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Evaluation of the Performance of Nitrate Reductase Assay for Rapid Drug-Susceptibility Testing of *Mycobacterium Tuberculosis* in South Gujarat Region

Smita D Rajani[1], Pratibha B Desai[1], Dhanji P Rajani[2]

[1]Departments of Microbiology, Shree Ramkrishna Institute of Computer Education & Applied Sciences, Veer Narmad South Gujarat University, Surat, Gujarat, India.

[2]Microcare Laboratory, 105, Manthan point, Unapani road, Surat, Gujarat, India

Abstract: ***The objective of the study was to evaluate the performance of nitrate reductase assay (NRA) as a rapid, reliable and inexpensive method for drug-susceptibility testing (DST) of Mycobacterium tuberculosis against first line antitubercular drugs, such as isoniazid (INH), rifampicin (RIF), ethambutol (EMB), streptomycin (STR). In total, 230 isolates were subjected to test by proportion method (PM) and NRA. By comparing the results of NRA with those of the gold standard PM, Sensitivity values for isoniazid, rifampicin, ethambutol and streptomycin were 98.7%, 100%, 98.3% and 97.3%, respectively and specificity values were 100%,100%, 100% and 96.7%, respectively. The median time of obtaining results was shorter using NRA (10 days) compared to PM (28 days). An excellent agreement was observed between the two phenotypic tests was 98.8%.The results demonstrated that NRA is suitable for the early determination of INH and RIF resistance and has the potential to be a useful tool for rapid drug-sensitivity test of M. tuberculosis in low resource settings.***

Keywords: Antitubercular agents, *Mycobacterium tuberculosis,* Nitrate reductase assay, Multi-drug-resistant, South Gujarat.

1. Introduction

Tuberculosis (TB) is one of the leading causes of illness and death in the world today. The World Health Organization (WHO) has estimated that 2 billion people have latent TB and that globally, in 2009, the disease killed 1.7 million people [1]. Approximately 3.9 million cases were sputum-smear positive, the most infectious form of the disease [2, 3]. The African region has the highest estimated incidence rate (345 per 100,000 populations annually), but the most populous countries of Asia harbor the largest number of cases: Bangladesh, China, India, Indonesia, and Pakistan together account for half the new cases arising each year. In terms of the total estimated number of new TB cases arising annually, about 80 percent of new cases occur in the top-ranking 22 countries. The world's two most populous countries, India and China, account for more than 50% of the world's MDR-TB cases and as such these countries are encountering a high and increasing TB disease burden, which is also expected because these two countries are two most populated countries of the world and a major part of their population cannot afford expensive treatment.

HIV is the most important known risk factor that promotes progression to active TB in people with *Mycobacterium tuberculosis* infection [4]. The life time risk of tuberculosis in immunocompetent persons is 5% to 10%, but in HIV positive individuals, there is a 5% to 15% annual risk of developing active TB disease. WHO estimated 9.2 million new cases of TB globally in 2006 (139 per 100,000), of whom 7,09,000 (7.7%) were HIV positive [4]. TB accounts for about one in four of the deaths that occur among HIV-positive people. Of the 9.4 million TB cases in 2009, 11–13% were HIV positive with approximately 80% of these co-infections confined to the African region [5]. In India, there were 2.5 million people living with HIV and AIDS at the end of 2007 while the incidence of TB was approximately 1.8 million cases per year [6].

Annually around 1 million people die from TB with MDR-TB defined as resistance to at least rifampicin (RIF) and isoniazid (INH) and XDR-TB (extensive drug-resistant-TB) occurs in MDR strains which are resistant to any fluoroquinolone and at least one of three injectable second-line aminoglycoside drugs, i.e. amikacin, kanamycin, or Capreomycin [7]. The sheer size of their TB case populations results in the highest estimated numbers of MDR-TB cases (about 100,000 each) emerging annually from these two countries. Moreover, the emergence of XDR strains of *M. tuberculosis* (5.4% of MDR-TB) cases are found to be XDR-TB is challenging TB treatment programmes in several other countries and even raises the possibility of a return to a situation akin to the pre-antibiotic TB era [7].

