

## Drug Susceptibility Testing of *Mycobacterium tuberculosis* by a Nitrate Reductase Assay Applied Directly on Microscopy-Positive Sputum Samples

Humberto R. Musa,<sup>1,2\*</sup> Marta Ambroggi,<sup>1,2</sup> Alejandro Souto,<sup>1,2</sup> and K. A. Kristian Ängeby<sup>3,4</sup>

Laboratorio de Micobacteriología, Hospital F. J. Muñiz, Buenos Aires, Argentina<sup>1</sup>; Instituto de Tisiología y Neumonología, Universidad Nacional de Buenos Aires, Argentina<sup>2</sup>; Department of Bacteriology, Swedish Institute for Infectious Disease Control, Solna, Sweden<sup>3</sup>; and Division of International Health, Karolinska Institute, Stockholm, Sweden<sup>4</sup>

Received 3 July 2004/Returned for modification 19 October 2004/Accepted 30 March 2005

**Current methods for drug susceptibility testing of *Mycobacterium tuberculosis* are either costly or slow. As the prevalence of multidrug-resistant strains increases, the need for fast, reliable, and inexpensive methods that can also be applied in settings with scarce resources is obvious. We evaluated a rapid colorimetric nitrate reductase assay (NRA) for direct drug susceptibility testing of *M. tuberculosis* directly from clinical sputum samples with positive microscopy results for acid-fast bacilli with more than 10 acid-fast bacilli per high-power field. We have saved valuable time by omitting the preisolation step. The sensitivity (ability to detect true drug resistance) and specificity (ability to detect true drug susceptibility) of the direct NRA, using the direct proportion method as the reference, were 100 and 100%, 93 and 100%, 76 and 100%, and 55 and 99% for rifampin, isoniazid, streptomycin, and ethambutol, respectively, when tested on *M. tuberculosis* strains present in 121 samples. The results were in most cases available in 14 days. The direct NRA could be used as a rapid, inexpensive, and accurate method to determine rifampin and isoniazid susceptibility directly from sputum. The technique might become a valid alternative to traditional methods, especially in low-income countries.**

There is no doubt that the global tuberculosis epidemic remains a problem for public health, and there is also an emergence of multidrug-resistant tuberculosis that is worsening the impact of this disease (10, 19). In order to fight this situation, we need a rapid and inexpensive drug susceptibility test to allow a prompt initiation of correct antibiotic therapy.

With traditional methods, such as the indirect or direct proportion method, it could take 3 to 4 weeks to obtain susceptibility results (4, 8). The time needed to obtain these results represents a potential danger to patients, health workers, and the community (11, 16). Using liquid medium-based culture systems such as the BACTEC 460 TB system (Becton Dickinson, Sparks, Md.), the Mycobacteria Growth Indicator Tube MGIT 960 (Becton Dickinson, Cockeysville, Md.), Bac/ALERT 3D (bioMérieux, Durham, NC), or ESP Culture System II (Trek Diagnostics, Inc., Westlake, Ohio) to perform indirect susceptibility tests (which require a pure culture of *Mycobacterium tuberculosis*), the results are available anywhere after 9 to 30 days. If susceptibility tests are performed directly from clinical specimens with these systems, the time needed for results is between 4 and 23 days (2, 3, 6, 15, 18). Although rapid, these methods require expensive substrates and equipment and are therefore not feasible in most developing countries. A recent article (1) described a rapid and inexpensive nitrate reductase assay (NRA) for the drug susceptibility testing (DST) of *M. tuberculosis*. This technique is based on the property of *M. tuberculosis* to reduce nitrate to nitrite, which is revealed as a color change of the culture media, using the

Griess method (1, 7). With the NRA, it was possible to obtain susceptibility results in 7 to 14 days, but since the test was performed indirectly (i.e., using *M. tuberculosis* strains isolated by culture), another 3 to 4 weeks needed for the isolation must be added to this time if the method is used in clinical praxis.

The aim of the present study was to evaluate the performance of a direct NRA, (i.e., with clinical sputum samples instead of bacterial isolates in order to shorten the turnaround time) in determining the susceptibilities to rifampin (RIF), isoniazid (INH), streptomycin (STR), and ethambutol (EMB) of *M. tuberculosis* strains in microscopy-positive clinical samples from patients with pulmonary tuberculosis in Buenos Aires.

