

# Tetrazolium Microplate Assay as a Rapid and Inexpensive Colorimetric Method for Determination of Antibiotic Susceptibility of *Mycobacterium tuberculosis*

Luz Caviedes,<sup>1</sup> Jose Delgado,<sup>2</sup> and Robert H. Gilman<sup>3\*</sup>

Infectious Diseases Laboratory, Pathology Department, Universidad Peruana Cayetano Heredia,<sup>1</sup> and Asociación Benéfica PRISMA,<sup>2</sup> Lima, Peru, and Department of International Health, Johns Hopkins School of Public Health, Baltimore, Maryland<sup>3</sup>

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**The emergence of multidrug-resistant tuberculosis underscores the need for low-cost, rapid methods to determine the susceptibility of *Mycobacterium tuberculosis* to antibiotics. A new, rapid, easily read, and inexpensive colorimetric method with a tetrazolium indicator performs this determination as quickly and accurately as the more expensive Alamar Blue technique.**

In recent years, tuberculosis (TB) has acquired a growing importance in developed and developing countries. The great toll of the disease, the emergence of multidrug-resistant (MDR) strains around the globe, and the close relationship between TB and human immunodeficiency virus infection underscore the need for simple, rapid, and affordable methods of detection and antibiotic susceptibility determination (2, 3, 5). Such methods could reduce the spread of resistant strains by determining more appropriate treatment regimens.

A rapid and low-cost method for the culture of *Mycobacterium tuberculosis* in clinical samples and determination of the susceptibilities of the strains to antibiotics was developed previously (1). This method, the microplate Alamar Blue assay (MABA), is based on the detection of colorimetric changes caused by the oxidation and reduction capabilities of Alamar Blue dye. With a pure culture, this method can determine antibiotic susceptibility in 6 to 7 days. We have recently tested a tetrazolium microplate assay (TEMA) that uses tetrazolium bromide [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] as an alternative, less expensive, and easier-to-read method for antibiotic susceptibility determination. This paper compares the results of this alternative method with those of the previously described MABA (1).

Briefly, the MABA involves the addition of Alamar Blue solution to microplates containing antibiotics in increasing concentrations and pure cultures of *M. tuberculosis* obtained from clinical samples. After an incubation period of 5 days, the growth of *M. tuberculosis* can be observed as a change in the coloration of the Alamar Blue solution due to reduction of the dye. MICs of each antibiotic tested can be determined by this change of color in the wells.

To perform the TEMA, suspensions of *M. tuberculosis* were prepared by emulsifying growth from slants with 100  $\mu$ l of Tween 80 into 0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). The turbidity was adjusted to McFarland standard no. 1 (approximately  $3 \times 10^7$  CFU/ml) by adding

Tween 80 and bovine serum albumin. Three hundred microliters of bacterial suspension was further transferred to 7.2 ml of 7H9GC broth (4.7 g of Middlebrook 7H9 broth base [Difco, Detroit, Mich.], 20 ml of 10% glycerol, 1 g of Bacto Casitone [Difco], 880 ml of distilled water, 100 ml [each] of oleic acid, albumin, dextrose, and catalase [Remel, Lenexa, Kans.]).

Antibiotic dilutions and 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, N.J.) were prepared as previously described for MABA (1). Briefly, 200  $\mu$ l of sterile water was added to all outer wells. Portions (100  $\mu$ l each) of 7H9GC broth were added to columns 3 to 11 in rows B to G (labeled as commercially stamped on the plates). One-hundred-microliter aliquots of  $2\times$  antibiotic solutions (isoniazid [INH], rifampin [RIF], streptomycin [STR], and ethambutol [ETB]) were added to the wells in columns 2 and 3. One hundred microliters of solution was transferred from column 3 to column 4 with a multichannel pipette. The antibiotics were serially diluted 1:2 in consecutive columns, except for column 10, where 100  $\mu$ l of excess medium was discarded. The final drug concentration ranges were as follows: 0.125 to 32  $\mu$ g/ml for INH, 0.062 to 16  $\mu$ g/ml for RIF, 0.125 to 32  $\mu$ g/ml for STR, and 0.5 to 128  $\mu$ g/ml for ETB.

