

Predictive value of direct nitrate reductase assay and its clinical performance in the detection of multi- and extensively drug-resistant tuberculosis

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Conventional culture and drug susceptibility testing (DST) methods for *Mycobacterium tuberculosis* are laborious and time consuming. For this reason alternative rapid culture and DST techniques are urgently needed to shorten the time for drug-resistance detection. A total of 222 smear-positive sputum samples were evaluated by the direct nitrate reductase assay (D-NRA) on Löwenstein–Jensen medium, for the rapid and simultaneous detection of resistance to isoniazid, rifampicin, kanamycin and ofloxacin. *p*-Nitrobenzoic acid was also included for identification of the *M. tuberculosis* complex. Results were compared with the BACTEC MGIT 960 as gold standard. The general performance of the D-NRA was very good, reaching a global value of 97%. D-NRA had a turn-around time of 16.9 days to obtain results while that of the indirect MGIT 960 system was 29 days. D-NRA is a low-cost technology, easy to set up in clinical laboratories and suitable to be used for DST of *M. tuberculosis* in all smear-positive samples.

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INTRODUCTION

Tuberculosis (TB) remains a major public health concern worldwide, especially in low- and middle-income countries (WHO, 2012, 2010). This situation is worsened by multidrug-resistant (MDR)-TB, caused by *Mycobacterium tuberculosis* resistant to at least isoniazid (INH) and rifampicin (RIF) and by extensively drug-resistant (XDR)-TB, caused by an MDR-TB strain also resistant to amikacin (AMK), kanamycin (KAN) or capreomycin (CAP) plus any fluoroquinolone. The World Health Organization (WHO) has estimated that around nine million new TB cases occurred in 2011 worldwide and 1.4 million deaths were due to the disease (WHO, 2009, 2012). Moreover, 3.7% of newly diagnosed TB patients and 20% of previously treated cases are estimated to be MDR. Estimates indicate that 5–9% of these MDR-TB cases will evolve to XDR-TB (WHO, 2009, 2008).

Argentina is considered a middle-income country with a TB incidence rate that has decreased to 22.3/100 000 in 2010 (Ministry of Health, 2011). The Buenos Aires Province has

the highest number of TB cases in the country ($n=5370$), with an incidence rate of 33.3/100 000. Almost 80% of these cases are concentrated in the suburbs of Buenos Aires City (Tuberculosis Control Program of Buenos Aires Province, 2012). It has been estimated that 15.5% of previously treated and 2.2% of new TB patients have MDR-TB (National Institute of Infectious Diseases, 2010).

Conventional culture of *M. tuberculosis* and drug susceptibility testing (DST) is time consuming and slow to give results. For this reason, alternative rapid DST methods are indispensable and urgently needed to shorten the time required for drug-resistance detection (Heifets, 1991; Morcillo *et al.*, 2008). The indirect proportion method on Löwenstein–Jensen medium (LJ) has a long turn-around time (TAT) of 28–42 days to obtain drug resistance profiles from *M. tuberculosis* isolates. The BACTEC MGIT 960 system and molecular detection methods are faster than the LJ but require equipment, and are more expensive to perform (Morcillo *et al.*, 2010).

The nitrate reductase assay (NRA) has been described by several authors as an inexpensive and rapid method to detect drug resistance in *M. tuberculosis* (Affolabi *et al.*, 2007; Angeby *et al.*, 2002; Coban *et al.*, 2004; Musa *et al.*, 2005; Martin *et al.*, 2008). The NRA is based on an enzymic reaction that detects the ability of *M. tuberculosis* to reduce

Abbreviations: AMK, amikacin; D-NRA, direct nitrate reductase assay; DST, drug susceptibility testing; INH, isoniazid; I-MGIT, indirect DST MGIT 960; KAN, kanamycin; MDR, multidrug-resistant; OFL, ofloxacin; PNB, *p*-nitrobenzoic acid; RIF, rifampicin; TAT, turn-around time; TB, tuberculosis; XDR, extensively drug-resistant.