### MATERIALS AND METHODS

**Setting.** Our laboratory is a mycobacteriology referral center for Municipals Hospitals of Buenos Aires city and its metropolitan area. It receives external quality control performed by the National Institute of Infectious Diseases "Carlos G. Malbran," Buenos Aires, Argentina.

**Samples and specimen processing.** From March 2002 to June 2003, 1,373 clinical specimens (only 1 per patient) submitted to our mycobacteriology laboratory were collected. The specimens were processed by Petroff digestion-decontamination method (12) and thereafter concentrated by centrifugation at 3,200 × g for 20 min. The supernatant was discarded, and a small portion of the sediment was used for preparation of microscopic slides, which were then stained with the Ziehl-Neelsen technique (8). Of these, we selected the samples that were microscopy positive with more than 10 acid-fast bacilli (AFB) per microscopic field (scored as "+++"). The rest of the remaining sediment was resuspended in 3 ml of sterile distilled water and then used to inoculate the culture medium used in both drug susceptibility tests (1.5 ml for direct NRA and 1.5 ml for the direct proportion method).

**Direct NRA DST.** The NRA was performed as described by Ängeby et al. (1) but with modifications regarding critical antibiotic concentrations, Griess reagent components, and inoculum. We used standard Löwenstein-Jensen (L-J) medium, with 1,000 µg of potassium nitrate (KNO<sub>3</sub>)/ml and with or without antimicrobial agents incorporated. The following critical concentrations were used:

\* Corresponding author. Mailing address: Department of Mycobacteriology, Hospital F. J. Muñiz, 1282, Buenos Aires, Argentina. Phone: (054) 11-49615015. Fax: (054) 11-49510244. E-mail: humbertomusa@hotmail.com.

0.2 µg/ml for INH, 20 µg/ml for RIF, 4.0 µg/ml for STR, and 3.0 µg/ml for EMB. The medium was prepared in 7-ml portions in 150-by-15.5-mm glass tubes with rubber plugs. Before NRA testing, part of the suspension was diluted 1:10 in sterile distilled water. For each strain, 0.2 ml of the undiluted suspension was inoculated into the tubes containing L-J medium with KNO<sub>3</sub> and the antibiotics, and 0.2 ml of the 1:10 dilution was inoculated into three drug-free tubes containing L-J medium with KNO<sub>3</sub> incorporated. The latter tubes served as growth controls. The tubes were incubated at 37°C, and 0.5 ml of Griess reagents was added to one drug-free control tube after 10 days. If any color change (strong or weak pink) could be seen, the corresponding antibiotic-containing tubes were also tested and the susceptibility results were read. If no color change was seen in the growth control tube, the remaining control tubes and the antibiotic tubes were reincubated. The procedure was then repeated at day 14 and, if needed, at day 18, using the last growth control tube. The Griess reagents were prepared as follows: reagent A consisted of 800 mg of sulfanilic acid plus 5 N acetic acid (100 ml), and reagent B consisted of 500 mg of α-naphthylamine plus 5 N acetic acid (100 ml) (14). Equal parts of reagents A and B were mixed shortly before use. The results were classified as negative (no color change) or positive (pink to violet). An isolate was considered resistant to a certain drug if there was a color change in the antibiotic tube in question greater than that in the 1:10-diluted growth control on the same day.

**Direct proportion method DST.** The technique was carried out on normal L-J medium according to the laboratory's standard procedure (4, 8). The medium was prepared in 7-ml portions in 150-by-15.5-mm glass tubes with rubber plugs, with or without antimicrobial agents incorporated. Critical concentrations of antituberculosis drugs were the same as were used for NRA. The critical proportion values were 10% for RIF and STR and 1% for INH and EMB. For each strain, part of the suspension was diluted 1:100, and 0.2 ml of the dilution was inoculated into two tubes of L-J medium without antibiotics. Then, 0.2 ml of the undiluted suspension was inoculated into the tubes containing L-J medium with antibiotics. The tubes were incubated at 37°C. Final susceptibility results were reported after 40 days following the laboratory's standard procedure, but preliminary results could be reported earlier for resistant strains, sometimes as early as after 20 days.

