

## Direct Nitrate Reductase Assay for detection of drug resistance in *Mycobacterium tuberculosis*: rapid, simple and inexpensive method for low resource laboratories

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### ABSTRACT

**INTRODUCTION:** The most common method for detection of drug-resistant-Tuberculosis (DR-TB) in resource-limited settings (RLSs) is indirect susceptibility testing on Lowenstein-Jensen (LJ) medium with results available only after 2-3 months. Rapid detection of drug resistance by direct Nitrate Reductase Assay (NRA) expedites Tuberculosis patient management. The objective of the study is to assess the feasibility and performance of Direct NRA for detection of DR-TB in National Tuberculosis Center under the National Tuberculosis Control Programme (NTP).

**MATERIALS AND METHODS:** Out of 416 previously treated and new pulmonary TB suspect cases; a total of 117 (28.1%) smear-positive sputa with a positivity score of 1+ or more were used in the study. The NRA results were compared with the gold standard LJ proportion method for 110 (94%) specimens while 7 were either contaminated or culture negative.

**RESULTS:** In comparison with LJ proportion method, the respective sensitivities, specificities, NPV, PPV and kappa agreement were 97.2% (95% CI, 86-100), 95.9% (95% CI, 89-99), 92.1% (95% CI, 78-99) 98.6% (95% CI, 92-100), and 0.92 for INH, 100% (95%CI, 90-100), 98.7% (95% CI, 93-100), 97.1% (95% CI, 85-100), 100% (95% CI, 95-100) and 0.98 for RFM, 97.1% (95% CI, 85-100), 96.1% (95%, 89-99), 91.7% (95% CI, 78-98), 98.7% ((95% CI, 93-100) and 0.92 for SM and 100% (95% CI, 88-100), 97.7% (95% CI, 91-100), 93.3% (95% CI, 78-99), 100% (95% CI, 95-100) and 0.93 for EMB.

**CONCLUSIONS:** The results obtained by direct NRA demonstrated excellent concordance for all drugs. Direct NRA is an assay which detects DR-TB directly from sputum rapidly and has the potential to become an alternative to existing methods particularly in resource-poor settings.

**KEY WORDS:** Drug resistant TB, Nitrate reductase assay, Sensitivity, Specificity, kappa agreement

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## INTRODUCTION

Tuberculosis (TB) remains a disease with an enormous impact on public health worldwide.<sup>1</sup> Globally, 22 “high-burden” countries account for over 80% of the active TB cases, highlighting the inequitable distribution of the disease. In 2010, among 8.8 million new TB cases 0.35 million deaths were with HIV positive and 1.2–1.5 million deaths with HIV negative.<sup>2-4</sup> The SAARC region with estimated annual incidence of 2.85 million TB cases, carries 32% of global burden.<sup>5</sup> Over the last decade multidrug-resistant TB (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) are of serious concern.<sup>6</sup>

TB remains a major public health problem in Nepal. About 50% of the total population is infected with TB. WHO estimates prevalence of all types of TB cases for Nepal at 71,000 while the number of all forms of incidence cases is estimated around 48,000. In Nepal, with the introduction of Directly Observed Treatment Short course (DOTS) number of deaths has dramatically reduced from 9,712 in 1990 to 6,200 in 2010. Till 2010, NTP registered 882 MDR-TB cases for treatment; largest number belonging to failures of CAT-II (83%) followed by CAT I failure (9%) with culture and DTS confirmed MDR-TB. Till July 2011, 27 XDR patients are under treatment in National TB control program.<sup>7,8</sup>

The rapid diagnosis of TB drug resistance is a priority to avoid the spread of resistant strains and for effective treatment of the cases. Early detection of XDR-TB and MDR-TB is more important to break the chain of transmission in the family of the patient and the community. The proportion method and other conventional tests, based on the measurement of growth in culture media containing antibiotics, require several weeks to give results. The BACTEC radiometric system has the advantage of being more rapid (5–10 days), but requires the use of radioisotopes and can be costly to be performed routinely.<sup>9</sup> Commercial tests (MGIT, ETest) and molecular tools (INNO-LiPA) are expensive and also impractical for routine use and is consequently not feasible in resource-poor settings. An alternative rapid method called Nitrate Reductase Assay (NRA) has been developed. This colorimetric assay is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, which is revealed as a color change in the culture medium, using the Griess method.<sup>10,11</sup> Direct NRA is expected to detect MDR-TB in resource-poor countries in short time period. In order to implement and maintain the quality of the new diagnostic services, an adequate certification or

quality assurance program needs to be inbuilt in terms of sensitivity, specificity and predictive values.

