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Prospective multicentre evaluation of the direct nitrate reductase assay for the rapid detection of extensively drug-resistant tuberculosis

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Objectives: To perform a multicentre study evaluating the performance of the direct nitrate reductase assay (NRA) for the detection of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis in sputum samples.

Methods: The study was conducted in six laboratories performing tuberculosis diagnosis that were located in six different countries. The NRA was performed directly on sputum samples in parallel with the reference method used at each site. Detection of resistance was performed for rifampicin, isoniazid, ofloxacin and kanamycin.

Results: Excellent agreement was obtained for all drugs tested at the majority of sites. The accuracy was 93.7%–100% for rifampicin, 88.2%–100% for isoniazid, 94.6%–100% for ofloxacin and 100% for kanamycin. The majority of NRA results were available at day 21 for sites 1, 2 and 5. Site 3 had a turnaround time of 13.9 days, at site 4 it was 18.4 days and at site 6 it was 16.2 days. The contamination rate ranged between 2.5% and 12%.

Conclusions: Rapid detection of drug resistance by the direct NRA on sputum smear-positive samples was accurate and easy to implement in clinical diagnostic laboratories, making it a good alternative for rapid screening for MDR and XDR tuberculosis.

Keywords: multidrug resistance, nitratase, *Mycobacterium tuberculosis*

Introduction

The increasing incidence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) highlights the need for rapid, affordable and accurate methods for testing susceptibility to first- and second-line anti-TB drugs. XDR *Mycobacterium tuberculosis* strains, by being resistant to several second-line drugs in addition to rifampicin and isoniazid, are a new threat to the effective control of the disease.[1] The current methods for drug susceptibility testing of *M. tuberculosis* are either slow or costly. Löwenstein–Jensen (LJ) medium is still the most commonly used and inexpensive solid medium for drug resistance detection in many clinical laboratories. Unfortunately, conventional drug susceptibility testing methods based on LJ medium are time consuming, taking up to 6 weeks to give results.[2,3] This is mainly due to the slow growth of *M. tuberculosis*. In order to reduce this turnaround time, commercial broth-based systems and molecular tests have been developed.[4–7] Nevertheless, these systems are still too expensive or have logistical problems preventing their broad adoption in low-income countries with a significant number of drug-resistant cases.

The nitrate reductase assay (NRA) is a rapid, low-cost, phenotypic method performed on solid media and based on the metabolic activity of *M. tuberculosis*;[8] it has been endorsed by the WHO for the rapid detection of MDR-TB.[9] The aim of this study was to evaluate in a multicentre study the feasibility of using the NRA for the simultaneous detection of MDR- and XDR-TB through the detection of resistance to isoniazid, rifampicin, ofloxacin and kanamycin directly from sputum samples.

Methods

Study design and setting

This prospective study was carried out in six laboratories situated in six different countries: Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India; Department of Pathology, Combined Military

Hospital, Dera Ismail Khan, Pakistan; Laboratorio de Referencia del Programa de Control de Tuberculosis, Hospital Dr Antonio Cetrángolo, Buenos Aires, Argentina; Clinical Microbiology Laboratory, Chest Diseases Hospital, Samsun, Turkey; Institut Pasteur de Madagascar, Antananarivo, Madagascar; and Laboratoire de Référence des Mycobactéries, Cotonou, Benin. The participating laboratories were randomly numbered from 1 to 6 for the purpose of this publication. The inclusion criteria were cough for >3 weeks, age >12 years, suspected pulmonary TB, and suspected treatment failure, suspected relapse or treatment default. Only those samples that were positive by Ziehl–Neelsen smear microscopy were included in the study. A total of 265 smear-positive (≥1+) sputum samples were collected prospectively at the six sites from January 2012 to October 2012. All participating laboratories had experience with the NRA or were previously trained.

Sputum samples

Smear microscopy was performed directly on all sputum samples. Those that were smear positive were selected and decontaminated by the sodium hydroxide–*N*-acetyl-L-cysteine method.[2] Samples were then concentrated by centrifugation at 3200 **g** for 20 min. The supernatant was discarded, and the remaining sediment was resuspended in 3 mL of sterile distilled water and used to inoculate, in parallel, either LJ medium or the Bactec MGIT 960 Mycobacterial Detection System (as the reference method) for indirect drug susceptibility testing, and NRA tubes containing antibiotics for direct testing.

