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ARTICLE

**J. Luna-Herrera · G. Martínez-Cabrera
R. Parra-Maldonado · J. A. Enciso-Moreno
J. Torres-López · F. Quesada-Pascual
R. Delgadillo-Polanco · S. G. Franzblau**

# Use of Receiver Operating Characteristic Curves to Assess the Performance of a Microdilution Assay for Determination of Drug Susceptibility of Clinical Isolates of *Mycobacterium tuberculosis*

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**Abstract** The aim of this study was to apply receiver operating characteristic (ROC) analysis to the microplate Alamar blue assay, a recently developed alternative for drug susceptibility testing of mycobacteria. As this is a quantitative assay, its performance can be determined by ROC analysis, in which the area under the ROC curve represents a summary of test performance (the higher the area, the better the test's performance). Sixty isolates of *Mycobacterium tuberculosis* were tested by the microcolorimetric assay against six twofold dilutions of streptomycin, isoniazid, rifampin, and ethambutol. For each isolate, the susceptibility pattern was simultaneously established by the agar proportion method, the result of which represented the gold standard value for the ROC analysis. The critical concentration, area under the curve, and *P* value for each drug were determined by ROC curve analysis. The results of the assay were obtained in an average of 8 days of incubation. The performance of the assay was excellent for all four drugs: the area under the curves was >0.97, the *P* values were 0.000, and sensitivity was 94%, specificity 97%, predictive value for resistance ≥92%, predictive value for susceptibility 97%, and test efficiency 97%. According to ROC analysis, the microplate Alamar blue assay is a reliable method for determination of drug-susceptibility. Rapidity and cost efficiency are two additional qualities that make this test an excellent alternative for the drug susceptibility testing of *Mycobacterium tuberculosis*. The ROC curve analysis is a robust statistical approach for evaluating the performance of new quantitative methods for determination of drug sensitivity of *Mycobacterium tuberculosis* isolates.

J. Luna-Herrera (✉) · G. Martínez-Cabrera · F. Quesada-Pascual
R. Delgadillo-Polanco
Departamento de Inmunología, Instituto Politécnico Nacional,
Escuela Nacional de Ciencias Biológicas,
Carpio y Plan de Ayala, Colonia Santo Tomás 11340,
Mexico City, Mexico
e-mail: jluna@encb.ipn.mx
Tel.: +52-5729-6300 ext 62371, Fax: +52-5396-3503

R. Parra-Maldonado · J. A. Enciso-Moreno · J. Torres-López
Hospital de Pediatria, Centro Médico Nacional Siglo XXI,
Instituto Mexicano del Seguro Social, Mexico City, Mexico

S. G. Franzblau
Pharmacology Department, University of Illinois at Chicago,
Chicago, IL, USA

## Introduction

A number of new methods for drug susceptibility testing of mycobacteria have been described over the last decade, including luciferase reporter phage [1, 2], mycobacterial growth indicator tube (MGIT) [3, 4], the E test [5], cytofluorometry [6, 7], PhaB, a bacteriophage-based assay [8], and colorimetric methods using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [9, 10] and Alamar blue [11, 12, 13]. Alamar blue (Trek Diagnostic Systems, USA) is an oxidation-reduction dye used as an indicator of cellular growth-viability. The blue oxidized form becomes pink upon reduction due to metabolic activity [14]. The use of Alamar blue as an indicator of microbial growth was originally introduced for drug-susceptibility testing of yeast [15] and was first used with mycobacteria by Yajko et al. [11], who performed the test in tubes and developed the color at high temperature. Collins and Franzblau [12] modified the assay format, the composition of the culture medium, and the reaction temperature to create the microplate Alamar blue assay, or MABA. Minimal inhibitory concentrations (MICs) determined using MABA were within one twofold dilution of those obtained in the Bactec 460 for 25–27 of 30 antimicrobial agents tested against both *Mycobacterium tuberculosis* H37Rv and *Mycobacterium avium*. Subsequently, the MABA was used to determine MICs for clinical *Mycobacterium tuberculosis* isolates from Peru [13].

