

Susceptibility of *Mycobacterium tuberculosis* to first-line antimycobacterial agents in a Brazilian hospital: assessing the utility of the tetrazolium (MTT) microplate assay

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We conducted a cross-sectional, hospital-based study between January 2006-March 2008 to estimate the resistance of *Mycobacterium tuberculosis* to first-line drugs in patients with tuberculosis at a Brazilian hospital. We evaluated the performance of the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) microplate assay compared with the Bactec-MGIT 960™ system for mycobacteria testing. The prevalence of resistance in *M. tuberculosis* was 6.7%. Multidrug-resistance [resistance to rifampicin (RMP) and isoniazid (INH)], INH-resistance and streptomycin (SM)-resistance accounted for 1%, 3.8% and 3.8% of all resistance, respectively, and all isolates were susceptible to ethambutol (EM). The resistance was primary in four cases and acquired in three cases and previous treatment was associated with resistance ($p = 0.0129$). Among the 119 *M. tuberculosis* isolates, complete concordance of the results for INH and EM was observed between the MTT microplate and Bactec-MGIT 960™ methods. The observed agreement for RMP was 99% (sensitivity: 90%) and 95.8% for SM (sensitivity 90.9%), lower than those for other drugs. The MTT colourimetric method is an accurate, simple and low-cost alternative in settings with limited resources.

Key words: tuberculosis - resistance - drug susceptibility test - tetrazolium - colourimetric method - hospital

Mycobacterium tuberculosis multidrug resistance (MDR) is an emerging global problem. The World Health Organization has estimated that 14% of all new tuberculosis (TB) cases involve MDR (WHO 2008). In the last decade, MDR-TB was observed in 0.9% of new Brazilian cases diagnosed in primary health care units (Natal et al. 1997). However, according to the Brazilian Ministry of Health Program, this epidemiological trend is changing: recent data indicate increased resistance and the initial standard treatment regimen now includes four drugs (MS/SVS 2009).

Early detection of *M. tuberculosis* resistance is a concern at local and international levels, especially for health care facilities in metropolitan areas with high population densities, high human immunodeficiency virus (HIV) infection rates and large numbers of institutionalized and homeless people. New drug susceptibility tests (DST) must be developed and implemented that are accurate enough to identify drug resistance, but that are also low-cost, sustainable and accessible. Many DSTs have attempted to meet these requirements and colorimetric assays have been proposed as cost-effec-

tive alternatives to high-cost, automated phenotyping and genotyping methods. Colorimetric methods using alamarBlue, resazurin or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reveal cellular viability in the presence of variable drug concentrations and allow the determination of the drugs' minimum inhibitory concentration (MIC) (Franzblau et al. 1998, Palomino & Portaels 1999, Caviades et al. 2002, Ribeiro et al. 2004).

In this study, we tested TB patients at a Brazilian hospital to estimate the prevalence of resistance of *M. tuberculosis* to first-line drugs and to evaluate the performance of the MTT microplate assay compared with Bactec-MGIT 960™.

PATIENTS, MATERIALS AND METHODS

A retrospective, cross-sectional study was performed at a Brazilian tertiary health-care facility in the Campinas metropolitan area, a region with a population of five million and a recent TB incidence rate of 48 per 100,000 inhabitants. We defined two study groups: the prevalence study group (P-group) and the MTT performance evaluation group (MTT-group). The P-group included patients of at least 14 years of age with a positive *M. tuberculosis* culture diagnosis reported to the surveillance system between January 2006-March 2008. To select the P-group, we evaluated 360 cases that were reported to the surveillance system and included 105 patients. The MTT-group was composed of all of the patients in the P-group and an additional 14 cases with confirmed resistance to first-line drugs prior to the study.

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The drug susceptibility patterns of *M. tuberculosis* were tested using the MTT assay and Bactec-MGIT 960™. Reference susceptibility patterns were obtained from Bactec-MGIT 960™ performed by Mycobacteriology Division of the Adolfo Lutz Institute, the regional laboratory of the state of São Paulo (SP). The MTT assay was performed at the Mycobacteriology Laboratory, Division of Clinical Pathology, State University of Campinas (UNICAMP) according to Franzblau et al. (1998) for the following drugs: isoniazid (INH), rifampicin (RMP), ethambutol (EM) and streptomycin (SM). *M. tuberculosis* suspensions were prepared by inoculating colonies from slants into 2 mL of 7H9 broth (Sigma Chemical Co, St. Louis, MO, USA) and incubating at 37°C for seven days. The turbidity was adjusted to McFarland Standard 3 by adding Middlebrook 7H9. Middlebrook 7H9 was prepared with 0.47 g of 7H9 broth base (Difco, Detroit, MI, USA), 0.2 mL of glycerol, 90 mL of distilled water and 10 mL of a mixture of oleic acid, albumin, dextrose and catalase (OADC) (Becton-Dickinson). RMP and INH stock solutions (10 mg/mL) were prepared in methanol and water, respectively. EM stock solutions were prepared in distilled water with and without 20% ethanol and SM solutions were prepared in distilled water with and without 5% phenol.