LJ proportion method

The proportion method was performed on LJ medium according to the standard procedure, with the recommended critical concentrations of 40 mg/L rifampicin, 0.2 mg/L isoniazid, 2 mg/L ofloxacin and 30 mg/L kanamycin. LJ tubes were incubated at 37°C and read at 28 and 42 days.[3]

MGIT 960 system

Testing using the MGIT 960 system was performed according to the manufacturer's instructions, with 1 mg/L rifampicin and 0.1 mg/L isoniazid. For the second-line drugs, 2 mg/L ofloxacin and 2.5 mg/L kanamycin were tested according to Martin *et al*.[10]

NRA

The LJ–NRA medium was prepared in-house and the method was carried out as previously described[11] with a minor modification in reading timepoints. LJ–NRA tubes contained 1 g/L potassium nitrate (KNO_3) and critical concentrations of 40.0 mg/L rifampicin, 0.2 mg/L isoniazid, 2.0 mg/L ofloxacin and 30 mg/L kanamycin. An additional LJ–NRA tube without drugs contained 0.5 g/L *para*-aminobenzoic acid (PNB) to identify the *M. tuberculosis* complex and three LJ–NRA drug-free tubes served as a growth control. Drug- and PNB-containing tubes were inoculated with 0.2 mL of the concentrated and decontaminated sputum, while growth controls were inoculated with 0.2 mL of a 1:10 dilution of the same sample. After 10 days of incubation at 37°C, the NRA was developed by adding 0.5 mL of freshly prepared Griess reagent consisting of 1 part 50% (v/v) hydrochloric acid, 2 parts 0.2% (w/v) sulfanilamide and 2 parts 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride to one of the growth controls. If any pink colour appeared, the Griess reagent was added to all drug-containing tubes. If there was no colour change, the remaining tubes were reincubated and the procedure repeated at days 14 and 28. An isolate was considered resistant if any colour change was observed in the drug-containing tube. If the control was negative at day 28, the NRA was considered invalid. All tests were performed blinded. Identification of the *M. tuberculosis* complex was made by reading the PNB-containing tube. If no colour change occurred, it was considered to be *M. tuberculosis* complex.

Statistical analysis

MedCalc statistical software version 12.3.0.0 (Mariakerke, Belgium) was used to calculate the statistical measures of sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) of the direct NRA using the LJ proportion method or the MGIT 960 system as the reference test. The time to positivity (TTP) of the NRA was also recorded.

Results

For all drugs, excellent agreement was obtained between the direct NRA and the gold standard method used at each site – either the LJ proportion method or the MGIT 960 system. The sole exception was ofloxacin at one site. Table 1 shows for each site the number of

Table 1. Susceptibility results for the direct NRA compared with the MGIT 960 system or LJ proportion method for each site

		MGIT 960 system or LJ proportion method							
		rifampicin		isoniazid		ofloxacin		kanamycin	
Site	Direct NRA	R	S	R	S	R	S	R	S
1	R	18	3	20	1	11	0	7	0
	S	0	31	0	31	0	41	0	45
2	R	12	0	15	0	1	0	1	0
	S	1	37	2	33	0	49	0	49
3	R	4	0	9	0	2	0	5	0
	S	0	53	0	48	0	55	0	52
4	R	0	0	6	0	2	1	0	0
	S	0	52	2	44	1	48	0	52
5	R	3	0	4	1	0	0	0	0
	S	1	12	1	11	0	17	0	17
6	R	2	0	2	0	0	2	0	0
	S	0	35	0	35	0	35	0	37

R, resistant; S, susceptible.

Table 2. Sensitivity, specificity, PPV, NPV and accuracy of the direct NRA for each drug tested at each study site

	Rifampicin (%)					Isoniazid (%)					Ofloxacin (%)					Kanamycin (%)				
Site	sens	spec	PPV	NPV	A	sens	spec	PPV	NPV	A	sens	spec	PPV	NPV	A	sens	spec	PPV	NPV	A
1	100	91.1	85.7	100	94.2	100	96.8	95.2	100	98.0	100	100	100	100	100	100	100	100	100	100
2	92.3	100	100	97.3	98.0	88.2	100	100	94.2	96.0	100	100	100	100	100	100	100	100	100	100
3	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
4	NA	100	NA	NA	100	75	100	100	95.6	96.2	66.7	98	66.6	97.9	96.2	NA	100	NA	100	100
5	75	100	100	92.3	93.7	80	91.6	80	91.6	88.2	NA	100	NA	100	100	NA	100	NA	100	100
6	100	100	100	100	100	100	100	100	100	100	NA	94.6	NA	100	94.6	NA	100	NA	100	100

sens, sensitivity; spec, specificity; A, accuracy; NA, not applicable.

resistant and susceptible samples detected by the NRA compared with results obtained by the reference method used at the site.

For rifampicin, only site 1 had false resistant results and two sites (site 2 and site 5) had one false susceptible result each. Site 5 had a sample contaminated for rifampicin and was discarded for the analysis. For isoniazid, two sites (site 1 and 5) had one false resistant result each, while sites 2 and 4 had two false susceptible and site 5 one false susceptible result.

