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Note

Simple Procedure for Drug Susceptibility Testing of *Mycobacterium tuberculosis* Using a Commercial Colorimetic Assay

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Abstract The aim of this study was to evaluate a simple method using a commercial colorimetric assay (Alamar Blue Oxidation-Reduction Indicator; Accumed, USA) in a microtiter format for testing the susceptibility of 94 strains of *Mycobacterium tuberculosis* to isoniazid, rifampicin, ethambutol and streptomycin. The method makes use of one critical concentration of each drug, and the results are available within 8–10 days. Overall, 97.1% agreement with the proportion method was obtained. Full agreement was obtained for isoniazid and rifampicin. The method is simple to perform, permits visual reading of results, and is practicable for laboratories with limited resources.

Introduction

Tuberculosis (TB) is still the major cause of death from a single infectious agent [1]. Of great concern for the control of the disease is the emergence of drug-resistant strains of *Mycobacterium tuberculosis*. As shown by the results of a global project on tuberculosis drug resistance surveillance, drug resistance was found in all 35 countries and regions surveyed, with an overall prevalence of 12.6% for single-drug resistance and 2.2% for multidrug resistance, making the disease incurable for those without access to better and more expensive health care systems [2]. Although the Directly Observed Treatment Short-course, the treatment strategy devised by the World Health Organization, has been successfully applied in several countries, there is still a need for other control measures such as new diagnostic tools, more effective drugs and treatment regimens, or even a more effective vaccine [3]. In particular, the emergence of multidrug resistant strains of *Mycobacterium tuberculosis* underscores the need for simple methods that enable rapid determination of the susceptibility of isolates to antimicrobial agents.

The proportion method is considered the gold standard for drug susceptibility testing of *Mycobacterium tuberculosis* [4, 5], but it requires 3–4 weeks of culture on solid media before results can be obtained; a radiometric adaptation is also in use in many laboratories but calls for expensive equipment and radioactive materials [6]. Several other methods have also been developed, with variable results [7–11].

The Alamar Blue Oxidation-Reduction (redox) Indicator (Accumed, USA) has been used in a variety of tests for measuring cell viability and toxicity or cellular growth [12–14]. This indicator changes from blue when in the oxidized state to pink when reduced, which can be easily differentiated visually, thereby providing a simple measure of viability. The method has already been used with some bacteria and yeasts [15, 16].

Yajko et al. [17] first applied this technique in a colorimetric method for determining minimal inhibitory concentrations (MICs) of some antimicrobial agents for *Mycobacterium tuberculosis*. More recently, Collins et al. [18] and Franzblau et al. [19] described a microplate adaptation for high throughput screening of compounds against mycobacteria and for MIC determination with clinical isolates of *Mycobacterium tuberculosis*.

The purpose of this study was to evaluate a simplified version of this technique for the drug susceptibility testing of clinical isolates of *Mycobacterium tuberculosis* using one concentration of isoniazid, rifampicin, ethambutol, and streptomycin and to compare the results with those obtained with the proportion method [5].

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Materials and Methods

Ninety-four *Mycobacterium tuberculosis* isolates from the collection of the Mycobacteriology Unit of the Institute of Tropical Medicine in Antwerp, Belgium, were evaluated. They were maintained on Löwenstein-Jensen medium and freshly subcultured before the evaluation. In addition, *Mycobacterium tuberculosis* strains ATCC 35822, ATCC 35838, ATCC 35837, and ATCC 35820 (American Type Culture Collection, Rockville, USA), resistant to isoniazid, rifampicin, ethambutol and streptomycin, respectively, were used for quality control.

The simplified version of the indirect proportion method was performed on Löwenstein-Jensen medium against isoniazid, rifampicin, ethambutol, and streptomycin, at 0.2, 40, 2, and 4 μg/ml, respectively, according to standard procedures [5].

For the Alamar Blue procedure, the inoculum was withdrawn from a fresh Löwenstein-Jensen tube and added to 5 ml of Middlebrook 7H9 broth containing 0.1% casitone and 0.5% glycerol and supplemented with oleic acid, albumin, dextrose, and catalase (Becton-Dickinson Microbiology Systems, USA) (7H9-S) in a tube containing several glass beads. After 7 days of incubation at 37 °C, the tube was vortexed for 2 min and allowed to sediment for 15 min. The supernatant was transferred to another tube and the turbidity adjusted to a McFarland tube no. 1 with a nephelometer. The suspension was further diluted 1:5 in 7H9-S and used as the inoculum for the test.

Antibiotic stock solutions were filter-sterilized and stored at –70 °C until used. For test performance, antibiotic stock solutions were thawed and diluted in 7H9-S; final concentrations were as follows: isoniazid, 0.1 μg/ml; rifampicin, 1 μg/ml; ethambutol, 5 μg/ml; and streptomycin, 0.5 μg/ml. For each strain evaluated, 180 μl of 7H9-S containing each antibiotic dilution was dispensed into the wells of a 96-well microtiter plate (Falcon, Becton Dickinson Labware, USA) and inoculated with 20 μl of the diluted inoculum. A growth control well containing 7H9-S without antibiotic and inoculated in the same way and a sterile control well containing 7H9-S alone were also included. The plate was covered, sealed in a polyethylene bag and incubated at 37 °C in air. After 8 days of incubation, the plate was taken from the incubator and bacterial growth measured by adding to each well 20 μl of the Alamar Blue indicator (10× sterile solution) and 25 μl of 10% Tween 20. The plate was incubated overnight at 37 °C; a change in color of the Alamar indicator from blue to pink as a result of reduction of the reagent indicates bacterial growth. In this way, susceptibility or resistance to each antibiotic was interpreted. If growth in the growth control well was insufficient (Alamar remained blue), the plate was reincubated for an additional 2 days and read again. The test and growth control wells were compared for the intensity of color development. Only wells matching the intensity of color of the growth control were interpreted as positive and thus resistant to the test antibiotic. Strains found to give results discordant with those of the proportion method were reevaluated by both methods. All manipulations were performed under a biological safety cabinet.

