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Multicenter Evaluation of Ethambutol Susceptibility Testing of *Mycobacterium tuberculosis* by Agar Proportion and Radiometric Methods

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Reproducibility of ethambutol (EMB) susceptibility test results for *Mycobacterium tuberculosis* has always been difficult for a variety of reasons, including the narrow range between the critical breakpoint for EMB resistance and the MIC for susceptible strains, borderline results obtained with the BACTEC 460TB method, the presence of microcolonies determined using the agar proportion (AP) method, and a lack of agreement between these two testing methods. To assess the frequency of these problems, *M. tuberculosis* drug susceptibility data were collected in a multicenter study involving four laboratories. Resistant, borderline, and susceptible isolates were shared among the laboratories to measure interlaboratory test agreement. Half of isolates determined by BACTEC 460TB to be resistant were determined to be susceptible by the AP method. Isolates determined to be resistant to EMB by both BACTEC 460TB and AP methods were almost always resistant to isoniazid. Results from isolates tested by the BACTEC 460TB method with an EMB concentration of 3.75 μg/ml in addition to the standard 2.5 μg/ml did not show improved agreement by the AP method. While these results do not indicate that the AP method is more accurate than the BACTEC 460TB method, laboratories should not report EMB monoresistance based on BACTEC 460TB results alone. Monoresistance to EMB should only be reported following confirmation by the AP method. Microcolonies could not be confirmed as resistant by the BACTEC 460TB method or by repeat testing with the AP method and do not appear to be indicative of resistance.

Radiometric detection of bacterial growth (BACTEC 460TB system; Becton Dickinson and Company, Sparks, Md.) is the most commonly used method in the United States for determining resistance to the primary drugs used to treat *Mycobacterium tuberculosis* disease (15). This technique was designed to provide rapid susceptibility test results for streptomycin (SM), isoniazid (INH), rifampin (RIF), and ethambutol (EMB) that are equivalent to those obtained by the reference agar proportion (AP) method using Middlebrook agar (7, 9, 10, 12, 13).

Testing of *M. tuberculosis* for susceptibility to EMB can be problematic by both the radiometric and AP methods. This may be due to the bacteriostatic nature of EMB, the reduced activity of the drug in a culture medium, or the narrow range between the MICs of susceptible and resistant isolates of *M. tuberculosis* (4, 6). While the radiometric method has been modified over the years, whether it accurately determines susceptibility to EMB remains in question (5, 9, 14, 16, 17). Decisions are unclear on the interpretation and reporting of small colonies of mycobacteria (microcolonies) as resistant mutants on the EMB drug quadrant by AP testing (11). To characterize the extent of these problems with EMB susceptibility testing and provide further information for assistance with test interpretation, we collected and analyzed data from four public health laboratories in a multicenter study.

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MATERIALS AND METHODS

Study design. Mycobacteriology laboratories enrolled in the study were a Veterans Administration TB Reference Laboratory, two state public health laboratories, and one large county public health laboratory. Results of susceptibility tests performed by the laboratories between January 1998 and December 1998 were collected retrospectively, while results of susceptibility tests performed by the laboratories between January 1999 and January 2000 were collected prospectively. During the prospective phase of the study, isolates found to be resistant or borderline by the BACTEC 460TB method to one or more of the SIRE drugs (i.e., SM, INH, RIF, and EMB), or pyrazinamide (PZA) by one of the four participating laboratories were distributed to the other three laboratories and to the Mycobacteriology Laboratory at the Centers for Disease Control and Prevention (CDC) for susceptibility testing. The CDC laboratory only performed AP testing.

Susceptibility testing methods. Test results were obtained for the SIRE drugs and PZA using the BACTEC 460TB testing system as described in the product and procedure manual (Becton Dickinson and Company). Drug concentrations tested in the BACTEC 460TB system were 0.1 μg/ml for INH, 2.5 μg/ml for EMB, 2.0 μg/ml for SM, 2.0 μg/ml for RIF, and 100 μg/ml for PZA. Susceptibility testing by the AP method was performed by the standard method (8). Two laboratories set up SIRE susceptibility testing with the AP method at the same time as the BACTEC 460TB method. One of these laboratories used a reduced AP panel consisting of INH (0.2 μg/ml), RIF (2.0 μg/ml), and EMB (5.0 μg/ml). The other two laboratories used the AP method only when resistance to at least one SIRE drug was detected by the BACTEC 460TB method and used the following drug concentrations: 0.2 and 1.0 μg/ml for INH, 5.0 μg/ml for EMB, 2.0 and 10.0 μg/ml for SM, and 1.0 μg/ml for RIF.

