Evaluation of rapid microplate assays using cellular-viability indicators to determine patterns of susceptibility to isoniazid and rifampin in *Mycobacterium tuberculosis* strains*

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Background: Knowledge of the rates of drug resistance is one of the pillars of tuberculosis control program evaluation. Data from low-resource countries are scarce and results are delayed due to the techniques employed. There is therefore an urgent need for evaluation of faster and less onerous testing methods.

Objective: To compare the performance of rapid colorimetric assays for phenotyping that employ oxidation-reduction indicators to determine the susceptibility profile of *Mycobacterium tuberculosis* with the gold-standard proportion method on Lowenstein-Jensen Medium.

Method: We analyzed 166 *M. tuberculosis* strains of known susceptibility. Minimal inhibition concentrations for isoniazid and rifampicin were determined in microplates, using a liquid medium and Alamar Blue and tetrazolium bromide indicators. To measure agreement the Kappa value was used. Cutoff values between sensitive and resistant strains were defined as 0.2µg/mL and 1.0µg/mL for isoniazid and rifampicin, respectively.

Results: There was 100% concordance between Alamar Blue and tetrazolium bromide methods in the determination of minimal inhibition concentrations. Agreement between the colorimetric method and the Lowenstein-Jensen was 95% for isoniazid and rifampicin. Using the colorimetric method, results were obtained within 7 days, in contrast to the 28 days required for the conventional method.

Conclusions: Assays to determine minimal inhibition concentrations in liquid medium and employing oxidation-reduction indicators proved to be rapid and inexpensive. This method has the potential to become a faster, alternative method for determining susceptibility of *M. tuberculosis* strains in developing countries.

Key words: Mycobacterium tubercolis. Disease susceptibility. Isoniazid/therapeutic use. Rifampin/therapeutic use.

INTRODUCTION

Tuberculosis (TB), known for thousands of years, continues to be a major public health problem[1]. Until the 1980s, it was expected that TB would soon be eradicated. However, the worldwide increase in the incidence of TB led the World Health Organization, in 1993, to declare TB a global emergency, requiring intensified efforts in order to combat this disease[2]. Although control measures and therapeutic regimes have been well established, there are difficulties that are inherent to the disease, chief among which is the appearance of drug-resistant strains[3,4].

Epidemiological studies of anti-TB drug resistance provide indicators, such as the prevalence of primary and acquired resistance, which are useful for the evaluation of the quality of treatment and of TB control programs. For such studies, the classically recommended method (gold standard) is the proportion method, performed on solid medium or on automated (MB/BacT and BACTEC 960) and semi-automated (BACTEC 460) systems. These methods are either prohibitively expensive or difficult to execute in the laboratory routine. There is therefore little information available regarding transmission and resistance patterns, whether for primary or acquired resistance[5]. In this study, we aimed to compare the susceptibility profile of *Mycobacterium tuberculosis* (Mtb) strains in relation to isoniazid (INH) and rifampin (RIF). This was done through a new method that uses cell-viability indicators to determine the minimal inhibitory concentrations (MIC) for INH and RIF.

METHODS

A total of 166 Mtb strains were evaluated. These strains were from the collections of the following laboratories: *Laboratório Central de Saúde Pública do Rio Grande do Sul* (Rio Grande do Sul Central Laboratory of Public Health); *Hospital Clementino Fraga Filho* (Clementino Fraga Filho Hospital) of the *Universidade Federal do Rio de Janeiro* (Rio de Janeiro Federal University); *Centro de Referência Professor Hélio Fraga* Professor Hélio Fraga Reference Center) in Rio de Janeiro; and the *Instituto Adolfo Lutz* (Adolfo Lutz Institute) in São Paulo. The standard Mtb H37Rv ATCC 27294 strain and *Mycobacterium fortuitum* ATCC 6841 (the latter as a standard for both INH and RIF resistance),

both of which were provided by the Professor Hélio Fraga Reference Center, were also used.

All strains were identified as belonging to the Mtb complex through standard colorimetric assays for phenotyping. The susceptibility test, using the Lowenstein-Jensen proportion method (LJPM), was carried out in accordance with the *Manual de Bacteriologia da Tuberculose* (Guidebook for Tuberculosis Bacteriology)[6]. When the Mtb began to multiply in the Lowenstein-Jensen medium, suspensions with turbidity equivalent to a McFarland turbidity standard of 3 were prepared in phosphate buffer at pH 7.0 and transferred to 2-mL cryotubes, where they were stored at –20°C.