The performance of the test was analyzed by determining the specificity and sensitivity values and the predictive values for susceptibility and resistance, using the proportion method as the gold standard.

Results and Discussion

Susceptibility testing of 94 *Mycobacterium tuberculosis* isolates against isoniazid, rifampicin, ethambutol, and streptomycin gave an overall agreement of 97.1% between the two methods (365 of 376 susceptibility tests). Of the concordant results, 195 results were susceptible and 170 results were resistant by both methods (Table 1).

Complete agreement was found for isoniazid and rifampicin: 37 isolates were susceptible and 57 resistant to isoniazid by both methods, and 58 were susceptible and 36 resistant to rifampicin by both methods. For ethambutol we found eight discordant results: five strains found resistant by the Alamar Blue procedure were susceptible by the proportion method, and three found resistant by the proportion method were susceptible by the Alamar Blue method. For streptomycin, we found three discordant results: one isolate was resistant by the Alamar Blue method but susceptible by the proportion method, and two were resistant by the proportion method but susceptible by the Alamar Blue method. These discrepant results were confirmed after repeated testing.

Table 2 shows the specificity, sensitivity, and predictive values for susceptibility and resistance with the Alamar Blue procedure. An accuracy of 1 was found for ison-

Table 1 Results of susceptibility testing of 94 isolates of *Mycobacterium tuberculosis* by the simplified Alamar Blue procedure and the proportion method

Antimicrobial agent	S, both methods	S, pro R, Ala	R, pro S, Ala	R, both methods
Isoniazid	37	0	0	57
Rifampicin	58	0	0	36
Ethambutol	55	5	3	31
Streptomycin	45	1	2	46

S, susceptible; R, resistant; Pro, proportion method; Ala, Alamar blue

Table 2 Performance of the simplified Alamar Blue procedure in the susceptibility testing of 94 isolates of *Mycobacterium tuberculosis* to four antimicrobial agents

Antimicrobial agent	Predictive value (%)				
	Specificity	Sensitivity	Susceptibility	Resistance	Accuracy
Isoniazid	100	100	100	100	1
Rifamipicin	100	100	100	100	1
Ethambutol	91.7	91.2	94.8	86.1	0.91
Streptomycin	97.8	95.8	95.7	97.9	0.97

iazid and rifampicin and 0.91 and 0.97 for ethambutol and streptomycin, respectively.

The emergence of multidrug resistant tuberculosis is of great concern for the proper control of tuberculosis, especially in developing countries. For this reason, monitoring of drug resistance patterns at a national level is of great importance, as has been recently emphasized [20]. It is also necessary to perform drug susceptibility testing on strains obtained from primary isolations or on those obtained from patients who fail treatment, so that appropriate chemotherapy can be administered promptly.

Current methods to this end are time consuming and laborious, and the alternative methods proposed are still expensive in some cases [9] or very complicated in others [8, 11].

We have evaluated a simple procedure based on the Alamar Blue Redox Indicator and found it very reliable, especially for testing susceptibility to isoniazid and rifampicin, the two most important drugs in the treatment of TB. The overall agreement obtained (97.1%) when compared with the proportion method was similar to the result obtained by Yajko et al. [17] with a tube macrodilution version of this method. Discrepancies were still found for ethambutol and streptomycin. On the basis of MIC values reported previously [17, 19], we selected one critical concentration of each antibiotic for this study to further simplify the technique and reported the results as susceptible or resistant. Additionally, and in contrast to the previous reported studies [17–19], Alamar Blue was added only once, after 8 days of incubation, avoiding continuous opening of the plates to reduce the risk of contamination and allowing less manipulation by the personnel. In a very few cases we found a weak positive reaction (purple) that turned completely positive (pink) after 48 h of additional incubation. Additional studies performed with an inoculum prepared directly from a Löwenstein-Jensen tube have produced similar results, helping to simplify even further the whole procedure and reducing the total time for the test from 15 days to 8–10 days (data not shown).

As has been recently reported by a quality assurance program for drug susceptibility testing of *Mycobacterium tuberculosis* [20], procedures for testing isoniazid and rifampicin are highly reliable. Testing susceptibility to these two drugs is of the highest importance for the detection of multidrug resistant TB. However, procedures for testing ethambutol and streptomycin require optimization, as has been highlighted in the present study, mainly to better define drug concentrations and the time required to read results.

The advantages and convenience of using a microtiter format for this assay have already been shown by Collins et al. [18] and Franzblau et al. [19]. This study shows that testing at a single concentration breakpoint is effective, particularly for isoniazid and rifampicin, and permits further cost reductions. Additional studies are needed to assess the usefulness of this method for testing susceptibility to other antituberculosis drugs.

In summary, susceptibility testing of *Mycobacterium tuberculosis* using the Alamar Blue indicator in a microtiter format with single breakpoint concentrations of antibiotics is simple, permits visual reading of results, and could be implemented in laboratories with limited personnel and equipment resources.

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