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Research Article

Evaluation of Nitrate Reductase Assay for Detection of Multidrug Resistant Tuberculosis

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Abstract

Background: Tuberculosis (TB) is an increasing public health problem in many parts of the world, especially in low-income countries, where most cases occur. Traditional drug susceptibility testing is either time consuming or expensive. In this study, a simple and cost effective method, the Nitrate Reductase Assay (NRA) was compared with the gold standard Proportion Method (PM) for drug susceptibility testing of *M. tuberculosis* (MTB).

Methods: A total of 71 clinical isolates confirmed to be *M. tuberculosis* were tested for their susceptibilities pattern to four primary anti-tubercular drugs by Nitrate Reductase Assay (NRA) and Standard Proportion method (PM).

Results: The sensitivity and specificity of NRA were 100% and 100% for INH, 100% and 100% for RMP, 84.37% and 100% for SM and 83.33% and 97.87% for EMB. Agreement between NRA and PM were 100%, 100%, 92.96% and 92.96% for INH, RMP, SM, and EMB respectively.

Conclusion: Drug susceptibility test of *M. tuberculosis* by the NRA is simple and sensitive with the shorter turnaround time of 10 to 14 days compared to 42 days by the Löwenstein–Jensen (LJ) proportion method.

Keywords: *Tuberculosis; MDR-TB; Drug Susceptibility Testing; M. tuberculosis; INH; RMP*

Introduction

Tuberculosis (TB) remains a serious infectious disease of worldwide distribution, with high morbidity and mortality, mainly in low socio-economic condition countries [1]. World Health Organization (WHO) estimates that one-third of the global population is infected with MTB, 9.4 million new cases of TB and 1.3 million deaths from TB occurring worldwide [2]. The most worrisome trend in recent years, which challenged the global prospects for TB control, is an increase in drug-resistant TB (DR-TB), particularly multidrug-resistant TB (MDR-TB), defined as TB caused by strains resistant to the first-line drugs (isoniazid and rifampicin).

Rapid detection of Drug Resistance (DR) is an urgent priority to identify patients who are not responding to the standard treatment and to avoid transmission of resistant strains [3]. Drug Susceptibility Testing (DST) methods are either very time consuming or too expensive to be broadly adopted in low income, high incidence settings [4]. A cost effective and rapid DST method is required to guide TB treatment. Nitrate Reductase Assay (NRA), previously reported as a useful tool for rapid and accurate detection of resistance to first-line

antitubercular drugs is an alternative method [5]. The method depends on the ability of MTB to reduce nitrate to nitrite which can be detected using a specific reagent producing a change in color [6].

Materials and Methods

This study was conducted at National Tuberculosis Centre (NTC) from May to October 2013. Sputum samples from all the suspected TB patients attending National Tuberculosis Centre (NTC), Thimi, Bhaktapur, during the study period were enrolled for the study. A total of 71 strains confirmed to be *M. tuberculosis* were studied. The PM was performed on Löwenstein–Jensen (LJ) medium according to the standard procedure with the recommended critical concentration [7].

The NRA was performed according to the protocol described previously [6]. The inoculum turbidity was adjusted to a McFarland tube no. 1 and diluted 1:10 in distilled water. Undiluted suspension (200 µl) was inoculated into tubes of Löwenstein–Jensen(LJ) medium with KNO_3 (1mg/mL) containing each of the drugs, such as isoniazid (INH), rifampicin (RMP), streptomycin (SM), and ethambutol (EMB) at the concentrations described above and 200 µl of the 1: 10 dilution was inoculated into three control tubes without antibiotics. The assay was advanced with reagent mix (50% conc. HCl, 0.2% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride at a ratio of 1: 2: 2) after 7 days of incubation at 37°C by adding 0.5 ml reagent mix to one control tube. If the clear reagent mix turned pink, the drug-containing tubes were subsequently developed. If there was no color change, the tubes were again incubated and the procedure repeated on days 10 and 14. An isolate was considered resistant if the drug-containing tube produced a color change that was more intense than the drug-free tube [8]. Statistical analysis of data was carried Statistical Package for Social Science (SPSS version 16.0).

Results

A total of 71 *M. tuberculosis* isolates as identified by Niacin test and growth on PNB (para- nitrobenzoic acid) containing medium were tested for their susceptibilities pattern to four primary anti-tubercular drugs by Nitrate Reductase Assay and Proportion method. The NRA test results were available for 8 isolates (11.3%) in seven days, 36 isolates (62.0%) in ten days and 27 isolates (100.0%) in fourteen days (Table 1).

No. of days	No. of specimen reported	Cumulative %
7	8	11.3
10	36	62.0
14	27	100

Table 1: *Number of days required for reportable results by NRA method.*

Of the total isolates, isolates showing resistance to INH, RMP, SM and EMB were 47.9%, 45.1%, 45.1% and 33.8% respectively by the PM while resistance to respective drugs was 47.9%, 45.1%, 29.6% and 38.0% by NRA (Table 2).