## MATERIALS AND METHODS

The study was conducted in combined laboratory of National TB centre and SAARC TB/ HIV-AIDS Centre. A total of 416 previously treated and new smear positive pulmonary TB patients visiting National Tuberculosis Centre, Thimi, Bhaktapur during March 2011 to May 2012 were enrolled in the study. Sputum specimens were initially processed by modified Petroff's method and Zhiel Neelsen (ZN) staining of the sediment was done according to standard operating procedures.<sup>12</sup> Of these, we selected the samples that were more than or equal to microscopy grade of “1+”. 0.2 ml of the sediment was inoculated into seven NRA drug susceptibility testing medium.

Processed specimen was cultured on two slopes of LJ-medium and drug susceptibility test of isolate was performed by 1% proportion method according to standard protocols.<sup>12</sup> For NRA, complete LJ-media was prepared and then 1g/1000 ml NaNO<sub>3</sub> was added to LJ medium and completely dissolved by stirring. The drugs were then added to the modified medium to prepare the drug containing medium and the medium was aliquoted and inspissated. Seven tubes with modified LJ medium are needed for each specimen: one containing INH at critical concentration (0.2 µg/ml), one containing RFM at critical concentration (40µg/ml), one containing SM at critical concentration (4µg/ml), one containing EMB at critical concentration(2 µg/ml) and three control tubes without any drugs added.

Each of the seven tubes described above was inoculated with 0.2 ml of the processed sputum sediments. After 14<sup>th</sup>, 21<sup>st</sup> and 28<sup>th</sup> days of incubation at 37°C, 0.5 ml of freshly prepared Griess reagent was transferred into one of the growth control tubes, and development of color was observed. If the color intensity was sufficient, the same amount of Griess reagent was transferred into each of the drug containing tube and other control tube. The color intensity in the drug-containing tube was then compared to the control tube.

The results were classified as negative if there were no color changes or a very pale pink color was observed. Positive results varied from pink to deep red or violet. The results were thus, interpreted as follows. Resistant (R); an isolate was considered resistant to a certain drug if there was a positive

color change in the drug tube in question and in the drug-free control tube. Sensitive (S); an isolate was considered sensitive to a drug if there was no color change in the drug tube in question and positive color change in the drug-free control tube. If no color changes or pale pink color were observed in the control tube, the test was considered to be invalid. For each batch of medium, internal quality control was done using the fully susceptible *M. tuberculosis* strain H<sub>37</sub>Rv.

All the collected data were processed and analyzed for calculation of p-value and kappa agreement using SPSS (Statistical package for Social Sciences) version 16.0. The performance of the NRA in comparison with that of the LJ proportion method was evaluated in terms of sensitivity (ability to detect true resistance) and specificity (ability to detect true susceptibility). The agreement between the two methods was estimated by the kappa value. Agreement between two tests were 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 agreement. Predictive values were also calculated.

## RESULTS

Out of 416 previously treated and new smear positive pulmonary TB cases; a total of 117 (28.1%) smear-positive sputa with a positivity score of 1+ or more were used in the study. Of 110 samples tested for drug susceptibility, 64 (58.2%) were sensitive to all drugs and 46 (41.8%) were resistant to one or more drugs. 11 (10%) were mono resistant, 4 (36.4%) were resistant to two drugs, 6 (54.6%) were resistant to 3 drugs, 25 (22.7%) were resistant to all four drugs and 34 (30.9%) were MDR.

The NRA results were compared with the gold standard LJ proportion method for 110 (94%) specimens while 7 were either contaminated or culture negative. NRA results were obtained at day 14 for 16 specimens (14.6%), results for 43 specimens (39.1%) were obtained at day 21, and results for the remaining 51 specimens (46.4%) were obtained at day 28 (p-value, <0.001). In comparison with LJ proportion method, the respective sensitivities, specificities, NPV, PPV

**Table 1.** Drug Susceptibility pattern of culture positive isolates (n=110) determined by the proportion method and Direct NRA method

Drugs	Direct NRA Method		Proportion Method	
	Resistant	Sensitive	Resistant	Sensitive
INH	38	72	36	74
RFM	35	75	34	76
SM	36	74	34	76
EMB	30	80	28	82

