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Multicentre study of nitrate reductase assay for rapid detection of rifampicin-resistant *M. tuberculosis*

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SUMMARY

SETTING: Four regional laboratories belonging to the Mycobacteria Reference Laboratory of São Paulo State, Brazil.

OBJECTIVE: To evaluate the nitrate reductase assay (NRA) for rifampicin (RMP) susceptibility testing of *Mycobacterium tuberculosis* directly from clinical sputum samples of patients with pulmonary tuberculosis (TB).

DESIGN: Performance of the NRA for detection of *M. tuberculosis* susceptibility to RMP was evaluated with 210 clinical sputum samples received by the participating laboratories during 2005 and 2006 and compared with the results of the direct proportion method.

RESULTS: Susceptibility tests performed using the NRA and the direct proportion method showed 204 susceptible isolates and six isolates resistant to RMP by both methods. NRA sensitivity and specificity for RMP was 100%. The NRA results of susceptibility tests against RMP were available in 15 days for 87% of the samples. The results showed that NRA may yield a rapid answer in determining resistance for the majority of sputum samples with smear results reported as 3+ and 2+.

CONCLUSION: The results demonstrate the feasibility of NRA for screening resistant strains in sputum samples from patients with pulmonary TB. NRA represents a rapid and low-cost alternative method that might be used in microbiological laboratories where resources are scarce.

KEY WORDS: *Mycobacterium tuberculosis*; susceptibility; nitrate reductase; proportion method

MULTIDRUG-RESISTANT (MDR) tuberculosis (TB), defined as resistance to at least isoniazid and rifampicin (RMP), is a threat to TB control.[1] The rapid detection of RMP resistance, which is an important marker of multidrug resistance, may be useful from the clinical point of view, as it identifies patients who do not respond to chemotherapy; this could also help reduce disease transmission. The screening of isolates for resistance to RMP can therefore be clearly valuable in settings where poor resources make it impossible to conduct ordinary drug susceptibility testing (DST) using all first-line drugs. The development of an alternative rapid and inexpensive method, which can be used directly from sputum, is important and beneficial for the National Tuberculosis Control Programmes, and would enable rapid monitoring of patients who may have MDR-TB.[2]

The conventional tests used in the detection of *Mycobacterium tuberculosis* susceptibility to drugs are lengthy and laborious. Automated systems enable rapid testing, but the costs make them impossible to apply in developing countries.[3]

The nitrate reductase assay (NRA), with chemical foundation in the Griess method,[4] has recently been described to evaluate *M. tuberculosis* drug susceptibility using Löwenstein-Jensen (LJ) medium.[5–9] The method is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite by the action of the nitrate reductase enzyme, which is indicated by the development of a dark rose to purple-rose colour after addition of the reagent. The NRA enables the rapid detection of bacterial growth.

The objective of this multicentre study was to evaluate the performance of the NRA in determining the susceptibility of *M. tuberculosis* to RMP directly from clinical sputum samples of patients with pulmonary TB.

MATERIAL AND METHODS

Setting

The results were generated by four regional laboratories belonging to the Instituto Adolfo Lutz: Campinas, Santo André, Sorocaba and Ribeirão Preto, all reference mycobacteria laboratories in the State of São Paulo, Brazil.

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Processing of clinical specimens

Clinical sputum samples received by the participating laboratories in 2005 and 2006 were selected for the study.

A total of 210 smear-positive sputum samples (only one per patient) with a positivity score of 1+ or more (>1 acid-fast bacilli [AFB] per field by Ziehl-Neelsen microscopy [magnification ×1000]),[10] were processed according to a modified Petroffs' method[11] and submitted simultaneously to NRA and the direct proportion method.

Antibiotic, media and reagent mixture

A stock solution of RMP was prepared from the chemically pure powder form (Sigma Chemical Co, St Louis, MO, USA) in methanol at a final concentration of 10 mg/ml. The solution was filtered through a 0.22 μm filter (Millipore, Woodbury, GA, USA) and stored at −20°C for not more than 6 months. For the NRA, the LJ medium was prepared containing 1000 μg/ml of potassium nitrate (KNO_3), with or without the addition of RMP (40 μg/ml). The reagent mixture (Griess reagent) used consisted of 1 part of 50% concentrated hydrochloric acid (HCl), 2 parts of 0.2% sulfanilamide and 2 parts of 01% N-1-naphthylethylenediamine dihydrochloride.