Conventional techniques for determination of drug sensitivity, such as the proportion method, are slow, la-

borious, and give only qualitative results. New rapid methods based on measurements of the metabolic activities of mycobacteria are therefore very attractive, but their accuracy must be clearly demonstrated. The statistical analyses to evaluate the performance of a qualitative diagnostic test (a test yielding results as yes/no or sensitive/resistant) are sensitivity and specificity calculations obtained from a contingency table. For quantitative tests with results in more than two ordinal categories or on a continuous scale, it is best to present all the information they provide in order to accurately determine the test's performance. It is also necessary to establish a cutoff point that separates negative from positive results; in this case, the cutoff will represent the ''critical concentration'' that will define susceptible and resistant strains. This type of test evaluation can be achieved with receiver operating characteristic (ROC) curves [16, 17, 18, 19], a method of analysis that is increasingly being used to evaluate medical tests and has been employed by the U.S. Food and Drug administration [20, 21].

In this study we applied the ROC curve concept to evaluate the performance of the MABA using the proportion method as the gold standard. To the best of our knowledge, this is the first attempt to apply ROC curve analysis to validate a drug sensitivity test for *Mycobacterium tuberculosis*.

## Materials and Methods

### Clinical Isolates, Growth Conditions, and Preparation of Bacterial Suspensions for the Microplate Alamar Blue Assay

Several *Mycobacterium tuberculosis* reference strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and included in the study: H37Ra (25177), H37Rv (27294), H37Rv rifampin resistant (35838), H37Rv isoniazid resistant (35822), H37Rv streptomycin resistant (35820), and H37Rv ethambutol resistant (35837). Fifty-four isolates of *Mycobacterium tuberculosis* were obtained from patients with pulmonary tuberculosis who attended different hospitals in Mexico City. Identification of the clinical isolates as *Mycobacterium tuberculosis* strains was performed with standard biochemical tests. Each isolate was checked for purity and subcultured at 37°C in Middlebrook 7H9 broth (Difco, USA) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% OADC enrichment (oleic acid, albumin, dextrose, catalase; Difco). To obtain even bacterial suspensions, the liquid culture tubes were incubated on an orbital shaker at low speed.

The inoculum for MABA was prepared by diluting log phase growth cultures with sterile Middlebrook 7H9 broth supplemented with 0.2% glycerol and 10% OADC enrichment without Tween 80 (7H9-T) to a McFarland no. 1 turbidity, and then further diluted 1:25 in 7H9-T medium. In the presence of pronounced clumping, the suspensions were allowed to settle, and then an even suspension was carefully withdrawn. The suspensions were prepared just prior to inoculation of the microplate. For some isolates, inocula for MABA were prepared simultaneously–both from broth and directly from the subculture–on slants (Middlebrook 7H11). Two to three loops of growth were transferred from slants to sterile screw-capped tubes containing three or four glass beads. Bacteria were dispersed in 7H9-T broth by mild agitation, and the suspension was left to settle to eliminate clumps. The suspensions were adjusted to match the turbidity of a McFarland tube no. 1 and then further diluted 1:25 in 7H9-T medium. Subsequent procedures were identical to those for the inocula taken from broth.

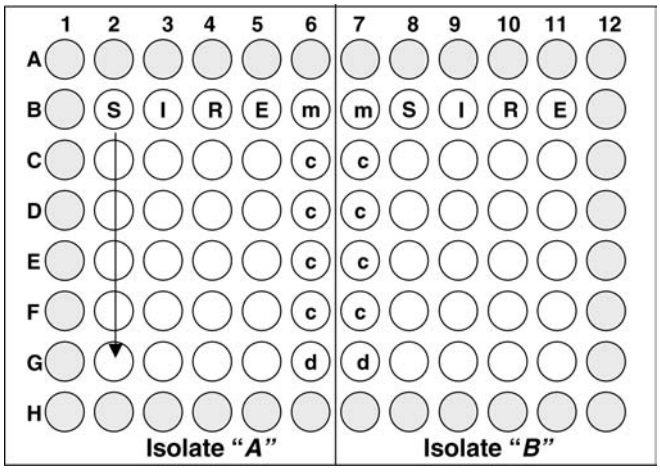


**Fig. 1** Layout for the microplate Alamar blue assay (MABA). Dark well contains water; arrow direction indicates decreasing drug dilution. S=streptomycin; I=isoniazid; R=rifampin; E=ethambutol; m=medium control; c=control free of drug; d=1/100-diluted control free of drug

