The Nitrate Reductase Assay in the Rapid Detection of Multidrug-Resistant and Extensively Drug- Resistant Tuberculosis in South Gujarat, India

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Abstract: _Introduction_: *The burden of Tuberculosis (TB) particularly with multi-drug-resistance (MDR) is increasing worldwide and has become a major public health concern. The increasing prevalence of MDR as well as extensively drug-resistant (XDR) tuberculosis highlights the need for simple, rapid, affordable and accurate methods for performing drug susceptibility testing against first- line and second- line anti- TB drugs.* _Aims_: *To evaluate the performance of NRA in the rapid detection of multi-drug resistant and extensively-drug resistant tuberculosis in the region of South Gujarat, India.* _Materials and Methods_: *234 samples were collected from clinically suspected TB patients from South Gujarat Region, India and were subjected to microscopy examination by ZN method, culturing on Lowenstein-Jensen medium and drug susceptibility testing by indirect NRA. Indirect NRA was performed on the culture isolates and the results were compared with that of the Proportion method.* _Results_: *Out of these 234 samples, 94 were smear positive by Ziehl Neelsen (ZN) method (40.17%) and 140 were smear negative. In case of culturing, out of 234 samples, 101 were culture positive (43.16%) and 133 were culture negative. By performing First-Line DST, MDR-TB was detected in a total of 37/101 (36.63%) and 35/101 (34.65%) by both Proportion method and NRA respectively. By performing Second-Line DST, one case of XDR-TB was detected by both the methods. NRA results were available for 33 samples on day 7, 58 samples on day 10 and 10 samples on day 14. As compared to Proportion method, the sensitivity of NRA was 87.5% and 100% and the specificity was 100% and 96.73% for RIF and INH respectively. Similarly, for K and OF, the sensitivity of NRA was 71.42% and 76.92% and the specificity was 100% and 100% respectively.* _Conclusion_: *NRA is a simple, accurate, inexpensive and rapid method for detection of MDR and XDR-TB, especially in poor-resource countries, with limited laboratory facilities. The technique may become a valid substitute to traditional time consuming methods.*

Keywords: *Mycobacterium tuberculosis*, multi-drug resistance, extensively-drug resistance, Proportion Method, Nitrate Reductase Assay

1. Introduction

Tuberculosis (TB) remains one of the world's deadliest communicable diseases. The disease caused by *Mycobacterium tuberculosis* resistant to two primary anti-tubercular drugs, rifampicin (RIF) and isoniazid (INH), is known as MDR-TB. The burden of tuberculosis (TB) particularly with multi-drug-resistance (MDR) is increasing worldwide and has become a major public health concern [1, 2]. According to the World Health Organization (WHO) report, most cases are estimated to be in Asia and Africa accounting for 58% and 27% respectively, with the highest incidence in India (range 2.0-2.4 million) and China (0.9 - 1.1 million), which together accounts for 38% of the total number of cases (Global TB Report, 2013). Current resurrection of TB is mainly due to increasing incidence of resistance of *M. tuberculosis* strains to first-line and important second-line anti-TB drugs and the association of active TB disease with HIV co-infection or other underlying immunosuppressive conditions [3, 4]. Accurate and timely diagnoses of MDR-TB cases with adequate treatment regimens are the essential key elements to stop primary transmission of MDR- TB. The increasing prevalence of multidrug- resistant (MDR) and extensively drug-resistant (XDR) tuberculosis highlights the need for simple, rapid, affordable and accurate methods for performing drug susceptibility testing against first- line and second- line anti-TB drugs.

Conventional culture method based on Lowenstein –Jensen (LJ) medium is still the most commonly used and inexpensive method for the growth of *M. tuberculosis.* Similarly, conventional drug susceptibility testing methods based on LJ medium are time consuming, requires up to 6 weeks giving results [5, 6]. Commercial broth based systems and molecular tests have been developed to reduce the turnaround time, but are still too expensive thus preventing them from being adopted in low resource countries [7, 8].

For developing countries, it would be useful to have a simple and inexpensive method for early and proper detection of drug resistant TB cases for the effective management and control of TB.