Nitrate reductase assay

The NRA was performed as described by Musa et al.[12] Briefly, 200 μl of the sediment obtained from decontamination procedures was inoculated into an RMP-containing tube, and 200 μl of the remaining sediment diluted 1/10 in sterile distilled water was then used to inoculate three antibiotic-free tubes used as growth controls. The tubes were incubated at 37°C and, after 10 days of incubation, 500 μl of the freshly made reagent mixture was added to one of the drug-free tubes. If any colour change was observed, the corresponding tubes with antibiotic were also tested. If no colour change was seen in the control tube, it was discarded and the remaining tubes were reincubated. The procedure was repeated at days 15 and 20. An isolate was considered resistant to RMP if the colour that developed in the test tube (pink or red or purple) was more intense than that in the 1/10-diluted growth control revealed on the same day. As an internal quality control of the assay, American Type Culture Collection (ATCC®, Manassas, VA, USA) strains of susceptible *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) and *M. tuberculosis* resistant to RMP (ATCC 35838) were used.

Direct proportion method

The direct proportion method on LJ medium was performed according to standard procedures with the recommended critical concentration of 40 μg/ml for RMP.[13] An isolate was reported as resistant if the number of colonies growing on the RMP-containing medium was 1% or more of the number of colonies developing on the drug-free control. The results obtained by the proportion method were used as the gold standard to compare to the results of NRA for susceptibility testing.

Table Time in days for the detection of *M. tuberculosis* isolates resistant or susceptible to rifampicin by NRA according to bacillary load

		NRA		
Bacillary load*	Isolates *n* (%)	Day 10 *n* (%)	Day 15 *n* (%)	Day 20 *n* (%)
1+	13 (6.2)	4 (1.9)	5 (2.3)	4 (1.9)
2+	72 (34.3)	18 (8.6)	39 (18.6)	15 (7.1)
3+	125 (59.5)	53 (25.3)	63 (30.0)	9 (4.3)
Total	210	75 (35.8)	107 (50.9)	28 (13.3)

* 1+ (10–99 bacilli per 100 fields examined), 2+ (an average of 1–10 bacilli per field in 50 examined fields), 3+ (an average of >10 bacilli per field in 20 examined fields).
NRA = nitrate reductase assay.

RESULTS

The susceptibility tests performed by the NRA and the direct proportion method showed 204 susceptible isolates and six isolates resistant to RMP by both methods. NRA sensitivity and specificity for RMP were 100%.

The NRA results of susceptibility tests to RMP were available in 10 days for 75 samples (35.8%), in 15 days for 107 samples (50.9%) and in 20 days for 28 samples (13.3%), whereas 30 days were needed to yield results using the proportion method.

The Table reports time in days for NRA results and the bacterial loads of the sputum samples. The majority of the results were available at day 15: 92.8% of the samples were reported as 3+, 79.2% of the samples as 2+ and 69.2% of the samples reported as 1+.

DISCUSSION

This study showed 100% agreement between the DST results of *M. tuberculosis* to RMP, obtained by direct NRA and by the direct proportion method. It was possible to obtain the DST results by NRA within 15 days for 87% of the studied sputum samples, whereas by the direct proportion method this was only possible in 30 days.

It has been suggested that quantitative microscopy gives a rather crude indication of the severity of disease and the degree of infectivity.[10] The results of this study showed that the NRA method may yield a quick answer in determining resistance for the majority of the sputum samples with smear results reported as 3+ and 2+.

Our results agree with those of Musa et al., who, in a study with 3+ positive sputum samples, showed 100% sensitivity and specificity in the DST results for RMP by the NRA method compared with the direct proportion method, and an average time of 14 days to obtain results.[12] Our results also agree with those of Affolabi et al. who performed NRA in liquid medium.[15]

Furthermore, 69.2% of NRA results for sputum samples that were smear-positive 1+ and 79.1% of smear-positive 2+ sputum samples were obtained within 15 days of incubation. Similar results were described by Affolabi et al.:[2] 50% of specimens with a low bacterial load (AFB 1+ or 2+) gave NRA results within 14.6 days. These results suggest that NRA can also be performed on smear-positive sputum samples with a low bacterial load.

