103	71	65	27	100	100	59
	R		26					
EMB (7.5)	S	156	33	84	25	100	100	83
	R		11					
PZA (100)[g]	S	158	8	92	77	98	90	95
	R	3	26					

[a] S, susceptible; R, resistant. Values are numbers of isolates.
[b] The sensitivity, i.e., the ability of MGIT 960 to detect true resistance compared with the RRM results.
[c] The specificity, i.e., the ability of MGIT 960 to detect true susceptibility compared with the RRM results.
[d] PPV, positive predictive value.
[e] NPV, negative predictive value.
[f] Mixed cultures (consisting of resistant and susceptible subpopulations).
[g] Five *M. tuberculosis* isolates did not grow in one of the systems used.

colonies. A resistance ratio of 2 or less confirms susceptibility, a resistance ratio of 4 indicates resistance, and 8 indicates that the strain is highly resistant (8, 14, 16).

(iii) **MPA.** Inoculated plates were sealed and then incubated at 37°C for 5 to 15 days. The MIC for a sample was defined as the antibiotic concentration immediately above that at which bacterial growth was last seen. For OFL and CIP, a MIC of ≥ 1 µg/ml, and for MOX, a MIC of ≥ 0.125 µg/ml was recorded as resistant.

Quality control strains. *M. tuberculosis* (H37 Rv, NCTC strain 7416, an isoniazid-resistant strain [MRU 03/9336], and MDR strains [MRU 02/3290 and MRU 97/1960]), *M. kansasii* (NCTC 13024), and *M. fortuitum* (NCTC 8573) were used for lot and batch quality control of commercial and homemade media.

Reproducibility testing. Prior to testing clinical isolates, a precharacterized panel of 10 strains of *M. tuberculosis* was tested. Altogether, this panel of strains was tested in duplicate at three cycles/time points during the study.

Study design. The study consisted of three phases.

Phase I. SIRE low and SIE high concentration and PZA susceptibility testing of challenge strains of *M. tuberculosis* was done as a comparison between MGIT 960 and the resistance ratio method. The panel consisted of 200 *M. tuberculosis* clinical isolates, which were subcultured on Löwenstein-Jensen medium prior to drug testing.

Phase II. Testing of susceptibility to second-line drugs was carried out with the MGIT 960 system and RRM for a total of 133 clinical isolates of *M. tuberculosis*. For ofloxacin testing, MPA was used in comparison with MGIT 960. Inocula for susceptibility testing were derived from the solid (Löwenstein-Jensen) media.

Phase III. The aim of phase III was to establish critical concentrations/breakpoints of ciprofloxacin and moxifloxacin for testing in the MGIT 960 system. For this purpose, MICs for 132 of the MDR *M. tuberculosis* clinical isolates used in phase II (one of the strains was contaminated) were determined by the MPA and MGIT 960 systems. For MPA, serial twofold dilutions of CIP and MOX were prepared with sterile water. The final test concentrations were 0.25, 0.5, 1.0, 2.0, and 4.0 µg/ml and 0.06, 0.125, 0.25, 0.5, and 1.0 µg/ml for CIP and MOX, respectively. The test concentrations selected for the MGIT 960 system were 1.0, 2.0, and 4.0 µg/ml for CIP and 0.125, 0.5, and 1.0 µg/ml for MOX. These initial values were chosen based on previously published MIC results (15). Plates were inoculated according to previously published recommendations (7).

Resolution of discrepant results. Arbitration of discrepant results was performed according to the study protocol, i.e., discrepant isolates were tested in duplicate in both systems, and the consensus results were obtained.

