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Canadian Multicenter Laboratory Study for Standardized Second-Line Antimicrobial Susceptibility Testing of *Mycobacterium tuberculosis*▽

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The purpose of this study was to establish a standardized protocol for second-line antimicrobial susceptibility testing of *Mycobacterium tuberculosis* using the Bactec MGIT 960 system in Canadian laboratories. Four Canadian public health laboratories compared the susceptibility testing results of 9 second-line antimicrobials between the Bactec 460 and Bactec MGIT 960 systems. Based on the data generated, we have established that the Bactec MGIT 960 system provides results comparable to those obtained with the previous Bactec 460 method. The critical concentrations established for the testing of the antimicrobials used are as follows: amikacin, 1 μg/ml; capreomycin, 2.5 μg/ml; ethionamide, 5 μg/ml; kanamycin, 2.5 μg/ml; linezolid, 1 μg/ml; moxifloxacin, 0.25 μg/ml; ofloxacin, 2 μg/ml; *p*-aminosalicylic acid, 4 μg/ml; rifabutin, 0.5 μg/ml.

The Public Health Agency of Canada (PHAC) publishes yearly statistics on the antimicrobial resistance patterns of all laboratory-isolated *Mycobacterium tuberculosis* strains in Canada. From 2000 to 2010, the number of *M. tuberculosis* isolates resistant to one or more of the first-line antimicrobials has varied between 8.0% and 11.0% of all tuberculosis (TB) cases per year in Canada (15). Between 0.9% and 1.6% of these cases were considered multidrug resistant (MDR) TB. Since 2005, TB cases in the foreign-born Canadian population have accounted for more than 60% of all Canadian TB cases and greater than 90% of MDR TB cases (10–14).

Currently it is recommended that after systematic testing against first-line anti-TB agents, isolates that are found to be monoresistant to rifampin or to demonstrate resistance to any two of the first-line antimicrobials be tested against a panel of second-line antimicrobials (3, 7, 21). Though the majority of resistant isolates in Canada show monoresistance to isoniazid (INH), recently published Clinical and Laboratory Standards Institute (CLSI) document M24-A2 recommends that second-line antimicrobial testing also be performed on isolates that are INH monoresistant in cases where fluoroquinolones may be added to the therapy. The current standard method for first-line antimicrobial sensitivity testing (AST) of *M. tuberculosis* in Canada is the Bactec MGIT 960 (M960) system (BD, Sparks, MD). Although the M960 system has been approved for first-line AST (2–4, 20), the Bactec 460 (B460; BD, Sparks, MD) and the agar proportion method are currently used for second-line AST since the M960 system had not been validated for this purpose (3, 8). Due to the increasing demand for second-line AST and the discontinuation of the B460 technology by the manufacturer, a multicenter validation of a second-line AST panel for the M960 system was undertaken. A validated and standardized method and antimicrobial panel for second-line AST are required to ensure that antimicrobial-resistant cases of *M. tuberculosis*, including MDR and extensively drug-resistant cases, are identified in an accurate and timely manner and that susceptibility testing and reporting of these isolates are standardized across Canada.

Several publications have suggested guidelines for the testing of second-line antimicrobials for *M. tuberculosis* in the M960 system (Table 1) (5, 6, 16, 17, 21). Our study objective was to design and perform a multicenter collaborative validation study of second-line AST for *M. tuberculosis* in the M960 system in four Canadian public health laboratories using *M. tuberculosis* strains with various antimicrobial resistance patterns, including pansusceptible strains, previously determined using the B460 system. This included the testing of a more comprehensive panel of antimicrobials than in previous studies. Validation of the method included a comparison of the results obtained with the B460 and M960 systems, as well as investigation of intralaboratory and interlaboratory reproducibility. This validation will facilitate the implementation of a standard second-line AST panel, using the M960 system, for *M. tuberculosis* isolates in Canada.

