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Colorimetric Method for Determining MICs of Antimicrobial Agents for *Mycobacterium tuberculosis*

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A colorimetric method for quantitative measurement of the susceptibility of *Mycobacterium tuberculosis* to antimicrobial agents is described. The method utilizes an oxidation-reduction dye, Alamar blue, as an indicator of growth. By this method, MICs of isoniazid, rifampin, streptomycin, and ethambutol were determined for 50 strains of *M. tuberculosis*. Colorimetric MIC results were available on the 7th, 10th, or 14th day of incubation for 29 (58%), 14 (28%), and 7 (14%) of the 50 strains, respectively. When MIC susceptibility results were compared with results obtained by the agar proportion method, increased levels of resistance detected by agar proportion were associated with higher MICs obtained by the colorimetric method. Tentative interpretive criteria for colorimetric MIC results which showed good agreement with results obtained by the agar proportion method were established. Interpretive agreement between the two methods was 98% for isoniazid, rifampin, and ethambutol and 94% for streptomycin. Overall, there was agreement between the two methods for 194 of 200 test results (97%). The colorimetric method is a rapid, quantitative, nonradiometric method for determining the antimicrobial susceptibility of *M. tuberculosis*.

The recent emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* underscores the need to rapidly determine the susceptibility of isolates of *M. tuberculosis* to antimicrobial agents. The commonly used agar proportion method for mycobacterial susceptibility testing (3) requires a 3-week period of incubation before an isolate can be reported as drug susceptible. The BACTEC radiometric susceptibility method (4) has the advantage of being more rapid than the agar proportion method (results are usually available in 5 to 10 days), but it requires the use of radioisotopes and can be costly to perform. Although it is possible to determine the MICs of drugs by the BACTEC method, most laboratories test only one or two critical concentrations of drug instead of determining the MIC. We have developed an alternative method for determining the antimicrobial susceptibility of *M. tuberculosis* that is rapid, quantitative, and nonradiometric and does not require the use of instrumentation. The method employs an oxidation-reduction indicator which changes color from blue to pink during growth (1, 2, 5, 6). This paper describes the colorimetric method and shows the relationship between colorimetric MIC results and susceptibility test results obtained by the established agar proportion method.

MATERIALS AND METHODS

Agar proportion tests and their interpretation. A collection of 50 strains of *M. tuberculosis* from San Francisco General Hospital, the San Francisco Department of Health, and the California State Department of Health were included in this study. Prior to the study, indirect agar proportion susceptibility tests were performed at the San Francisco Department of Health or at the California State Department of Health by established procedures (3). The following drugs and concentrations were included in agar proportion susceptibility tests: isoniazid, 0.2 and 1 μg/ml; rifampin, 1 μg/ml; ethambutol, 5 μg/ml; and streptomycin, 2 and 10 μg/ml.

In the agar proportion method, an isolate was classified as susceptible to a drug if the number of colonies that grew on the drug-containing plate was <1% of the number of colonies that grew on a control plate without drug, partially resistant if the number was between 1 and 10%, and resistant if the number was >10%. In cases where two drug concentrations were tested in the agar proportion method, an isolate was classified as partially resistant if it showed resistance at the lower concentration but was susceptible at the higher of the two concentrations tested.

By using these interpretive criteria for agar proportion test results, 7 of the 50 strains of *M. tuberculosis* were susceptible to all four of the drugs tested and 43 strains were partially resistant or resistant to at least one of the drugs. The interpretive results obtained from agar proportion tests were used as the standard with which results of the new colorimetric method were compared.

Determination of colorimetric MICs. As a consequence of sufficient bacterial metabolism and growth, the oxidation-reduction indicator Alamar blue changes color from blue to pink. This color change is observed by adding the indicator to test tubes after a period of incubation. Because the length of incubation that is needed for sufficient metabolic activity to occur can vary from strain to strain, three control tubes (containing no drug) were included in each susceptibility test run. Control tubes were tested after 7, 10, or 14 days of incubation by adding Alamar blue (Sensititre/Alamar, Westlake, Ohio) and noting whether a color change occurred. If the color in the control tube turned pink, it meant that sufficient metabolic activity had occurred to allow the test tubes to be read. In this case, Alamar blue was added to each of the drug-containing tubes, the color in each of these tubes was recorded, and the experiment was terminated. Thus, depending on the length of time it took for the control tubes to show sufficient metabolic activity, susceptibility test results were available on the 7th, 10th, or 14th day of incubation.