Microdilution plates were prepared by adding 200 µL of sterile water to all outer wells and 100 µL of 7H9 broth to columns 2-11 in rows B-G. Then, 100 µL of 4X antibiotic solutions (INH, RMP, SM or EM) were added to rows B-G of column 2. To perform the dilution, 100 µL were transferred from row B-G of column 2 to column 3 with a multi-channel pipette. The antibiotics were serially diluted 1:2 in this manner except in column 11, where the 100 µL of excess media was discarded. The final drug concentration ranges were 0.015-16 µg/mL for INH, 0.015-16 µg/mL for RMP, 0.01-16 µg/mL for SM and 0.03-16 µg/mL for EM. At this point, 100 µL of a log-phase *M. tuberculosis* bacterial suspension (20-30 days old) were added to rows B-G in columns 2-11. Bacteria were not added to row H of columns 10 and 11 and these wells served as inoculum controls. The plates were sealed with parafilm in plastic bags and incubated at 37°C for six or seven days. On day six, 15 µL of 5 mg/mL MTT (Sigma-Aldrich) in a 1:9 ratio of ethanol:water was added to well H10. The plate was incubated at 37°C for 24 h and, if the color of H10 turned purple, MTT was added to all wells and the color was recorded at 24 h. If the color of well H10 remained yellow, the plates were incubated for another 24 h. At this point, MTT solution was added to well H11 and the plate was incubated for another 24 h to observe the color change. Isolates were considered to be drug susceptible if their MICs were below or equal to the critical concentrations reported by Martin et al. (2005): INH, 0.25 µg/mL; RMP, 0.5 µg/mL; EM, 4 µg/mL; SM, 1 µg/mL. The critical concentrations of Bactec-MGIT 960™ reported by the manufacturer's protocol were as follows: INH, 0.10 µg/mL; RMP, 1.0 µg/mL; EM, 5.0 µg/mL; SM, 1.0 µg/mL.

Statistical analysis was performed with Epi-Info version 6.04b (Centers for Disease Control and Prevention, Atlanta, GA, USA). Categorical variables were analyzed with Chi-square (Yates' correction) and two-tailed Fisher's

exact tests. P values of < 0.05 were considered statistically significant. The study was approved by the Ethical Committee of the Faculty of Medical Sciences of UNICAMP.

RESULTS

Among the 105 P-group patients, 78 (74.3%) were male and the median age was 36 years. Pulmonary presentation alone was observed in 68 (64.8%) cases, extra-pulmonary in 11 (10.5%) cases and combined (pulmonary and extra-pulmonary) in 26 (24.7%) patients. Disseminated disease occurred in seven (18.9%) cases. Thoracic X-rays were suggestive of TB in 82 (78.1%) cases. Among the 94 (89.5%) patients with pulmonary involvement, 66 (62.9%) had sputum positive for acid-fast bacilli and 82 (78.1%) had *M. tuberculosis*-positive sputum cultures. In other specimens, acid-fast bacilli were observed in 13 (12.4%) cases and the culture was positive in 39 (37.1%) cases. HIV co-infection was diagnosed in 39 (37.1%) cases. Of the 10 patients that had previous treatment, six (5.7%) were cured, three (2.8%) were in default of treatment and one case failed.

The prevalence of drug-resistant *M. tuberculosis* was 6.7%, MDR was detected in a single case and resistance to INH and SM was detected in 3.8% of cases (Table I). The resistance was primary in four cases and acquired in three. Previous treatment was associated with resistance ($p = 0.0129$); however, HIV was not related to resistance. Favorable treatment outcomes occurred in 67 (63.8%) cases, death in 26 (24.8%) cases and default of treatment in 12 (11.4%) cases. Of the seven patients with drug-resistance, four were cured, two died and follow-up data was not available for one. The patient with MDR-TB was cured at the 18th month of the treatment.

Analysis of the MTT-group (119 *M. tuberculosis* isolates) showed agreement between the MTT and Bactec-MGIT 960™ methods in 100% of cases for INH and EM, 99% for RMP and 95.8% for SM (Table II). Final results (resistant or susceptible) were similar when ethanol or phenol was added to solubilise EM or SM, respectively.