There is an urgent demand for early and proper detection of MDR and XDR-TB cases for the effective management and control of TB. Conventional method, i.e. proportion method (PM), is the gold standard for DST of *Mycobacterium tuberculosis* (MTB) but has some limitations, such as cumbersomeness and long-turnaround time (TAT). In recent years, a many other techniques for rapid DST has been designed and evaluated, such as colorimetric redox method [8], radiometric method BACTEC 460-TB [9], commercial MGIT, E-tests [10,11], and molecular methods—Genotype MTBDRplus and INNO-LiPA [12,13]. Most of these have proved their reliability and accuracy but are limited to developed countries only because of high expenses while

others, such as colorimetric redox methods or nitrate reductase assay, need more evaluation in the field. NRA—also called Griess method [14]—was first described by Angeby et al. [15]. NRA is based on the principle that MTB has the capability of reducing nitrate to nitrite. The presence of nitrite can be detected by addition of Griess reagent which changes the colour of the culture medium [16]. Several studies evaluated the performance of NRA but only a few were in India [17-22]. The present study was, therefore, conducted to evaluate the performance of NRA compared to the gold standard PM for DST of MTB. Susceptibility tests were performed for all four first-line antitubercular drugs, i.e. rifampicin (RIF), isoniazid (INH), streptomycin (STR), and ethambutol (EMB).

2. Material and Methods

2.1 Settings

Early morning sputum samples from 230 clinically suspected TB patients were collected from different hospitals of south Gujarat region, India, a period of two year from July 2009 – June 2011. All Mycobacterial investigations were carried out at the Microcare Laboratory & Tuberculosis Research Centre, Surat. The laboratory 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.

2.2 Sample Collection and Processing:

230 patients suspected to be suffering from tuberculosis having clinical symptoms viz. Coughing, loss of weigh, night sweat, fever, chest pain, dysponea and anaemia were selected for study. Early morning sputum samples were collected in a sterile, leak-proof container. 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 [23]. All the samples were subjected to smear examination for detection of acid fast bacilli (AFB) and culturing. Smears were made from the mucopurulent portion of sputum and stained by the conventional Ziehl Neelsen method. The smears were graded according to the number of bacilli seen on the slide, as per recommendations of the World Health Organization (WHO). For culturing of the specimen, two McCartney Bottles of Lowenstein – Jensen medium were inoculated with each sample and incubated at 37^0C until growth of mycobacterium was observed or were discarded as negative after 8 weeks.

H37Rv (ATCC 27294), and M. intracellulare (ATCC 13950) strains served as nitrate-positive and nitrate-negative controls respectively. A known MDR strain was also used as control. All the strains were first confirmed for their nitrate reductase activity [23], which is part of the routine biochemical tests. All the culture isolates were identified as *Mycobacterium tuberculosis* by their slow growth rate, colony morphology, inability to grow on L-J media containing p-nitro benzoic acid (500 mg/ml), niacin positive and catalase negative tests [24]. All the strains were sub-cultured in Lowenstein-Jensen (LJ) medium for four weeks before NRA was performed.

2.3 Antitubercular Drugs

INH, STR, RIF, and EMB were obtained in powder form from Sigma (St Louis, Missouri, USA). Each drug was prepared at a concentration of 10 mg/mL in sterile distilled water, except RIF, which was dis-solved in di-methyl formamide (DMF). Stock solutions were filter-sterilized and stored at -20^0C for not more than one month.

2.4 Media

Conventional LJ medium was prepared as described by Canetti et al. [25]. The LJ medium for NRA was prepared with a slight modification: 1 mg/mL KNO_3 was added to the LJ medium, with or without antibiotics, dissolved by stirring and then aliquoted and inspissated once for 50 minutes at 80^0C. For NRA, the bottles containing LJ with antibiotics and KNO_3 were used in duplicates while control tubes were used in triplicate.