RESULTS

Phase I. Two hundred clinical isolates of *M. tuberculosis* were tested for susceptibility to the five first-line anti-TB drugs at the critical concentration and also at higher concentrations (for STR, INH, and EMB) (Table 1). The rifampin results were concordant (200/200 samples; 100%). The isoniazid results agreed for 197 of 200 isolates tested at the critical concentration and at the higher concentration (99% agreement). Three isolates with discordant results against INH were mixed cultures and consisted of resistant and susceptible subpopulations. The ethambutol results agreed for 129 of 200 (65% agreement) isolates tested at the critical concentration and for 167 of 200 (84% agreement) isolates at the higher concentration. The streptomycin results agreed for 194 of 200 (97% agreement) isolates at the critical concentration and for 184 of 200 (92% agreement) at the higher concentration. The pyrazinamide results obtained by the two methods agreed for 184 of 195 (92% agreement) isolates tested with both methods.

Comparison of MGIT 960 with RRM for all drugs yielded 120 clinical isolates with discordant results overall, amounting to 210 (13%) discrepant results out of a total of 1,595 tests. Of these, 20 isolates were resistant according to MGIT 960 but susceptible by RRM (STR [$n = 10$], EMB [$n = 7$], and PZA [$n = 3$]) and 190 were susceptible according to MGIT 960 but resistant by the RRM (STR [$n = 35$], INH [$n = 7$], RIF [$n = 8$], and EMB [$n = 130$]) (Table 2). Most of the discordant results tested susceptible by MGIT 960 and resistant by the RRM. The false-resistant ($n = 3$) and false-susceptible ($n = 138$) results remaining after arbitration of discrepant results are

TABLE 2. Resolution of discrepant results by arbitration

Drug (concn [µg/ml])	No. of tests	Initial result		Arbitrated result		No. R by MGIT 960 but S by RRM[b]	No. S by MGIT 960 but R by RRM[c]
		No. R by MGIT 960 but S by RRM[a]	No. S by MGIT 960 but R by RRM	No. R by MGIT 960 and RRM (true resistant)	No. S by MGIT 960 and RRM (true susceptible)		
STR (1.0)	17	9	8	10	1	0	6
STR (4.0)	28	1	27	6	6	0	16
INH (0.1)	4	0	4	1	0	0	3
INH (1.0)	3	0	3	0	0	0	3
RIF (1.0)	8	0	8	0	8	0	0
EMB (5.0)	85	5	80	7	9	0	69
EMB (7.5)	52	2	50	2	17	0	33
PZA (100)	13	3	10	1	1	3	8
Total	210	20	190	27	42	3	138

[a] R, resistant; S, susceptible.
[b] Classified as a false-resistant result (major error).
[c] Classified as a false-susceptible result (very major error).

presented in Table 2 and are mostly associated with EMB resistance detection ($n = 69$ and 33 at low and high EMB concentrations, respectively).

The accuracy of the MGIT 960 system compared to that of the RRM is presented in Table 1. The sensitivity, i.e., the ability to detect true resistance, was 100% for RIF, and for other drugs it ranged from 77 to 98% at the critical concentrations and from 86 to 98% at the high concentrations for PZA, STR, and INH but was only 25 to 27% for EMB at both concentrations. In contrast, specificity, i.e., the ability to detect true susceptibility, was 100% for all of the drugs and concentrations tested except PZA (98%).

Phase II. The susceptibility results for the 133 MDR *M. tuberculosis* strains are presented in Table 3. For susceptibility to OFL, the results obtained by the MGIT 960 and MPA systems were in full agreement. The AMI and RIFB results obtained by MGIT 960 agreed with the RRM in 131 (99%) cases, and for CAP, the results agreed for 129 of 133 isolates tested (97% agreement). Two concentrations of PRO were applied in testing PRO susceptibility. With a PRO concentration of 2.5 µg/ml in MGIT 960, the test results of MGIT 960 agreed with the RRM results in 128 (96%) cases. When a PRO concentration of 5.0 µg/ml was used, the results obtained by MGIT 960 agreed with the RRM results in 129 (97%) cases. Among the discordant results, nine isolates tested as resistant by MGIT 960 but as susceptible by the RRM. Twenty-five out of 133 clinical isolates of *M. tuberculosis* tested as susceptible by MGIT 960 but resistant by the RRM prior to arbitration (Table 4). Following arbitration, six results appeared to give false resistance and nine appeared to give false sensitivity in the MGIT960 system.