**Table 2.** Comparison of Direct Nitrate Reductase Assay results with Conventional Proportion method

Drugs	Direct Nitrate Reductase Assay	Conventional Proportion method		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	Kappa value
		Resistant	Sensitive					
INH	Resistant=38	35	3	97.2 (86 -100)	95.9 (89-99%)	92.1 (78-99)	98.6 (92-100)	0.92
	Sensitive=72	1	71					
RFM	Resistant=35	34	1	100 (90-100)	98.7 (93-100)	97.1 (85-100)	100 (95-100)	0.98
	Sensitive=75	0	75					
SM	Resistant=36	33	3	97.1 (85-100)	96.1 (89-99)	91.7 (78-98)	98.7 (93-100)	0.92
	Sensitive=74	1	73					
EMB	Resistant=30	28	2	100 (88-100)	97.7 (91-100)	93.3 (78-99)	100 (95-100)	0.93
	Sensitive=80	0	80					

and kappa agreement were 97.2% (95% CI, 86-100), 95.9% (95% CI, 89-99), 92.1% (95% CI, 78-99) 98.6% (95% CI, 92-100), and 0.92 for INH, 100% (95% CI, 90-100), 98.7% (95% CI, 93-100), 97.1% (95% CI, 85-100), 100% (95% CI, 95-100) and 0.98 for RFM, 97.1% (95% CI, 85-100), 96.1% (95%, 89-99), 91.7% (95% CI, 78-98), 98.7% ((95% CI, 93-100) and 0.92 for SM and 100% (95% CI, 88-100), 97.7% (95% CI, 91-100), 93.3% (95% CI, 78-99), 100% (95% CI, 95-100) and 0.93 for EMB, respectively.

### DISCUSSION

To our knowledge, this is the second evaluation of the direct NRA in Nepal. Full agreement concerning the sensitivity results of the direct NRA and proportion method was seen for all four drugs in the study. The method revealed the best agreement for RFM showing 100% sensitivity, 98.7% specificity with a PPV of 97.1% and NPV of 100% and kappa agreement of 0.98. RFM also showed best agreement conducted by Mandal *et al.* in Nepal with 100% sensitivity and 98.9% specificity.<sup>13</sup> However, indirect NRA method conducted by Sah *et al.* in NTC, Nepal showed the sensitivities, specificities and kappa agreement of 98.1%, 100% and 99.1 for INH, 95.1%, 98.6% and 97.3 for RFM, 91.4%, 94.90% and 93.8 for SM and 78.6%, 97.9% and 95.6% for EMB respectively.<sup>14</sup> Similar result was seen in study conducted by Musa *et al.* which showed best agreement for RFM with 100% sensitivity and 100% specificity RFM together with INH are the most important anti tuberculosis drug.<sup>11</sup> Resistance to RFM is also almost always associated with MDR and can thus serve as a marker of MDR-TB strains if resources are limited.<sup>15</sup>

Study by Affolabi *et al.* showed the sensitivity, specificity, NPV, PPV and kappa agreement of NRA with comparison to proportion method as 87.5%, 100%, 98.6%, 100% and 0.93 for RFM.<sup>10</sup> Syre *et al.* in Norway, compared colorimetric nitrate reductase based antibiotic susceptibility (CONRAS) test with radiometric BACTEC 460TB system in determining the susceptibilities to INH and RFM by using the BACTEC 460TB system as the "gold standard," the sensitivity, specificity and kappa agreement of the CONRAS test were 100%, 95% and 0.9 for INH and 94%, 100% and 0.9 for RFM respectively.<sup>9</sup> Similar study conducted by Rosales *et al.* evaluated sensitivity, specificity, PPV, NPV and kappa agreement of NRA compared to LJ-PM as 100%, 99%, 91%, 100%, 0.95 and 80%, 100%, 100%, 99%, 0.88 respectively.<sup>16</sup>

NRA results were obtained at day 14 for 16 specimens (14.55%), results for 43 specimens (39.1%) were obtained at day 21, and results for the remaining 51 specimens (46.4%) were obtained at day 28 with  $p < 0.001$ . In a similar study conducted by Affolabi *et al.* to determine RFM resistance, 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 8 specimens (4%) were obtained at day 28.<sup>10</sup> The shorter turnaround time (14 to 28 days) is an advantage over the proportion method, which requires  $\geq 28$  days for culture and 42 days for DST, while the direct NRA excludes a period of 3 to 8 weeks of Mycobacterial cultivation. Moreover, NRA can be performed on smear positive sputa grades of AFB 1+ or 2+. In this study, these sputa represented 47.27% of the samples. The simple, cost effective and rapid NRA is suitable for large-scale surveillance studies in resource-limited settings.<sup>11</sup> With these advantages, NRA has some limitations such as some strains (<1%) of *M. tuberculosis* lack nitrate reductase rendering the test invalid. The direct NRA can therefore be complemented with p-nitrobenzoic acid to confirm the presence of *M. tuberculosis* complex isolates.<sup>10,13</sup>

### CONCLUSION

Direct NRA is simple to perform and provides a rapid, accurate, and cost-effective means for the detection of drug resistance in *M. tuberculosis*. Even though more studies are needed to further assess the accuracy and applicability of this method, the direct NRA has the potential to become an inexpensive alternative for DST where resources are scarce.

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