Evaluation of Indirect Susceptibility Testing of *Mycobacterium tuberculosis* to the First- and Second-line, and Alternative Drugs by the Newer MB/BacT System

Angela Maria Werneck Barreto+, Joselba Borges Melo Araújo, Reginalda Ferreira de Melo Medeiros, Paulo César de Souza Caldas

Serviço de Laboratório, Centro de Referência Professor Hélio Fraga, Estrada de Curicica 2000, 22710-552 Rio de Janeiro, RJ, Brasil

In order to evaluate the Organon Teknika MB/BacT system used for testing indirect susceptibility to the alternative drugs ofloxacin (OFLO), amikacin (AMI), and rifabutin (RIF), and to the usual drugs of standard treatment regimes such as rifampin (RMP), isoniazid (INH), pyrazinamide (PZA), streptomycin (SM), ethambutol (EMB), and ethionamide (ETH), cultures of clinical specimens from 117 patients with pulmonary tuberculosis under multidrug-resistant investigation, admitted sequentially for examination from 2001 to 2002, were studied. Fifty of the Mycobacterium tuberculosis *cultures were inoculated into the gold-standard BACTEC 460 TB (Becton Dickinson) for studying resistance to AMI, RIF, and OFLO, and the remaining 67 were inoculated into Lowenstein Jensen (LJ) medium (the gold standard currently used in Brazil) for studying resistance to RMP, INH, PZA, SM, EMB, and ETH. We observed 100% sensitivity for AMI (80.8-100), RIF (80.8-100), and OFLO (78.1-100); and 100% specificity for AMI (85.4-100), RIF (85.4-100), and OFLO (86.7-100) compared to the BACTEC system. Comparing the results obtained in LJ we observed 100% sensitivity for RMP (80-100), followed by INH - 95% (81.8-99.1), EMB - 94.7% (71.9-99.7), and 100% specificity for all drugs tested except for PZA - 98.3 (89.5-99.9) at 95% confidence interval. The results showed a high level of accuracy and demonstrated that the fully automated, non-radiometric MB/BacT system is indicated for routine use in susceptibility testing in public health laboratories.*

Key words: tuberculosis - multidrug - resistant - laboratory diagnostics

Since 1979, Brazil has adopted a short-course chemotherapy which uses rifampin (RMP), isoniazid (INH), and pyrazinamide (PZA) for pulmonary and extrapulmonary tuberculosis as the first choice of treatment; if the short-course treatment fails, ethambutol (EMB), ethionamide (ETH), streptomycin (SM), and PZA are used for retreatment. When patients, after treatment with the standard regimes, are not cured and their mycobacteria show in vitro resistance to RMP, INH, and one additional drug, they constitute the group of multidrug-resistant patients (TBMDR). This definition was established by the Brazilian Consensus on Tuberculosis of 1997, and reiterated in the recently revised standards (Brasil 2002b).

In 2000, the TBMDR epidemiological surveillance system was created in order to determine the magnitude of the problem and to test the feasibility of treatment regimes to be adopted to control it, in a cooperative effort among the federal Ministry of Health and the state Secretaries of Health. In the context of treatment for TBMDR patients, new drugs associated with old tuberculostatics and drugs remaining from other regimes made it possible to implement an alternative therapeutic scheme.

+Corresponding author. Fax: +55-21-2441.4715. E-mail : crphf.labtb@funasa.gov.br
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Prominent among the new drugs is ofloxacin (OFLO), a quinolone; and among the older drugs now being used again are amikacin (AMI), an aminoglycoside; and terizidone (TZ), a cycloserine (CS) analog (Dalcolmo 2000).

The MB/BacT (Organon Teknika Corp., Durham, North Carolina, US), a fully automated continuous monitoring system, is used to detect growth of mycobacteria, based on readings by reflectometers sensitive to the CO_2 produced by the microorganisms. The system was evaluated on a preliminary basis for use not only as a method for primary isolation, but also to determine susceptibility to the drugs used for the standard treatment of tuberculosis, such as RMP, SM, EMB, INH, and PZA (Beer et al. 1997a, b).

The BACTEC 460 TB radiometric system (Becton Dickinson Diagnostic Instrument Systems, Sparks, Maryland, US) has been used as the gold standard by different investigators and has yielded results that were in close agreement with the MB/BacT (Beer et al. 1997a, b, Tortoli et al. 2000, Brunello & Fontana 2000).

Most public health laboratories in Brazil uses the proportion method according to Canetti et al. (1969), in Lowenstein Jensen (LJ) medium, to diagnose resistance to tuberculosis. This method is inexpensive and relatively simple although requires at least three weeks from cultured islolates to provide results.

The aim of this study was to evaluate the automated MB/BacT system to test the susceptibility of OFLO, AMI, and RIF, which are used as an alternative therapeutic scheme for TBMDR in Brazil, comparing the results with the BACTEC 460 TB radiometric system, and also to

evaluate the MB/BacT system susceptibility results to the drugs usually employed in the standardized treatment regimes of the Ministry of Health (RMP, INH, PZA, SM, EMB, and ETH), comparing it to the proportion method in LJ medium.

