

Evaluation of Direct Detection of *Mycobacterium tuberculosis* Rifampin Resistance by a Nitrate Reductase Assay Applied to Sputum Samples in Cotonou, Benin[∇]

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The aim of this study was to evaluate a nitrate reductase assay (NRA) performed on smear-positive sputa for the direct detection of rifampin resistance in *Mycobacterium tuberculosis*. A total of 213 smear-positive sputa with a positivity score of 1+ or more (>1 acid-fast bacillus per field by fluorescence microscopy) were used in the study. The samples were decontaminated using the modified Petroff method, and portions of the resulting suspension were used to perform the NRA. The NRA results were compared with the reference indirect proportion method for 177 specimens for which comparable results were available. NRA results were obtained at day 10 for 15 specimens (9%), results for 88 specimens (50%) were obtained at day 14, results for 66 specimens (37%) were obtained at day 18, and results for the remaining 8 specimens (4%) were obtained at day 28. Thus, 96% of NRA results were obtained in 18 days. Of the 177 specimens, there was only one discrepancy (susceptible according to the NRA and resistant according to the indirect proportion method). NRA is simple to perform and provides a rapid, accurate, and cost-effective means for the detection of rifampin resistance in *M. tuberculosis* isolates.

Tuberculosis (TB) remains a major public health problem worldwide. In recent years, the incidence of TB has been rising. There is also an emergence of multidrug-resistant (MDR) tuberculosis (defined as resistance to at least rifampin [RMP] and isoniazid) that is worsening the impact of this disease (1, 4, 21).

Previous studies suggest that RMP resistance could be a surrogate marker for multidrug resistance, especially in settings with a high prevalence of drug resistance (8, 18). Therefore, the detection of resistance to this major anti-TB drug is essential for the optimal control of TB.

Conventional tests for the detection of drug resistance require several weeks to yield results (5). Recently, alternative rapid methods have been developed (13). Among them, the nitrate reductase assay (NRA) on Löwenstein-Jensen (LJ) medium is simple to perform and has been successfully implemented in low-income countries (7, 13, 14). This test is based on the ability of *Mycobacterium tuberculosis* to reduce nitrate to nitrite, which is revealed as a color change in the culture medium, using the Griess method (10). The indirect (using isolates) NRA yields results in less than 14 days but requires an initial 3 to 4 weeks for cultivation of the isolate (7, 13, 14).

So far, only a few studies have evaluated the NRA applied directly to sputum samples. The results of these studies (which were done in high-incidence settings) were concordant with results obtained by the reference method (15, 17). However, to our knowledge, no direct NRA study has been done in a setting with low resistance prevalence or in Africa, where there is

potentially a high frequency of nitrate reductase-negative *M. tuberculosis* complex strains (11, 12).

The objective of this study was to evaluate NRA applied directly to smear-positive sputa in the West African country of Benin in order to rapidly detect *M. tuberculosis* complex resistance to RMP.

MATERIALS AND METHODS

Setting. Our laboratory is the mycobacteriology reference laboratory for Benin. It receives samples from patients living in Cotonou and suburbs and also from neighboring countries. External quality control of the laboratory is performed by the Institute of Tropical Medicine (ITM), Antwerp, Belgium.

Specimen processing. From January to November 2006, a total of 213 smear-positive sputa from new and previously treated patients, with a positivity score of 1+ or more (>1 acid-fast bacillus [AFB] per field by fluorescence microscopy [magnification, ×250]) were collected (20). The samples (one per patient) were processed using the modified Petroff digestion decontamination method (19). The sediment was resuspended in 1 ml of sterile distilled water, and portions were inoculated into NRA drug susceptibility testing medium and into an LJ tube without nitrate, which was later used for the indirect proportion method (IPM).

Direct NRA drug susceptibility test. The NRA was performed as described previously by Musa et al. (15). We used standard LJ medium with 1,000 µg of potassium nitrate (KNO₃)/ml and with or without RMP. For LJ medium with RMP, the critical concentration of 40 µg/ml was used. Before NRA, part of the decontaminated suspension was diluted 1:10 in sterile distilled water. For each specimen, 0.2 ml of the undiluted suspension was inoculated into LJ medium containing KNO₃ and RMP, and 0.2 ml of the 1:10 dilution was inoculated into four drug-free LJ medium tubes containing KNO₃. The tubes were incubated at 37°C.

