EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 6
Issue: 6
564-567
Year: 2019

RAPID DETECTION OF DRUG RESISTANT TUBERCULOSIS BY DIRECT NITRATE REDUCTASE ASSAY IN RESOURCE POOR SETTINGS

Dristi Halwai[*1], Rajendra Gurung[1] and S. K. Bhattacharya[1]

Department of Microbiology and Infectious Diseases, B.P Koirala Institute of Health Sciences, Dharan-18, Sunsari, 56700 Nepal.

***Corresponding Author: Dr. Dristi Halwai**
Department of Microbiology and Infectious Diseases, B.P Koirala Institute of Health Sciences, Dharan-18, Sunsari, 56700 Nepal.

Article Received on 21/04/2019 **Article Revised on 11/05/2019** **Article Accepted on 01/06/2019**

ABSTRACT
Background: The most common method for detection of drug resistant tuberculosis is conventional proportion method requiring three months to yield the results. Delay in the proper diagnosis may hinder the effective treatment and increase the transmission of the drug resistant tuberculosis. Hence, this study was conducted to evaluate nitrate reductase assay (NRA) as a simple and rapid alternative method for detecting drug resistance tuberculosis in resource limited settings. **Methods:** A total of fifty eight new smear positive sputum samples were processed as per the standard guidelines. After decontamination, sputum samples were inoculated on NRA medium for rapid drug susceptibility testing and conventional culture medium. **Results:** NRA detected 13.2% isoniazid resistance, 11.3% rifampicin resistance and 5.7% multidrug resistant strains of *Mycobacterium tuberculosis*. A total of 44 samples yielded results within 21 days and 19 samples yielded results in 14 days. The mean duration for detection and drug susceptibility testing of *Mycobacterium tuberculosis* by NRA was 19.2 days. **Conclusions:** The present study concludes that NRA is a simple and rapid alternative method for detection of drug resistance to isoniazid and rifampicin in resource strain settings.

KEYWORDS: drug resistant tuberculosis, nitrate reductase assay, tuberculosis.

INTRODUCTION
Tuberculosis (TB) still remains a major public health challenge in Nepal, as it is responsible for disease and disability among thousands of people each year. Almost half of Nepal's population are infected with TB with 31,723 new TB cases and 1023 TB deaths reported to National Tuberculosis Programme, Nepal in the year 2017.[1] The increasing occurrence of multidrug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) have further worsened the impact of this disease. In Nepal, there are estimated around 1500 cases of drug resistant TB (DR-TB) and 450 cases of MDR-TB notified annually.[1]

Molecular methods like Gene Xpert MTB/RIF and mycobacterial growth indicator tube (MGIT) 960 has substantially increased the sensitivity and reduced the time for drug susceptibility testing (DST) but due to the expensive cost it is difficult to be used as a reference method in developing countries with limited resources.[2] The conventional methods of DST in Lowenstein Jensen media are slow and tedious.[3] Therefore, the development and availability of rapid, simple and inexpensive methods for early screening of DR-TB cases is needed especially in low resource settings.

WHO in its publication in 2008, "New laboratory Diagnostic Tools for Tuberculosis Control", has proposed nitrate reductase assay as a simple and inexpensive method for rapid detection of drug resistant tuberculosis.[4] NRA is a phenotypic method based on the ability of *Mycobacterium tuberculosis* (MTB) to reduce nitrate present in the medium to nitrite which can be detected by colour change in the culture medium after adding Griess reagent. NRA can be done directly on the sputum samples thereby significantly reducing the turnaround time needed to obtain the results of culture and DST of MTB.[5] The present study aimed for rapid drug susceptibility testing of MTB by NRA directly on smear positive sputum samples in a tertiary care hospital of Nepal.

MATERIALS AND METHODS
This was a comparative cross-sectional study conducted at Department of Microbiology, BPKIHS, Dharan, Nepal over a period of one year. A total of 58 new smear-positive sputum samples received in the laboratory were included in our study.

The sputum samples were decontaminated by modified Petroff's method.[6] The sediments were used to inoculate

the culture media middlebrook 7H11 media for NRA and Lowenstein Jensen (LJ) medium.

Conventional Proportion method

The sediments were inoculated in LJ medium and para nitro benzoic acid containing LJ medium (PNBLJ). The isolates were confirmed as MTB isolates by slow growth rate, standard biochemical tests and inhibition of its growth on PNBLJ.[6]

DST for both the drugs was done by conventional proportion method in LJ media as per the standard guidelines with recommended critical concentration of INH and RIF. The reference strain *Mycobacterium tuberculosis* H37Rv was used as a quality control strain for both culture and DST.[7]

Nitrate reductase assay[8,9]

Nitrate reductase assay was performed directly from sputum samples in NRA tubes containing 7ml of media incorporated with 1000 µg/ml KNO_3 in the medium. The sputum sediments were inoculated in three NRA tubes containing the following: one as a growth control without drugs; one with 0.2µg/ml of INH and one with 1µg/ml of RIF. All three types of NRA bottles were inoculated and incubated at 37ºC according to the standard procedure manual.