**Quality control.** For medium batch quality control we used the following *M. tuberculosis* reference strains: fully susceptible H37Rv (ATCC 27294) and INH-resistant H37Rv (ATCC 35822).

**Statistical analysis.** In the present study, the term sensitivity reflects the ability to detect a true drug resistance in a strain, whereas specificity reflects the ability to detect a true drug susceptibility. Statistical analysis of data was carried out by using McNemar's test (5b).

## RESULTS

We selected 130 of 1,373 sputum samples, of which smear results for AFB were positive with more than 10 AFB per high-power field (+++), for the present study. Of the 130 smear microscopy-positive results, 9 had a negative growth control as determined by the NRA method at day 18 and could thus not be used in the comparison. Eight of them were later shown to grow *M. tuberculosis* and were from patients that were already undergoing antituberculosis chemotherapy, and one of them grew *Mycobacterium avium*, which usually does not reduce nitrate. Consequently, 121 samples could be used for the comparison between the two methods.

Testing of the *M. tuberculosis* strains present in these 121 samples for their susceptibilities to INH, RIF, STR, and EMB gave an overall agreement of 98% between the NRA and proportion methods (473 of 484 individual susceptibility tests). The susceptibility results were completely concordant for 110 of 121 individual samples (91%). Full concordance was found in results for RIF. A fine association was also seen for INH, whereas the sensitivity in identifying STR and EMB resistance was less impressive (Table 1). The agreement of the direct NRA DST was excellent for RIF (kappa = 1 and  $P < 0.001$ ) and for INH, ( $\kappa = 0.958$  and  $P < 0.001$ ). The results were

TABLE 1. Susceptibility results of the direct NRA method compared to those of the direct proportion method for *M. tuberculosis* strains present in 121 microscopy-positive sputum samples<sup>a</sup>

Drug	Direct proportion method determination	Direct NRA results <sup>a</sup>			
		No.		%	
		Res	Susc	Sensitivity	Specificity
RIF	Res	11	0	100	
	Susc	0	110		100
INH	Res	13	1	93	
	Susc	0	107		100
STR	Res	16	5	76	
	Susc	0	100		100
EMB	Res	5	4	55	
	Susc	1	111		99
Total	Res	45	10	82	
	Susc	1	428		100

<sup>a</sup> Sensitivity reflects the ability to detect true resistance, while specificity reflects the ability to detect true susceptibility. Res, resistant; Susc, susceptible.

available in 10 days for 20 samples (16%), in 14 days for 58 samples (64%), and in 18 days for 43 samples (100%).

## DISCUSSION

Full agreement concerning the results of the direct NRA and proportion methods was seen for RIF. This is essential, for the reason that RIF, jointly with INH, is the most valuable antituberculosis agent. In addition, RIF resistance is mostly combined with INH resistance (17) (in our study 77%) and might therefore be used as a marker of multidrug resistance if resources are inadequate. The direct NRA was comparable to the direct proportion method regarding susceptibility testing of INH (sensitivity in detecting resistance, 93%; specificity, 100%). However, the sensitivities in detecting resistance to STR and EMB were far too low to be acceptable (76 and 55%, respectively), whereas the specificities (abilities to find true drug susceptibility) were excellent (100%) for both these drugs. This seemingly systematic discordance might possibly be overcome by adjusting the critical drug concentrations used in the NRA test, although the susceptibility of *M. tuberculosis* to STR and EMB is more complicated to determine also by recommended standard methods (19). Our results for RIF and INH susceptibility were similar to those obtained previously by an indirect NRA-based assay (1, 5a, 9, 13, 20). Moreover, one of these studies also reported concordant results for susceptibility testing of STR and EMB (20).

For practical and economic reasons, the direct proportion method was used as the "gold standard" in our study instead of the internationally more commonly applied indirect proportion method. The direct method has been used as the standard method for DST of *M. tuberculosis* in our laboratory for more than 30 years, and it has been shown to give adequate results as long as the number of bacilli in the sputum is not too low (4).