Nitrate Reductase Assay (NRA), also called Griess Method [9], was first described by Angeby *et al.* [10]. It has been endorsed by the WHO for the rapid detection of MDR-TB [11]. NRA is a simple, rapid, low-cost, phenotypic method performed on solid media and is based on the ability of *Mycobacterium tuberculosis* to reduce nitrate to nitrite, the presence of which can be easily detected with specific reagent, so called Griess Reagent, that produce a color change [10].

The main aim of the present study was to evaluate the performance of Nitrate Reductase Assay in the rapid detection of multi-drug resistant and extensively-drug resistant tuberculosis in South Gujarat, India.

2. Materials and Methods

In this study, 234 samples, pulmonary or extrapulmonary,

were collected from clinically suspected TB patients from South Gujarat Region, India, for 18 months during June 2013 to December 2014. All mycobacterial investigations were carried out at the Microcare Tuberculosis Laboratory, Surat, which is accredited for carrying out culture and Drug Susceptibility Testing (DST) by the Central TB Division, Ministry of Health and Family Welfare, Govt. of India.

Microscopy and Culture

All the specimens were handled in class II bio safety cabinet in a bio-safety level (BSL) – 3 laboratory and were decontaminated by Modified Petroff's Method [12]. All the samples were subjected to microscopic examination by using the conventional Ziehl Neelsen method for detection of acid fast bacilli (AFB) and culturing. The smears were graded according to the number of bacilli, as per recommendations of the World Health Organization (WHO).

All the culture isolates were confirmed as *Mycobacterium tuberculosis* by their slow growth rate, colony morphology, and pigmentation, inability to grow on L-J media containing p-nitro benzoic acid (PNB), niacin positive and catalase negative tests [13].

Drug Susceptibility Testing

In this study, drug susceptibility testing (DST) was performed by Nitrate Reductase Assay and the performance was compared to that of the Conventional Lowenstein – Jensen Proportion Method (LJPM). Since the LJ Proportion method is widely used across laboratories in India under RNTCP as a standardized method for DST, this method was used as a reference standard method in this study [14].

LJ Proportion Method

The Lowenstein – Jensen Proportion Method was carried out on solid LJ medium according to the standard operating procedure of RNTCP with the recommended critical concentrations of 40μg/ml rifampicin, 0.2μg/ml isoniazid, 2μg/ml ofloxacin and 30μg/ml kanamycin. LJ media were incubated at 37°C and read at 28 and 42 days [6].

Nitrate Reduction Assay (NRA)

The method was carried out as described by Angeby *et al.* [10]. This method is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, which is routinely used for biochemical identification of mycobacterial species. The presence of nitrite can easily be detected with specific reagent, Griess reagent, which produces a color change. The nitrate reduction assay uses the detection of nitrite as an indication of growth when used as a drug susceptibility test. The rifampicin, isoniazid, ofloxacin and kanamycin were added in the LJ medium at a concentration of 40μg/ml, 0.2μg/ml, 2μg/ml and 30μg/ml respectively with 1000 mg/L potassium nitrate (KNO_3). The inoculum turbidity was adjusted to a McFarland tube no. 1 and diluted 1:10 in PBS. The Griess reagent mixture consisted of 1 part 50% concentrated hydrochloric acid (HCl), 2 parts 0.2% sulfanilamide and 2 parts 0.1% n-1-naphthylethylenediamine dihydrochloride. For each strain, 200μl of the undiluted suspension was inoculated into the drug containing medium and 200μl of the 1:10 dilution into the drug-free medium. All the media were incubated at 37°C. After 7 days, 500μl of reagent mixture was added to one drug-free medium. If any

color occurred, all the media were developed by the reagent mixture. If no color change was observed in the growth control medium, the medium was discarded, and the other two growth control media and the antibiotic-containing media were re-incubated. The procedure was then repeated at day 10, using the second growth control, and if needed, also at day 14, using the last growth control medium.

Statistical analysis

The results of NRA and Proportion method were analyzed and their sensitivity (ability to detect true resistance) and specificity (ability to detect true susceptibility) were calculated using standard formula.