MATERIALS AND METHODS

Study design. Four Canadian public health laboratories designed and participated in a multisite evaluation and validation of the M960 methodology for second-line AST. These laboratories included the PHAC National Reference Centre for Mycobacteriology (NRCM), Winnipeg, Manitoba; the Public Health Laboratory, Public Health Ontario (PHO), Toronto, Ontario; the Laboratoire de Santé Publique du Québec, Institut National de Santé Publique du Québec (LSPQ-INSPQ), Quebéc, and the Provincial Laboratory for Public Health, Edmonton, Alberta. The protocol for the study was designed on the basis of previously published studies investigating second-line AST using the M960 method (5, 6, 16, 17, 21). These data were used to select a broad and relevant

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TABLE 1. CCs used for second-line antimicrobials in previously published studies and the CCs/test concentrations used in this study

Antimicrobial	CC (μg/ml)							
	B460	Lab 1[b]	Lab 2[c]	Lab 3[d]	Lab 4[e]	Lab 5[f]	NRCM lab	Study participant labs
Amikacin	1	1.5	1	1	1	1	0.5, 1.0, 2.0	1
Capreomycin	1.25	3	2.5	2.5	2.5	NT[a]	1.25, 2.5, 5.0	2.5
Ethionamide	2.5	5	5	5	5	NT	2.5, 5.0, 7.5	5
Kanamycin	5	NT	2.5	NT	NT	NT	2.5, 5.0, 7.5	2.5, 5.0
Linezolid	1	NT	NT	1	1	NT	0.5, 1.0, 2.0	0.5, 1.0, 2.0
Moxifloxacin	0.5	NT	1.0	0.25	NT	0.125	0.25, 0.5, 1.0	0.25, 0.5, 1.0
Ofloxacin	2	NT	2	2	2	1	1.0, 2.0, 4.0	2
PAS	4	NT	4	NT	NT	NT	2.0, 4.0, 8.0	2.0, 4.0, 8.0
Rifabutin	0.5	NT	NT	NT	0.5	0.5	0.25, 0.5, 1.0	0.5

[a] NT, not tested.
[b] Reference 6.
[c] Reference 16.
[d] Reference 21.
[e] Reference 17.
[f] Reference 5.

panel of antimicrobials and to select a range of appropriate antimicrobial concentrations or a single critical concentration (CC) to be tested (Table 1).

The study consisted of two phases. All strains were initially tested by the NRCM for first- and second-line AST using known CCs for the B460 system (8, 9, 16, 17) and three test concentrations for second-line AST in the M960 system. Second, the blinded panel of strains was tested by the other three collaborating laboratories using CCs as published for all antimicrobials in the B460 system and investigational CCs for all antimicrobials in the M960 system, except for *p*-aminosalicylic acid (PAS), moxifloxacin, and linezolid, which were tested against three antimicrobial concentrations due to the lack of published data. Tests that produced discordant results between the methods or laboratories were repeated in order to verify the results obtained. Final results were analyzed for inter- and intralaboratory reproducibility and concordance with B460 system results.

Strains. A total of 36 *M. tuberculosis* strains were tested. Seven resistant and 10 pansensitive clinical isolates were selected from the NRCM repository. Four strains obtained from the American Type Culture Collection (ATCC) were also tested, i.e., ATCC 700457 (rifabutin resistant), ATCC 35827 (capreomycin, kanamycin, and amikacin resistant), ATCC 35824 (PAS resistant), and ATCC 35825 (PAS resistant). Due to the limited variety of MDR TB strains currently available in Canada, six MDR strains with resistance to moxifloxacin and linezolid were provided courtesy of Sabine Rüsch-Gerdes (National Reference Center for Mycobacteria, Borstel, Germany) (17). Nine of the above-listed antimicrobial-resistant strains were tested in duplicate in order to test for intralaboratory reproducibility. A blinded panel of these 36 strains was designed at the NRCM and tested in-house for all first- and second-line antimicrobials using both the B460 and M960 systems before shipment to other participants. The predicted distribution of antimicrobial resistance can be found in Table 2. *M. tuberculosis* strain H37Rv (ATCC 27294) was run in parallel with all test batches as a pansensitive quality control. Only tests that met quality control parameters were included in this study.

Antimicrobials. The nine antimicrobials included in the panel were ofloxacin (Sigma-Aldrich), ethionamide (Sigma-Aldrich), amikacin hydrate (Sigma-Aldrich), kanamycin sulfate (Sigma-Aldrich), PAS (Sigma-Aldrich), capreomycin sulfate (Sigma-Aldrich), rifabutin (Tecoland, TX), linezolid (Pfizer, Groton, CT), and moxifloxacin HCl (Bayer, Toronto, Ontario, Canada). All antimicrobials, with the exception of linezolid, were prepared in the manufacturer-recommended solvent to a stock concentration of 10,000 μg/ml as calculated using the potency of the antibiotic powder, which was provided by the manufacturer. Linezolid was prepared to a concentration of 1,000 μg/ml due to solubility issues. Antimicrobial stocks were stored at or below −70°C prior to preparation of the working stock concentrations (3).