nitrate to nitrite. In this assay the presence of nitrite is detected by adding the Griess reagent [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 a fresh culture on LJ containing KNO₃. In a positive test, before macroscopic growth can be seen, the Griess reagent turns pink allowing easy interpretation. The incorporation of drugs into the culture medium allows use of the NRA as an alternative method to rapidly detect drug resistance. High sensitivity and specificity for INH and RIF have been previously reported in several studies, with easy implementation in TB diagnostic laboratories (Affolabi *et al.*, 2007). The NRA has also been used directly (D-NRA) on sputum samples, reducing the final TAT to obtain the DST results (Martin *et al.*, 2014). This method has been recently approved by WHO to quickly detect MDR-TB patients (WHO, 2011).

The main aim of this study was to evaluate the clinical performance of D-NRA for detecting M/XDR TB cases. More specifically, the objectives were to estimate the predictive value of D-NRA for a rapid detection of M/XDR cases in comparison with either phenotypic and/or genotypic drug-resistance methods and to analyse the relationship between D-NRA results and clinical data of the detected cases.

METHODS

Setting. Reference Laboratory of Tuberculosis Control Program (RLTBPCP) at Dr Cetrángolo Hospital, Buenos Aires, Argentina.

Clinical specimens. This prospective study included 222 smear-positive sputum samples obtained from January 2012 to July 2013 from new and previously treated patients at the RLTBPCP. Each sample originated from a different patient. The included smear-positive sputa ranged in bacillary load from 1+ ($n=47$; 21.2 %) to 2+ ($n=66$; 29.7 %) to 3+ ($n=109$; 49.1 %).

Samples were decontaminated using the sodium hydroxide-*N*-acetyl-L-cysteine (NaOH-NALC) method (Morcillo *et al.*, 2008) and cultures were performed by inoculating 500 µl on a BACTEC MGIT 960 tube, and 200 µl each on LJ, Stonebrink solid medium and D-NRA, the latter for direct DST (LJ-KNO₃-containing tube) (Morcillo *et al.*, 2008; Musa *et al.*, 2005; Martin *et al.*, 2008).

Nitrate reductase assay applied directly on sputum samples (D-NRA).

The D-NRA protocol was adapted from that previously described by Musa *et al.* (2005). Decontaminated samples were made up to 4.0 ml with sterile distilled water and 200 µl was inoculated into LJ tubes containing 1000 µg KNO₃ ml⁻¹ and the different drugs: INH, RIF, ofloxacin (OFL) and KAN (concentrations indicated in Table 1). *p*-Nitrobenzoic acid (PNB) was also included in an LJ-KNO₃ tube in order to identify *M. tuberculosis* complex. Four drug-free LJ-KNO₃ tubes were also inoculated with 200 µl of the sample and used as growth control. All tubes were incubated at 37 °C for 7 days and then 1 ml of freshly prepared Griess reagent was added to one control tube. If the colour turned to pink, indicating mycobacterial growth, the reagent was added to the drug-containing tubes. If the drug-containing tube turned to pink (same or greater intensity than the control tube), the sample was considered as 'resistant' to that drug. If the colour change was less than in the control, the result was considered as 'non-interpretable' and

Table 1. Drug concentrations used in each test

Drug	Concentration (µg ml ⁻¹)	
	D-NRA	I-MGIT
INH	0.2	0.1
RIF	40.0	1.0
OFL	2.0	1.0
KAN	30.0	5.0
PNB*	500.0	500.0

*PNB was included for the simultaneous identification of *M. tuberculosis* complex.

drug-resistance needed to be confirmed by a different DST method. A sample was considered as 'susceptible' to a particular drug if no colour change appeared in the drug-containing tubes. In the case of no colour change in the control tube, the remaining tubes were reincubated and the procedure repeated on days 14, 21 and 28. If no colour change was observed after that time, the D-NRA result was considered 'invalid'.

