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Rapid, Low-Technology MIC Determination with Clinical *Mycobacterium tuberculosis* Isolates by Using the Microplate Alamar Blue Assay

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A colorimetric, microplate-based Alamar Blue assay (MABA) method was used to determine the MICs of isoniazid (INH), rifampin, streptomycin (SM), and ethambutol (EMB) for 34 Peruvian *Mycobacterium tuberculosis* isolates (including both pansensitive and multidrug-resistant strains) and the H_{37}Rv strain by using bacterial suspensions prepared directly from solid media. Results for all isolates were available within 8 days. Discordant results were observed on initial tests for 3 of 16 INH-susceptible isolates, 5 of 31 EMB-susceptible isolates, and 2 of 4 SM-resistant isolates (by the BACTEC 460 system). The overall agreements between the MICs obtained by MABA and the results obtained with the BACTEC 460 system were 87.9% for initial results and 93.6% after retesting 12 of 17 samples with discrepant results. Interpretation of MABA endpoints improved with technical experience. The MABA is a simple, rapid, low-cost, appropriate technology which does not require expensive instrumentation and which makes use of a nontoxic, temperature-stable reagent.

Tuberculosis is estimated to have caused the deaths of 1 billion people in the last 200 years (10). Both the current human immunodeficiency virus pandemic and multidrug-resistant *Mycobacterium tuberculosis* have emerged as major obstacles to treatment and public health control of tuberculosis (5). Many developing countries have difficulty obtaining drug susceptibility information for *M. tuberculosis* isolates for financial or technical reasons. Treatment of tuberculosis without the benefit of susceptibility information increases the risk of treatment failure and the spread of resistant strains, as well as the development of resistance to additional drugs. Global surveillance of the drug resistance of *M. tuberculosis* isolates has been proposed as a means of augmenting databases of drug-resistant *M. tuberculosis* isolates to help with the development of future program policy recommendations (1).

The agar proportion susceptibility method (4) is labor-intensive, and results may take up to 2 months, often making the result clinically irrelevant. Commercially available systems such as the BACTEC system (7) and the newer Mycobacteria Growth Indicator Tubes (9, 15) and the Etest (14) are simple and rapid but expensive, making them impractical for use in developing countries.

Oxidation-reduction dyes, e.g., tetrazoliums, have been used to obtain drug susceptibility measurements for bacteria (12) including mycobacteria (3, 16). Yajko et al. (16) reported as a result of tests with clinical isolates a good correlation between the proportion technique and a broth method with Alamar Blue, a novel proprietary, resazurin-based (11) oxidation-reduction indicator which delivered colorimetric MICs for *M. tuberculosis* isolates in 14 days. A microplate version of the Alamar Blue assay (MABA) with modified medium composition, reaction time and temperature, and inoculum preparation was evaluated as a high-throughput screen by comparing the MICs of 30 antimicrobial agents for *M. tuberculosis* H_{37}Ra and H_{37}Rv obtained by MABA to the MICs obtained with the BACTEC 460 system (2).

This study evaluated the performance of MABA with 34 clinical *M. tuberculosis* isolates and *M. tuberculosis* H_{37}Rv. MABA was performed in a university laboratory in Peru, a country that has a high incidence of tuberculosis and multidrug-resistant *M. tuberculosis* isolates (8, 13), in order to determine the feasibility of using the assay under conditions resembling those existing in areas of the world with a high prevalence of tuberculosis and minimal financial resources. The results were compared with those obtained in the United States at the New Mexico State Department of Health with the BACTEC 460 system.

(This study was presented in part at the 45th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Baltimore, Md., 1996 [2a].)

MATERIALS AND METHODS

Isolates and drug preparation. Thirty-four clinical isolates of *M. tuberculosis* obtained from Cayetano Heredia University Hospital and *M. tuberculosis* H_{37}Rv ATCC 27294 (American Type Culture Collection, Rockville, Md.) were subcultured on Middlebrook 7H11 agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Suspensions were prepared in 0.04% (vol/vol) Tween 80–0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) so that their turbidities matched that of a McFarland no. 1 turbidity standard (6). Suspensions were further diluted 1:25 in 7H9GC broth (4.7 g of Middlebrook 7H9 broth base [Difco, Detroit, Mich.], 20 ml of 10% [vol/vol] glycerol, 1 g of Bacto Casitone [Difco], 880 ml of distilled water, 100 ml of oleic acid, albumin, dextrose, and catalase [Remel, Lenexa, Kans.]).