TABLE 2. Predicted susceptibility results for strains used in this study as determined in the B460 system at the NRCM

Antimicrobial agent	No. of strains:	
	Resistant	Sensitive
Amikacin	10	26
Capreomycin	12	24
Ethionamide	16	20
Kanamycin	10	26
Linezolid	3	33
Moxifloxacin	11	25
Ofloxacin	11	25
PAS	5	31
Rifabutin	15	21

Strain preparation and inoculation. Strains were subcultured by participating laboratories to Löwenstein-Jensen slants or to MGIT liquid medium and incubated at 37°C ± 1°C. Tests were inoculated and incubated according to the procedure for M960 system first-line AST as described in the Becton Dickinson Bactec M960 system manual in 1999 (1). The Becton Dickinson Bactec TB System product and procedure manual (1996) was followed for B460 system testing (18). Interpretation of results was completed in accordance with established BD procedures for each specific method.

Testing protocol. The panel of strains was tested by the NRCM against the nine antimicrobials using the existing B460 method (18) with CCs and the M960 method using three concentrations of each antimicrobial (Table 1). The other participating laboratories tested the same strains using CCs for the B460 method. For the M960 method, published CCs were used for six antimicrobials and three test concentrations each for moxifloxacin, PAS, and linezolid since limited published data were available for these antimicrobials (Table 1). Results obtained from all labs for the M960 method were then examined for concordance with B460 system results (Table 2) in order to define the CC appropriate for M960 system testing for each antimicrobial. In order to accommodate the extended panel of antimicrobials for routine testing, two AST carriers were required. Tests were loaded onto an eight-position and a four-position AST carrier in the order growth control, capreomycin, ethionamide, kanamycin, ofloxacin, PAS, rifabutin, and amikacin for the eight-position AST carrier and in the order growth control, moxifloxacin, linezolid, and streptomycin for the four-position AST carrier. Antimicrobials were manually entered during the analysis of test results.

After the establishment of a single CC for each antimicrobial in the M960 system, the data were also analyzed for inter- and intralaboratory reproducibility. Interlaboratory reproducibility was calculated as 1 − (number of discrepant M960 system results/total number of M960 system results) for each antimicrobial ($n = 144$) and expressed as a percentage. Intralaboratory reproducibility for each antimicrobial was calculated as 1 − (total number of discrepant M960 system results between replicates within individual laboratories/total number of replicates tested [$n = 36$]) and expressed as a percentage.

RESULTS

Determination of CCs. Previously published CCs of linezolid, moxifloxacin, and PAS are either lacking or contradictory. Three concentrations of each antimicrobial were tested to help establish a CC which allowed the highest concordance

TABLE 3. Comparison of B460 system results to M960 system results listed by antimicrobial agent and by participating laboratory site[a]

Antimicrobial agent[b] (CC B460/CC M960)[c]	No. of strains at:																Overall % reproducibility of B460 vs M960
	Site 1				Site 2				Site 3				Site 4				
	S/S	S/R	R/R	R/S	S/S	S/R	R/R	R/S	S/S	S/R	R/R	R/S	S/S	S/R	R/R	R/S	
Amikacin (1.0/1.0)	26	0	10	0	26	0	10	0	26	0	10	0	26	0	10	0	100
Capreomycin (1.25/2.5)	24	0	12	0	24	0	12	0	24	0	12	0	24	0	12	0	100
Ethionamide (2.5/5.0)	19	1	16	0	17	6	13	0	19	1	16	0	19	0	17	0	94.4
Kanamycin (5.0/5.0)	26	0	10	0	25	0	11	0	26	0	10	0	25	0	10	1	100
Linezolid (1.0/1.0)	33	0	3	0	33	0	3	0	33	0	3	0	33	0	3	0	100
Ofloxacin (2.0/2.0)	25	0	11	0	25	0	11	0	25	0	11	0	25	1	9	1	98.6
Moxifloxacin (0.5/0.25)	25	0	11	0	25	0	11	0	25	0	11	0	25	0	11	0	100
PAS (4.0/4.0)	30	1	5	0	27	1	4	4	31	0	5	0	25	0	6	5	92.3[d]
Rifabutin (0.5/0.5)	21	0	15	0	21	0	15	0	21	0	15	0	21	0	15	0	100

[a] S, sensitive; R, resistant.
[b] Results are expressed as B460 system interpretation/M960 system interpretation.
[c] CCs are in μg/ml.
[d] Nine discrepancies associated with PAS testing were due to erroneous results obtained with the B460 system, likely caused by overinoculation due to clumping.

with B460 system results. Based on MIC data for moxifloxacin, PAS, and linezolid, the ideal CC for each of the antimicrobials was determined as described below.

