Evaluation of the nitrate reductase assay for the rapid detection of resistance to first-line medications in *Mycobacterium tuberculosis* strains isolated from patients in a general hospital*

Avaliação do teste de nitrato redutase para a detecção rápida de resistência aos medicamentos de primeira linha em cepas de *Mycobacterium tuberculosis* isoladas de pacientes em um hospital geral

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Abstract

We compared the nitrate reductase assay with the proportion method, which is considered the gold standard, in 57 *Mycobacterium tuberculosis* strains isolated from patients treated at the Federal University of Minas Gerais *Hospital das Clínicas*, located in the city of Belo Horizonte, Brazil. For rifampin and isoniazid, the sensitivity, specificity, and accuracy of the nitrate reductase assay were all 100%, whereas they were 100%, 88.9%, and 66.7%, respectively, for streptomycin and 98.0%, 100%, and 98.2%, respectively, for ethambutol. The mean time to results was ten days. In the study sample, the nitrate reductase assay proved highly accurate and showed excellent concordance with the gold standard.

Keywords: Mycobacterium tuberculosis; Microbial sensitivity tests; Tuberculosis, multidrug-resistant; Nitrate reductase.

Resumo

Comparamos o teste de nitrato redutase com o método de proporções, considerado como padrão ouro, em 57 cepas de *Mycobacterium tuberculosis* isoladas de pacientes atendidos no Hospital das Clínicas da Universidade Federal de Minas Gerais, em Belo Horizonte (MG). A sensibilidade, a especificidade e a acurácia para rifampicina e isoniazida foram de 100% para todas, enquanto essas foram, respectivamente, de 88,9%, 66,7% e 96,5% para estreptomicina e de 98,0%, 100% e 98,2% para etambutol. A média de tempo para a obtenção dos resultados foi de dez dias. Na amostra estudada, o teste de nitrato redutase mostrou grande acurácia e excelente concordância com o padrão ouro.

Descritores: Mycobacterium tuberculosis; Testes de sensibilidade microbiana; Tuberculose resistente a múltiplos medicamentos; Nitrato redutase.

Tuberculosis remains one of the major causes of morbidity and mortality from infection in humans. The World Health Organization estimates that one third of the world population is infected with *Mycobacterium tuberculosis*, 9.4 million new cases of tuberculosis and 1.3 million deaths from tuberculosis occurring worldwide. The worldwide incidence is 140 cases per 100,000 population. [1] The emergence of multidrug-resistant (MDR) tuberculosis, defined as tuberculosis caused by strains resistant to the two first-line drugs (isoniazid and rifampin), and extensively drug-resistant (XDR) tuberculosis, defined as tuberculosis caused by strains resistant to the two abovementioned

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drugs, to at least one fluoroquinolone, and to at least one of the three injectable second-line drugs (amikacin, kanamycin, and capreomycin), makes for a worrisome scenario.[1] According to the World Health Organization, there were approximately 440,000 cases of MDR tuberculosis in 2008, those cases accounting for 3.6% of all cases of tuberculosis worldwide.[1] In Brazil, the numbers of new cases of MDR tuberculosis in 2002 and 2009 were 350 and 394, respectively.[2] In the last decade, several rapid molecular and automated methods for first-line drug susceptibility testing, such as BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 (Becton Dickinson, Sparks, MD, USA) and ESP II (TREK Diagnostics Systems Inc., Westlake, OH, USA), were described. However, such methods are expensive, especially in countries with limited resources. Rapid phenotypic methods, such as colorimetric methods—which are inexpensive and easy to perform and include the nitrate reductase assay—represent alternatives to the abovementioned methods.[3-7]

The objective of the present study was to evaluate the performance of the nitrate reductase assay in detecting resistance to first-line drugs in *M. tuberculosis* strains. Forty-three strains of *M. tuberculosis* were isolated from patients treated at the *Hospital das Clínicas da Universidade Federal de Minas Gerais* (HC-UFMG, Federal University of Minas Gerais *Hospital das Clínicas*), located in the city of Belo Horizonte, Brazil. We included strains isolated in the HC-UFMG Mycobacteriology Laboratory between January of 2009 and June of 2010. All strains were identified as *M. tuberculosis*, and drug susceptibility testing was performed at the Ezequiel Dias Foundation referral center in the state of Minas Gerais. We used 14 strains of *M. tuberculosis*, which are part of an international panel of drug susceptibility testing proficiency and were provided by the Ezequiel Dias Foundation. The susceptibility profiles of those strains were as follows: 1 XDR strain (no. 1008); 4 MDR strains (nos. 192, 193, 213, and 230); 3 strains resistant to isoniazid and streptomycin (nos. 1578, 1669, and 1670); 3 strains resistant to rifampin and streptomycin (nos. 445, 1695, and 1643); 1 strain resistant to rifampin only (no. 639); 1 strain resistant to isoniazid only (no. 551), and 1 strain resistant to streptomycin only (no. 202). The reference strain H37Rv (ATCC 27294) was used as a susceptible control.