Recent studies conducted in Western Africa and Latin America have shown the usefulness of NRA in determining the susceptibility profile of *M. tuberculosis*.[7–9,14,15] A review performed by Palomino et al. of the different methods used for the detection of susceptibility profiles reported that colorimetric methods, such as those that use stain indicators of oxide reduction and the NRA, can be used in limited-resource countries as they do not require sophisticated equipment and can be performed in a simple and rapid manner.[16]

In a review of the use of NRA in Latin American laboratories, Barrera and Montoro concluded that the introduction of the method is simple, especially in laboratories that perform the direct proportion method, as most of the supplies required are the same for both methods.[17] They also stressed the biosafety advantage of it being a method performed on LJ medium, which is responsible for a lesser amount of aerosol generation compared with the methods that use liquid medium and oxide-reduction staining. Compared to the proportion method, the NRA generates fewer aerosols as there is no preparation of serial dilutions, a practice that creates substantial aerosols.

The timely diagnosis of RMP resistance in *M. tuberculosis* isolates enables the clinician to prescribe the appropriate therapeutic regimen and prevent the circulation of resistant bacilli in the community.

With the results obtained in this study, it was possible to demonstrate the feasibility of NRA for the direct screening of resistant strains in sputum samples from patients with pulmonary TB.

NRA represents a rapid alternative method that might be used in microbiological laboratories where resources are scarce.

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RÉSUMÉ

CONTEXTE : Au Brésil, quatre laboratoires régionaux appartenant au laboratoire de référence pour les mycobactéries de l'Etat de Sao Paulo.

OBJECTIF : Evaluer le test de nitrate réductase (NRA) pour la recherche de la sensibilité de *Mycobacterium tuberculosis* à la rifampicine (RMP), directement à partir des échantillons cliniques de crachats de patients atteints de tuberculose (TB) pulmonaire.

SCHÉMA : On a évalué les performances du NRA pour la détection de la sensibilité de *M. tuberculosis* à la RMP dans 210 échantillons cliniques de crachats reçus par les laboratoires participants au cours des années 2005 et 2006, et on les a comparés avec les résultats de la méthode directe des proportions.

RÉSULTATS : Les tests de sensibilité réalisés tant par la méthode NRA que par la méthode directe des proportions ont révélé 204 isolats sensibles et six isolats résistants à la RMP par les deux méthodes. La sensibilité et la spécificité du NRA pour la RMP ont été de 100%. Les résultats du NRA concernant les tests de sensibilité à la RMP ont été obtenus en 15 jours dans 87% des échantillons. Les résultats ont démontré que le NRA peut fournir une réponse rapide de détermination de la résistance pour la majorité des échantillons de crachats où les résultats signalés pour les frottis étaient 3+ et 2+.

CONCLUSION : Les résultats démontrent la faisabilité du NRA pour le dépistage des souches résistantes dans les crachats de patients atteints de TB pulmonaire. Le NRA représente une méthode alternative rapide et de faible coût qui pourrait être utilisée dans les laboratoires de microbiologie à ressources limitées.

RESUMEN

MARCO DE REFERENCIA : Los cuatro laboratorios regionales que pertenecen al laboratorio de referencia de micobacterias en el estado de San Paulo, Brasil.

OBJETIVO : Evaluar la prueba de la nitrato reductasa (NRA) en el estudio de sensibilidad de *Mycobacterium tuberculosis* a rifampicina (RMP), directamente en las muestras de esputo de pacientes con tuberculosis (TB) pulmonar.

MÉTODOS : Se evaluó el rendimiento diagnóstico de la NRA en el estudio de la sensibilidad de *M. tuberculosis* a RMP en 210 muestras clínicas de esputo recibidas en los laboratorios participantes durante los años 2005 y 2006 y se comparó el resultado con el método directo de las proporciones.

RESULTADOS : El estudio de sensibilidad se llevó a cabo mediante la NRA y el método directo de las proporciones y se encontraron 204 aislados sensibles y seis aislados resistentes a RMP por ambos métodos. La NRA ofreció una sensibilidad y una especificidad del 100% y sus resultados se obtuvieron en 15 días en el 87% de las muestras. Estos datos indican que la NRA puede dar respuestas rápidas sobre la resistencia en la mayoría de las muestras de esputo, cuya baciloscopia se ha informado como 3+ y 2+.

CONCLUSIÓN : Los resultados demuestran la factibilidad del uso de la NRA en la detección de cepas resistentes en el esputo de pacientes con TB pulmonar. Esta prueba representa una opción rápida y de bajo costo, que se puede aplicar en los laboratorios de microbiología que cuentan con escasos recursos.