Since the NRA method makes use of the recognition of nitrate reduction as a sign of growth, results are acquired

earlier than by eye examination of colonies. The time needed to complete the NRA DST directly from a decontaminated sputum sample with a positive AFB (+++) smear was in most cases available in 14 days (range, 10 to 18 days); which comply with the Centers for Disease Control and Prevention turnaround times for susceptibility testing of *M. tuberculosis*, expected for a rapid test (5). With the direct proportion method (4, 8), which was used as the reference method, it took 40 days to get the final results, even though preliminary results could be obtained earlier for some resistant strains. In previously published NRA studies (1, 5a, 9, 20) the results were available after 7 to 14 days, and when Syre et al. (13) applied the NRA test with liquid medium most results were available after 5 days. However, in these three studies NRA tests were performed indirectly, i.e., with cultured material instead of clinical samples, and then the 3 to 4 weeks it takes to obtain an isolate by culture must be added to the total turnaround time. We have earlier experimented with the direct NRA technique using sputum samples with a lower number of bacilli, i.e., 1 to 10 AFB per high-power field (++), but the majority of tests were ready only after 18 days (data not shown). Possibly, a liquid medium-based NRA (13) could reduce this time if applied directly on smear microscopy-positive samples.

Today's methods for DST are either costly, as are automatic culture systems, or slow, as are methods based on culture on solid media. Other low-cost methods have been proposed, such as the MTT or reazurin assays (9). They have been shown to be comparable to the NRA, at least for RIF when performed indirectly (9). However, they make use of liquid medium in a microplate format and that makes the techniques more complex and might also constitute a biohazard. Instead, the NRA utilizes standard solid L-J medium, although with KNO<sub>3</sub> incorporated, and (apart from being safer), it could therefore be easily adopted in any culture laboratory.

Even though more studies are needed to further assess the accuracy and applicability of the method, the direct nitrate reductase assay has the potential to become an inexpensive alternative for DST where resources are scarce, especially for INH and RIF, the two most important antituberculosis drugs. It might then be used either as a rapid screening tool alone or in combination with other methods. We believe that in the future this technique could improve the performance of tuberculosis control programs in low-income countries.

#### ACKNOWLEDGMENTS

We thank Sven E. Hoffner at the Department of Bacteriology, Swedish Institute for Infectious Disease Control, for valuable comments on the manuscript.

We have no financial conflicts of interest or other relationships that are relevant to this study.