Inocula were prepared by growing strains of *M. tuberculosis* in 7H9 broth (Middlebrook 7H9 broth base [Difco, Detroit, Mich.] supplemented with 0.5 ml of Tween 80 and 10% ADC enrichment [Difco] per liter) to a turbidity equal to that of a no. 1 McFarland standard (~3×10^7 *M. tuberculosis* CFU/ml) and diluting the culture 1:5 in broth. Serial twofold dilutions of drug were prepared in 7H9 broth, and tubes containing 0.2 ml of diluted drug were inoculated with 0.02 ml of the 1:5-diluted culture. The final concentration of mycobacteria in the susceptibility test tubes was ~6×10^5 CFU/ml. Three control tubes (no drug) were also inoculated in the same manner. All tubes were incubated at 35°C in ambient atmosphere. Control tubes were tested after 7, 10, or 14 days to determine whether the drug-containing tubes were ready to be read. This was accomplished by adding 0.02 ml of 10× Alamar blue solution and 0.05 ml of 5% Tween 20 (or Tween 80) to the control tube and incubating it for 2 h at 50°C. If the color in the control tube changed from blue to pink after 2 h of incubation, then Tween and Alamar blue were added to the drug-containing tubes and these were

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incubated for 2 h at 50°C before the MIC was read. The MIC was defined as the lowest concentration of drug that prevented a color change.

RESULTS

For 29 (58%) of the 50 strains of *M. tuberculosis* included in the study, colorimetric MIC test results were available on the seventh day of incubation. Results were available on the 10th day for 14 (28%) strains and on the 14th day for the remaining 7 (14%) strains.

The relationships between MIC results obtained by the Alamar blue colorimetric method and results obtained by the agar proportion method are shown in the figures. Vertical dotted lines in the figures show the interpretive breakpoints for the agar proportion method. Two vertical lines were drawn to define the partially resistant category. In cases where two concentrations of a drug were tested by the agar proportion method, the first line indicates the interpretive breakpoint for susceptibility to the lower concentration of drug and the second line indicates the interpretive breakpoint for susceptibility to the higher concentration of drug. An isolate with results falling between these two vertical lines was considered partially resistant to the drug.

The patterns obtained by plotting colorimetric MICs versus agar proportion results permitted the assignment of tentative interpretive criteria for the MICs. The interpretive breakpoints for colorimetric MICs (indicated in the figures by horizontal dotted lines) were selected on the basis of the best fit of these results with the agar proportion test results. Two horizontal lines were drawn to define a partially resistant MIC category corresponding to the agar proportion partially resistant category.

Isoniazid susceptibility tests. The relationship between isoniazid colorimetric MICs and isoniazid agar proportion results is shown in Fig. 1. For all of 20 strains susceptible to isoniazid at 0.2 μg/ml by the agar proportion method, the colorimetric MIC was ≤0.06 μg/ml (panel A). Among 16 strains that were partially resistant by the agar proportion method, for 15 the colorimetric MIC was in the range of 0.12 to 1 μg/ml (panel B). For all of 14 strains that were resistant to 1 μg/ml of isoniazid by the agar proportion method, the colorimetric MIC was ≥2 μg/ml (panel C). The following colorimetric MIC interpretive criteria were therefore proposed for isoniazid: susceptible, ≤0.06 μg/ml; partially resistant, 0.12 to 1 μg/ml; and resistant, ≥2 μg/ml. By using these criteria, there was interpretive agreement between the agar proportion method and the Alamar blue method for 49 of the 50 strains (98%) tested for susceptibility to isoniazid.

The one discrepant result was with a strain that was classified as partially resistant to isoniazid by the agar proportion method and resistant by the colorimetric method (MIC = 16 μg/ml). When this isolate was retested by the agar proportion method, it gave results which put it in the resistant category; the number of colonies that grew on agar containing 0.2 μg of isoniazid per ml was >50% of the number on the control plate, and the number of colonies that grew on agar containing 1 μg of isoniazid per ml was 1% of the number on the control plate. The retest agar proportion results would have placed this isolate in panel C of Fig. 1 (resistant category).