DST on BACTEC MGIT 960. The indirect DST was performed on the MGIT 960 system using INH and RIF following the procedure recommended by the manufacturer (Becton Dickinson and Company, 2005). OFL and KAN were assessed following the protocol provided by Rüsich-Gerdes *et al.* with modifications (Morcillo *et al.*, 2010; Rüsich-Gerdes *et al.*, 2006) and PNB was used as previously described (Sharma *et al.*, 2010). The tested drug concentrations are shown in Table 1.

Molecular detection of INH and RIF resistance. As part of the routine laboratory procedures, those isolates showing INH and/or RIF resistance were analysed by the GenoTypeMTBDR*plus* assay (Imperiale *et al.*, 2012) in order to identify mutations conferring drug resistance. Technology to explore mutations related to OFL and KAN resistance was not available in the laboratory when these studies were performed.

Statistical methods. MedCalc software v. 12.4.0 (Mariakerke) was used to determine the summary statistic and the parameters sensitivity (S), specificity (SP), and positive and negative predictive values (PPV and NPV) of the D-NRA using the MGIT 960 system as a reference DST method.

RESULTS

Three samples (1.4 %) had contamination in both solid and liquid media as well as in the D-NRA; therefore they were excluded from the study. Interpretable and valid D-NRA results were obtained in 95.0 % (211/222) of the specimens included in the study. *M. tuberculosis* was identified in 99.0 % (209/211) of the specimens, while in 2 (1.4 %), non-tuberculous mycobacteria were identified since they grew in the PNB-containing tube. Both samples were further identified as *Mycobacterium avium* subspecies by the GenoType Mycobacterium CM (HAIN) system (Richter *et al.*, 2006). Eight samples (8/222; 3.6 %) gave invalid results because they failed to grow either during the D-NRA or in the other culture media.

No significant differences in the yield of valid results were observed among samples containing different bacillary

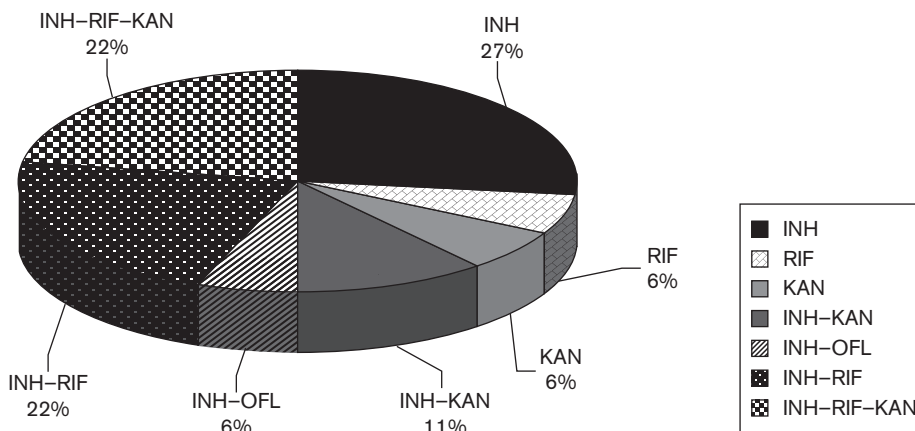


Fig. 1. Drug-resistance patterns by D-NRA directly applied to sputum samples.

loads. A total of 89.4% (42/47) of 1+, 96.9% (64/66) of 2+ and 96.3% (105/109) of 3+ gave valid results.

According to the D-NRA, 8.6% (18/209) of specimens showed resistance to one or more anti-TB drug. Seven were mono-resistant (five to INH, one to RIF and one to KAN). Three were poly-resistant (two to INH plus KAN and one to INH plus OFL), and eight were MDR (four of them with KAN resistance). The remaining 191 (91.3%) specimens were fully drug-susceptible. The 209 samples identified as *M. tuberculosis* showed no growth on the PNB-containing tube. Fig. 1 shows the drug-resistance patterns detected by D-NRA.

Samples containing 3+ of bacillary load yielded results in a mean time of 16.3 days, while those samples with 2+ or 1+ showed results in a mean time of 17.5 days; nevertheless this difference was not significant $P=0.1377$. Table 2 shows the TAT to obtain D-NRA results.