The assay was performed as described previously by Angeby et al. (3). After 10 days of incubation, 0.5 ml of freshly prepared reagent mixture (1 part 50% concentrated hydrochloric acid, 2 parts 0.2% sulfanilamide, and 2 parts 0.1% *n*-1-naphthylethylenediamine dihydrochloride) was added to one drug-free tube. If any color appeared, the tube with RMP was developed with the reagent mixture. Otherwise, the other tubes were reincubated, and the procedure was repeated at day 14, day 18, and finally at day 28. An isolate was considered to be resistant if there was a color change in the RMP tube equal or greater than that in the 1:10-diluted growth control. An isolate was considered to be susceptible if

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TABLE 1. Drug susceptibility testing of 177 specimens by NRA and IPM

NRA	No. of specimens with IPM result of:		%				Agreement (kappa value)
	Resistant	Susceptible	Sensitivity	Specificity	Negative predictive value	Positive predictive value	
Resistant	7	0					
Susceptible	1	169					
Result			87.5	100	98.6	100	0.93

there was no color change or a color change less than that in the 1:10-diluted growth control. NRA was considered to be invalid if the nitrate reaction was negative in the drug-free medium at day 28 despite the presence of colonies.

IPM. An LJ tube without nitrate was inoculated with 0.2 ml of undiluted decontaminated suspension and incubated for up to 42 days. Isolates from this tube were used for IPM blindly performed using LJ medium according to standard procedures, with a critical RMP concentration of 40 µg/ml (6).

Quality control. For each batch of medium, internal quality control was done using the fully susceptible *M. tuberculosis* strain H37Rv and a known MDR *M. tuberculosis* isolate. External quality control was done at the ITM on one-quarter of isolates of the study and yielded complete agreement.

Sequencing. On the isolate with discordant result, *rpoB* gene sequencing was performed at the ITM as described previously (16).

Data analysis. The performance of the NRA in comparison with that of the IPM was evaluated in terms of sensitivity (ability to detect true resistance) and specificity (ability to detect true susceptibility). The agreement between the two methods, estimated by the kappa value, was interpreted as follows: <0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; ≥0.81, excellent (1). Predictive values were calculated by using the prevalence of RMP resistance in all TB cases in Cotonou (2; D. Affolabi, unpublished data).

RESULTS

Of the 213 specimens processed, the NRA was completed for 177 of them. The remaining 36 specimens were culture negative (25 samples, 2 of which had an AFB score of 3+, 16 of which had a score of 2+, and 7 of which had a score of 1+), were contaminated (five samples), or gave invalid results (six samples).

AFB results for the 177 sputum specimens were 1+ for 32 (18%) specimens, 2+ for 110 (62%) specimens, and 3+ for 35 (20%) specimens.

NRA results were obtained at day 10 for 15 specimens (9%), and results were obtained for 88 (50%) specimens at day 14, 66 (37%) specimens at day 18, and the remaining 8 (4%) specimens at day 28. Thus, 96% of NRA results were obtained in 18 days.

The comparison of NRA and IPM (Table 1) showed a discrepancy for one specimen, which was susceptible to RMP by NRA but resistant by IPM. For this specimen, only IPM was repeated and showed the same result. It was not possible to retest the sputum specimen with the NRA since the IPM result was available after 10 weeks.

In this isolate, *rpoB* sequencing showed a deletion of codon 518 of the *rpoB* gene. Based on these data, the sensitivity and specificity were 87.5% and 100%, respectively. Moreover, using the prevalence of RMP resistance in our setting (10%), negative predictive values and positive predictive values were 98.6% and 100%, respectively (Affolabi, unpublished).