Nitrate reduction was tested by adding 0.5ml of the Griess reagent in drug free NRA bottle on 10^{th} day of incubation. If the medium turned pink, then the corresponding NRA bottles with INH and RIF were tested for any colour changes. If there were no colour changes, then the NRA bottles were re-incubated and the procedure was repeated on 14^{th}, 21^{st} and finally on 28^{th} day of incubation. The isolates were classified as resistant to a drug if there was pink or purple colour development in drug containing media. *Mycobacterium tuberculosis* H37Rv strain was used as a positive control and *Mycobacterium intracellulare* strain was used as a negative control strain for NRA.

RESULTS

Nitrate reductase assay was implemented for direct detection and drug susceptibility testing of Mycobacterium tuberculosis from sputum samples. Out of total 58 smear positive sputum samples, mycobacterial growth was detected in 53 (91.4%) samples, contamination was seen in 4 (6.9%) and a single sample did not grow in the culture media. Five sputum samples were excluded from our study for drug susceptibility testing as there were contamination and no growth. Fifty three sputum samples were included for the drug susceptibility testing and turnaround time by direct NRA.

Among 53 sputum samples tested by direct NRA, 18.9% were resistant to any drugs. NRA detected 13.2% isoniazid resistance, 11.3% rifampicin resistance and 5.7% multidrug resistant strains of *Mycobacterium tuberculosis* as depicted in **Table 1**. The results showed that NRA and conventional proportion method do not vary significantly (p>0.05) for both the drugs.

Table 1: Percentage of drug resistance (Total n=53).

Antibiotics	Resistant
Isoniazid	7 (13.2%)
Rifampicin	6 (11.3%)
MDR-TB	3 (5.7%)

Nitrate reductase assay gives rapid results of drug susceptibility testing compared to conventional proportion method. NRA detected resistance to INH and RIF within 28^{th} day whereas MDR-TB was detected within 21^{st} day of the culture as shown in **Table 2**. The results of this study indicates that NRA could be used for rapid diagnosis of MDR-TB. Conventional proportion method takes 28-42 days for primary isolation and additional 28-42 days for drug susceptibility testing of the MTB isolates.

Table 2: Detection of drug resistance by NRA (n=53).

Resistance	10^{th} day	14^{th} day	21^{st} day	28^{th} day	Total
INH	1	2	3	1	7
RIF	1	2	2	1	6
MDR	0	1	2	0	3

A total of 19 (35.8%) samples yielded results in 14 days, 44 (83%) samples yielded results in 21 days and the results for all the samples were available within 28 days. As NRA could detect mycobacterial growth and drug resistant simultaneously, the mean duration for detection and DST of MTB by NRA was found to be 19.2 days (range, 10-28 days).

As shown in **Table 3**, the results of drug susceptibility testing were faster in sputum samples with higher bacillary load. On day 21, 100% of sputum samples with ZN score of 3+ yielded results whereas only 89.5% and 63.2% of the results were available for sputum samples with ZN score of 2+ and 1+ respectively. The mean turnaround time of NRA for drug susceptibility testing was compared with respect to the grading of the sputum smear. Significant differences (p<0.05) in mean turnaround time was observed in different grades of sputum.

Table 3: Turnaround time of drug susceptibility testing by direct nitrate reductase assay in relation to ZN smear score (n=53).

ZN Score	Direct Nitrate Reductase Assay					Total samples
	10th day n (%)	14th day n (%)	21st day n (%)	28th day n (%)	Mean turnaround days	
1+	1 (5.3)	0 (0)	11(57.9)	7 (36.8)	23	19 (35.8%)
2+	3 (15.8)	3 (15.8)	11 (57.9)	2 (10.5)	18.9	19 (35.8%)
3+	2 (13.3)	10 (66.7)	3(20)	0 (0)	14.8	15 (28.3%)
Total	6 (11.3)	13 (24.5)	25 (47.2)	9 (17)	19.2	53 (100%)

DISCUSSION

New diagnostic tools for rapid detection of drug resistant TB are the need of time. WHO has endorsed non-commercial culture and DST methods for implementation in low income countries for rapid detection of MDR-TB.[10] Many studies have shown that NRA test gives concordant results with proportion method. Hence, in this study sputum samples were processed in direct nitrate reductase assay for rapid diagnosis of drug resistant TB.