3. Results

In total, 234 samples were collected from TB suspected patients from South Gujarat region, India. Out of these 234 samples, 94 were smear positive by Ziehl Neelsen (ZN) method (40.17%) and 140 were smear negative. In case of culturing, out of 234 samples, 101 were culture positive on Lowenstein-Jensen medium (43.16%) and 133 were culture negative [Table 1]. In total 101 TB positive patients, 70 were males (69%) and the rest 31 were females (31%). The youngest patient included in the study was 9 years old while the oldest was 86 years old. The maximum number of patients were found to be in the age-group of 20-29 (31.68%), followed by in the age-group of 30-39 (24.75%).

Table 1: Comparison of results of Microscopy Versus Culture on LJ medium

Comparison of results of Microscopy versus culture on LJ medium			
	Smear +ve	Smear -ve	Total
Culture +ve	94	7	101
Culture -ve	0	133	133
Total	94	140	234

Table 2: Primary Culture results

Sr. No.	Culture results	Patient's Number
1	Scanty	3
2	1+	14
3	2+	14
4	3+	70
	Total	101

ZN microscopy results for the 94 TB positive samples were scanty for 2 samples, 1+ for 25 samples, 2+ for 27 samples and 3+ for 40 samples. Primary culture results for the 101 samples were scanty for 3 samples, 1+ for 14 samples, 2+ for 14 samples and 3+ for 70 samples [Table 2]. The minimum time required for the growth of *Mycobacterium tuberculosis* on Lowenstein – Jensen medium was 2 weeks and the maximum was 8 weeks.

By performing First-Line drug susceptibility testing, MDR-TB was detected in a total of 37/101 (36.63%) and 35/101 (34.65%) by both Proportion method and Nitrate Reductase

Assay respectively [Table 3]. By performing Second-Line drug susceptibility testing, one case of XDR-TB was detected by both Proportion method and NRA [Table 4]. The Nitrate Reductase Assay was easy to interpret. NRA results were available for 33 samples at day 7, 58 samples at day 10 and 10 samples at day 14.

Table 3: Drug susceptibility testing results against First-Line Drugs by the NRA compared to Proportion Method

Susceptibility Test results for 101 *M. tuberculosis* strains		
Drug Sensitivity Pattern	Proportion method	Nitrate Reductase Assay
Susceptible to RIF and INH	47	47
RIF-Monoresistant	8	7
INH-Monoresistant	9	12
MDR (Resistant to RIF and INH)	37	35
Total	101	101

Table 4: Drug susceptibility testing results against Second Line Drugs by the NRA compared to Proportion Method

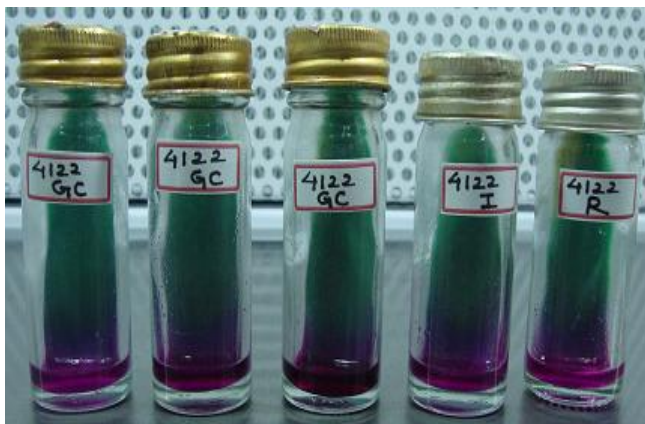| Drug Susceptibility Pattern	Proportion Method	Nitrate Reductase Assay
Susceptible to K & OF	17	22
K-Monoresistant	7	5
OF-Monoresistant	13	10
XDR	1	1
In total, 38 samples were tested for XDR-TB.		