Linezolid. Each laboratory tested linezolid at concentrations of 0.5, 1, and 2 μg/ml in the M960 system, and these results were compared to those obtained with a CC of 1 μg/ml in the B460 system (17). Three strains yielded resistant results using the B460 system, and the corresponding MIC in the M960 system was >2 μg/ml. Sensitive strains had MICs ranging from ≤0.5 μg/ml to 1 μg/ml in the M960 system. Based on these data, a CC of 1.0 μg/ml was determined to be appropriate for the testing of linezolid resistance in the M960 system.

Moxifloxacin. Both the reference laboratory and the participant laboratories tested moxifloxacin concentrations of 0.25, 0.5, and 1 μg/ml in the M960 system. A CC of 0.5 μg/ml in the B460 system (5) was used as a reference. The MICs for resistant strains ranged from 0.5 μg/ml to 1 μg/ml. The MICs for the sensitive strains were all ≤0.25 μg/ml. Based on this information, the CC of moxifloxacin in the M960 system was determined to be 0.25 μg/ml.

PAS. Based on initial testing, 10 of the 31 strains that were susceptible to PAS in the B460 system had a PAS MIC of 8 μg/ml or greater in the M960 system. A high level of discordance was found between the PAS test results obtained with the B460 system and those obtained with the M960 system by all of the laboratories, as well as low interlaboratory reproducibility. The technique used to inoculate the tests was reevaluated. The known clumping properties and lack of homogeneity of mycobacterial suspensions in cultures can result in inconsistent inocula and false-resistant susceptibility test results (3, 19). This problem appeared to be particularly evident in the testing of PAS. If laboratories encountered this problem, cultures were mixed thoroughly and allowed to settle for 15 min and an aliquot of the supernatant was used for the inoculation of the PAS test. After the implementation of this procedure, the MIC for all resistant strains was ≥8 μg/ml. The establishment of a CC of 4 μg/ml resolved all but one instance of unexpected resistance in the M960 system. Erroneous resistance results remained problematic for two of the laboratories using the B460 technology. It may be noted that this inoculation method was used only for PAS repeat testing in this study. Using the M960 system, the interlaboratory reproducibility was 99.3% and the intralaboratory reproducibility was 100% (see Table 4).

Comparison of M960 and B460 system data. After the determination of the CCs of all of the antimicrobials, the M960 system AST results were analyzed for reproducibility between methods (Table 3), between laboratories (interlaboratory reproducibility), and between replicates (intralaboratory reproducibility) (Table 4).

Amikacin, capreomycin, linezolid, moxifloxacin, and rifabutin showed 100% concordance between B460 and M960 system AST results. Inter- and intralaboratory reproducibility was also found to be 100% for these antimicrobials (Table 4).

Ethionamide. One of the 20 ethionamide-susceptible strains in the B460 system gave inconsistent results among the testing laboratories, with all four laboratories finding this strain to be repeatedly resistant in the M960 system and only one of the four laboratories obtaining a resistant result using the B460 system. The MIC for this strain was determined to be >7.5 μg/ml in the M960 system. Based on this result, the sensitive result from the B460 system is likely in error. The overall concordance between the B460 and M960 system AST results

TABLE 4. Inter- and intralaboratory reproducibility of second-line antimicrobial testing in the M960 system

Antimicrobial agent	% Reproducibility	
	Interlaboratory	Intralaboratory
Amikacin	100	100
Capreomycin	100	100
Ethionamide	98.6	100
Kanamycin at:		
5.0 μg/ml	99.3	100
2.5 μg/ml	100[a]	100[a]
Linezolid	100	100
Moxifloxacin	100	100
Ofloxacin	99.3	97.2
PAS	99.3	100
Rifabutin	100	100

[a] Two laboratories tested kanamycin at a CC of 2.5 μg/ml.

for ethionamide was 94.4%. Inter- and intralaboratory reproducibility was calculated to be 98.6% and 100%, respectively (Table 4).