### Preparation of Drugs

All drugs were obtained from Sigma Chemical (USA). Streptomycin (STR), isoniazid (INH), and ethambutol (EMB) stock solutions were prepared in deionized water and filter sterilized (0.22 µm). Rifampin (RIF) stock was prepared in dimethyl sulfoxide. All stocks were stored at −70°C until use. Working drug solutions were prepared just prior to use by diluting drug stocks to four times (4×) the maximum desired final testing concentration in sterile 7H9-T broth.

### Drug Susceptibility Testing by Conventional Methods

For each isolate, drug-susceptibility testing was done by the proportion method on 7H10 agar according to standard procedures [22, 23, 24].

### Microplate Alamar Blue Assay

Susceptibility testing with Alamar blue was performed in 96-well flat-bottom plates (Nunc, USA) as described by Franzblau et al. [13], with some modifications. In each plate two different isolates were tested for susceptibility to STR, INH, RIF, and EMB; isolate ''A'' was tested in columns 2 to 6 (rows B to G) and isolate ''B'' in columns 7 to 11 (rows B to G), as shown in Fig. 1. All test wells received 100 µl of 7H9-T medium. One hundred microliters of 4X drug solution was added to wells with the highest concentration: STR-4X solution (16 µg/ml), INH-4X solution (4.0 µg/ml), RIF-4X solution (8.0 µg/ml), and EMB-4X solution (64.0 µg/ml). Using a multichannel pipette, the contents of these wells were mixed thoroughly and 100 µl transferred into the next well; the process was then repeated, thus creating serial twofold-dilutions.

One hundred microliters was taken from the last wells in the series and discarded. One hundred microliters of the *Mycobacterium tuberculosis* inocula was added to each drug-containing well and also to drug-free controls. The ranges of the final drug concentrations were as follows: STR, 0.125–4.0 µg/ml; INH, 0.031–1.0 µg/ml; RIF, 0.062–2.0 µg/ml; and EMB 0.5–16 µg/ml. One hundred microliters of 7H9-T medium was added to wells B6 and B7, the latter a bacteria-free control. To facilitate interpretation of results, we introduced an additional control containing 1% of the test inoculum. This control was prepared by adding 99 µl of 7H9-T medium and 1 µl of the respective 1:25 bacterial suspen-

**Table 1** Minimal inhibitory concentrations as determined by the microplate Alamar blue assay for reference H37RV strains or for clinical isolates of *Mycobacterium tuberculosis* (growth in different media)

| Isolate/strain no. | Source | MIC (μg/ml) | | | |
|---|---|---|---|---|---|
| | | STR | INH | RIF | EMB |
| 82 | 7H11 slant | 2.0 | 0.125 | 0.125 | 8.0 |
| | 7H9 broth | 2.0 | 0.25 | 0.25 | n.d. |
| 83 | 7H11 slant | 1.0 | <0.031 | 0.125 | 2.0 |
| | 7H9 broth | 1.0 | <0.031 | 0.062 | 2.0 |
| 91 | 7H11 slant | 1.0 | >1.0 | 0.125 | 8.0 |
| | 7H9 broth | 1.0 | >1.0 | 0.062 | n.d. |
| H37Rv-INH r (ATCC 35822) | 7H9 broth | 0.5 | >1.0 | <0.062 | 2 |
| H37Rv-RIF r (ATCC 35838) | 7H9 broth | <0.125 | 0.25 | >2 | 2 |
| H37Rv-EMB r (ATCC 35837) | 7H9 broth | <0.125 | <0.031 | <0.062 | >16 |
| H37Rv-STR r (ATCC 35820) | 7H9 broth | >4 | <0.031 | <0.062 | 4 |
| H37Rv (ATCC 27294) | 7H9 broth | <0.125 | <0.031 | <0.062 | <0.5 |
| H37Ra (ATCC 25177) | 7H9 broth | <0.125 | <0.031 | <0.062 | <0.5 |