Rifampin susceptibility tests. The relationship between rifampin colorimetric MICs and rifampin agar proportion results is shown in Fig. 2. For 40 of 41 strains susceptible to 1 μg of rifampin per ml by the agar proportion method, the colorimetric MIC was ≤0.25 μg/ml (panel A). For one susceptible strain the colorimetric rifampin MIC was 0.5 μg/ml, and for one partially resistant strain the colorimetric MIC was 0.5 μg/ml. For all of eight strains resistant to rifampin by the agar proportion method, the colorimetric MIC was ≥1 μg/ml (panel C). The following colorimetric MIC interpretive criteria were proposed for rifampin: susceptible, ≤0.25 μg/ml; partially re-

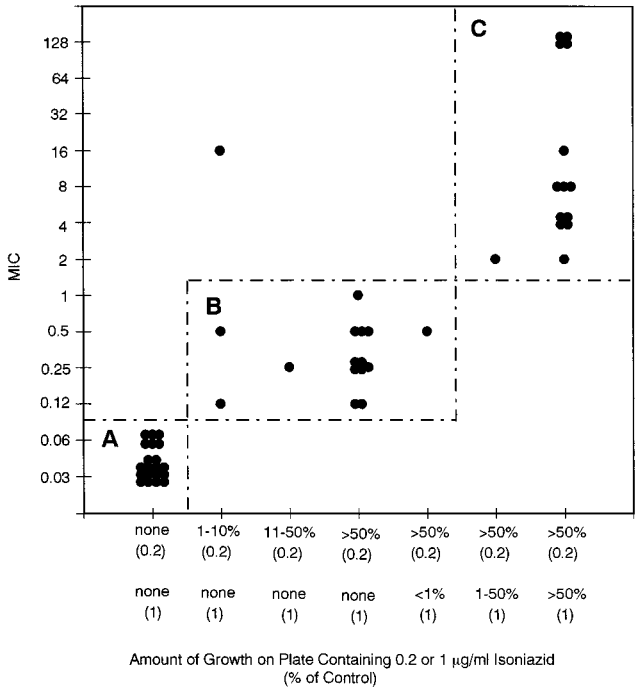

FIG. 1. Plot of the susceptibilities of 50 isolates of *M. tuberculosis* to isoniazid by the agar proportion method versus a broth colorimetric MIC method. Interpretive breakpoints are indicated by vertical dotted lines for the agar proportion method and horizontal dotted lines for the colorimetric method. (A) Susceptible isolates; (B) partially resistant isolates; (C) resistant isolates.

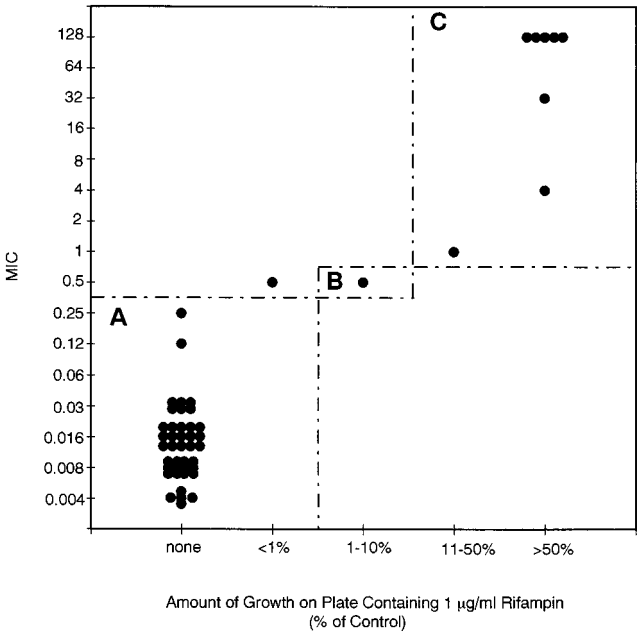

FIG. 2. Plot of the susceptibilities of 50 isolates of *M. tuberculosis* to rifampin by the agar proportion method versus a broth colorimetric MIC method. See the legend to Fig. 1 for details.