One hundred microliters of a log-phase *M. tuberculosis* bacterial suspension (20 to 30 days old) was added to wells in rows B to G in columns 2 to 11 with an Eppendorf repeating pipette. The wells in column 11 served as inoculum-only controls.

The plates were sealed with Parafilm and then incubated at 37°C for 5 days. On day 5, 50  $\mu$ l of the tetrazolium-Tween 80 mixture {1.5 ml of tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] [Aldrich Chemical Co., Milwaukee, Wis.] at a dilution of 1 mg/ml in absolute ethanol and 1.5 ml of 10% Tween 80} was added to well B11 and the plate was then incubated at 37°C for 24 h. If well B11 turned purple, tetrazolium-Tween 80 was added to all wells and the color was recorded at 24 h. If well B11 remained yellow, the plates were incubated for another 24 h, after which tetrazolium-Tween 80 solution was added to well C11 before the plate was incubated for another 24 h. If well C11 remained yellow, incubation was continued and tetrazolium-Tween 80 solution was added to wells D11, E11, F11, and G11 on days 9, 11, 13, and 15, respectively.

\* Corresponding author. Mailing address: Department of International Health, Johns Hopkins School of Public Health, 615 N. Wolfe St., Room 3501, Baltimore, MD 21205. Phone: (410) 614-3639. Fax: (410) 614-6060. E-mail: rgilman@jhsph.edu.

TABLE 1. Results of and agreement between MABA and TEMA

Antibiotic	No. of samples processed by MABA that were:			No. of samples processed by TEMA that were:			Kappa value <sup>a</sup>	P value
	Sensitive	Partially resistant	Resistant	Sensitive	Partially resistant	Resistant		
INH	17	0	18	17	0	18	1.000	<0.001
RIF	21	NA	14	21	NA	14	1.000	<0.001
STR	24	10	1	25	10	0	0.733	<0.001
ETB	14	10	11	15	6	14	0.480	<0.001

<sup>a</sup> The kappa value is a measure of test reliability, with values interpreted as follows: <0.4, poor; 0.4 to 0.75, fair to good; and >0.75, strong.

<sup>b</sup> NA, not applicable.

Thirty-five sputum samples that tested positive for *M. tuberculosis* were obtained from patients at Cayetano Heredia Hospital in Lima, Peru, and processed by MABA and TEMA. All plates were read the first day after incubation with tetrazolium or Alamar Blue solution. The antibiotic susceptibility results are summarized in Table 1. Major agreement between the MABA and TEMA results was found for the INH and RIF MICs. On the other hand, some discordance appeared in the data on ETB susceptibility in resistant and partially resistant strains.

The TEMA developed by our group has proven to be a reliable method for the determination of MDR strains of *M. tuberculosis*. The procedure is faster than other tetrazolium-based methods (4), because it uses ethanol instead of a lysis buffer, thus eliminating one step and shortening the total testing time by 1 day. At the same time, the method replaces Alamar Blue with an inexpensive tetrazolium indicator, thus reducing the cost compared to that of the previously developed MABA. The use of TEMA for the determination of susceptibilities to antibiotics (INH, RIF, STR, and ETB) costs 5.04 U.S. dollars for each strain tested compared to 7.54 U.S. dollars for each strain tested by MABA. These characteristics make the TEMA a promising alternative method for use in

developing countries, where tuberculosis is an important and often fatal disease. An easy, rapid, and cheap assay could help to reduce the transmission of MDRTB by increasing the detection capacities of local laboratories and improving treatment regimens, thus reducing the amount of time patients remain contagious.

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