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Rapid detection of resistant tuberculosis by nitrate reductase assay performed in three settings in Brazil

Maria de Lourdes Shikama[1], Regina Ruivo Ferro E. Silva[2], Maria Conceição Martins[3], Carmen Maria Saraiva Giampaglia[3]*, Rosângela Siqueira Oliveira[3], Rosmari F. A. M. Silva[1], Paula Ferro E. Silva[2], Maria Alice da Silva Telles[3], Anandi Martin[4] and Juan Carlos Palomino[4]

[1]Regional Laboratory Sorocaba, Brazil; [2]Regional Laboratory Santo André, Brazil; [3]Instituto Adolfo Lutz Mycobacteria Laboratory, São Paulo, Brazil; [4]Mycobacteriology Unit, Institute of Tropical Medicine, Antwerpen, Belgium

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***Objectives*: To evaluate nitrate reductase assay (NRA) efficacy for streptomycin, isoniazid, rifampicin and ethambutol susceptibility testing of *Mycobacterium tuberculosis* strains.**

***Methods*: Results were generated by three laboratories: the Instituto Adolfo Lutz (IAL) Mycobacteria Reference Laboratory and two IAL Regional Laboratories in Santo André and Sorocaba, São Paulo State, Brazil. One hundred and twenty *M. tuberculosis* strains were simultaneously tested using NRA and the proportion method (PM), while 117 strains were tested using both NRA and BACTEC MGIT 960 (M960).**

***Results*: Repeatability analysis of NRA results showed rates of 100% for isoniazid and ethambutol and 97% for streptomycin and rifampicin susceptibility detection, representing substantial agreement. McNemar testing of the data also indicates that NRA and PM, as well as NRA and M960, do not differ significantly. On average, NRA results were available after 10 days.**

***Conclusions*: The data demonstrate that NRA is reliable for susceptibility testing of isoniazid and rifampicin, the two most important drugs for the treatment of tuberculosis. In addition, the reduction in the time necessary to obtain susceptibility results is of fundamental importance.**

Keywords: drug susceptibility testing, *M. tuberculosis* diagnostic, NRA, MGIT 960, proportion method

Introduction

Multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* (TB) pose a serious problem to TB control programmes.[1] The current spread of MDR TB demands new tools for rapid yet accurate drug susceptibility testing. In Brazil, the proportion method (PM) performed on Löwenstein–Jensen (LJ) medium is the most frequently used susceptibility test,[2] but it is characterized by a long delay before yielding results. Methods using liquid medium, such as the BACTEC MGIT 960 (M960) system, have reduced detection time but require costly reagents and equipment. Thus, it is essential to continue searching for a fast, reliable and inexpensive method of TB susceptibility testing.[3,4] The objective of this study was to evaluate the nitrate reductase assay (NRA) as a rapid alternative for determining *M. tuberculosis* susceptibility to rifampicin, isoniazid, streptomycin and ethambutol in three settings in Brazil.

Methods

During the initial phase of the study, a panel of 10 *M. tuberculosis* strains was tested in triplicate at the Instituto Adolfo Lutz (IAL) Mycobacteria Reference Laboratory to establish NRA repeatability. Each of the strains was tested for susceptibility to rifampicin, isoniazid, streptomycin and ethambutol by three technicians. The results of these repeatability tests were then analysed using Fleiss' kappa and agreement was interpreted as follows: <0.0, poor; 0.0–0.20, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial; and 0.81–1.00, nearly perfect agreement.[5]

*Corresponding author. Mycobacteria Department, Instituto Adolfo Lutz, Av Dr. Arnaldo, 351 São Paulo, Brazil. Tel: +55 11 3068 2986; Fax: +55 11 3085 3505; E-mail: hrgiampa@uol.com.br

In the next phase of the study, results were generated by three laboratories: the IAL Mycobacteria Reference Laboratory and two IAL Regional Laboratories in Santo André and Sorocaba, São Paulo State, Brazil. The tested *M. tuberculosis* strains were clinical isolates from TB patients, with 67 from IAL, 50 from Santo André and 53 from Sorocaba. The IAL and Sorocaba laboratories tested 120 strains simultaneously by NRA and PM, while the IAL and Santo André laboratories tested 117 strains simultaneously by NRA and M960.