#### REFERENCES

1. Angeby, K. A. K., L. Klintz, and S. E. Hoffner. 2002. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. *J. Clin. Microbiol.* **40**:553–555.
2. Angeby, K. A. K., J. Werngren, J. C. Toro, G. Hedstrom, B. Petrini, and S. E. Hoffner. 2003. Evaluation of the BacT/ALERT 3D system for recovery and drug susceptibility testing of *Mycobacterium tuberculosis*. *Clin. Microbiol. Infect.* **9**:1148–1152.
3. Bemer, P., F. Palicova, S. Rüscher-Gerdes, H. B. Drugeon, and G. E. Pfyffer. 2002. Multicenter evaluation of fully automated BACTEC Mycobacteria Growth Indicator Tube 960 system for susceptibility testing of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **40**:150–154.
4. Canetti, G., N. Rist, and J. Grosset. 1963. Mesure de la sensibilité du bacille tuberculeux aux drogues antibacillaires par la méthode des proportions. *Revue Tuberc. Pneumol.* **T27** 23:217–272.
5. Centers for Disease Control and Prevention. 2002. TB elimination cooperative agreement. Centers for Disease Control and Prevention, Atlanta, Ga.
- 5a. Coban, A. Y., A. Birinci, B. Ekinci, and B. Durupinar. 2004. Drug susceptibility testing of *Mycobacterium tuberculosis* with nitrate reductase assay. *Int. J. Antimicrob. Agents* **24**:304–306.
- 5b. Fleiss, J. L. 1981. Statistical methods for rates and proportions, 2nd ed. Wiley Interscience, New York, N.Y.
6. Goloubeva, V., M. Lecocq, P. Lassowsky, F. Matthys, F. Portaels, and I. Bastian. 2001. Evaluation of mycobacteria growth indicator tube for direct and indirect drug susceptibility testing of *Mycobacterium tuberculosis* from respiratory specimens in a Siberian prison hospital. *J. Clin. Microbiol.* **39**:1501–1505.
7. Golyshvskaia, V. I., A. A. Korneev, L. N. Chernousova, L. G. Selina, T. A. Kazarova, T. D. Grishina, S. G. Safonova, V. A. Puzanov, G. M. Nikolaeva, and N. I. Fadeeva. 1996. New microbiological techniques in diagnosis of tuberculosis. *Probl. Tuberc.* **6**:22–25.
8. Kent, P. T., and G. P. Kubica. 1985. Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, Ga.
9. Lemus, D., A. Martin, E. Montoro, F. Portaels, and J. C. Palomino. 2004. Rapid alternative methods for detection of rifampicin resistance in *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* [Online.] <http://jac.oupjournals.org/>.
10. Pablos-Mendez, A., M. C. Raviglione, A. Laszlo, N. Binkin, H. L. Rieder, F. Bustreo, D. L. Cohn, C. S. Lambregts-van Weezenbeeck, S. J. Kim, P. Chaulet, P. Nunn, et al. 1998. Global surveillance for antituberculosis-drug resistance, 1994–1997. *N. Engl. J. Med.* **338**:1641–1649.
11. Palmero, D., V. Ritacco, M. Ambroggi, M. Natiello, L. Barrera, L. Capone, A. Dambrosi, M. di Lonardo, N. Isola, S. Poggi, M. Vescovo, and E. Abbate. 2003. Multidrug-resistant tuberculosis in HIV-negative patients, Buenos Aires, Argentina. *Emerg. Infect. Dis.* **9**:965–969.
12. Pan American Health Organization. 1973. Manual de bacteriología de la TBC: técnicas y procedimientos básicos. OPS, Washington, D.C.
13. Syre, H., S. Phyu, P. Sandven, B. Bjorvatn, and H. M. S. Grewal. 2003. Rapid colorimetric method for testing susceptibility of *Mycobacterium tuberculosis* to isoniazid and rifampin in liquid Culture. *J. Clin. Microbiol.* **41**:5173–5177.
14. Tison F., and B. Carboneille. 1972. Recherche, isolament et etude du bacille tuberculeux et des autres mycobacteries eu pratique courante, p. 239–240. Crohan et Roques, Paris, France.
15. Tortoli, E., M. Benedetti, A. Fontanelli, and T. Simonetti. 2002. Evaluation of automated BACTEC MGIT 960 system for testing susceptibility of *Mycobacterium tuberculosis* to four major antituberculous drugs: comparison with the radiometric BACTEC 460TB method and the agar plate method of proportion. *J. Clin. Microbiol.* **40**:607–610.
16. Turett, G. S., E. E. Telzak, L. V. Torian, S. Blum, D. Alland, I. Weisfuse, and B. A. Fazzal. 1995. Improved outcomes for patients with multidrug-resistant tuberculosis. *Clin. Infect. Dis.* **21**:1238–1244.
17. Varedzisz, B. P., J. Grosset, I. De Kantor, J. Crofton, A. Laszlo, M. Felten, M. C. Raviglione, and A. Kochi. 1994. Drug-resistant tuberculosis: laboratory issues: World Health Organization recommendations. *Tuberc. Lung Dis.* **75**:1–7.
18. Williams-Bouyer, N., R. Yorke, H. I. Lee, and G. L. Woods. 2000. Comparison of the BACTEC MGIT 960 and ESP culture system II for growth and detection of mycobacteria. *J. Clin. Microbiol.* **38**:4167–4170.
19. World Health Organization. 2000. The W.H.O./IUATLD global project on anti-tuberculosis drug resistance surveillance. Anti-tuberculosis drug resistance in the world, report no. 2: prevalence and trends. World Health Organization, Geneva, Switzerland.
20. Sethi, S., S. Sharma, S. K. Sharma, S. K. Meharwal, S. K. Jindal, and M. Sharma. 2004. Drug susceptibility of *Mycobacterium tuberculosis* to primary antitubercular drugs by nitrate reductase assay. *Indian J. Med. Res.* **120**:468–471.