Phase III. Prior to DST of clinical isolates, a panel of 10 *M. tuberculosis* strains precharacterized as fully drug susceptible and 20 precharacterized as fluoroquinolone resistant were tested against various concentrations of ciprofloxacin and moxifloxacin. All of the precharacterized strains had been tested using the BACTEC 460TB system or the RRM. The MICs for all of the fluoroquinolone-resistant strains were ≥1.0 µg/ml for

TABLE 3. Test results for clinical strains of MDR *M. tuberculosis* ($n = 133$) for susceptibility to second-line drugs

Antibiotic (concn [µg/ml])	MGIT 960 result[a]	RRM/MPA[a,b] result		Rate of agreement (%)	Sensitivity[c] (%)	Specificity[d] (%)	PPV[e] (%)	NPV[f] (%)
		S	R					
AMI (1.0)	S	108	1	99	96	99	96	99
	R		23					
CAP (1.25)	S	105	2	97	92	98	92	98
	R	2	24					
OFL (1.0)[b]	S	97		100	100	100	100	100
	R		34					
RIFB (0.5)	S	6	2	99	98	100	100	75
	R		125					
PRO (2.5)	S	119	3	96	75	98	82	98
	R	2	9					
PRO (5.0)	S	124	3	97	63	99	83	98
	R		5					

[a] S, susceptible; R, resistant.
[b] Values are numbers of isolates. A total of 132 *M. tuberculosis* clinical isolates were tested.
[c] The sensitivity, i.e., the ability of MGIT 960 to detect true resistance compared with the RRM results.
[d] The specificity, i.e., the ability of MGIT 960 to detect true susceptibility compared with the RRM results.
[e] PPV, positive predictive value.
[f] NPV, negative predictive value.

TABLE 4. Resolution of discrepant results by arbitration

Drug (concn [µg/ml])	No. of tests	Initial result		Arbitrated result		No. R by MGIT 960 but S by RRM[a]	No. S by MGIT 960 but R by RRM[b]
		No. R by MGIT 960 but S by RRM	No. S by MGIT 960 but R by RRM	No. R by MGIT 960 and RRM (true resistant)	No. S by MGIT 960 and RRM (true susceptible)		
AMI (1.0)	13	0	13	2	9	1	1
CAP (1.25)	7	5	2	0	3	2	2
OFL (1.0)	0	0	0	0	0	0	0
PRO (2.5)	9	3	6	3	1	2	3
PRO (5.0)	3	1	2	0	0	1	2
RIFB (0.5)	2	0	2	0	1	0	1
Total	34	9	25	5	14	6	9

[a] Classified as a false-resistant result (major error).
[b] Classified as a false-susceptible result (very major error).

CIP and ≥ 0.125 µg/ml for MOX. The MICs for all of the pansusceptible strains were ≤ 1.0 µg/ml for CIP and ≤ 0.125 µg/ml for MOX.

The CIP and MOX MICs were determined for 132 MDR *M. tuberculosis* clinical isolates by MGIT 960 and MPA. The results are shown in Fig. 2. In total, 35 clinical isolates tested resistant to both CIP and MOX. For the MGIT 960 method, critical testing concentrations of 1.0 µg of CIP/ml and 0.125 µg of MOX/ml appear to be valid testing concentrations, because these drug levels reliably tested pansusceptible strains as susceptible and resistant strains as resistant by both methods.