Isoniazid (INH), rifampin (RMP), streptomycin (SM), and ethambutol (EMB) were obtained from Sigma. Stock solutions of INH, SM, and EMB were prepared in deionized water, and RMP was prepared in dimethyl sulfoxide. Stock solutions were diluted in 7H9GC broth to two times the maximum desired final testing concentrations prior to their addition to microplates.

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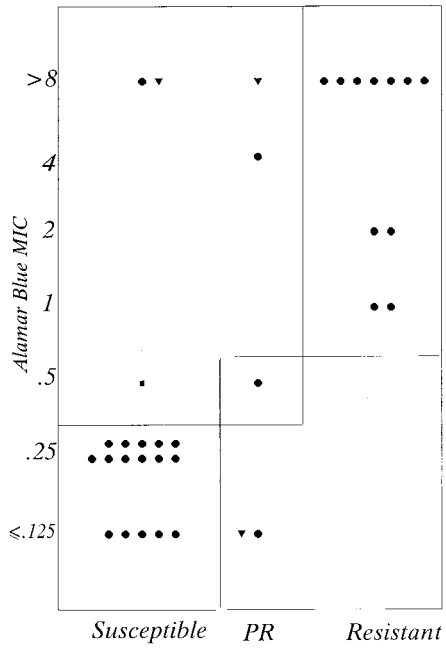

FIG. 1. Correlation between MICs by MABA and BACTEC 460 system classification for INH. ●, isolates for which results were concordant (and isolates for which discordant results were confirmed upon repeat testing by MABA); ■, isolates for which results were discordant and for which MICs by MABA were not redetermined; ▼, isolates for which results were discordant but for which results were concordant upon repeat MABA testing by MABA. The vertical lines in the figures separate the standard breakpoints for the BACTEC 460 system. PR, partially resistant. The horizontal lines separate the interpretive breakpoints for colorimetric MICs, which were selected on the basis of the best fit of the MABA results with the BACTEC 460 system results.

MABA. Two hundred microliters of sterile deionized water was added to all outer-perimeter wells of sterile 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, N.J.) to minimize evaporation of the medium in the test wells during incubation. The wells in rows B to G in columns 3 to 11 received 100 μl of 7H9GC broth. One hundred microliters of 2× drug solutions were added to the wells in rows B to G in columns 2 and 3. By using a multichannel pipette, 100 μl was transferred from column 3 to column 4, and the contents of the wells were mixed well. Identical serial 1:2 dilutions were continued through column 10, and 100 μl of excess medium was discarded from the wells in column 10. Final drug concentration ranges were as follows: for INH, 0.031 to 8.0 μg/ml; for RMP, 0.0156 to 4 μg/ml in initial tests and 0.062 to 16 μg/ml on repeat testing; for SM, 0.125 to 32 μg/ml; and for EMB, 0.5 to 128 μg/ml.

One hundred microliters of *M. tuberculosis* inoculum was added to the wells in rows B to G in columns 2 to 11 by using an Eppendorf repeating pipette (yielding a final volume of 200 μl per well). Thus, the wells in column 11 served as drug-free (inoculum-only) controls.

The plates were sealed with Parafilm and were incubated at 37°C for 5 days. Fifty microliters of a freshly prepared 1:1 mixture of 10× Alamar Blue (Accumed International, Westlake, Ohio) reagent and 10% Tween 80 was added to well B11. The plates were reincubated at 37°C for 24 h. If well B11 turned pink, the reagent mixture was added to all wells in the microplate (if the well remained blue, the reagent mixture would be added to another control well and the result would be read on the following day). The microplates were resealed with Parafilm and were incubated for an additional 24 h at 37°C, and the colors of all wells were recorded. A blue color in the well was interpreted as no growth, and a pink color was scored as growth. A few wells appeared violet after 24 h of incubation, but they invariably changed to pink after another day of incubation and thus were scored as growth (while the adjacent blue wells remained blue). The MIC was defined as the lowest drug concentration which prevented a color change from blue to pink.

BACTEC assay. Drug susceptibility was determined in the BACTEC 460 instrument (Becton Dickinson, Sparks, Md.) by standard procedures (7) but using the following critical concentrations: INH, 0.1 and 0.4 μg/ml; RMP, 1 μg/ml; SM, 2 and 6 μg/ml; and EMB, 2.5 and 5 μg/ml. Isolates which were susceptible to the higher concentration of drug but resistant to the lower concentration were termed partially resistant.