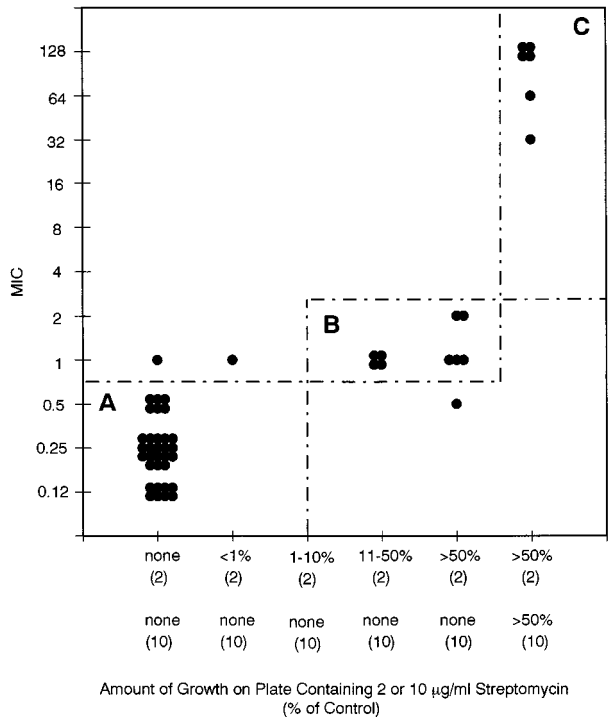

FIG. 3. Plot of the susceptibilities of 50 isolates of *M. tuberculosis* to streptomycin by the agar proportion method versus a broth colorimetric MIC method. See the legend to Fig. 1 for details.

sistant, 0.5 μg/ml; and resistant, ≥1 μg/ml. By using these criteria, there was interpretive agreement between the agar proportion method and the colorimetric method for 49 of the 50 strains (98%) tested for susceptibility to rifampin.

The one discrepant result was with a strain classified as susceptible to rifampin by the agar proportion method (the amount of growth on the drug-containing plate was <1% of the amount of growth on the control plate) but classified as partially resistant by the colorimetric method (MIC = 0.5 μg/ml).

Streptomycin susceptibility tests. The relationship between streptomycin colorimetric MICs and streptomycin agar proportion results is shown in Fig. 3. Among 34 strains susceptible to streptomycin at 2 μg/ml by the agar proportion method, for 32 the colorimetric MIC was ≤0.5 μg/ml (panel A). For 2 of the 34 susceptible strains the colorimetric MIC was 1 μg/ml. Among 10 strains that were partially resistant to streptomycin by the agar proportion method (panel B), for 9 the colorimetric MIC was in the range of 1 to 2 μg/ml and for 1 the MIC was 0.5 μg/ml. For all of six strains resistant to 10 μg of streptomycin per ml by the agar proportion method the colorimetric MIC was ≥32 μg/ml (panel C). The following colorimetric MIC interpretive criteria were therefore proposed for streptomycin: susceptible, ≤0.5 μg/ml; partially resistant, 1 to 2 μg/ml; and resistant, ≥32 μg/ml. By using these criteria, there was interpretive agreement between the agar proportion method and the colorimetric method for 47 of the 50 strains (94%) tested for susceptibility to streptomycin.

The three discrepant results included two strains classified as susceptible to 2 μg of streptomycin per ml by the agar proportion method that were partially resistant by the colorimetric method (MIC = 1 μg/ml) and one strain classified as partially resistant by the agar proportion method (the number of colonies on agar containing 2 μg of streptomycin per ml was >50% of the number of colonies on the control plate, and there was no growth on the plate containing 10 μg/ml) that was susceptible by the colorimetric method (MIC = 0.5 μg/ml).

Ethambutol susceptibility tests. The relationship between ethambutol colorimetric MICs and ethambutol agar proportion results is shown in Fig. 4. For all of 46 strains susceptible to 5 μg of ethambutol per ml by the agar proportion method, the colorimetric MIC was ≤4 μg/ml (panel A). Among four strains resistant to ethambutol by the agar proportion method, for three the colorimetric MIC was ≥8 μg/ml (panel B). The following colorimetric MIC interpretive criteria were proposed for ethambutol: susceptible, ≤4 μg/ml, and resistant, ≥8 μg/ml. By using these criteria, there was interpretive agreement between the agar proportion method and the colorimetric method for 49 of the 50 strains (98%) tested for susceptibility to ethambutol.