DISCUSSION

The aims were to (i) compare the performance of the MGIT 960 automated liquid-culture system with a methodology that had been standardized in clinical trials (RRM), relating DST results to clinical outcome; (ii) compare the performances of the MGIT 960, RRM, and MPA methodologies for second-line drugs using a predominantly drug-resistant strain population; and (iii) compare the MGIT 960 and MPA methodologies, using the results to define appropriate critical concentrations for ciprofloxacin and moxifloxacin.
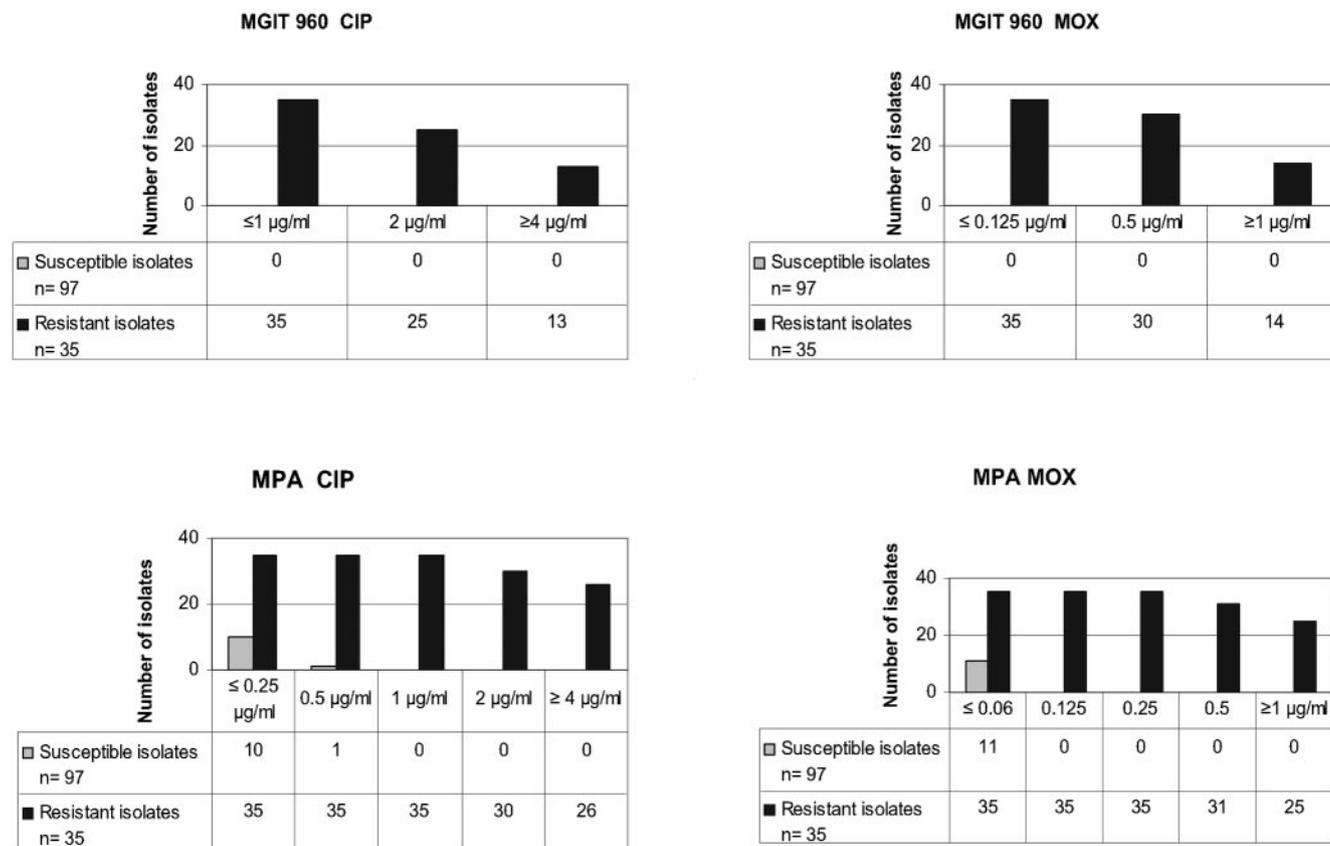

FIG. 2. CIP and MOX MIC results for 132 MDR *M. tuberculosis* clinical isolates tested by MGIT 960 and compared with MPA.