STR, streptomycin; INH, isoniazid; RIF, rifampin; EMB, ethambutol
n.d., not done; r, resistant

sion. Two hundred microliters of sterile water was added to all outer-perimeter wells.

The plates were placed in a plastic bag and incubated at 37°C for 5 days. On day 5, 20 μl of Alamar blue solution (Trek Diagnostics, Westlake, OH, USA) and 12 μl of sterile 10% Tween 80 solution were then added to one drug-free well corresponding to each isolate. The plates were then replaced in the bag and reincubated at 37°C for 24 h. If, after this incubation, the drug-free well turned pink, all the wells corresponding to that isolate received the Alamar blue and Tween solutions. After a further 24-h incubation, the colors of all wells were recorded, using the color of the 1% well as a reference. Test wells were scored as no growth if they remained blue or had the same shade-intensity as the 1% well. The MIC was defined as the lowest drug concentration that prevented a color change to pink and gave an intensity of color equal to or less than that of the 1% control. If on day 5 there was no change on the drug-free control, the plate was reincubated for 2, 4, or 5 more days, and Alamar blue and Tween solutions were added to the respective drug-free control following the procedure described previously.

### Analysis of Data

The performance of MABA was determined by ROC curve analysis using the Stata software for Windows (Stata, USA). Area under the curve (AUC), *P* value, and cutoff point were obtained from the curve. Specificity, sensitivity, predictive values for sensitivity (PVS) and resistance (PVR), and test-efficiency were calculated in contingency tables [18, 19].

## Results

### General Results for the Microplate Alamar Blue Assay

Colorimetric and proportion susceptibility determinations were performed on 54 *Mycobacterium tuberculosis* clinical isolates and 6 reference strains. Results of MABA were available after an average of 8 days of incubation and those of the proportion method after 3 weeks. Forty-seven isolates were tested colorimetrically at least twice. For three isolates inocula were prepared both from broth and directly from the slant (7H11). There was no significant effect of inoculum source on the time to complete the test or on the susceptibility pattern obtained (Table 1). To assess the importance of the observer on the interpretation of MABA results, the STR, INH, RIF, and EMB MABA determinations for 25 isolates were read by four to six different observers, two of whom had no prior experience with the method. Observers were asked to use the 1% control as a reference for determining the MIC. Differences among the observers were found in 7.5% of the results. In most cases these differences were a single twofold dilution difference from the mean obtained for each isolate (data not shown); there was no correlation between the results obtained and the observers' experience with the method. The drug sensitivity pattern for H37Rv variants utilized in this study, obtained by the proportion method and MABA, was according to their pattern reported by the ATCC (Table 1).

### Isoniazid Susceptibility Test

For 35 of the 36 isolates susceptible to INH by the proportion method, the MIC as determined by MABA was ≤0.25 μg/ml (0.25 μg/ml for 2 isolates, 0.125 μg/ml for 4 isolates, and ≤0.062 μg/ml for the rest). The only isolate for which discordant results were obtained was susceptible by the proportion test but resistant by MABA (MIC>1.0 μg/ml). Of 24 isolates that were resistant by the proportion method, the MABA MICs for 21 were >1.0 μg/ml and for two were 0.5 μg/ml. For the one discordant isolate, the MABA MIC was 0.25 μg/ml (Fig. 2A). ROC curve analysis (Fig. 2B), showed an AUC of 0.98, a *P* value of 0.000, and a cutoff value of 0.25 μg/ml (Table 2).