TABLE I

Prevalence of resistance to first-line antimycobacterial agents among tuberculosis cases in prevalence study group performed by MTT microplate assay and Bactec-MGIT 960™^a

Drug	Resistant		Susceptible	
	n = 7	%	n = 98	%
Isoniazid	4	3.8	101	96.2
Rifampicin	1	1	104	99
Ethambutol	0	0	105	100
Streptomycin	4	3.8	101	96.2
Isoniazid + rifampicin	1	1	104	99
Total	7	6.7	98	93.3

^a: the benchmark test when the results were discrepant; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay.

TABLE II
Performance of MTT microplate assay compared with Bactec-MGIT 960™ among tuberculosis cases in MTT-group

Drug	Bactec-MGIT 960™	MTT		Agreement %
		Susceptible n	Resistant n	
Isoniazid	S	104	0	100
	R	0	15	
Rifampicin	S	109	0	99.1
	R	1	9	
Ethambutol	S	117	0	100
	R	0	2	
Streptomycin	S	108	4	95.8
	R	1	6	

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay.

DISCUSSION

In our study, the presence of resistant-TB was lower than in other Brazilian studies performed at tertiary care facilities. Although few Brazilian hospital-based studies have specifically evaluated resistance, the resistance rates reported by other studies were higher. For RMP, INH or any drug, the resistance rates varied from 3.6-4.3%, 10.9-13% and 13.3-19.2%, respectively (Brito et al. 2004, de Souza et al. 2006, Aguiar et al. 2009). Our results can be explained by the characteristics of our study population, such as the number of cases evaluated, the absence of previous treatment in the majority of patients and the TB incidence rate in the Campinas metropolitan area being lower than in Rio de Janeiro, where most Brazilian studies have been conducted.

MDR-TB has been evaluated in other Brazilian hospitals at higher rates than observed in the general population. In the 1990s, a national surveillance of resistance in basic health care units reported that 0.9% of cases were MDR-TB and 8.6% of all cases were resistant to some drug. Similar results were observed in SP (Pablos-Méndez et al. 1998, Telles et al. 2005).

An association between resistance and previous treatment was found in our study, as well as in other studies (Fandinho et al. 1999, de Melo et al. 2003, Aguiar et al. 2009). In Brazil, acquired resistance was related to inadequate adherence to treatment, supporting the movement to extend directly observed therapy.

The general performance of the MTT microplate assay was adequate to detect resistance to INH, RMP and EM. The assay results were in agreement with Bactec-MGIT 960™, but few TB resistance isolates were tested. These results are compatible with results reported by others (WHO 1997, Palomino & Portaels 1999, Caviedes et al. 2002, Ribeiro et al. 2004, Mengatto et al. 2006). DST for EM and SM has been reported to have low reproducibility (WHO 1997). In a multicentric study of the

MTT assay, Martin et al. (2005) reported specificity and sensitivity for SM of 85% and 90.5%, respectively. These results are probably associated with the drug's stability. In our study, we added ethanol in small concentrations to improve the stability of EM in the stock solutions and phenol was added for SM solubility. This approach possibly improves the evaluation of EM and SM resistance through MTT evaluation.

In addition, the MTT microplate assay has been widely used to test second-line antimycobacterial drugs. Expertise with this method assumes great importance in the context of the global rise in MDR, primarily in developing countries.

Reported overall efficiencies of the MTT and resazurin assays have been similar with high accuracy, except for SM (Martin et al. 2005, Montoro et al. 2005). According to previous studies, MTT allows for a better interpretation of colors, which facilitates its standardization (Ribeiro et al. 2004). One test for resistance to four drugs using MTT or resazurin costs \$5 (US) or \$3 (US), respectively, whereas the test performed by Bactec-MGIT 960™ costs \$39 (US) (Mengatto et al. 2006, WHO 2008). The use of colorimetric methods to detect drug resistance in *M. tuberculosis* has recently been endorsed by the Strategic and Technical Advisory Group for Tuberculosis (WHO 2009).

The next steps to take towards standardizing the MTT and resazurin colorimetric tests for clinical evaluation depend on phases III (performance) and phase IV (cost-effectiveness) clinical trials and involve comparisons with reference methods (Palomino et al. 2007). A study analyzing the cost-effectiveness of five DST methods, including the MTT assay, was performed in Peru and they found that all of the methods to identify MDR-TB in middle-income countries were cost-effective. Including the cost savings from reduced TB transmission, the MTT assay had the second-lowest gained cost per disability-adjusted life-year (\$95 US) (Acuna-Villaorduna et al. 2008). New colorimetric methods now enable the direct diagnosis of resistance in clinical specimens (Pai et al. 2006).

The MTT colorimetric method is an accurate, simple test that costs less than the automated phenotyping and genotyping methodologies. Colorimetric tests are a boon for settings with limited resources and make possible sustainable resistance surveillance, a critical step in global TB control.

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