The present study has several advantages and differences in comparison with previous studies (1, 2, 4, 5, 19, 21, 31, 32). First, the large collection of clinical isolates of *M. tuberculosis* ($n = 247$) tested included 176 (71%) MDR cases, representing the most challenging panel of clinical isolates tested in evaluation studies so far. Second, in all published studies to date, the MGIT 960 system was evaluated against the proportion method, and in seven studies out of eight, the BACTEC 460TB system was used as the standard method. In this study, the RRM was used as the reference methodology.

According to our results, encompassing more than 4,400 individual susceptibility test results generated by testing 247 *M. tuberculosis* clinical isolates, MGIT 960 is appropriate for drug susceptibility testing of most of the drugs examined, including five first-line and five second-line drugs. In particular, the comparative results for MGIT 960 and the RRM for rifampin and isoniazid were in excellent agreement for drug-susceptible and MDR strains. The pyrazinamide results were also highly concordant. The PZA susceptibility results generated by MGIT 960 disagreed in 8% of the cases with the semisolid method. Very similar results were reported by Scarparo et al. for first-line drugs (31). The concordance of INH and RIF susceptibility results obtained in the studies that compared the MGIT 960 and BACTEC 460 methods were also similar to our findings (1, 4, 32). However, in the case of EMB, fewer than 85% (65% for the critical concentration and 84% for the high concentration) of the results obtained by the MGIT method were in agreement with the RRM.

A similar finding was reported in the smaller study of Adjers-Koskela and Katila, when a panel of 36 *M. tuberculosis* strains with various susceptibilities to first-line drugs was distributed for external quality control by the WHO. The authors explained the nature of discordant results as heteroresistance to EMB (1). As all other previous evaluation studies included only small numbers of MDR *M. tuberculosis* strains, they would have missed this finding; for example, in the multicenter evaluation study reported by Bemer et al. (4), only three ME (major error–false-resistant results) for EMB (5.0 μg/ml) were reported. With a specificity of almost 97% at the critical concentration with 100% sensitivity, the authors concluded that the EMB testing in the MGIT 960 system was very reliable.

It is important to emphasize that in our study, in nearly all (94%) of the instances of disagreement, the reference method reported resistance, whereas MGIT 960 reported susceptibility. This gives us a false-susceptible (very major error [VME], which leads to treatment with ineffective drugs) rate of up to 147 results in total across all drugs. Moreover, 69% of VME cases were generated by EMB testing alone. It is obvious that the challenging panel of isolates tested in this study comprised a very heterogeneous population of clinical isolates, including heteroresistant cases.

It is generally accepted that the price of mutational change producing drug resistance is reduced bacterial fitness, i.e., reduced growth within and outside hosts. Mariam et al. (23) demonstrated by using three different assays that rifampin-resistant mutants of *M. tuberculosis* have decreased growth rates in vitro compared to an isogenic susceptible parent strain. We speculate that the same phenomenon would also apply to EMB-resistant mutants and thus explain our discordant results. In 16 out of 17 discordant EMB cases where the MGIT

tubes were reentered (after being taken out as a susceptible test) into the MGIT analyzer, they became resistant 2 to 10 days later (data not shown). Our experience confirms the findings of Adjers-Koskela and Katila that heteroresistance to EMB is the main source of discordant results, and also that the growth rates of resistant mutants are slowed down and that they grow in the liquid-medium system 2 to 5 days later than the susceptible subpopulation of bacteria. Similarly, MGIT 960 also missed three INH-heteroresistant cultures, and those were clearly defined as mixed cultures by RRM. Since the RRM results are recorded after 4 weeks of growth, the time window allows not only the dominant susceptible population to be detected, but also the mutant subpopulation of bacteria.

In agreement with previously published data (1, 4, 31), problems were also experienced in STR testing. There were six VME overall at the critical concentration and 16 VME at high drug testing concentrations.