### Rifampin Susceptibility Test

Of 35 isolates susceptible by the proportion method, 34 were susceptible to RIF by MABA, with MICs of ≤0.25 μg/ml (Fig. 3). One isolate deemed susceptible by the proportion method was resistant by MABA, with an MIC of >2 μg/ml. One isolate was resistant to RIF by the proportion method and susceptible by MABA (MIC, 0.125 μg/ml). Of the remaining 24 isolates resistant by the proportion method, 22 were also resistant by MABA, with MICs ≥2.0 μg/ml, 1 had an MIC of 0.5 μg/ml, and

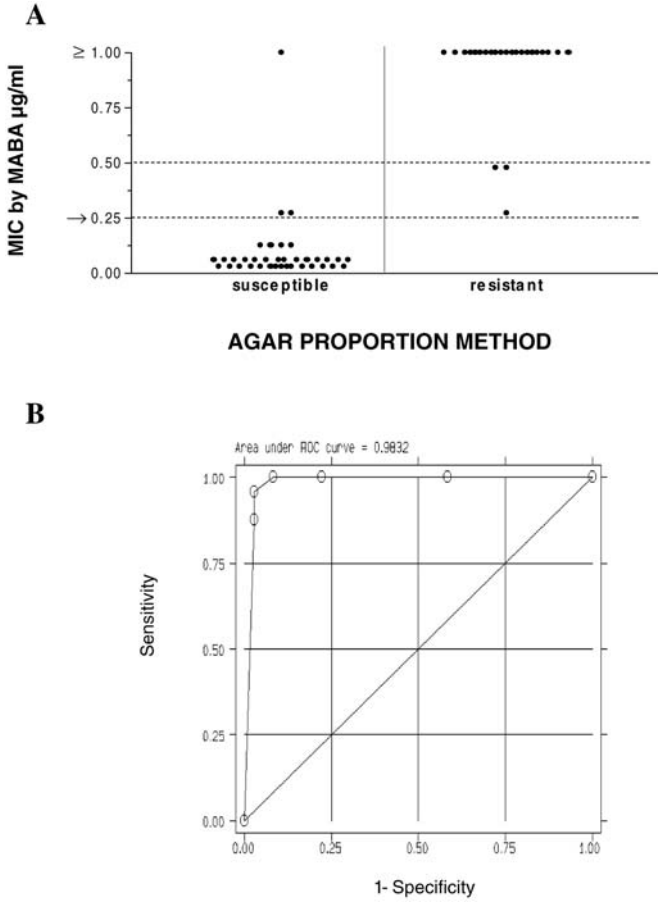


**Fig. 2** **A** Correlation between MABA and the agar proportion method for isoniazid. Each point depicts one isolate. Susceptible/resistant criterion was established on the basis of agar proportion results. Dotted lines represent intermediate resistance range. Arrow indicates cutoff for MABA determination. **B** Receiver operating characteristic curve for isoniazid determination

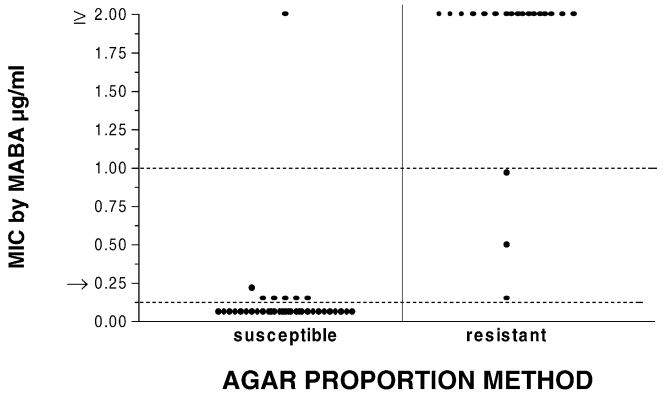


**Fig. 3** Correlation between MABA and the agar proportion method for rifampin. Each point depicts one isolate. Susceptible/resistant criterion was established on the basis of agar proportion results. Dotted lines represent intermediate resistance range. Arrow indicates cutoff for MABA determination