MATERIALS AND METHODS

Strains - A total of 117 strains obtained from individual patients at National Reference Laboratory (NRL) were studied. All of them were isolated from sputum and grew at 35/37 °C on LJ medium after three weeks incubation. The isolates were identified as *M. tuberculosis* complex using the AccuProbe® genetic probe (Gen Probe Incorporated, San Diego, California, US). Fifty isolates were MDR and recovered in the period of 2001 and 2002 from patientes under alternative treatment but with suspicion of failure. These strains were used to evaluate the ability of MB/BacT system for detect resistance to AMI, RIF, and OFLO using as the gold standard the BACTEC 460 TB radiometric system results. The remain 67 strains were recovered from patients suspected to be TBMDR and MB/BacT system for detect resistance to RMP, INH, PZA, SM, EMB, and ETH was assayed, comparing the results with the gold standard currently used in Brazil, the LJ proportion method. Susceptible standard strain *M. tuberculosis* H37Rv (ATCC 27294) was used for quality control of the all tests.

Drugs and concentrations used - The concentrations of these drugs in the LJ (Canetti et al. 1969) and the MB/BacT followed the recommendations of Beer et al. (1997b) and Barreto et al. (2002) in the proportions indicated in Table I. Except for RIF, which was obtained from Pharmacia (Spa, Italy), all the drugs were obtained from Sigma Chemical Co. (St. Louis, Missouri, US).

The drugs were diluted in sterile distilled water, except RMP, ETH, and RIF for which ethylene glycol was used, before being added to the culture medium under aseptic conditions.

Test in BACTEC 460 TB - The flasks were seeded into the BACTEC system as recommended by the manufacturer (Siddiqui 1996). The test inoculum was standardized from the active-phase bacterial growth in LJ medium, in distilled water with turbidity compared to tube no.1 on the McFarland scale; 0.1 ml volumes were inoculated into the system flasks, pre-prepared with drugs in the above-mentioned concentrations (Table I). The flasks were incubated at 35/37ºC; daily readings were taken and interpretation was done after comparing the changes in the growth index of the inoculated control with the test drug .

Test in LJ medium - The standardized inoculum from the same cultures used in the BACTEC was serially diluted to 10^{-6} and the dilutions of 10^{-3}, 10^{-5}, and 10^{-6} (control) were added to the culture media containing the drugs. The tubes were incubated at 35/37ºC and read after 21 days of incubation. The results was considered sensitive when the proportion of up to 1% and 10% (only for SM, ETH, and PZA) of colony growth was seen in the tubes containing drugs (Canetti et al. 1969).

Test in the MB/BacT system - Supplements were added to the flasks containing Middlebrook 7H9 liquid medium, as recommended by the manufacturer (Organon Teknika 1997). The test inoculum is described in the BACTEC 460TB test, with two decimal dilutions made. From the first dilution, 0.1 ml volumes were inoculated into flasks of the MB/BacT system containing the drugs in the above-mentioned concentrations (Table I), and one flask was inoculated with 0.1 ml of 1/100 suspension, used as a control. The test tubes with PZA, and PZA control were adjusted to pH 5.8. The flasks were incubated at 35/37ºC and monitored every 10 min to detect growth. The test was finalized when the 1/100 tube showed growth.

Analysis of the data - The following characteristics were determined for each drug in the tests as recommended by IUATLD (2001): sensitivity, the capacity of the test method correctly to identify resistant strains [=A/(A+C)]; specificity, the capacity of the test correctly to identify susceptible strains [= D/(B+D)]; positive predictive value (PPV), the ratio of strains classified as resistant by the test method which were truly resistant [=A/(A+B)]; and negative predictive value (NPV), the ratio of strains classified as susceptible by the test method which were truly susceptible [= D/(C+D)]. A is the number of strains found to be resistant by both methods, B is the number of strains found to be susceptible by the standard method and resistant by the test method, C is the number of strains found to be resistant by the standard method and susceptible by the test method, and D is the number of strains found to be susceptible by both methods.

TABLE I

Concentrations of the drugs in the culture media of each system, in µg/ml

Drugs	Lowenstein Jensen	MB/BacT	BACTEC
Ofloxacin	-	2	2
Amikacin	-	2	2
Rifabutin	-	1	1
Rifampin	40	2	-
Pyrazinamide	100	100	-
Ethionamide	20	1.25	-
Streptomycin	4	2	-
Isoniazid	0.2	0.2	-
Ethambutol	2	2.5	-

RESULTS

The results obtained by comparing the MB/BacT with the BACTEC system were in complete agreement showing 100% sensitivity and specificity at the 95% confidence interval for AMI (80.8-100 and 85.4-100), RIF (80.8-100, and 85.4-100), OFLO (78.1-100, and 86.7-100), respectively. Table II lists the results of the tests for susceptibility to the drugs RMP, INH, PZA, EMB, SM, and ETH with the MB/BacT system and with the proportion method in LJ medium. The best results for sensitivity were obtained with RMP - 100% (80-100), PZA - 100% (59.8-100), INH - 95% (81.8-99.1), and EMB - 94.7% (71.9-99.7) followed by ETH - 90.9 (57.1-99.5), and SM - 88.5 (65.5-98.2), and for