RESULTS

Colorimetric MIC test results for all 34 of the clinical *M. tuberculosis* isolates and the H_{37}Rv strain were available by the 8th day of incubation. After 5 days of incubation, the Alamar Blue reagent was added to the control wells. Following incubation at 37°C for 24 h, most control wells became pink. For those that remained blue, Alamar Blue was added to the next control well and the plates were reincubated for another 24 h until all control wells were pink (indicating sufficient growth to determine drug susceptibility). Alamar Blue was then added to all remaining wells, and the results were determined on the following day (day 7 or 8). The correlations between MIC results obtained by MABA and the results obtained by the BACTEC 460 system are illustrated in Fig. 1 to 4. Although we intended to retest all isolates for which discordant results were obtained, for logistical reasons this was not possible in all cases.

INH susceptibility tests. For 16 of the 19 isolates that were susceptible to 0.1 μg of INH per ml in the BACTEC 460 system, the MIC by MABA was ≤0.25 μg/ml (Fig. 1). Of the three isolates with discordant results, the MIC for one isolate was 0.5 μg/ml (one dilution off, which was interpreted as partial resistance) and the MICs for two isolates were 8 μg/ml (one of the two isolates was retested and the MIC was 0.125 μg/ml, which was classified as susceptible; the other isolate was retested five times, with all results indicating resistance). Of five isolates that were partially resistant in tests with the BACTEC system (resistant at 0.1 μg/ml and susceptible at 0.4 μg/ml), the MICs by MABA for one isolate were in agreement with those of the BACTEC system (0.5 μg/ml); the MICs for the other four isolates were discordant between the two systems (MICs, 0.125, 0.125, 4, and 8 μg/ml, respectively; (two isolates were retested by MABA, and the MICs were in agreement with those obtained with the BACTEC system [0.5 μg/ml]). For all 12 isolates which appeared to be resistant to 0.4

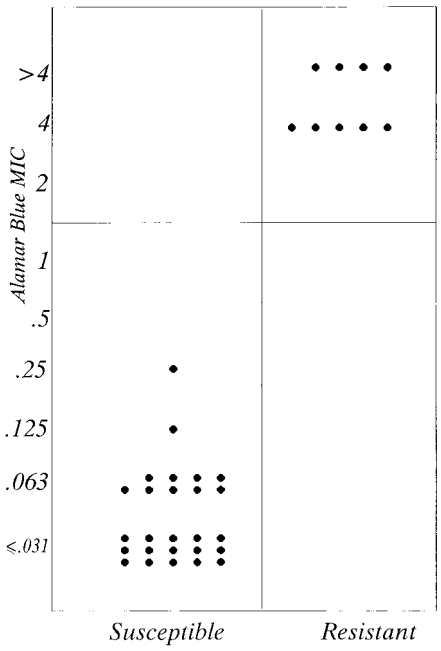

FIG. 2. Correlation between MICs by MABA and BACTEC 460 system classification for RMP. The vertical lines separate the standard breakpoints for the BACTEC 460 system. The horizontal lines separate the interpretive breakpoints for colorimetric MICs, which were selected on the basis of the best fit of the MABA results with the BACTEC 460 system results.

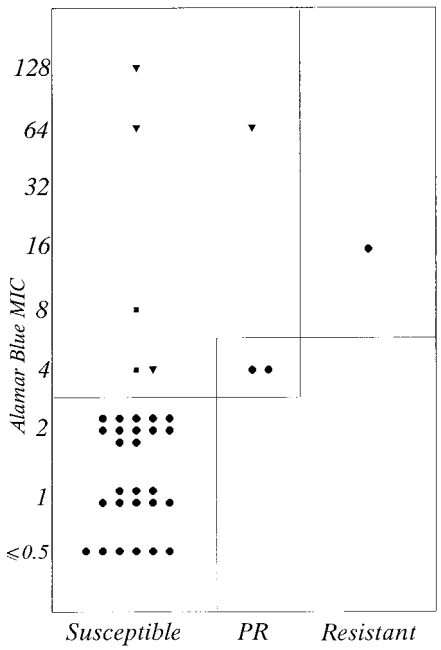

FIG. 3. Correlation between MABA MICs and BACTEC 460 system classification for EMB. ●, isolates for which results were concordant (and isolates for which discordant results were confirmed upon repeat testing by MABA); ■, isolates for which results were discordant and for which MICs by MABA were not redetermined; ▼, isolates for which results were discordant but for which results were concordant upon repeat testing by MABA. The vertical lines in the figures separate the standard breakpoints for the BACTEC 460 system. PR, partially resistant. The horizontal lines separate the interpretive breakpoints for colorimetric MICs, which were selected on the basis of the best fit of the MABA results with the BACTEC 460 system results.