TAT was defined as the total time to obtain results from processing the specimen for culture to drug-resistance result. In this sense, D-NRA showed a mean TAT of 16.9 days for DST results while with the indirect DST MGIT 960 (I-MGIT) system the mean TAT was 29 days (taking into account time for isolation plus time for DST); the difference in TAT was significant, $P<0.0001$. Fig. 2

Table 2. Turn-around time obtained with D-NRA

Bacillary load	Number (%)	Turn-around time (days)					P*
		7	14	21	28	Mean	
+	42 (19.9)	2	19	19	2	17.5	0.1377
++	64 (30.3)	2	34	23	5	17.5	
+++	105 (49.8)	6	62	35	2	16.3	
Total	211 (100.0)	10	115	77	9	16.9	

*P value between mean of 17.5 and 16.3 days.

shows the TAT for DST results from D-NRA and I-MGIT. A total of 14 and 24 days, respectively, were necessary to obtain 75% of DST results while 100% of results were available at 25 days by D-NRA and 35 days by I-MGIT.

Table 3 shows the sensitivity, specificity positive predictive value (PPV) and negative predictive value (NPV) for the tested drugs. The global parameters for all the tested drugs were higher than 90%.

Table 4 shows the statistical parameters calculated by the receiver operating characteristic (ROC) method for KAN and RIF for specimens with a bacillary load of 2+ and 3+. Statistical parameters are not shown for specimens with 1+ because the statistical software did not calculate the ROC curve because there was only one sample with a drug-resistance profile. One isolate each was found resistant to RIF and KAN by D-NRA but susceptible by I-MGIT.

Drug resistance was also confirmed by the GenoType MTBDRplus assay, which showed that 13/16 INH-resistant

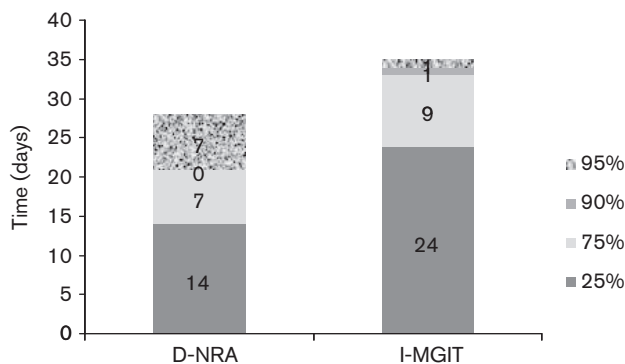


Fig. 2. Comparison of turn-around time obtained with D-NRA directly applied to sputum samples and I-MGIT testing with the BACTEC MGIT 960 system.

Table 3. Statistical parameters obtained for different drugs

Drug	Sensitivity	Specificity	PPV	NPV	r^2 *	AUC
INH	100.0	100.0	100.0	100.0	1.0	1 (0.98–1.0)
KAN	87.5	99.5	87.5	99.5	0.87 (0.83–0.90)	0.94 (0.89–0.96)
OFL	100.0	100.0	100.0	100.0	1.0 (0.98–1.0)	1.0 (0.98–1.0)
RIF	100.0	99.0	77.8	100.0	0.88 (0.84–0.91)	0.95 (0.97–1.0)
Global	96.9	99.6	91.3	99.9	0.93 (0.83–1.00)	0.97 (0.89–1.00)

PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve.

* r^2 , Correlation between D-NRA and I-MGIT.

samples had a *katG* S315T mutation while the other three had C 15T in the *inhA* gene promoter region. Regarding RIF resistance, eight samples had the *rpoB* S531L mutation and the remaining one harboured the *rpoB* D516V.