Each laboratory site obtained all antituberculosis drugs, susceptibility test media, and other components for susceptibility testing individually. Middlebrook 7H10 agar was either purchased or prepared in-house by each laboratory.

TABLE 1. EMB susceptibility results for *M. tuberculosis* isolates tested by BACTEC 460TB and AP methods[a]

BACTEC 460TB result	No. of isolates with AP result		Total no. of isolates
	Resistant	Susceptible	
Resistant	85	80	165
Borderline	1	21	22
Susceptible	8	1,737	1,745
Total	94	1,838	1,932

[a] EMB was used at 2.5 and 5.0 μg/ml in the BACTEC 460TB and AP methods, respectively.

TABLE 2. Association between EMB resistance as determined by the BACTEC 460TB and AP methods and INH (0.2 μg/ml) resistance as determined by the AP method

EMB susceptibility[a] as determined by:		No. of isolates	% Isolates INH resistant by the AP method
BACTEC 460TB	AP		
R	R	29	96.6
S	R	5	60.0
R	S	43	46.5
B or S	S	1,157	11.0

[a] Abbreviations: R, resistant; S, susceptible; B, borderline.

BACTEC 460TB supplies and antituberculosis drugs were obtained from Becton Dickinson and Company. Quality control procedures were performed according to recommended guidelines and standard protocols for culture media, antituberculosis drugs, and other test components (8; see also the product and procedure manual from Becton Dickinson and Company).

Data collection and analysis. Variables collected for BACTEC 460TB susceptibility results included the number of days to an interpretable result on initial test, control delta growth index (ΔGI), EMB ΔGI, and susceptibility interpretation for each antituberculosis drug. AP data included the percent resistance and interpretation, along with the presence of any microcolonies on the EMB drug quadrant. When borderline results obtained using BACTEC 460TB were resolved, variables recorded included the number of additional days of incubation required to obtain a resistant or susceptible result and the control ΔGI and the EMB ΔGI at the time of resolution. Resolved results were used in the analysis.

All statistical analyses were performed using Fisher's exact methods.

Comparison of EMB 2.5- and 3.75-μg/ml vials in the BACTEC 460TB system. For 6 months, patient isolates at each of the four laboratory sites were tested in a bi-level study using EMB at concentrations of both 2.5 and 3.75 μg/ml in the BACTEC 460TB system and EMB at 5.0 μg/ml in the AP method. During this stage of the study, the same drug lot number of EMB was used in all four of the participating laboratories. Twenty-one frozen isolates previously determined by the BACTEC 460TB method to be resistant or borderline to EMB at 2.5 μg/ml were retested by the BACTEC 460TB and AP methods in the same manner to determine resistance to the higher concentration of EMB. Frozen isolates were retested only in the source laboratory.

RESULTS

Intralaboratory agreement between BACTEC 460TB and AP method results. The number of isolates in the study was 2,184. One isolate per patient was used in the study. There were 989 isolates in the retrospective year and 1,195 isolates in the prospective part of the study. Both BACTEC 460TB and AP EMB susceptibility test results were collected for 1,932 isolates of *M. tuberculosis* (Table 1). Of the 94 isolates found by the AP method to be resistant to EMB at 5.0 μg/ml, 85 (90.4%; 95% confidence interval, 82.3 to 95.8%) were found by the BACTEC 460TB method to be resistant to EMB at 2.5 μg/ml. Of the 1,838 isolates that were shown by the AP method to be susceptible to EMB, 1,737 (94.5%; 95% confidence interval, 93.3 to 95.5%) were also found to be susceptible by BACTEC 460TB. Of the 53 isolates (data not shown) that initially revealed borderline results in the BACTEC 460TB system, 22 were incubated 1 to 3 days longer, at which point 21 isolates resolved as susceptible and 1 resolved as resistant. Based on these results, three of the four study laboratories adopted the practice of routinely holding and resolving borderline tests upon the conclusion of the study.

Interlaboratory agreement of BACTEC 460TB and AP method results. The four enrolled laboratories shared 183 isolates of *M. tuberculosis.* Of the BACTEC 460TB results for the shared isolates, 127 were found to be susceptible to EMB at 2.5 μg/ml by every laboratory, 21 isolates were found to be resistant by every laboratory, and 35 isolates had discordant results. For the BACTEC 460TB method, we found 91.4% pairwise interlaboratory agreement.