The one discrepant result was with a strain classified as resistant by the agar proportion method (the number of colonies on the drug-containing plate was >50% of the number of colonies on the control plate) and classified as susceptible by the Alamar blue method (MIC = 4 μg/ml). When this isolate was retested, the colorimetric MIC was 16 μg/ml. This value would have placed the isolate in the resistant category in Fig. 4 (panel B).

DISCUSSION

When tested with a collection of 50 strains of *M. tuberculosis*, 43 of which were drug resistant or partially drug resistant, antimicrobial susceptibility test results obtained by the colorimetric Alamar blue method showed good agreement with results obtained by the established agar proportion method. Interpretive agreement between the two methods occurred among 194 of 200 susceptibility tests (97%). All but one of six discrepant MIC results were within a single MIC dilution tube of being in agreement with the agar proportion result. The one exception, an isolate classified as resistant to isoniazid by the

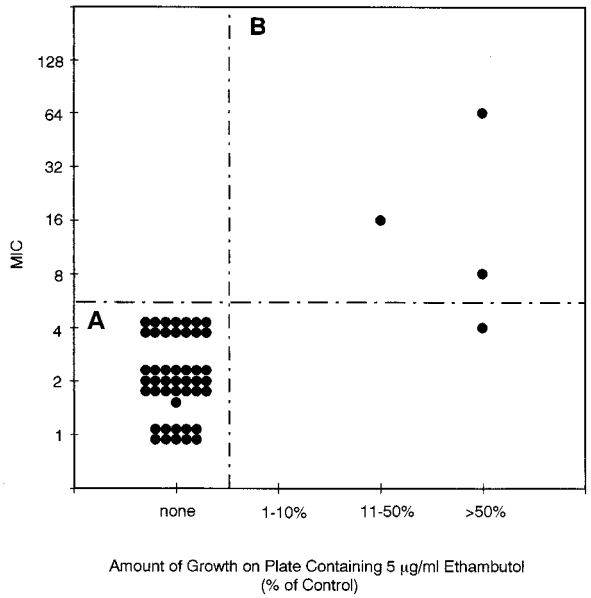

FIG. 4. Plot of the susceptibilities of 50 isolates of *M. tuberculosis* to ethambutol by the agar proportion method versus a broth colorimetric MIC method. See the legend to Fig. 1 for details. (A) Susceptible isolates; (B) resistant isolates.

colorimetric method (MIC = 16 μg/ml) but showing only low-level partial resistance by the agar proportion method, was resolved in favor of the MIC result when the agar proportion test was repeated.

Colorimetric MIC results were available after 7 days of incubation for 58% of the strains and within 14 days for all 50 strains of *M. tuberculosis*. This time frame compares favorably with the length of incubation required to obtain BACTEC radiometric susceptibility test results. During a 6-month period in 1994, BACTEC susceptibility results at San Francisco General Hospital were available within 7 days of incubation for 60% of all *M. tuberculosis* isolates and within 12 days for 100% of the isolates, using a nonweekend BACTEC reading schedule. The advantages of the colorimetric MIC method are that it does not use radioisotopes or require expensive instrumentation and it yields quantitative (MIC) susceptibility results. The ability to rapidly determine MICs may aid in the early detection of drug resistance during therapy of *M. tuberculosis* infections.

The colorimetric MICs obtained here for isoniazid-, rifampin-, streptomycin-, and ethambutol-susceptible strains of *M. tuberculosis* are in close agreement with the MICs for susceptible strains reported by Lee and Heifets (4) using radiometric BACTEC 7H12 broth. These authors obtained slightly higher MICs of isoniazid, rifampin, and ethambutol on 7H11 agar than in 7H12 broth.

The colorimetric method was tested here with only four antimycobacterial agents. However, Alamar blue has been used successfully to determine the susceptibility of gram-positive (2) and gram-negative (1) bacteria and yeasts (5) to a wide range of antimicrobial agents, and it is anticipated that the method can be used for additional antimycobacterial agents as well. The simplicity of the colorimetric method may make it possible for laboratories to test first-, second-, and third-line agents on a routine basis, allowing rapid detection of first-line multidrug-resistant strains of *M. tuberculosis* and the ability to provide susceptibility data for second- and third-line agents at the same time.

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