DISCUSSION

The slow growth of *M. tuberculosis* remains a major obstacle for the timely determination of its susceptibility or resistance to anti-TB drugs. Drug-resistant strains when disseminated can infect people without a past history of TB, causing the acquisition of already resistant strains, delays in cure, and frequently, treatment failures. For this reason it is of paramount importance to have a rapid and inexpensive method for early detection of drug resistance in all TB-diagnosed cases. D-NRA is a low-cost technology, easy to set up in laboratories experienced in cultures and DST using LJ solid medium. Furthermore, this method allows the replacement and/or the addition of as many drugs to be tested as necessary and is potentially useful to detect M/XDR strains. These characteristics make it

suitable to be used for DST in all smear-positive TB cases. For these reasons, we decided to explore the predictive values of D-NRA in an area with a medium incidence of TB and MDR-TB.

In this study only 11 samples (5.0%) gave invalid D-NRA results. The contaminated cultures (3/222, 1.4%) were fewer than expected for cultures on solid medium used for primary isolation. Those samples that failed to grow were probably obtained from patients under treatment, and theoretically, despite inhibition of growth the mycobacteria should still be detectable by direct smear examination.

The D-NRA was easy to perform and implement in the routine activities of the reference laboratory. It was shown to be a rapid alternative for drug-resistance detection (mean time 16.9 days), while conventional methods were more time consuming (the mean time using MGIT for culture and for DST was 29 days). Moreover, the D-NRA could be applied directly on smear-positive sputum samples independent of the bacillary load.

According to the predictive values obtained, 90% of M/XDR strains detected by D-NRA will be truly positive

Table 4. Statistical parameters obtained for RIF and KAN from AFB smears 2+ and 3+

	Drug	Sensitivity	Specificity	PPV	NPV	r^2 *	AUC
AFB 2+	KAN	66.7	98.4	66.7	98.4	0.65	0.83
		(9.4–99.2)	(91.2–100.0)	(9.4–99.2)	(91.2–100.0)	(0.48–0.77)	(0.71–0.91)
AFB 3+		100.0	100.0	100.0	100.0	1	1
		(39.8–100.0)	(96.4–100)	(39.8–100.0)	(96.4–100)	(0.97–1.00)	(0.97–1.00)
Global		83.4	99.2	83.4	99.2	0.81	0.92
		(9.4–100.0)	(91.2–100)	(9.4–100.0)	(91.2–100.0)	(0.48–1.0)	(0.71–1.00)
AFB 2+	RIF	100.0	98.4	66.7	100.0	0.81	0.99
		(15.8–100.0)	(91.3–100.0)	(4.0–99.8)	(94.1–100.0)	(0.70–0.88)	(0.93–1.00)
AFB 3+		100.0	99.0	80.0	100.0	0.89	0.99
		(39.8–100)	(94.6–100.0)	(28.4–99.5)	(96.4–100.0)	(0.84–0.92)	(0.96–1.00)
Global		100.0	98.7	73.4	100.0	0.85	0.99
		(15.8–100.0)	(91.3–100.0)	(4.0–99.8)	(94.1–100.0)	(0.84–0.91)	(0.97–1.00)

AFB, acid-fast bacilli; PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve.

* r^2 , Correlation between D-NRA and I-MGIT.

(resistant) and 100 % truly negative (susceptible) to all the tested drugs. Furthermore, the general performance of the method was very good, reaching a global value of 97 %. These figures are clinically important at the moment of making a therapeutic decision based on *in vitro* DST results.

The difference in TATs between I-MGIT and D-NRA can be partially explained by the different timing for the application of both techniques. The approved protocols for SIRE (streptomycin, isoniazid, rifampin and ethambutol) and second-line drugs DST using MGIT 960 with the EpiCenter and Tb-eXiST, respectively, determine drug resistance indirectly, after isolation of the bacteria, while the D-NRA avoids the isolation step and is performed directly on the smear-positive sputum sample.

All INH- and RIF-resistant specimens were confirmed by molecular testing and the mutations found were the most relevant found in previous studies (*katG* S315T and *rpoB* S531L) (Imperiale *et al.*, 2011, 2013).

In summary, we found that the D-NRA is a low-cost technology, easy to set up in clinical laboratories and suitable for accurate DST of *M. tuberculosis* in all smear-positive samples. Additionally, the D-NRA can be prepared in-house with low-cost reagents and culture medium while doing away with costly equipment.

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