TABLE II

Accuracy of the MB/BacT results to the first-line drugs compared with the Lowenstein Jensen proportion method in 67 patients

Drugs	Sensitivity	Specificity	Predictive positive value	Predictive negative value
Rifampin	100 (80-100)	100 (90.6-100)	100 (80-100)	100 (90.6-100)
Isoniazid	95 (81.8-99.1)	100 (84.5-100)	100 (88.6-100)	93 (75.8-98.8)
Pyrazinamide	100 (59.8-100)	98.3 (89.5-99.9)	88.9 (50.7-99.4)	100 (92.1-100)
Streptomycin	88.5 (65.5-98.2)	100 (90.8-100)	100 (77.1-100)	96 (85.1-99.3)
Ethambutol	94.7 (71.9-99.7)	100(90.8-100)	100 (78.1-100)	98 (87.8-99.9)
Ethionamide	90.9 (57.1-99.5)	100 (92-100)	100 (65.5-100)	98.2 (89.4-99.9)

specificity with all drugs was 100%: RMP - (90.6-100), INH - (84.5-100), SM - (90.8-100), EMB - (90.8-100), ETH - (92-100), followed by PZA with 98.3% (89.5-99.9), at the 95% confidence interval. The spectrum of the 47 multidrug-resistant strains analized in this investigation was: 9 (19%) were resistant to all drugs tested (first-, second-line, and alternative drugs); 7 (14.8%) were resistant to 8 drugs; 5 (10.6%) were resistant to 7 drugs. In relation to drugs from the first- and second-line regimes 13 (27.6%) were resistant to all 6 drugs, 15 (31.9%) were resistant to 4 or 5 drugs, and the 19 (40.4%) remaining were resistant to 3 drugs. In our experiments all readings of the tests in LJ were finalized after 3 weeks of incubation, and those in the BACTEC system after 7 days. In the experiments with the MB/BacT, 80% of the tests were finalized in 10 days and the remainder in up to 13 days.

DISCUSSION

In Brazil, patients with multidrug-resistant tuberculosis are treated using OFLO, TZ, AMI, and RIF in combination with those drugs remaining from standardized treatment schemes which show susceptibility when tested in vitro. Since the establishment of the TBMDR surveillance program, in effect since 2000, about 1 100 patients have been enrolled as multidrug-resistant, and at the moment 800 patients are undergoing treatment (Brasil 2002a). The majority of the patients analized in this study came from Rio de Janeiro (34.72%) and could reflect the prevalence of the problem in that state.

The results of the antibiogram are fundamental to notification and adequate treatment of these patients. The automated methods developed to isolate mycobacteria, which use Middlebrook 7H9 liquid medium with a system for early detection of growth, greatly shorten the time required to obtain the antibiogram compared to the proportion method in LJ or Agar. In our experiments, all readings of the tests in the MB/BacT were made for a few more days than in BACTEC. This small difference in the final reading test time compared to the BACTEC was also observed by Tortoli et al. (2000). The availability of the susceptibility results obtained in our study is within the time limit suggested by Tenover et al. (1993) for rapid intervention in the disease transmission chain, acceptable for public health laboratories in this era of TBMDR: no more than 30 days should pass from collection of the clinical material to the time that the results become available.

Pfyffer et al. (1999) based in their multi-center study recommended the AMI, OFLO, and RIF concentrations for BACTEC method of 1, 2, and 0.5 µg/ml, respectively.

In our study the same concentrations were used except for RMP (2 µg/ml) in which Siddiqui's (1996) guidelines were followed.

Terizidone is an analog of cycloserine, which is used to treat TBMDR patients, however it was not included in this study. We do not routinely test CS because it shows wide variability in test readings, giving inconsistent results, as observed by Pfyffer et al. (1999).

The results obtained with the OFLO, AMI, and RIF showed a high degree of accuracy compared to the BACTEC, as did the results obtained with RMP, INH, and EMB in relation to the proportion method.

Some discordant results between the standard test and the test method could be attributed to the presence of borderline-resistant strains, mainly in relation to LJ tests where the final results depend on an accurate count of colonies. This was previously observed for INH (WHO 1997), and EMB (Madison et al. 2002).

Our observations are similar to those of Beer et al. (1997 a, b), Diaz-Infantes et al. (2000), Brunello and Fontana (2000), Tortoli et al. (2000), and Yew et al. (2001), using the same systems. The results of the tests in the MB/BacT indicate that this system is an accurate method for use in public health laboratories to deal with the workload of susceptibility-testing routines to diagnose multidrug-resistant tuberculosis mainly in those states that the prevalence of the disease is high and where the laboratories act as a reference facility to the national network.

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