The proportion method was performed in accordance with the study by Canetti et al.[8] Drug susceptibility testing by the nitrate reductase assay was performed with the following drugs: rifampin (Sigma Chemical, St. Louis, MO, USA); isonicotinic acid hydrazide (Sigma Chemical); ethambutol dihydrochloride (Sigma Chemical); and streptomycin sulfate (Sigma Chemical).

The nitrate reductase assay was performed in accordance with the protocol described by Angeby and the procedure manual of the Institute of Tropical Medicine Mycobacteriology Unit, located in Antwerp, Belgium.[9]

We prepared stock solutions of isoniazid, ethambutol, and streptomycin in water (at a concentration of 10,000 µg/mL), two dilutions having subsequently been prepared in order to achieve the critical concentration of the drugs on the medium. We prepared only one 4,000-µg/mL stock solution of rifampin in ethylene glycol, an adequate volume of the solution having been used in order to achieve the critical concentration on the medium. The critical concentrations of isoniazid, rifampin, streptomycin, and ethambutol on Löwenstein-Jensen (LJ) medium were 0.2 µg/mL, 40.0 µg/mL, 4.0 µg/mL, and 2.0 µg/mL, respectively.

The LJ medium was prepared in accordance with the manufacturer specifications (DIFCO™; Becton Dickinson), a solution of potassium nitrate having been added to the culture medium in order to achieve a final concentration of 1.0 mg/mL. We prepared two batches of LJ medium. One was prepared as described above in order to be used as a control, and the other was prepared by adding isoniazid, rifampin, streptomycin, and ethambutol.

The test was performed as follows:

- Each drug-containing LJ medium was inoculated with 200 µL of the bacterial suspension corresponding to a McFarland standard of 1.
- A total of 200 µL of the suspension (diluted at 1:10) was inoculated into three tubes containing drug-free LJ medium, which were used as growth controls.

After 7 days of incubation at 37°C, 500 µL of developing solution—50% (v/v) concentrated hydrochloric acid, 0.2% (w/v) sulfanilamide, and 0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride—were inoculated into one of the control (drug-free) tubes. If there was a change in

Table 1 - Comparison between the proportion method and the nitrate reductase assay in terms of the results of drug susceptibility of 57 *Mycobacterium tuberculosis* strains, as well as in terms of sensitivity and specificity.

Drug	Proportion method		Nitrate reductase assay		Sensitivity	Specificity
	Drug susceptibility of the strains	Strains	Drug-resistant strains	Drug-susceptible strains		
		n	n	n	%	%
Rifampin	Resistant	11	11	0	100	100
	Susceptible	46	0	46		
Isoniazid	Resistant	12	12	0	100	100
	Susceptible	45	0	45		
Streptomycin*	Resistant	9	8	1	88.9[a]	98.0[b]
	Susceptible	48	1	47		
Ethambutol**	Resistant	1	2	0	66.7[c]	100
	Susceptible	56	1	54		

[a]95% CI: 0.68-1.09; [b]95% CI: 0.94-1.02; [c]95% CI: 0.13-1.20; *Kappa = 0.87; **Kappa = 0.79.

color (from colorless to pink), the corresponding tubes with drug-containing LJ medium were inoculated with the developing solution. The control tube was discarded if there was no change in color, and the others were reincubated. The procedure was repeated 10 days later with the second control tube and, if necessary, 14 days later with the third control tube.

A strain was considered resistant if the color of the tube with drug-containing LJ medium became more intense than that of the control tube, being considered susceptible if there was no change in color in the tubes with drug-containing LJ medium or if the color of those tubes became lighter than that of the control tube. The present study was approved by the Research Ethics Committee of the UFMG.

Table 1 shows the results for the 57 *M. tuberculosis* strains tested. For rifampin and isoniazid, concordance between the proportion method and the nitrate reductase assay was 100%. Discrepancies were found for streptomycin and ethambutol. The mean time to results was 10 days.

There is a great need for rapid tests for the detection of drug resistance and for epidemiological surveillance of tuberculosis, especially in the context of a teaching hospital providing advanced medical care, where complex procedures such as solid organ transplantation and bone marrow transplantation are performed and where treatment is provided to patients with liver disease, kidney disease, diabetes mellitus, or other comorbidities, as well as to immunocompromised patients, principally those infected with HIV, all of which are factors that contribute to the development of MDR tuberculosis.

In the present study, the results obtained were similar to those reported in other studies,[4,10-12] demonstrating the excellent performance of the nitrate reductase assay for drug susceptibility testing. The method showed excellent concordance, accuracy, and speed in obtaining results, therefore representing an alternative for the diagnosis of tuberculosis in laboratories in countries with limited resources.

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