For ofloxacin, site 4 had one false resistant and one false susceptible result. Because of the very low number of strains resistant to ofloxacin by the NRA, the sensitivity was lower for this drug. Site 6 found two samples resistant to ofloxacin by the NRA; however, the proportion method found those samples to be susceptible. For kanamycin, all results were concordant at all sites. Site 4 did not find any samples resistant to either rifampicin or kanamycin during the study period. Similarly, site 5 did not have any ofloxacin- or kanamycin-resistant samples.

Based on these results, Table 2 shows the specificity, sensitivity, PPV, NPV and accuracy of the NRA method for all sites. For rifampicin the accuracy ranged between 93.7% and 100%, for isoniazid the accuracy ranged between 88.2% and 100%, for ofloxacin the accuracy ranged between 94.6% and 100%, and for kanamycin the accuracy was 100%. The majority of NRA results were available at day 21 for sites 1, 2 and 5, with an average of 72% positivity at day 21, 12% at day 14 and 16% at day 28. Site 3 had a TTP of 13.9 days, at site 6 it was 16.2 days and at site 4 it was 18.4 days. For site 3, out of 61 consecutive samples, 2 did not yield growth and another 2 were identified as non-tuberculous mycobacteria (*Mycobacterium avium*). The contamination rate was between 2.5% and 12% for the NRA.

Discussion

We performed the first multicentre prospective evaluation to assess the performance of the direct NRA assay for the detection of MDR and XDR-TB. The assay was easy to implement in the field setting, showed low or acceptable contamination levels and a shorter turnaround time than the proportion method on LJ medium, and a similar TTP compared with the MGIT 960 system. The direct NRA showed overall good sensitivity and specificity for the four drugs tested. Furthermore, incorporating PNB in the assay allowed very simple identification of the *M. tuberculosis* complex.

Although considered as the gold standard, it is well known that drug susceptibility testing performed on LJ medium or Middlebrook agar by the proportion method is very slow, requiring 3–6 weeks to produce results. The development of affordable and rapid drug susceptibility testing methods for low-resource countries is then a continuing priority. The NRA described here has the advantage of a shorter TTP (10–14 days) compared with the conventional proportion method. The NRA is based on the detection of nitrate reduction as an indicator of growth, and the results are therefore obtained before any macroscopic growth can be visually detected.

A number of studies have reported the usefulness of the NRA for determining susceptibility or resistance to rifampicin and isoniazid, the two most important drugs for the treatment of TB, and has shown high sensitivity and specificity.[12–14] The NRA also has the potential to be used for the detection of resistance to second-line drugs. In 2005, our group reported the first evaluation of the NRA for the detection of ofloxacin resistance and found complete concordance with the proportion method.[15] Rosales *et al.*[16] evaluated the NRA for the rapid detection of resistance to ofloxacin and kanamycin in Honduras. They found good specificity for both drugs, but lower sensitivity for detecting resistance to kanamycin. They also showed how the NRA could easily be adapted to the local conditions of a diagnostic laboratory that was already using the proportion method on LJ medium. More recently, Visalakshi *et al.*[17] evaluated the NRA for rapid detection of resistance to kanamycin, ethambutol, ofloxacin, cycloserine and *para*-aminosalicylic acid. They obtained sensitivity that ranged between 86.4% and 100%, and specificity ranging between 98.4% and 100%.

In the present study, the sensitivity and specificity for detecting resistance to rifampicin and isoniazid were excellent and >94% for the second-line drugs. This is the first multicentre study that confirms that the direct NRA can also be used to screen XDR-TB. This study was limited by the relatively small number of XDR-TB cases found. The NRA has been adapted for the direct detection of MDR-TB using smear-positive sputum samples in a number of countries such as Peru, Argentina, Benin, Brazil, India, Nigeria and Pakistan.[16,18–23] The main advantage of the direct NRA is its much shorter turnaround time. Also, the simplicity and cost-effectiveness of the test is suitable for large-scale surveillance studies in resource-limited settings. It furthermore offers the advantage of using solid media, which minimizes cross-contamination and biohazard risks. Additional studies of direct NRA testing for second-line drugs are warranted.

Our study was limited by the low number of drug-resistant samples found in the different countries. Nevertheless, the direct NRA can be used as a rapid and low-cost screening method to detect drug resistance.

In conclusion, rapid detection of drug resistance by the direct NRA on sputum smear-positive samples was accurate and easy to implement in clinical diagnostic laboratories, making it a good option for rapid screening for MDR- and XDR-TB.

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Transparency declarations

None to declare.

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