Drugs	Proportion Method		NRA Method	
	Resistant	Sensitive	Resistant	Sensitive
INH	34 (47.9%)	37 (52.1%)	34 (47.9%)	37 (52.1%)
RMP	32 (45.1%)	39 (54.9%)	32 (45.1%)	39 (54.9%)
SM	32 (45.1%)	39 (54.9%)	27 (29.6%)	44 (70.4%)
EMB	24 (33.8%)	47 (66.2%)	21 (38.0%)	50 (62.0%)

Table 2: *Drug Susceptibility Pattern of M. tuberculosis isolates (n = 71) determined by proportion method and NRA method.*

The sensitivities and specificities of NRA compared to those of PM were observed to be 100 and 100%, 100 and 100%, 84.37 and 100%, 83.33 and 97.87% for INH, RMP, SM, and EMB respectively. Positive Predictive values were 100%, 100%, 100% and 95.28 % for INH, RMP, SM, and EMB. Negative Predictive Values were 100%, 100%, 88.64% and 92% for INH, RMP, SM and EMB respectively (Table 3).

Drugs	Conventional Proportion method	Nitrate Reductase Assay					
		Resistant	Sensitive	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
INH	Resistant = 34	34	0	100	100	100	100
	Sensitive = 37	0	37				
RMP	Resistant = 32	32	0	100	100	100	100
	Sensitive = 39	0	39				
SM	Resistant = 32	27	5	84.37	100	100	88.64
	Sensitive = 39	0	39				
EMB	Resistant = 24	20	4	83.33	97.87	95.23	92
	Sensitive = 47	1	46				

Table 3: *Comparison of Indirect Nitrate Reductase Assay results with Conventional Proportion Method.*

The results showed that NRA and PM do not differ significantly ($P > 0.05$ for all the drugs). An excellent agreement was found between the two methods when tested against INH, RMP, SM and EMB with kappa values of k = 1, 1, 0.86 and 0.84 respectively (Table 4).

S. No	Drugs	No. of isolates with following results		Percentage Agreement	Kappa value
		PR method Susceptible NRA method Susceptible	PR method Resistant NRA method Resistant		
1	INH	37	34	100	1.00
2	RMP	39	32	100	1.00
3	SM	39	27	92.96	0.86
4	EMB	46	20	92.96	0.84

Table 4: *Percentage agreement between the Proportion and the NRA methods for susceptibility testing of M. tuberculosis to each drug used.*

Discussion

The time lag to diagnose this is a significant threat to the patient, the community, and health care workers. So, early identification of MDR-TB cases would decrease the risk of disease and possible amplification of drug resistance.

The sensitivity (the ability to detect a true drug resistance in a strain) and specificity (the ability to detect a true drug susceptibility in a strain) for NRA were 100% and 100% for isoniazid, 100% and 100% for rifampicin, 84.37% and 100% for streptomycin, and 83.33% and 97.87% for ethambutol. The results obtained in this study are in agreement with previous studies presented in a meta-analysis that evaluated the accuracy of the NRA for the detection of MDR-TB. The meta-analysis of NRA suggests that the NRA is highly sensitive and specific for determining RMP- and INH-resistant TB in both culture isolates and directly on clinical sputum specimens [9,10]. Most of the studies had a sensitivity of 95% or greater, and nearly all were 100% specific with a high degree of accuracy.

The inversion time for indirect NRA was between 7-14 days, in contrast to 28 - 42 days for the conventional proportion method [10]. In the present study, a complete agreement for the results of the indirect NRA and PM was seen against INH and RMP with a kappa value of 1 and an excellent agreement was also found in the results among the same for EMB and SM with kappa values of 0.86 and 0.84 respectively. The present study focused mainly on RMP and INH, the most valuable first line anti-TB drugs and also the marker of MDR-TB. The results obtained by NRA method were successful and in accordance with the same previous studies reported by Sah., *et al.* [10].

Conclusion

NRA has some limitations, such as some strains (< 1%) of MTB lack nitrate reductase but false susceptible results would, in this case, be detected by the lack of a positive reaction also in drug-free growth. Also, the culture is killed by the mixed reagent used to develop the assay, requiring that multiple cultures be prepared if the comparative study is to be performed and only fresh cultures must be used (< 14 days) [10]. 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 while *M. bovis* is nitrate-negative. In our study, interpretable results were obtained for 71 of the 75 strains tested. Four strains (5.3%) had invalid results for NRA, and did not show a color change in the control tube after 14 days of incubation. This might be due to the reduction of nitric oxide beyond nitrite which cannot be detected by Griess reagent. Zinc-dust should be added to all negative tubes as zinc reduces nitrate rapidly, and a true-negative test will directly turn red while there will be no change in color in a tube where reduction has passed beyond nitrite. Reports on drug susceptibility testing by NRA are generally favorable for INH and RMP [10]. Noting that both drugs are major first-line anti-tuberculosis agents and considering that rifampicin is a surrogate marker for MDR- *M. tuberculosis*, the NRA could be used to screen for resistance to both drugs to enable prompt assessment of MDR prevalence particularly in highly endemic regions [11]. The rather low sensitivity rates for EMB and SM observed in this study require further evaluation.

On the basis of the findings, we conclude that the NRA constitutes a useful tool for detection of MDR-TB in low-resource countries with limited laboratory facilities due to its low cost, rapidness, ease of performance and lack of requirement for sophisticated equipment.

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