About 5.7% of mycobacterial strains were multi drug resistant, which were detected within 21st day of the culture. In our study, 11.3% results were obtained at day 10, 24.5% at day 14, 47.2% at day 21 and 17% of the results were obtained at day 28. Similar study done by Mishra et al reported that results for 28.1% strains were available at day 14 and for 65.6% strains the results were available at day 21 which is concordant to our findings.[11] In contrast to our findings, Mashaly et al obtained 87% of the results at day 15 and Rosales et al reported that 83.7% results were available within 14 days.[10,12] The difference in the results may be explained by difference in the bacillary load of the inoculums.

The results of drug susceptibility testing were obtained significantly faster in sputum samples with higher bacillary load. We found that 100% of sputum samples with ZN score of 3+ yielded results on day 21, whereas only 89.5% and 63.2% of the results were available on day 21 for sputum samples with ZN score of 2+ and 1+ respectively. Similar findings were reported by Mashaly et al, who reported that 100% of the results were available at day 14 for samples with ZN score of 3+ and at day 18 for sputum samples with ZN score 2+.[12] Our study have shown that NRA can be performed even on sputum samples with low ZN score (1+ or 2+), which comprised the 73% of the samples in the present study. The ZN scoring of the samples included in the study and the final volume of the sediments may have influenced the results.

CONCLUSIONS

This study demonstrated that NRA is a simple and rapid alternative method for detecting drug resistant tuberculosis and needs no additional equipment. NRA being a simple and rapid method, can be implemented in low resource settings for drug susceptibility testing of *Mycobacterium tuberculosis*.

ACKNOWLEDGEMENTS

The authors thank all the faculty members and laboratory staffs of Department of Microbiology for their support throughout the study period.

REFERENCES

1. National Tuberculosis Centre. Annual Report 2018. Nepal National TB Programme.Department of Health Services, Bhaktapur, Nepal.; 2018.
2. Sagili K, Muniyandi M, Nilgiriwala KS, Shringarpure S, Satyanarayana S, Kirubakaran R, et al. Cost-effectiveness of GeneXpert and LED-FM for diagnosis of pulmonary tuberculosis : A systematic review. PLoS One. 2018; 1–16.
3. Wells CD, Cegielski JP, Nelson LJ, Laserson KF, Holtz TH, Finlay A, et al. HIV Infection and Multidrug-Resistant Tuberculosis — The Perfect Storm. J Infect Dis. 2007; 196(Suppl 1): 86–107.
4. World Health Organization. New Laboratory Diagnostic Tools for Tuberculosis Control. Geneva: World Health Organization; 2008.
5. Musa HR, Ambroggi M, Souto A, Ängeby KAK. Drug Susceptibility Testing of *Mycobacterium tuberculosis* by a Nitrate Reductase Assay Applied Directly on Microscopy-Positive Sputum Samples. J Clin Microbiol. 2005; 43(7): 3159–61.
6. World Health Organization. Laboratory services in Tuberculosis Control: Culture. Geneva: World Health Organization; 1998.
7. Canetti G, Froman S, Grosset J, Hauduroy P, Langerova M, Mahler HT, et al. Mycobacteria : Laboratory Methods for Testing Drug Sensitivity and Resistance. Bull World Heal Organ. 1963; 29(5): 565–78.
8. Martin A, Palomino JC. Procedure manual:Nitrate reductase assay: Drug susceptibility testing for *Mycobacterium tuberculosis*. Belgium; 2012.
9. Singh S, Kumar P, Sharma S, Mumbowa F, Martin A, Durier N. Rapid Identification and Drug Susceptibility Testing of *Mycobacterium tuberculosis* : Standard Operating Procedure for Non-Commercial Assays : Part 2:Nitrate Reductase Assay. J Lab Physicians. 2012; 4(2): 112–9.
10. Rosales S, Almendarez N, Membreño H, Hoffner SE, Pineda-Garcia L. Field assessment of the direct nitrate reductase assay for rapid detection of multidrug-resistant tuberculosis in Honduras. Int J Tuberc lung Dis. 2011; 15(9): 1206–10.
11. Mishra B, Murlidharan S, Srinivastav H. Direct drug susceptibility testing of *Mycobacterium tuberculosis*

to primary anti-tubercular drugs by nitarte reductase assay. Indian J Pathol Microbiol. 2019; 52(3): 343–4.

12. Mashaly M, Fathy M, Elazeim DA, Abdelaal A. Rapid detection of Drug Resistant *Mycobacterium tuberculosis* in Sputum Samples by Direct Nitrate Reductase Assay Method. Curr Res Microbiol Biotechnol. 2014; 2(2): 334–9.