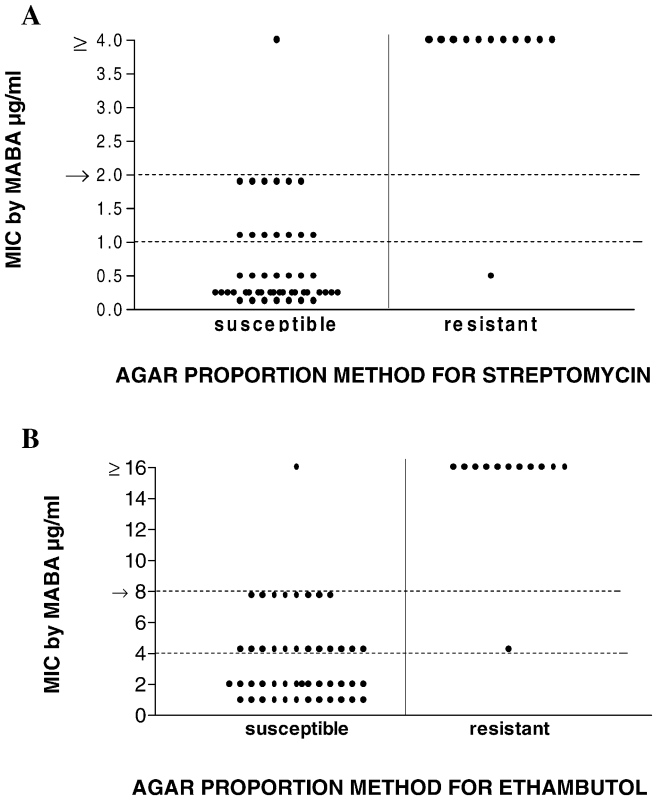


**Fig. 4** Correlation between MABA and the agar proportion method for (**A**) streptomycin and (**B**) ethambutol. Each point depicts one isolate. Susceptible/resistant criterion was established on the basis of agar proportion results. Dotted lines represent intermediate resistance range. Arrow indicates cutoff for MABA determination

**Table 2** Receiver operating characteristic curve analysis for the microplate Alamar blue assay (MABA)

| Drug | Comparison | AUC | *P* value | Cutoff[a] value (µg/ml) |
|---|---|---|---|---|
| INH | MABA vs. PROP | 0.98 | 0.000 | 0.250 |
| RIF | MABA vs. PROP | 0.97 | 0.000 | 0.250 |
| STR | MABA vs. PROP | 0.96 | 0.000 | 2.0 |
| EMB | MABA vs. PROP | 0.97 | 0.000 | 8.0 |

AUC, area under the curve; EMB, ethambutol; INH, isoniazid; PROP, proportion method (gold standard); RIF, rifampin; STR, streptomycin

[a] An isolate for which the MIC of a drug is higher than the cutoff is considered resistant

1 had an MIC of 1.0 µg/ml. The ROC curve for RIF (not shown) gave an AUC of 0.979, a *P* value of 0.000, and a cutoff of 0.25 µg/ml (Table 2).

## Streptomycin Susceptibility Test

Of 60 isolates analyzed, 48 were susceptible by the proportion-method, and, for 47 of these, MICs as determined by MABA were ≤2.0 µg/ml, with a wide distribution of the MIC values (Fig. 4A). Only one isolate sus-

**Table 3** Performance of the microplate Alamar blue assay (MABA) in comparison with the proportion method, as determined by contingency tables

| Drug | No. of isolates | | | | Sensitivity (%) | Specificity (%) | PVR (%) | PVS (%) | Efficacy (%) | Prevalence of resistance (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Both S | Pro-S, MABA-R | Pro-R, MABA-S | Both R | | | | | | |
| INH (*n*=60) | 35 | 1 | 1 | 23 | 96 | 97 | 96 | 97 | 97 | 40 |
| RIF(*n*=60) | 34 | 1 | 1 | 24 | 96 | 97 | 96 | 97 | 97 | 44 |
| STR (*n*=60) | 47 | 1 | 1 | 11 | 92 | 98 | 92 | 98 | 97 | 20 |
| EMB (*n*=60) | 47 | 1 | 1 | 11 | 92 | 98 | 92 | 98 | 97 | 20 |