2.5 Griess reagent

Fifty percent (vol/vol) concentrated hydrochloric acid (HCl), 0.2% (wt/vol) sulphanilamide, and 0.1% (wt/vol) n-1-naphthylethylenediamine dihydrochloride were prepared in small volumes and were mixed shortly before use in the ratio: 1 part: 2 part: 2 part respectively.

2.6 DST by PM

The PM was carried out on the LJ medium according to the laboratory's standard procedures with the recommended critical concentrations of 40 µg/mL for RIF, 0.2 µg/mL for INH, 2 µg/mL for EMB, and 4 µg/mL for STR [25, 26].

2.7 DST by NRA

NRA was performed according to the method described by Angeby et al.[15]. Bacterial suspensions were made from a subcultured LJ tube by dispensing two 1-µL loopfuls of bacteria in 0.5 mL of phosphate-buffered saline (PBS) (pH 7.4) in 7.5-mL screw-cap bottles containing a few 3-mm diameter glass beads and vortexed to obtain a uniform solution. To obtain the turbidity of McFarland standard no. 1, approximately 2.5 mL of PBS was added. Part of the suspension was diluted at 1:10 in PBS. For each strain, 0.2 mL of the undiluted suspension was inoculated into tubes containing the LJ medium with KNO_3 and the antibiotics in duplicates to reduce the risk of contamination and to assess the reproducibility of the test while 0.2 mL of the 1:10 dilution was inoculated into three drug-free tubes containing LJ with KNO3. The latter tubes served as growth controls. The tubes were incubated at 37 °C. After seven days, 0.5 mL of Griess reagent was added to one drug-free control tube. If any colour change could be observed, the corresponding antibiotic-containing tubes were also tested, and susceptibility results were read. If no colour change was observed in the growth control tube, this tube was discarded, and the other two control tubes and the antibiotic-containing tubes 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 tube.

The results were classified as negative if no colour change of the medium was observed and positive if pink to violet colour appeared in the medium. An isolate was considered to be resistant to a certain drug if there was a colour change in the antibiotic-containing tube in question greater than that in the 1:10 diluted growth control on the same day.

3. Result

DST for the first-line antitubercular drugs, i.e. RIF, INH, STR, and EMB, was performed with 230 clinical MTB isolates. The results of 101, 115, and 14 isolates were obtained after 7, 10, and 14 days respectively and were compared with those produced by the gold standard PM. The results are shown in the Table. For isoniazid, 77 isolates were found resistant and 152susceptible by both methods. One isolate gave discordant results since this was resistant by PM but susceptible by NRA. For rifampicin, all isolates gave concordant results with 75 resistant and 155 susceptible isolates. For ethambutol, 60 isolates were resistant and 169 susceptible by both methods; one strain gave a discordant result being resistant by PM but susceptible by NRA. For streptomycin, 73 isolates were resistant and 150 susceptible by both methods. Five isolates were susceptible by PM but resistant by NRA, and two isolates were resistant by PM but susceptible by NRA. Sensitivity values for isoniazid, rifampicin, ethambutol and streptomycin were 98.7%, 100%, 98.3% and 97.3%, respectively and specificity values were 100%,100%, 100% and 96.7%, respectively. The overall concordance was 98.8%.

Table 1: First-line drug susceptibility testing of isolates of *M.tuberculosis* by the nitrate reductase assay & the proportion method

	Proportion method							
	INH		RIF		EMB		SM	
NRA	R	S	R	S	R	S	R	S
Resistant	77	0	75	0	60	0	73	5
Susceptible	1	152	0	155	1	169	2	150
Total	78	152	75	155	61	169	75	155

The accuracy of NRA was high with the PM for INH (99.3%), RIF (100%), EMB (99.1%), and STR (98.3%). An excellent agreement was observed n the results for RIF and INH. A good correlation was also found for EMB and STR resistance and susceptibility (Table).

