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Comparison of Flow Cytometric and Alamar Blue Tests with the Proportional Method for Testing Susceptibility of *Mycobacterium tuberculosis* to Rifampin and Isoniazid

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The performance of flow cytometry and the microplate Alamar Blue assay in determining susceptibility of *Mycobacterium tuberculosis* was assessed by testing 150 Brazilian isolates. The overall agreement was 97.3 and 98% for isoniazid and 94.7 and 100% for rifampin by flow cytometry and MABA, respectively. This study was entirely done in a developing country.

Many developing countries have serious difficulties obtaining drug susceptibility information for *Mycobacterium tuberculosis* isolates for financial or technical reasons. Treatment of tuberculosis without susceptibility information increases the risk of treatment failure and of the spread of resistant strains as well as the risk of the development of resistance to additional drugs (2, 8).

The commonly used agar proportion method for mycobacterial susceptibility testing requires a 3- to 4-week period of incubation before a pattern of susceptibility is established (3, 9). Flow cytometry that relies on fluoresceine diacetate (FDA) for detection has been used to perform susceptibility testing of *M. tuberculosis* and can yield results within 24 h. The inhibition by rifampin (RIF) and isoniazid (INH) of the ability of viable *M. tuberculosis* to hydrolyze FDA can be measured by flow cytometry. In addition, multiplication of the mycobacteria is not required (10, 11, 15).

The microplate Alamar Blue assay (MABA) (7) has been reported to show very good correlations with the proportional and BACTEC methods. MABA is a resarzurin-based oxidation-reduction indicator which measures colorimetric drug MICs for *M. tuberculosis* for up to 7 days. In this study, we compared the flow cytometric and MABA tests with the standard proportional method to assess INH and RIF susceptibilities of 150 clinical isolates from a community in Rio de Janeiro, Brazil; 100 isolates were susceptible to both drugs, 50 were resistant to INH, and 37 of these 50 were also RIF resistant (multidrug resistant).

The proportional method was performed according to the method of Canetti, Rist, and Grosset (3). The results obtained by the proportional method were used as a reference to compare the results of flow cytometry and MABA.

For mycobacterium preparation, each isolate was grown in two Löwenstein-Jensen tubes (Difco, Detroit, Mich.) at 37°C in aerobic conditions for 30 days. After incubation, colonies were suspended in Middlebrook 7H9 medium (Difco) directly from solid medium and adjusted to a no. 1 McFarland standard ($\sim 3 \times 10^7$ CFU/ml).

Flow cytometric susceptibility testing was performed according to the method of Norden et al. (11). The only modification was implemented to assure bacterial inactivation before analysis with an XL-MCL flow cytometer (Coulter, Miami, Fla.). In a previous study (data not published), we showed that *M. tuberculosis* cells can be killed by formaldehyde at a final concentration of 10% for 1 h.

Final drug concentrations were 0.5, 1.0, and 2.0 μg/ml for RIF and 0.1, 0.2, and 0.3 μg/ml for INH. For each isolate the relative fluorescence value of each drug-containing sample was divided by the relative fluorescence value of the drug-free control to obtain the susceptibility index. An isolate of *M. tuberculosis* was considered susceptible to an antimycobacterial agent when the susceptibility index of all three drug concentrations was 0.75 or less. The calculation eliminates the variability among isolates of *M. tuberculosis* in their abilities to hydrolyze FDA in the absence of antimycobacterial agents (11).

MABA susceptibility testing was performed according to the method of Franzblau et al. (7). Final concentrations ranged from 2.5 to 0.156 μg/ml for RIF and 0.5 to 0.031 μg/ml for INH. The $H_{37}Rv$ (ATCC 27294) strain was used as a control for all methodologies.

Each of the 100 pan-susceptible isolates (except for 6 isolates) had a susceptibility index value of 0.75 or less. The discordant results occurred in one sample resistant to INH and five samples resistant to RIF by flow cytometry (susceptibility index values ranged from 0.76 to 0.96).

For the 50 INH-resistant samples detected by the proportional method, 47 (94%) were concordant, showing a susceptibility index of >0.75. The three samples that were discordant were found to be susceptible to INH (susceptibility index ≤ 0.75) by flow cytometry.

For the 37 samples also resistant to RIF (multidrug resistant) by the proportional method, 34 (91.9%) were concordant. The three samples that were discordant were found to be susceptible to RIF (susceptibility index ≤ 0.75) by flow cytometry.

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The overall agreement between the proportional method and flow cytometry was 146 and 142 of the 150 samples for INH (97.3%) and RIF (94.7%), respectively.

MABA colorimetric drug MIC results for all 150 clinical *M. tuberculosis* isolates were available by the 7th day of incubation. MICs $\leq$ 0.25 and 1.25 μg/ml in MABA were considered to signify susceptibility to INH and RIF, respectively.

A total of 99 of 100 susceptible samples detected by the proportional method were concordant with the MABA results (drug MIC $\leq$ 0.25 μg/ml). The drug MIC was >0.5 μg/ml for the one sample with a discordant result. For 50 INH-resistant isolates, 48 (96%) resulted in drug MICs $\geq$ 0.25 μg/ml. For the two discordant samples, the drug MIC was lower than 0.25 μg/ml.

For all 37 isolates resistant to RIF, the drug MIC results by the proportional method were concordant (MIC $\geq$ 1.25 μg/ml). The overall agreement was 147 and 150 for the 150 isolates for INH (98%) and RIF (100%), respectively. None of the discordant samples were the same by flow cytometry and MABA. All methodologies were repeated for the discordant results; none of the results changed.

The time required to obtain in vitro susceptibility results with *M. tuberculosis* is frustratingly long (6, 9, 19). Molecular methods can provide very good results in resistance detection, especially for RIF (14, 17, 18), but they are very expensive, often making it impossible to perform the tests in developing countries.

In this study we tested three concentrations for each drug (RIF and INH) and a drug-free control for each sample. Although we could only use one concentration (a breakpoint), we decided to investigate three to choose the best for use in clinical routines and for correlation to established breakpoints. The study shows that the intermediate concentration of both drugs (the same breakpoint established with a BACTEC 460TB system [Becton Dickinson, Sparks, Md.]) can be used. Flow cytometry also allowed rapid measurement of the samples' susceptibility.

The high cost of a flow cytometer should not be a problem for many hospitals, since even in developing countries like Brazil many facilities already have the equipment for other uses, such as CD_4^+/CD_8^+ quantitative determination, leukemia classification, and many other applications. Once we can assure biosafety by adding formaldehyde before acquiring results in the flow cytometer, further containment would not be required. Microbiological use of a flow cytometer should be considered (even if only for screening of drug resistance to help avoiding the spread of drug-resistant tuberculosis) (1, 4, 5, 16). We could confirm the feasibility of use of flow cytometry with clinical samples of *M. tuberculosis* in 24 h.

MABA can yield results in a short period of time (7 days). It is also less expensive, although a cost-benefit study should be done to confirm this. O'Brien et al. (12) demonstrated that Alamar Blue is mostly resarzurin and showed that resarzurin yields results similar to those obtained with Alamar Blue for mycobacteria susceptibility assays; the cost was even lower with the former (13). MABA is simpler to execute than the proportion method, and up to four samples can be tested in a single 96-well plate with five different concentrations of each INH and RIF. The ability to rapidly determine MICs may aid in the early detection of drug resistance during therapy and, as in flow cytometry, can provide better treatment schemes to prevent further spread of drug-resistant strains.

MABA or resarzurin testing has the potential to become one of the standard tests for *M. tuberculosis* susceptibility determination in developing countries.

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