Pro, proportion method; S, susceptible; R, resistant; PVR, predictive value for resistance; PVS, predictive value for susceptibility; INH, isoniazid; RIF, rifampin; STR, streptomycin; EMB, ethambutol

ceptible by the proportion method was found to be resistant by MABA, with an MIC of ≥4.0 μg/ml. Of 12 isolates resistant to STR by the proportion method, MICs as determined by MABA were ≥4.0 μg/ml for 11. For the twelfth isolate, a discordant result was obtained: the MIC as determined by MABA as 0.5 μg/ml. ROC curve analysis (not shown) gave an AUC of 0.960, a *P* value of 0.000, and a cutoff value of 2.0 μg/ml (Table 2).

### Ethambutol Susceptibility Test

For 47 of the 48 isolates susceptible by the proportion method, MICs as determined by MABA ranged from 1 to 8 μg/ml; for one discrepant isolate, the MABA-MIC was ≥16 μg/ml (Fig. 4B). For 11 of 12 isolates resistant by the proportion method, MICs as determined by MABA were ≥16 μg/ml. One isolate was resistant by the proportion method but susceptible by MABA (MIC, 4 μg/ml). ROC curve analysis gave an AUC value of 0.964, a *P* value of 0.000, and a cutoff value ≤8 μg/ml (not shown).

### Sensitivity, Specificity, Predictive Values, and Test Efficiency

The overall comparison of the MABA with the proportion method produced a total of 8 (3.3%) discordant results out of 240 individual determinations. Contingency tables showing correlation of the MABA with the proportion method at the cutoff values obtained from the ROC curves are presented in Table 3. The mean values for the correlation of results from the MABA and the proportion methods for the four drugs were as follows: for sensitivity and specificity, 94 and 97.5%, respectively; for predictive value for resistance, 94%, for predictive value for sensitivity, 97.5%, and for test efficiency, 97%.

## Discussion

In low-income countries there is a great need for effective and economical new methods for drug susceptibility testing of *Mycobacterium tuberculosis*. In these countries the proportion and Bactec methods are the standard procedures for drug susceptibility testing and are performed only in reference or perhaps tertiary-facility laboratories. The conventional proportion method is slow and laborious. The Bactec system reduces time for obtaining susceptibility results, but the machine, the tubes, and the additives are all expensive, and perhaps the best system, the Bactec 460, generates radioactive waste. In addition, Bactec gives only qualitative results (resistant or susceptible). Antimicrobial susceptibility testing in the microdilution format [25, 26] offers many advantages, including economy of reagents and space due to the miniaturization of the test. Considering all the advantages of microdilution assays, e.g. cost-effectiveness, rapidity, and quantitative (MIC) results, MABA represents an excellent option for antimycobacterial drug-susceptibility testing.

Previous use of MABA with clinical isolates demonstrated a good correlation with the Bactec method, especially for RIF and INH but showed some discrepancies with EMB and STR [12]. In our comparison of MABA with the proportion method, we tried to reduce discrepancies by including an extra control containing 1% of the test inoculum. The use of this 1% control as a reference facilitated naked-eye interpretation, even for people not familiar with the procedure, and reduced doubtful results due to different shades or intensities of blue-purple-pink colors.

In our study we found that MABA is much faster than the proportion method and can be as rapid as Bactec. Inocula for MABA could be obtained from bacteria growing in 7H9 broth or on Middlebrook agar without affecting the time required or the susceptibility results of the test. Thus, a clinical isolate could be used from the original Middlebrook agar slant, eliminating the time, labor, expense, and biosafety risk required for liquid subcultures.