PM has been described in detail previously.[2] This technique was conducted on LJ medium at the recommended critical concentrations of 4.0 mg/L for streptomycin, 0.2 mg/L for isoniazid, 40.0 mg/L for rifampicin and 2.0 mg/L for ethambutol. M960 was performed using standard procedures according to the MGIT Procedure Manual (Geneva).[6] Final drug concentrations were 1.0 mg/L for streptomycin, 0.1 mg/L for isoniazid, 1.0 mg/L for rifampicin and 5.0 mg/L for ethambutol. Meanwhile, NRA was performed as described by Ängeby *et al.*[4] with minor modifications. Briefly, LJ medium containing 1000 mg/L potassium nitrate was prepared. Antibiotics were then included at a concentration of 40.0 mg/L for rifampicin, 0.2 mg/L for isoniazid, 2.0 mg/L for ethambutol and 4.0 mg/L for streptomycin.

The inoculum was adjusted to a turbidity equivalent to that of a no. 1 McFarland standard and diluted 1:10 in sterile water. For each strain, 0.2 mL of the undiluted inoculum was added to an antibiotic-containing tube and 0.2 mL of the 1:10 inoculum dilution was added to three antibiotic-free tubes as growth controls. As an internal quality control, the ATCC (Manassas, VA, USA) strain of susceptible *M. tuberculosis* $H_{37}R_V$ (ATCC 27294) was used in each batch of media prepared with antibiotics.

After 7 days of incubation at 37°C, 0.5 mL of a reagent mixture consisting of one part 50% concentrated hydrochloric acid (HCl), two parts 0.2% sulfanilamide and two parts 0.1% *n*-1-naphthylethylenediamine dihydrochloride was added to one control tube. If any colour appeared, all tubes were developed using the reagent mixture; otherwise, the tubes were re-incubated and the procedure was repeated at days 10 and 14. An isolate was considered drug resistant if the colour that developed in the antibiotic-containing tube (pink to red or purple) was comparable to or darker than the colour appearing in the growth control.

The results obtained using the standard methods of M960 or PM were compared with those obtained using NRA. Statistical analysis of NRA performance was specifically conducted using McNemar's test.

Results

Repeatability analysis

Among 30 NRAs performed for streptomycin and rifampicin, respectively, only one of each group yielded an unexpected result. Meanwhile, there were no discrepant results when NRA was performed for isoniazid and ethambutol. Thus, the NRA repeatability rate was 100% for isoniazid and ethambutol and 97% for streptomycin and rifampicin. Statistical analysis of the results indicated substantial agreement, with a Fleiss' kappa value of 0.70.

Inter-laboratory agreement of NRA results as compared with PM results

Comparison of the results of testing 120 isolates by NRA with the results of analysing the same isolates by PM, which is considered the gold standard for susceptibility testing, is shown in Table 1. TB resistance to isoniazid, rifampicin, streptomycin and ethambutol indicated by NRA was 54.1%, 63.3%, 70.0% and 87.5%, respectively. Resistance values for PM were comparable: 54.1%, 63.3%, 62.5% and 85.8%, respectively. McNemar testing confirmed that NRA and PM susceptibility testing did not differ significantly ($P > 0.05$ for all drugs).

Table 1. Susceptibility results of NRA compared with those of PM for 120 *M. tuberculosis* strains

Drug	Susceptible by both methods	Resistant by both methods	Susceptible only by NRA	Susceptible only by PM	Percentage agreement
Isoniazid	54	64	1	1	98.3
Rifampicin	43	75	1	1	98.3
Streptomycin	35	74	1	10	90.8
Ethambutol	12	100	3	5	93.3

NRA, nitrate reductase assay; PM, proportion method.
No significant difference between the two tests ($P > 0.05$).

Table 2. Susceptibility results of NRA compared with those of M960 for 117 *M. tuberculosis* strains

Drug	Susceptible by both methods	Resistant by both methods	Susceptible only by NRA	Susceptible only by M960	Percentage agreement
Isoniazid	64	50	3	0	97.4
Rifampicin	32	81	3	1	96.6
Streptomycin	29	69	2	17	83.8
Ethambutol	10	100	2	5	94.0

NRA, nitrate reductase assay; M960, BACTEC MGIT 960.
No significant difference between the two tests ($P > 0.05$).

Inter-laboratory agreement of NRA results as compared with M960 results

Table 2 shows the results of 117 isolates tested by NRA as well as M960, which is also a standard method for susceptibility testing. TB resistance to isoniazid, rifampicin, streptomycin and ethambutol, as demonstrated by NRA, was 42.7%, 70.1%, 73.5% and 89.7%, respectively. For M960, these values were comparable: 45.3%, 71.8%, 60.7% and 87.2%, respectively. The results of NRA and M960 did not differ significantly, as calculated using McNemar's test ($P > 0.05$ for all drugs).

NRA results were available for 113 (66.5%) isolates at day 7, 38 (22.3%) isolates at day 10, and 19 (11.2%) isolates at day 14.