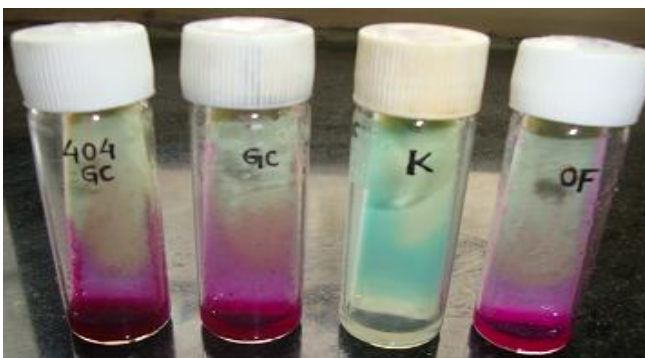**Figure 1:** Drug susceptibility testing of *M. tuberculosis* for First Line Drugs by NRA

Figure 2: Drug susceptibility testing of *M. tuberculosis* for Second Line Drugs by NRA

Table 5: Sensitivity and Specificity for Rifampicin by NRA

	RIF	Proportion Method		
		Positive	Negative	Total
Nitrate Reductase Assay	Positive	7 (TP)	0 (FP)	7
	Negative	1 (FN)	93 (TN)	94
	Total	8	93	101

Sensitivity of Rifampicin (RIF) = TP/ TP+FN×100, 7/7+1×100 = 87.5%

Specificity of Rifampicin (RIF) = TN/TN+FP×100, 93/93+0×100 = 100%

As compared to Proportion method, the sensitivity of the NRA was found to be 87.5% and 100% whereas specificity of the NRA was found to be 100% and 96.73% for RIF and INH respectively.

The sensitivity of the NRA was found to be 71.42% and 76.92% whereas specificity of the NRA was found to be 100% and 100% for K and OF respectively.

4. Discussion

In recent years, a major emphasis has been given on rapid diagnosis and timely treatment of MDR-TB. Simple, efficient, fast and low cost methods for detection of drug resistant TB is highly desirable to cut the transmission of the disease. In this context, we evaluated the performance of Nitrate Reductase Assay in the rapid detection of multi-drug resistant and extensively-drug resistant tuberculosis in South Gujarat region.

This study shows that men are more commonly affected than women. It was also found that TB cases reported in most countries were higher in males than in females [15]. Our findings were found to be similar with Vanisree *et al.* (2014). In their finding, they reported out of 100 cases, 71 were males and 29 were females

Although considered as the gold-standard method, the drug susceptibility testing performed on LJ medium by the Proportion method is very slow and requires 4-6 weeks to produce the results.

The drug susceptibility results for the NRA were obtained within 7-14 days after isolation of the bacterium [16]. Similar time period is also taken by BACTEC TB-460 or MGIT methods with the disadvantage of requiring high cost equipment and radioactive substances [17]. In our study, the indirect NRA showed overall good sensitivity and specificity for the drugs Isoniazid, Rifampicin, Kanamycin and Ofloxacin to be tested. Many studies have also reported the importance of the NRA for determining susceptibility or

resistance to rifampicin and isoniazid and have shown high sensitivity and specificity [18, 19, 20]. The accuracy for Rifampicin is an attractive feature of the NRA so it is used as a screening method for detection of MDR strains, as resistance to Rifampicin is a marker for MDR detection [21, 22]. The NRA also has the potential to be used for the detection of resistance to second line drugs. In 2005, Martin *et al.* reported the first evaluation of the NRA for the detection of ofloxacin resistance and found it to be in complete agreement with the proportion method [16].

Rosales *et al.* evaluated the NRA for the rapid detection of resistance to ofloxacin and kanamycin and found good specificity for both drugs, but lower sensitivity for detecting resistance to kanamycin which is in concordance with our results [23]. Our study confirms that the NRA can also be used to screen XDR-TB. But this study was limited by the relatively small number of XDR-TB cases found.

5. Conclusion

From the results of the present study, it can be concluded that the NRA is a simple, accurate, inexpensive and rapid method for detection of MDR and XDR-TB, especially in poor-resource countries, with limited laboratory facilities because it does not require expensive reagents and sophisticated equipments. The technique may become a valid substitute to traditional time consuming methods.

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