Of the AP results for the shared isolates, 157 were found to be susceptible to EMB at 5 μg/ml by every laboratory, 10 isolates were found to be resistant by every laboratory, and 16 isolates had discordant results. For the AP method, we found 95.8% pairwise interlaboratory agreement.

Association between resistance to EMB and INH. A total of 1,234 isolates (first isolate per patient) were evaluated for associations among EMB resistance as determined by the BACTEC 460TB and AP methods and INH resistance as determined by the AP method. As shown in Table 2, 28 of 29 (96.6%) isolates resistant to EMB by both the BACTEC 460TB and AP methods were also resistant to INH, 3 of 5 (60.0%) isolates resistant to EMB by the AP method and susceptible to EMB by the BACTEC 460TB were resistant to INH, 20 of 43 (46.5%) isolates resistant to EMB by the BACTEC 460TB method and susceptible by the AP method were resistant to INH, and 127 of 1,157 (11.0%) isolates borderline or susceptible by the BACTEC 460TB method and susceptible by the AP method were resistant to INH. Only 1 of 39 (2.6%) isolates (data not presented) found to be resistant to EMB and susceptible to INH using the BACTEC 460TB method appeared to be monoresistant to EMB using AP method results as confirmation, but even this apparently EMB-monoresistant shared isolate was found by three of the four laboratories to be susceptible to EMB when the AP method was used. Isolates confirmed as EMB resistant by the AP and BACTEC 460TB methods by all four laboratories were always resistant to INH.

Bi-level study of EMB (2.5 and 3.75 μg/ml) in the BACTEC 460TB system. A number of isolates that were tested by both the BACTEC 460TB and AP methods were EMB resistant or borderline by the BACTEC 460TB method but were EMB susceptible by the AP method (Table 1). In a bi-level study, a total of 408 isolates were tested with EMB at concentrations of 3.75 and 2.5 μg/ml in the BACTEC 460TB system and compared with results obtained using EMB at 5.0 μg/ml in the AP method. As shown in Table 3, 36 of 38 (94.7%) isolates shown to be resistant by the AP method were found to be resistant to EMB at 2.5 μg/ml in the BACTEC 460TB system, and 30 (78.9%) isolates were found to be resistant to EMB at 3.75 μg/ml. Of the 370 isolates shown to be susceptible by the AP

TABLE 3. EMB susceptibility results for *M. tuberculosis* isolates tested by BACTEC 460TB and AP methods[a]

BACTEC 460TB result	No. of isolates with AP result		Total no. of isolates
	Resistant	Susceptible	
EMB, 2.5 μg/ml			
Resistant	36	27	63
Borderline	1	3	4
Susceptible	1	340	341
EMB, 3.75 μg/ml			
Resistant	30	18	48
Borderline	1	0	1
Susceptible	7	352	359
Total	38	370	408

[a] EMB was used at 2.5 and 3.75 μg/ml in the BACTEC 460TB method and at 5.0 μg/ml in the AP method.

method, 340 (91.9%) were found to be susceptible to EMB at 2.5 μg/ml in the BACTEC 460TB system and 352 (95.1%) were found to be susceptible to EMB at 3.75 μg/ml. Results were equivalent whether the isolates had previously been frozen or whether they were fresh culture isolates.

Microcolonies in AP method. Microcolonies were reported for 46 of 890 (5.2%) test results from the 183 shared isolates. Of the 46 isolates showing microcolonies, 37 (80.4%) were susceptible to EMB by radiometric detection. Of the remaining nine isolates that produced microcolonies among the five laboratories, three produced only microcolonies without sufficient numbers of mature colonies to indicate resistance, and only one of these isolates was found to be resistant according to the AP method by all of the other laboratories. In addition, the presence of microcolonies on EMB was not associated with INH resistance.

DISCUSSION

EMB susceptibility test results among the laboratories in this study have greater interlaboratory agreement for results obtained by the AP method than for those obtained by the BACTEC 460TB method. Isolates which were found to be resistant by the BACTEC 460TB method and susceptible by the AP method were seen in both inter- and intralaboratory studies. The increased agreement among AP results does not indicate that the AP method is a more accurate test. Because resistance in the BACTEC 460TB system is reflective of growth and differences in the amount of ^{14}C-labeled CO_2 in the control and EMB vials (as described in the product and procedure manual), one could postulate that the radiometric method is a more sensitive method than the AP method for detecting resistant organisms in susceptibility testing. In addition, the smaller number of discrepancies observed with AP testing may be because there are only two possible AP results (susceptible or resistant), while there are three possible results with the BACTEC 460TB system (susceptible, resistant, and borderline), thus increasing the potential for discordant BACTEC 460TB results among the laboratories and for discordant results between the two methods within laboratories.