μg/ml, in the BACTEC system, MICs by MABA were ≥1 μg/ml.

RMP susceptibility tests. For all 26 isolates susceptible to RMP at 1 μg/ml in the BACTEC system, the MIC by MABA was ≤0.25 μg/ml (Fig. 2). For the nine isolates resistant to RMP at 1 μg/ml in the BACTEC system, the MICs by MABA for five isolates were >4 μg/ml on initial testing and >16 μg/ml on repeat testing (when the concentration range was extended). The other four isolates were initially tested at the higher concentration range, and the RMP MICs for all four isolates were >16 μg/ml.

EMB susceptibility tests. For 26 of the 31 isolates susceptible to EMB at 5 μg/ml in the BACTEC system, the MIC by MABA was ≤2 μg/ml (Fig. 3). For five isolates with discordant results between the two systems, the MICs by MABA were 4, 4, 8, 64, and 128 μg/ml, respectively. The MICs by MABA were redetermined for three of the isolates, and all repeat MICs were ≤2 μg/ml, putting them into agreement with those obtained with the BACTEC system. The other two isolates were not retested. For two of the three isolates resistant to EMB at 5 μg/ml but susceptible to EMB at 10 μg/ml in the BACTEC system, the MIC by MABA was 4 μg/ml. The MIC for the third isolate was originally 64 μg/ml, but on retesting the MIC by MABA was 4 μg/ml. For the one isolate completely resistant to EMB at 10 μg/ml in the BACTEC system, the MIC by MABA was 16 μg/ml.

SM susceptibility tests. For all 18 isolates susceptible to SM at 2 μg/ml in the BACTEC system, the MIC by MABA was ≤1 μg/ml (Fig. 4). For 11 of the 13 isolates resistant to SM at 2 μg/ml but susceptible to SM at 6 μg/ml in the BACTEC system (partial resistance), the MIC by MABA was 2 to 8 μg/ml. For the two isolates with discordant results, the MICs by MABA were 1 μg/ml. For two of the four isolates resistant to SM at 6 μg/ml in the BACTEC system, the MIC by MABA was ≥32 μg/ml. The MIC for one isolate with discordant results was 4 μg/ml, but upon retesting by MABA the MIC was 16 μg/ml.

DISCUSSION

The overall agreements between the results obtained with the BACTEC system and by MABA were 87.9% upon initial testing and 93.6% after retesting 12 of the 17 isolates with discordant results (Table 1). Although initial concordance was relatively low for INH-susceptible, EMB-susceptible, and SM-resistant (by the BACTEC system) isolates, these values improved upon retesting, especially for EMB-susceptible isolates. For INH, RMP, and SM, the breakpoints were sharp (the wells were either blue or pink), while with EMB testing with some isolates, violet wells were observed, but on extended incubation these became pink. In general the repeat test results were considered to be more accurate as a result of the additional experience obtained by the technicians, for whom this study represented the first attempt at performing susceptibility studies in a microplate format.

The tube microdilution format used by Yajko et al. (16) offers a rapid Alamar Blue reaction by incubating the tubes at 50°C and providing results in 2 h (versus overnight when incubation is at 37°C). A tube format may also have an advantage over microplates with respect to biosafety, although sealing of the microplates with Parafilm should minimize the biohazard potential in the event that a plate is mishandled. On the other