The techniques used in the determination of MICs were performed according to the protocols devised by various authors[7-9]. The test was carried out in 96-well flat-bottom microplates, with sterile covers (Corning Costar, Cambridge, MA, USA). We used Middlebrook 7H9 broth medium (Difco, Detroit, MI, USA), containing glycerol and enriched with 10% oleic acid-albumin-dextrose-catalase (OADC), as well as with Bacto Casitone (Difco). The INH and RIF (Sigma, St. Louis, MO, USA) were prepared as stock solutions at concentrations of 10,000 μ g/mL in sterile distilled water and ethylene glycol (Difco), respectively. The concentrations of INH and RIF were chosen based on the cutoff values, standardized for the BACTEC 460 TB System: 0.2 μ g/mL for INH and 1.0 μ g/mL for RIF[10]. After the serialized dilutions, the final concentrations of INH in the microplate wells were 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 μ g/mL. The final concentrations for RIF were 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.062 μ g/mL[9]. Preparation of the Mtb suspension began at the moment that growth was first observed on the solid medium and continued for up to 30 days. The Mtb

suspension was prepared in tubes containing glass beads and sterile distilled water. After agitation and subsequent settling, the suspension was transferred by eyedropper into another tube containing sterile distilled water, until turbidity similar to the McFarland turbidity standard of 1 was achieved. The inoculate was diluted at 1:25 in Middlebrook 7H9 medium.

The solution used for the indicators was prepared one day prior to the test. Using Alamar Blue® dye (Fisher Scientific, Pittsburgh, PA, USA) as a base, a mixture of 10% Tween 80 solution in water was prepared in volume to volume proportions[7]. A volume of 25 µ L was used in each well of the microplate. Alamar Blue® is a fluorescent/colorimetric indicator with redox properties. The oxidized form (non-fluorescent/ non-viable cell) is blue, and the reduced form (fluorescent/viable cell) is rose-colored. To prepare the solution for use, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma), which is a tetrazolium salt that is reduced by dehydrogenase enzymes, forming formazan crystals soluble in ethanol, was used. The MTT was diluted in 1 mg/mL of absolute ethanol and equivalent amounts (v/v) were added to 10% Tween 80 solution. The volume used in each microplate well was 50 µ L. The oxidized form of MTT is yellow, and the reduced form is purple.

Standard strains were tested for each battery of tests. The peripheral microplate wells were filled with 200 µ L of sterile distilled water to avoid evaporation of the medium in the incubator. Wells designated as controls for drugs and for the culture medium were included. With the exception of the peripheral wells, all wells received 100 µ L of 7H9 liquid medium. To the initial wells in each row, 100 µ L of each drug was added. Using these wells and a multichannel pipette, serial dilutions were made. No drugs were added to the last column, with the intention of preserving it as a control for bacterial growth. Therefore, all wells had a volume of 100 µ L. We then added 100 µ L of suspension of each strain, for a final volume of 200 µ L.

Each microplate was sealed with plastic film and remained in the incubator at 37°C for five days. On the fifth day, the respective indicators were added to the bacterial control wells as well as to the drug and medium control wells. On the following day, if the indicators in the bacterial growth control wells had changed color, an indicator was added to all remaining wells. The microplates were placed in the incubator for an additional 24 hours. The MIC was then determined, defined as the lowest drug concentration capable of impeding the change in color, i.e. of inhibiting cell growth.

RESULTS

The susceptibility profile to INH and RIF was determined using the LJPM. Of the 166 strains studied, 34 were sensitive and 91 were resistant to both drugs. There were 38 strains resistant to INH only and 3 strains resistant to RIF only. Considering each drug separately, there were 129 INH-resistant strains and 37 INH-sensitive strains versus 94 RIF-resistant strains and 72 RIF-sensitive strains.

All 166 Mtb strains were tested using the Alamar Blue® method (ABM) and the MTT method (MTTM) for determining MIC. The 37 INH-sensitive strains (determined by LJPM) presented the same MICs for ABM as for MTTM. Of the 129 INH-resistant strains (LJPM), 23 presented divergence between ABM and MTTM MICs at only one dilution. However, using a cutoff value of 0.2 µ g/mL between sensitive and resistant strains, there was no discordance between the two methods. The 72 RIF-sensitive strains presented the same MICs for ABM as for MTTM. Of the 94 RIF-resistant strains, 5 presented divergence between ABM and MTTM MICs at only one dilution. However, similar to the results obtained for INH, there was no divergence in the sensitive/ resistant classification when a cutoff value of 1.0 µ g/mL was used. The methods studied for determining MIC (ABM and MTTM) presented 100% concordance when cutoff values of 0.2 µ g/ mL for INH and 1.0 µ g/mL for RIF were applied.

We evaluated the degree of concordance of the LJPM with both methods by calculating the kappa value. The indicator kappa values were κ = 0.89 for INH and κ = 0.95 for RIF, which allows us to assert that there was a near-perfect concordance for both methods and for both drugs. Table 1 shows the comparisons between the LJPM and the results of the ABM and MTTM. The specificity and sensitivity values for INH were 95.3% and 97.3%, respectively, versus 91.5% and 100% for RIF.