Despite the publication of guidelines for second-line drug susceptibility testing by the WHO (34), the reliability of such testing has been widely questioned (20), suggesting that the original attempt at standardization may have been premature. With the exception of the "older" second-line drugs, such as para-amino-salicylic acid and streptomycin, which were extensively analyzed in the 1940s and 1950s as parts of clinical trials, most second-line agents have not been analyzed in trials for microbiological end points, let alone clinical ones.

Furthermore, there has been only limited analysis with the new automated liquid-culture systems for second-line and new drug classes. There has been only one well-designed multicenter study published on this topic using semiautomated or automated liquid-culture systems (28). There have been few new anti-TB drugs tested, and most of them have been fluoroquinolones or rifamycins. The MGIT 960 system has been used successfully for testing one of the new antituberculosis agents (30); Sanders et al. tested 32 pansusceptible and 14 quinolone-resistant isolates of *M. tuberculosis* against levofloxacin using three different methods, including MGIT 960.

Several important conclusions can be drawn from the present study for testing second-line and newer antituberculosis drugs. First, the MGIT 960 automated version is a straightforward and rapid method which can reliably generate drug susceptibility results for most second-line and newer antituberculosis drugs. Most (except for ofloxacin and prothionamide) of the critical test concentrations used in the study were the same as defined by Pfyffer et al. for the BACTEC 460TB broth-based system in 1999 (28). For ofloxacin, a critical concentration of 1 μg/ml instead of 2 μg/ml was used, and no discordant results were obtained. Although two test concentrations were used for prothionamide testing (2.5 and 5.0 μg/ml), the highest number (three ME and five VME) of discordant results for second-line drugs was seen in testing this drug. As only a limited number of prothionamide-resistant strains were included, further testing is needed. Some VME and ME occurred in testing AMI (one ME and one VME), CAP (two ME and two VME), and RIFB (one VME). Since there are no comparable studies available, it is difficult to say if the number of ME or VME seen in this phase of the study should be considered satisfactory, although we believe that they are. Clearly, future studies are needed to verify our conclusions.

Also, the critical concentrations for ciprofloxacin and moxi-

floxacin testing were proposed for the MGIT 960 system. Moxifloxacin is a recently introduced 8-methoxyfluoroquinolone with greater activity than ofloxacin in vitro and in a murine tuberculosis model (17). Furthermore, in experimental murine tuberculosis, moxifloxacin has been found to have sterilizing activity, whereas ofloxacin has none. Therefore, it is predicted that this new quinolone might be able to shorten the treatment of tuberculosis.

Our study results indicate that critical testing concentrations of 1.0 μg/ml of CIP and 0.125 μg/ml of MOX appear to be valid for testing, because both drug levels reliably tested pansusceptible strains as susceptible and resistant strains as resistant by the MGIT 960 system and by the microtiter plate assay. We propose critical concentrations of 1.0 μg/ml and 0.125 μg/ml for ciprofloxacin and moxifloxacin, respectively, for liquid-culture-based testing.

In conclusion, the MGIT 960 automated system gave results comparable to those of the reference resistance ratio method for isoniazid, rifampin, and pyrazinamide testing and performed adequately for streptomycin DST. Under the challenging conditions of this study, which included a high number of MDR *M. tuberculosis* strains, ethambutol testing did not give the expected results. This could be due to heteroresistance of the tested isolates, as suggested by Adjers-Koskela and Katila. One approach might be to continue to incubate EMB tubes in the MGIT 960 instrument in the case of MDR *M. tuberculosis*, but this requires further study to establish the most appropriate protocol. The MGIT 960, RRM, and MPA assays correlated well for most second-line drugs tested.

We believe that this study contributes significantly to the debate on the value and standardization of methodology for second-line drugs since, with the exception of prothionamide, a substantial panel of highly drug-resistant isolates was analyzed.

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