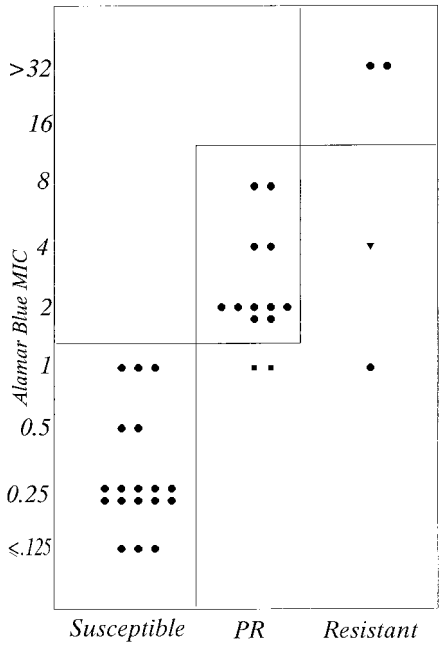

FIG. 4. Correlation between MABA MICs and BACTEC 460 system classification for SM. ●, isolates for which results were concordant (and isolates for which discordant results were confirmed upon repeat testing by MABA); □ isolates for which results were discordant and for which MICs by MABA were not redetermined; ▼, isolates for which results were discordant but for which results were concordant upon repeat testing by MABA. The vertical lines in the figures separate the standard breakpoints for the BACTEC 460 system. PR, partially resistant. The horizontal lines separate the interpretive breakpoints for colorimetric MICs, which were selected on the basis of the best fit of the MABA results with the BACTEC 460 system results.

TABLE 1. Concordance between MABA with BACTEC 460 system results

Drug and BACTEC 460 system result	No. of isolates for which results were concordant/total no. of isolates (% agreement)	
	Initial test	After retesting
INH		
Susceptible	16/19	17/19
Partially resistant	1/5	3/5
Resistant	11/11	11/11
RMP		
Susceptible	26/26	26/26
Resistant	9/9	9/9
EMB		
Susceptible	26/31	29/31
Partially resistant	2/3	3/3
Resistant	1/1	1/1
SM		
Susceptible	18/18	18/18
Partially resistant	11/13	11/13
Resistant	2/4	3/4
Total	123/140 (87.9)	131/140 (93.6)

hand, the MABA used in the present study facilitates liquid handling and should be economical, especially when (as anticipated for routine clinical testing) only two critical concentrations of each drug are used. This would allow at least three specimens to be tested per plate. Contamination was not found to be a problem. Our use of a 37°C reaction temperature obviates the need for a second incubator or water bath or the use of separate vessels for control samples, factors advantageous to a scheme that can be set up at minimum cost.

The fact that 100% of results were available within 8 days in this study, compared to 58% of results in 7 days in the study of Yajko et al. (16), was somewhat surprising considering that our use of 37°C for the Alamar Blue reaction lengthens the total assay time by 2 days, whereas the use of 50°C shortens the Alamar Blue reaction time to 2 h. Moreover, our use of an inoculum prepared from solid medium (in order to simulate the use of a primary culture and to reduce the overall turnaround time) would not be expected to shorten the lag phase of growth in the susceptibility test. This is more likely due to the use of glycerol in the culture medium in the present study.

Existing methods for drug susceptibility testing of clinical *M. tuberculosis* isolates are either inexpensive with long turnaround times or rapid but too expensive for all but the most affluent institutions. We have also successfully used the Mycobacteria Growth Indicator Tube and Etest (unpublished data) in Peru to determine drug susceptibilities, but the cost of materials is substantially higher than the cost of materials for the MABA. The MABA or the Alamar Blue tube microdilution version of Yajko et al. (16) offer a superior combination of rapidity and affordability. Results from this study, that of Yajko et al. (16), and others to be performed may allow the selection of one or two critical concentrations of each drug for use in differentiating susceptible, partially resistant, and fully resistant strains. This would further reduce the cost of the assay by allowing the drug susceptibilities of up to three isolates to be determined on a single 96-well plate. The minimum major equipment needed to perform MABA consists of a biosafety cabinet, an autoclave, and a 37°C incubator.

Preliminary results (1a) suggest that the less expensive nonproprietary oxidation-reduction indicator dimethylthiazoldiphenyltetrazolium bromide (MTT) in a microplate assay would give results similar to those obtained by MABA and thus could further reduce the costs of such assays (1a). MTT has already been shown to be of value in susceptibility testing of *Mycobacterium avium-Mycobacterium intracellulare* (3). Another nonproprietary oxidation-reduction indicator, 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride, has shown promise for use in drug susceptibility testing of *M. tuberculosis* isolates (17).

Considering their rapidity, their use of low technology, and their low cost, microplate assays that use Alamar Blue or tetrazolium-type compounds have the potential of becoming the methods of choice for drug susceptibility testing of *M. tuberculosis* isolates for much of the world where tuberculosis is a major problem.

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