DISCUSSION

To our knowledge, this is the first evaluation of the direct NRA in Africa, where some previous studies suggested a high frequency of nitrate reductase-negative *M. tuberculosis* leading to an invalid NRA result (11, 12). In our case, only 3% of specimens yielded an invalid result. The six isolates cultivated from these specimens were all identified as belonging to the *M. tuberculosis* complex. This low frequency of nitrate-negative *M. tuberculosis* was also found previously by Martin et al. (14). For these specimens, another drug susceptibility test is warranted.

NRA is simple to perform and does not require additional equipment and reagents besides those used for IPM. Moreover, biosafety problems are limited because of the use of solid medium. The NRA was therefore easy to implement in our laboratory.

In this study, 96% of the results were obtained in 18 days. This represents an advantage, since indirect methods require 21 to 28 days for primary isolation prior to performing the drug sensitivity test and about 28 to 42 additional days for the final results. Moreover, NRA can be performed on smear-positive sputa with low positivity of AFB (1+ or 2+). In our study, these sputa represented 80% of the samples. Therefore, it is not necessary to perform the NRA in highly-smear-positive sputa (3+), as for the direct proportion method (15). Moreover, the turnaround time for the NRA is lower than that of the direct proportion method, which can take up to 40 days to give a final result (15). As shown in Table 1, there was an excellent concordance between the NRA and the IPM. In addition, the specificity was excellent (100%). However, out of the eight isolates found to be resistant by the IPM, one was found to be sensitive by the NRA. Therefore, the sensitivity of the test was 87.5%. The use of sequencing in this study confirmed that the NRA gave a truly falsely susceptible result. The observed AAC deletion at codon 518 is rare but was previously described as being associated with RMP resistance (22). The AFB smear result of the discordant specimen was 2+. Solis et al., who also tested low- and high-degree AFB-positive sputa (17), also found some falsely susceptible specimens, contrary to data reported previously by Musa et al., who tested only highly positive sputa and did not find any discordant results (15). Further studies are needed to improve the NRA for drug susceptibility testing of RMP on low-smear-positive sputa. Other antibiotics could also be tested in our setting to assess the usefulness of this rapid method. Moreover, although nitrate-positive mycobacteria other than TB occur less frequently in sputum, these species can be identified by testing

their susceptibility to *p*-nitrobenzoic acid. The direct NRA can therefore be complemented with *p*-nitrobenzoic acid to confirm the presence of *M. tuberculosis* complex isolates (9).

In conclusion, the NRA is simple to perform and provides a rapid, accurate, and cost-effective means for the detection of RMP resistance in *M. tuberculosis*. However, further studies are needed to improve its sensitivity in our setting.