Borderline and monoresistant results in the BACTEC 460TB system may be seen with EMB because of the slow activity of EMB on the mycobacterial cell wall (2, 3, 4). This slower activity of EMB on the mycobacterial cell wall may result in a higher GI, which is further enhanced if the test inoculum is too heavy. This may be why almost all of the borderline results in this study resolved as susceptible results with continued incubation for 1 to 3 days as suggested by the manufacturer's guidelines. The large number of isolates that were resistant according to the BACTEC 460TB method and susceptible according to the AP method suggests that the critical concentration of drugs in the two methods may not be equivalent. When the concentration of EMB was increased to 3.75 μg/ml in the bi-level study, the rate of isolates determined to be resistant by the BACTEC 460TB method and susceptible by the AP method decreased, while the rate of isolates determined to be susceptible by the BACTEC 460TB method and resistant by the AP method increased compared with the results obtained with EMB at 2.5 μg/ml. Among those isolates found to be susceptible by the AP method, the rate of resistance determined by the BACTEC 460TB method with EMB at 2.5 μg/ml was significantly higher in the bi-level study (7.3%) than in the original study (4.5%) ($P = 0.035$). One might attribute this difference to using a sample of EMB with less than the expected activity, since all the laboratories in the bi-level study used the same lot of EMB, or the possibility that the equivalent concentration for the BACTEC 460TB method is somewhere between 2.5 and 3.75 μg/ml. Increasing the concentration of EMB in this study yielded more results that were determined to be susceptible by the BACTEC 460TB method and resistant by the AP method at the expense of fewer results that were resistant by the BACTEC 460TB method and susceptible by the AP method.

Isolates found to be EMB resistant by both the BACTEC 460TB and AP methods were almost always resistant to INH. Approximately 50% of isolates that were EMB resistant by the BACTEC 460TB method and susceptible by the AP method were found to be resistant to INH. These results suggest that the BACTEC 460TB method might be more sensitive at detecting resistant organisms in a population and may be detecting early emerging EMB resistance accompanied by INH resistance.

Microcolonies are more commonly associated with EMB than any of the other first-line drugs (J. C. Ridderhof et al., Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 865, 1999). Deciding whether microcolonies detected on AP susceptibility test media represent a population of resistant mycobacteria, partially resistant mycobacteria, or drug degradation followed by an overgrowth of susceptible organisms (1, 2, 11) is challenging. The presence of microcolonies may be due to one or more factors, which could include variability in Middlebrook agar and oleic albumin-dextrose-catalase enrichment components and/or an inoculum that is too concentrated (3, 4) and thus requires expertise and careful assessment of all of the quality control components of the test.

Although the molecular mechanisms of EMB resistance are not yet completely understood, a genotypic association between small colonies of *M. tuberculosis* and EMB resistance has not yet been established. In this study, the presence of the microcolonies on EMB drug media was not confirmed with

BACTEC or repeat AP testing. Based on analyzing these study results, we found strong evidence that the presence of microcolonies alone did not indicate resistance.

In summary, the increased number of EMB-borderline and resistant results with the BACTEC 460TB system may be due in part to the borderline category for defining populations of resistant mycobacteria in BACTEC 460TB. For borderline results, we recommend that all laboratories follow the manufacturer's suggested guideline of observing the vials an additional 1 to 3 days and reporting the results only when a definite pattern has been established (as discussed in the product and procedure manual). An increase in the concentration of EMB in BACTEC 460TB vials did not improve the agreement between BACTEC 460TB and AP method results, because the decrease in isolates that were determined to be resistant by the BACTEC 460TB method and susceptible by the AP method was offset by a corresponding increase in isolates that were determined to be susceptible by the BACTEC 460TB method and resistant by the AP method.

In addition, EMB resistance was accompanied by 96.6% resistance to INH. In view of our findings, laboratories should not report resistance to EMB alone (monoresistance) based on BACTEC 460TB results. Results should be confirmed by another method. When EMB resistance is associated with resistance to INH or other first-line drugs, results should be reported immediately and then confirmed by another method as recommended by NCCLS guidelines for *M. tuberculosis* susceptibility testing (11).

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