4. Discussion

The most worrisome trend during recent years is an increase in multidrug-resistant (i.e. resistant to RIF and INH) TB strains. Rapid detection of MDR strains is very important to restrict their spread in the population. Current methods for DST of MTB are either costly or very slow. So, a cost-effective and rapid drug-susceptibility method is required to guide the treatment of TB. Results of meta-analysis of Martin et al. about NRA suggest that the NRA is highly sensitive and specific for determining RIF- and INH-resistant TB in both culture isolates and directly on clinical sputum specimens [27]. Most studies had a sensitivity of 95% or greater, and nearly all were 100% specific with high degree of accuracy. The average TAT was 5-12 days with indirect NRA and 14-21 days with direct NRA. The biggest asset of NRA is that there is no need to change the laboratory infrastructure as it is performed in the classical LJ medium, routinely used in TB laboratories, with the addition of KNO3. There is no need of any sophisticated equipment or expensive reagent, making it a widely used method. Results are easy to observe by a colour change of the medium. In the present study, an excellent agreement between the results of NRA and PM was observed.

RIF together with INH are the most important drugs for the treatment of TB. Sensitivities of NRA for RIF and INH were 100% and 97% respectively whereas specificity was 100% for both the drugs. However, an acceptable result of NRA was also found for EMB and STR, with 98.3% and 97.3% sensitivity and 100% and 96.7%, specificity. Other studies have also reported high sensitivity and specificity values for INH and RIF [28, 29]. The accuracy with the PM was high for all the drugs used in this study, according to the criteria established by the WHO/IUATLD Supranational Laboratory Network, which has proposed the accuracy levels of 99.0% and 97.0% for RIF and INH respectively and 92.0% for EMB and STR as reasonable performance goals for reference laboratories.

In another study, Martin et al. reported the evaluation of NRA for ofloxacin, a second-line drug, and found complete agreement with the agar PM [30]. Therefore, NRA has the capability to be used also for the evaluation of second-line drugs. In addition, Lemus et al. evaluated indirect NRA with 320 strains of MTB and found an overall agreement of 98.8% between the NRA and the PM [31].

With the objective of reducing the TAT of DST by omitting the pre-isolation step, the NRA has been applied directly on clinical sputum specimens. A full agreement was observed for the detection of RIF resistance while some discordant results were obtained for other drugs [32].Visalakshi et al. observed sensitivity and specificity of the direct NRA and indirect PM to be 94% and 98%, and 100% and 98% for RIF and INH respectively with an excellent agreement between the two tests [22]. Moreover, Shikama et al. stated 100% sensitivity and specificiy of NRA for RIF[33]. In another study of Shikama et al., reproducibility of NRA was 100% for INH and EMB and 97% for STR and RIF, with 98.3% agreement between the results of NRA and PM for INH and RIF[34].

Syre et al. used colorimetric nitrate reductase-based antibiotic-susceptibility (CONRAS) test for DST of MTB against INH and RIF in liquid cultures [35]. The results were produced within five days, indicating that the CONRAS test is an alternative in all settings, particularly for resource-poor countries. Being advantageous, NRA has some limitations, such as some strains (<1%) of MTB lack nitrate reductase [36] rendering the test invalid. However, in our setting, we could not find any such strain in routine biochemical tests, including nitrate reductase test. In addition, nitrate might be reduced to nitric oxide beyond nitrite which cannot be detected by Griess reagent. Hence, zinc-dust was added to all negative tubes[23]. Zinc reduces nitrate rapidly, and a true-negative test will directly turn red while there will be no change in colour in a tube where reduction has passed beyond nitrite. Another possible limitation of NRA is that it can give positive results with atypical mycobacteria *M.*

kansasii, M.szulgai, M. flavescens, M. terrae complex, and some rapid growers [36] while *M. bovis* is nitrate-negative.

5. Conclusion

Our results indicate that NRA is suitable for the early determination of INH and RIF resistance. On the basis of the findings, we conclude that NRA has the potential to be a useful tool for rapid DST of MTB in resource-poor countries with limited laboratory facilities because of its low-cost, rapidness, reproducibility of results, simplicity and lack of requirement of expensive reagents and equipment.

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