REFERENCES

- Altman, D. G. 1999. Practical statistics for medical research. Chapman & Hall/CRC, London, United Kingdom.
- Altman, D. G., and J. M. Bland. 1994. Diagnostic tests 2: predictive values. *BMJ* **309**:102.
- Angeby, K. A., 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.
- Aziz, M. A., A. Wright, A. Laszlo, A. De Muynck, F. Portaels, A. Van Deun, C. Wells, P. Nunn, L. Blanc, M. Raviglione, and WHO/International Union against Tuberculosis and Lung Disease Global Project on Anti-Tuberculosis Drug Resistance Surveillance. 2006. Epidemiology of antituberculosis drug resistance (the Global Project on Anti-Tuberculosis Drug Resistance Surveillance): an updated analysis. *Lancet* **368**:2142–2154.
- Canetti, G., W. Fox, A. Khomenko, H. T. Malher, N. K. Menon, D. A. Mitchison, N. Rist, and N. A. Smelev. 1969. Advances in techniques of testing mycobacterial drug sensitivity and the use of sensitivity tests in tuberculosis control programmes. *Bull. W. H. O.* **41**:21–43.
- Canetti, G., N. Rist, and J. Grosset. 1963. Mesure de la sensibilité du bacille tuberculeux aux drogues antibacillaires par la méthode des proportions. *Rev. Tuberc. Pneumol.* **27**:217–272.
- 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.
- Gamboa, F., P. J. Cardona, J. M. Manterola, J. Lonca, L. Matas, E. Padilla, J. R. Manzano, and V. Ausina. 1998. Evaluation of a commercial probe assay for detection of rifampin resistance in *Mycobacterium tuberculosis* directly from respiratory and non respiratory clinical samples. *Eur. J. Clin. Microbiol. Infect. Dis.* **17**:189–192.
- Giampaglia, C. M., M. C. Martins, V. T. Inumaru, I. V. Butuem, and M. A. Telles. 2005. Evaluation of a rapid differentiation test for the *Mycobacterium tuberculosis* complex by selective inhibition with *p*-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide. *Int. J. Tuberc. Lung Dis.* **9**:206–209.
- Golyshevskaja, 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. Tuberk.* **6**:22–25. (In Russian.)
- Hoffner, S. E., S. B. Svenson, R. Norberg, F. Dias, S. Ghebremichael, and G. Kallenius. 1993. Biochemical heterogeneity of *Mycobacterium tuberculosis* complex isolates in Guinea-Bissau. *J. Clin. Microbiol.* **31**:2215–2217.
- Kallenius, G., T. Koivula, S. Ghebremichael, S. E. Hoffner, R. Norberg, E. Svensson, F. Dias, B. I. Marklund, and S. B. Svenson. 1999. Evolution and clonal traits of *Mycobacterium tuberculosis* complex in Guinea-Bissau. *J. Clin. Microbiol.* **37**:3872–3878.
- Lemus, D., E. Montoro, M. Echemendia, A. Martin, F. Portaels, and J. C. Palomino. 2006. Nitrate reductase assay for detection of drug resistance in *Mycobacterium tuberculosis*: simple and inexpensive method for low-resource laboratories. *J. Med. Microbiol.* **55**:861–863.
- Martin, A., J. C. Palomino, and F. Portaels. 2005. Rapid detection of ofloxacin resistance in *Mycobacterium tuberculosis* by two low-cost colorimetric methods: resazurin and nitrate reductase assays. *J. Clin. Microbiol.* **43**:1612–1616.
- Musa, H. R., M. Ambroggi, A. Souto, and K. A. Angeby. 2005. Drug susceptibility testing of *Mycobacterium tuberculosis* by a nitrate reductase assay applied directly on microscopy-positive sputum samples. *J. Clin. Microbiol.* **43**:3159–3561.
- Rigouts, L., O. Nolasco, P. de Rijk, E. Nduwamahoro, A. Van Deun, A. Ramsay, J. Arevalo, and F. Portaels. 2007. Newly developed primers for comprehensive amplification of the *rpoB* gene and detection of rifampin resistance in *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **45**:252–254.
- Solis, L. A., S. S. Shin, L. L. Han, F. Llanos, M. Stowell, and A. Sloutsky. 2005. Validation of a rapid method for detection of *M. tuberculosis* resistance to isoniazid and rifampin in Lima, Peru. *Int. J. Tuberc. Lung Dis.* **9**:760–764.
- Traore, H., K. Fissette, I. Bastian, M. Devleeschouwer, and F. Portaels. 2000. Detection of rifampicin resistance in *Mycobacterium tuberculosis* isolates from diverse countries by a commercial line probe assay as an initial indicator of multidrug resistance. *Int. J. Tuberc. Lung Dis.* **4**:481–484.
- World Health Organization. 1998. Laboratory services in tuberculosis control. Part III. Culture. WHO, Geneva, Switzerland.
- World Health Organization. 1998. Laboratory services in tuberculosis control. Part II. Microscopy. WHO, Geneva, Switzerland.
- World Health Organization. 2004. The WHO/IUATLD global project on antituberculosis drug resistance surveillance. Antituberculosis drug resistance in the world, report no. 3. WHO/HTM/TB/2005.349. WHO, Geneva, Switzerland.
- Yang, B., H. Koga, H. Ohno, K. Ogawa, M. Fukuda, Y. Hirakata, S. Maesaki, K. Tomono, T. Tashiro, and S. Kohno. 1999. Relationship between antimycobacterial activities of rifampicin, rifabutin and KRM-1648 and *rpoB* mutations of *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* **42**:621–628.