The time required to obtain drug susceptibility results from M960 ranged from 4 to 12 days, with a mean of 6.9 days. In the case of PM the results were available after 28 days.

Discussion

Any new method of TB drug susceptibility testing must provide repeatable results that are consistent with data obtained using reference methods. During the first stage of this study, we demonstrated the substantial repeatability of NRA for all drugs examined. Statistical analyses also indicated substantial agreement between NRA and PM results as well as between the outcomes of NRA and M960 testing for all anti-TB drugs studied. More than 95% agreement between NRA and PM and between NRA and M960 was determined for isoniazid and rifampicin, two of the most powerful drugs against TB.[1] For ethambutol susceptibility testing, NRA was also very effective, with an agreement with PM (93.3%) and M960 (94%) higher than the value (92%) proposed by the WHO.[7] However, despite agreement between NRA and reference methods (PM 90.8%, M960 83.8%) for streptomycin susceptibility testing, NRA performance was not acceptable since the expected level of agreement (92%) was not attained.

It is known that ethambutol and streptomycin susceptibility is difficult to test even by conventional methods. Thornsberry and Gavan[8] suggested that when evaluating a new method of drug susceptibility testing, the total of the very major errors (VMEs; false susceptible result) and major errors (MEs; false resistant result) should be $<5\%$.[8] As expected, the total of the VMEs and MEs was $>5\%$ for streptomycin and ethambutol when the results of NRA and both standard methods were compared. However, the most frequent error observed was MEs, representing a less serious problem than VMEs, which may result in the failure of anti-TB chemotherapy.

Our results are reliable according to Jorgensen's guidelines,[9] suggesting use of at least 35 isolates resistant to each drug to accurately verify and compare *M. tuberculosis* susceptibility tests. The number of tested strains was also sufficient to detect the real VMEs.

Traditional drug susceptibility testing, such as PM on solid media, is time consuming, while M960 is rapid but expensive. This study demonstrated an alternative method of susceptibility testing that is both inexpensive and easy to perform, characteristics that are particularly important in countries with a high prevalence of MDR TB and limited laboratory facilities. Our data indicate that NRA is reliable for testing isoniazid and rifampicin, the two most important drugs for TB treatment, in agreement with Martin *et al.*[10] On average, the results obtained by M960 and NRA were available in 10 days while those of PM were usually obtained only after 28 days. For streptomycin and ethambutol testing, further studies are required to optimize preparation of adequate inoculum and drug concentration in order to avoid strain misclassification.

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Transparency declarations

The authors have no conflicting interests to declare.

References

1. World Health Organization. *The WHO/International Union Against Tuberculosis and Lung Disease Global Project on Anti-tuberculosis Drug Resistance Surveillance. Antituberculosis Drug Resistance in the World, report no. 3*. Geneva, Switzerland: WHO, 2004.

2. Canetti G, Froman F, Grosset J *et al*. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. *Bull World Health Organ* 1963; **29**: 565–78.

3. Palomino JC, Martin A, Portaels F. Rapid drug resistance detection in *Mycobacterium tuberculosis*: a review of colourimetric methods. *Clin Microbiol Infect* 2007; **13**: 754–62.

4. Ängeby KAK, Klintz L, Hoffner SE. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. *J Clin Microbiol* 2002; **40**: 553–5.

5. Fleiss' kappa. http://en.wikipedia.org/wiki/Fleiss'_kappa (25 June 2009, date last accessed).

6. Siddiqi SH, Rüsch-Gerdes S. *MGIT Procedure Manual*. Geneva, Switzerland: Foundation for Innovative New Diagnostics, 2006.

7. Laszlo A, Rahman M, Espinal M *et al*. Quality assurance programme for drug susceptibility testing of *Mycobacterium tuberculosis* in the WHO/IUATLD Supranational Reference Laboratory Network: five rounds of proficiency testing, 1994–1998. *Int J Tuberc Lung Dis* 2002; **6**: 748–56.

8. Thornsberry C, Gavan L. Automated procedures for antimicrobial susceptibility tests. In: Lenette EH, Balows AL, Hausler WJ Jr *et al.*, eds. *Manual of Clinical Microbiology*. 3rd edn. Washington, DC: American Society for Microbiology, 1980; 491–4.

9. Jorgensen JH. Selection criteria for an antimicrobial susceptibility testing system. *J Clin Microbiol* 1993; **31**: 2841–4.

10. Martin A, Montoro E, Lemus D *et al*. Multicenter evaluation of the nitrate reductase assay for drug resistance detection of *Mycobacterium tuberculosis*. *J Microbiol Methods* 2005; **63**: 145–50.