Kanamycin. The initial study design was for kanamycin to be tested at 2.5, 5.0, and 7.5 μg/ml by the reference laboratory and at a CC of 5.0 μg/ml by the participant laboratories in the M960 system. Based on the testing done at the reference laboratory, one of the B460 system sensitive strains tested was determined to have an MIC of kanamycin at the proposed CC of 5.0 μg/ml in the M960 system. One participant laboratory found this isolate to be sensitive at the CC of 5.0 μg/ml in the B460 system, while the other two participant laboratories found it to be resistant. Only one laboratory found the isolate to be resistant in the M960 system. Based on the inconsistent sensitivity results, it could be concluded that this strain exhibits emerging/borderline resistance to kanamycin. Decreasing the CC in the M960 system to 2.5 μg/ml ensures that strains exhibiting this type of emerging resistance are classified as resistant and treatment is adjusted accordingly. All other strains found to be sensitive in the B460 system were tested at a CC of 2.5 μg/ml by the reference laboratory and one of the participants. This testing yielded 100% reproducibility between the laboratories and between the methods. Based on the above data, we are recommending that kanamycin be tested in the M960 system at a CC of 2.5 μg/ml. This recommendation is in line with the recently published CLSI guidelines (3).

Ofloxacin. All strains previously determined to be sensitive in the B460 system at a CC of 2.0 μg/ml were also determined to be sensitive at a CC of 2.0 μg/ml in the M960 system. One laboratory found one strain to be repeatedly discordant between the two methods. This isolate was determined to have an MIC of 4 μg/ml by the reference laboratory, a result that was consistent with the other two laboratories. As a result, the inter- and intralaboratory reproducibility for ofloxacin was 99.3% and 97.2%, respectively (Table 4).

DISCUSSION

First-line AST of *M. tuberculosis* using the M960 system is U.S. FDA and Health Canada approved, and a standardized kit for AST using the B960 system is provided by the manufacturer, BD. However, there are no commercial kits or approved methods for rapid, broth-based second-line AST. Although MDR TB in Canada is still rare, there is an increasing demand for second-line AST of *M. tuberculosis*. It is critical that a standardized method for second-line testing be developed and validated.

Based on current literature regarding the use of the M960 system for second-line AST of *M. tuberculosis*, including recommendations by the CLSI and the World Health Organization (Table 3), we selected a panel of 9 second-line antimicrobials for testing with either CCs, when sufficient data were available, or three test concentrations when published data were lacking or contradictory. These results were then compared to CC testing data from the B460 system. Overall, our results showed good concordance between the methods (98.2%) and between the participating laboratories (99.5%). Intralaboratory reproducibility with replicate isolates was also high (99.7%), with only a single replicate for ofloxacin testing having a discordant result. Table 5 lists the recommended second-line AST panel and CCs determined by this study.

TABLE 5. Final determination of CCs for standardized testing of second-line antimicrobials in Canada with the M960 system versus the B460 system

Antimicrobial agent	CC (μg/ml)	
	B460	M960
Amikacin	1	1
Capreomycin	1.25	2.5
Ethionamide	2.5	5
Kanamycin	5	2.5
Linezolid	1	1
Moxifloxacin	0.5	0.25
Ofloxacin	2	2
PAS	4	4
Rifabutin	0.5	0.5

The literature is limited with respect to M960 system CCs for moxifloxacin, linezolid, and PAS AST. Our data further support the establishment of a moxifloxacin CC of 0.25 μg/ml, as published in the CLSI guidelines. Unfortunately, there are a limited number of linezolid-resistant *M. tuberculosis* strains available for testing. This study proposes that the CC be set at 1.0 μg/ml, but as more resistant strains become available, this concentration may require reevaluation. For PAS, the suggested CC is 4.0 μg/ml. Our experience with PAS testing showed that the test inoculation procedure is critical for accurate results. In order to overcome the problems associated with clumping and overinoculation, we suggest that all laboratories consider the inoculation method described in this report.

We have validated the M960 method using a standardized extended panel of second-line antimicrobials for susceptibility testing of *M. tuberculosis* strains in Canadian laboratories. We were able to obtain very high levels of inter- and intralaboratory reproducibility with the M960 methodology for all nine antimicrobials tested. The CCs recommended by this study demonstrated good concordance with the previous B460 methodology, as well as with the suggested values in previously published studies.

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