TABLE 1

Comparison of Alamar Blue/3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method results with those from the proportion method in 166 *Mycobacterium tuberculosis* strains

	Proportion Method			
	Isoniazid		Rifampin	
ABM/MTTM (μ g/mL)	Resistant	Sensitive	Resistant	Sensitive
Resistant to INH (≥ 0.2)	123 (96%)	1 (2.6%)	-	-
Sensitive to INH (< 0.2)	6 (4%)	36 (97.4%)	-	-
Resistant to RIF (≥ 1.0)	-	-	86 (93.5%)	-
Sensitive to RIF (< 1.0)	-	-	8 (8.7%)	72 (100%)
TOTAL	129 (100%)	37 (100%)	94 (100%)	72 (100%)

ABM: Alamar Blue method; MTTM: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method; INH: isoniazid; RIF: rifampin

DISCUSSION

Due to concern about emergency control and transmission of resistant strains, it is essential that new phenotyping methods used to determine strain susceptibility to anti-TB drugs, especially INH and RIF, be tested. These methods should be practical and effective and should allow a faster result than that provided by the conventional method. Therefore, with the objective of decreasing the cost of these tests and the time spent waiting for results, fast, reliable and easily performed phenotyping methods have been developed. Susceptibility tests conducted in microplates represent one such alternative. The use of indicators, such as resazurin and tetrazolium salts, to determine cell viability is an alternative for better viewing during the reading of these tests in microplates. These compounds function as chromogenic substrates of dehydrogenase enzymes, acting as oxidation-reduction indicators, and are reduced (through gaining hydrogen molecules) by flavins bound to transport system enzymes present during the cell metabolism process[11].

Since 1995, studies have been conducted using Alamar Blue® (resazurin salt) as an indicator of cell growth. The authors of one such study[12] dubbed the method MABA (*Microplate Alamar Blue Assay*) and compares favorably with the agar proportion method, obtaining a concordance of 97% in determining the MIC of Mtb strains. Other studies applying changes in the way the test is read of the test, using fluorescence, also showed positive results[13,14]. When compared to the BACTEC 460[7] system and to the proportion method on solid medium method, MABA presented concordances of 93.6% and 97.1%, respectively, and has the added advantage of the fact that the results remain available for a period of 8 to 10 days[8].

The MTT dye has been used as a cell growth indicator to determine MIC for the *Mycobacterium avium* complex[15]. Guidelines for detection of RIF resistance in Mtb strains and for the use of microplates were established in 1998, in a study that determined the appropriate cutoff values for defining resistance and sensitivity to RIF[16]. The technique of performing this method in tubes was standardized through comparison with the BACTEC 460 system, used to detect resistance to RIF, with concordant results[17].

In this study, 166 Mtb strains were evaluated with the objective of analyzing the performance of new MIC-determining methodologies, which were evaluated with regard to sensitivity and concordance in comparison with the standard method on solid medium. In order to evaluate the degree of concordance between the new methods and the conventional methods, we used predetermined cutoff values of 0.2 μ g/mL for INH and 1.0 μ g/mL for RIF. The new methods showed 100% concordance when compared with each other. When the methods ABM and MTT were compared to PMLJ, we observed a concordance of 95% for both drugs. The sensitivity and specificity values for INH were 95.3% and 97.3%, respectively, whereas those for RIF were 91.5% and 100%, respectively.

In two previously published studies[9,18], the cutoff values for these two drugs were established after the analysis of the study performance. In the first study, 80 Mtb strains were evaluated and the

resazurin microtiter assay plate method, which uses resazurin as an indicator of cell viability, was used for determining MIC. The LJPM method was used as the gold standard. Sensitivity and specificity for INH were 96.2% and 100%, respectively. For RIF, all 80 results showed a 100% concordance. In the second study, the authors compared the MABA method with the tetrazolium microplate assay (MTT) method in determining the MICs of 35 Mtb strains for INH, RIF, streptomycin and ethambutol. The authors found a strong concordance between the two methods, although it was unclear whether there was a gold-standard method used for comparison or the new methods were simply compared with each other.

In tests designed to determine MIC and conducted in liquid medium, technical and biological variables, such as size of the inoculate, phase of bacterial growth, characteristics of the medium and drug stability, hinder reproducibility[19]. In this study, we tried to control some of these variables, using the same inoculate, the same culture medium and the same drug preparations. The technical manipulation of the serial dilutions and the characteristics of each indicator were identified as the possible causes for the differences in the MICs observed. We found that the MTT method facilitates the visualization of color change, since yellow changes to purple, with no possible confusion caused by nuances of colors intermediate between the two.

In conclusion, susceptibility tests in microplates, using oxidation-reduction indicators, are rapid, are easily performed, and present a high rate of concordance with the gold-standard method (LJPM) used in the present study. These tests may represent a timesaving alternative for basic laboratories, since most possess the infrastructure necessary for culturing Mtb.

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