To statistically evaluate the performance of quantitative assays such as MABA, ROC curves and logistic regression are recommended [16, 17, 18, 19, 20]. ROC curve analysis [19] assesses the ability of a test to discriminate the diseased from the normal condition and can also be used to compare the diagnostic performance of two or more laboratory diagnostic tests [20]. The ROC curve is a plot of the true positive rate against the

false positive rate for the different possible criterion values of a diagnostic test. The accuracy of the test is based on how well the new test discriminates the group being tested into positive or negatives, as defined by a ''gold standard'' used for evaluation. Accuracy is measured by the area under the ROC curve. The closer the curve follows the left-hand border and then the top border of the ROC space, the more accurate the test. The closer the curve comes to the 45-degree diagonal of the ROC space, the less accurate the test. An area of 1 represents a perfect test; an area of 0.5 represents a worthless test. The following is a rough guide for classifying the accuracy of a diagnostic test based on area under the ROC curve: 1–0.9=excellent; 0.9–0.8=good; 0.8–0.7=fair; 0.7–0.6=poor; and 0.6–0.5=fail. In our case the ROC curve displayed sensitivity versus 1 minus specificity at each MIC obtained by the colorimetric method.

In a quantitative test like the MABA, it is also important to establish which of the concentrations involved in the test will separate the susceptible from the resistant isolates. The ROC curve will show this value (denominated as the cutoff concentration) and will be represented by the concentration found at the inflection point of the curve, that is, the concentration with the highest sensitivity and specificity. In the case of the MABA, this value (cutoff) could be considered the critical concentration used for other drug sensitivity tests to separate susceptible from resistant isolates [27].

Analysis of ROC curves was very successful for MABA: the AUC was close to 1.0 for the four drugs (≥0.96), and the *P* value was also highly significant (0.000). The cutoff value for INH and RIF was 0.25 μg/ml, so isolates for which the MIC is ≥0.5 μg/ml could be considered resistant. The cutoff value for STR was 2 μg/ml and for EMB 8 μg/ml; thus, isolates for which the MICs are ≥4 μg/ml and ≥16 μg/ml could be considered resistant to these drugs, respectively.

However, care must be taken when interpreting MIC values similar or close to the cutoff value, especially for STR and EMB, since, like in our study, 15 and 10% of the values obtained, respectively, fell at the cutoff MIC. One possibility could be to consider isolates for which borderline MICs are obtained as partially resistant. On the basis of this observation, and, since other authors have used partially resistant ranges for Alamar blue drug sensitivity determinations [11, 13, 28], we proposed a partially resistant range that was close to that obtained with the ROC curve for each drug; these ranges are indicated in Figs. 2, 3, and 4. Considering these ranges, the MIC values necessary to designate an isolate as highly resistant (or highly susceptible) are very close to those proposed by Yajko and Franzblau in the previous studies of MABA for the same four drugs [11, 13].

Contingency tables elaborated with the cutoff obtained from the ROC curve analysis showed an excellent test performance: 92% sensitivity and 98% specificity for both STR and EMB determinations; and 96% sensitivity and 97% specificity for RIF and INH determinations. The predictive value for resistance was ≥92% and for sensitivity ≥97% for the four determinations, confirming the good performance of the test previously demonstrated by ROC analysis.

The cost of materials and supplies for determination of mycobacterial sensitivity to STR, INH, RIF, and EMB by MABA is approximately USD $8.00 (labor expenses excluded) when performed in Mexico; the most expensive materials are the microplate and the OADC medium enrichment. Sophisticated equipment such as plate readers or plate fluorometers are not required. Placement of the microplate in a transparent plastic bag, appropriately secured, adds an element of biosafety and avoids loss of water. For most of the isolates, the Alamar blue added after 5 days of incubation gives a positive result (pink color), avoiding further handling of the plate and reducing risks factors. For all of these reasons, the MABA represents an excellent option for drug sensitivity testing of *Mycobacterium tuberculosis* isolates.

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