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Evaluation of Nitrate Reductase Assay for Rapid Detection of Drug Resistant Tuberculosis

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ABSTRACT

Emergence of multidrug-resistant tuberculosis (MDR-TB) urgently demands for simple, rapid and inexpensive methods of its detection for the effective treatment of drug resistant tuberculosis, particularly in low-income countries. A total of 113 clinical isolates of *M. tuberculosis* were tested for four first line antitubercular drugs by nitrate reductase assay (NRA) and were compared with standard proportion method to evaluate NRA efficacy. Results were available in 7 - 14 days by NRA as compared to proportion method which generally takes 4 - 6 weeks. The sensitivity and specificity of NRA were 98.1% and 100% for isoniazid, 95.1% and 98.6% for rifampicin, 91.4% and 94.9% for streptomycin, and 78.6% and 97.9% for ethambutol, respectively. Agreement between NRA and proportion method were 99.1%, 97.3%, 93.8%, 95.6% for isoniazid, rifampicin, streptomycin and ethambutol, respectively. NRA is easier, inexpensive and reliable method for susceptibility testing of *Mycobacterum tuberculosis* for isoniazid and rifampicin, the two most important drugs for the treatment of tuberculosis. The reduction in susceptibility testing time, and higher sensitivity and specificity of NRA method is of fundamental importance in detecting MDR-TB.

Keywords: Drug Susceptibility; MDR-TB; NRA; Proportion Method

1. Introduction

Emergence of multidrug resistant tuberculosis (MDR-TB) during the past ten years in higher rate represents a major public health problem, especially in low-resource countries where the burden of the disease is higher. MDR-TB constitutes a serious threat for the effective control of the disease stressing the need for the rapid detection of drug resistance [1]. Conventional methods like proportion method (PM), the resistance ratio method and the absolute concentration method to detect drug resistance in *Mycobacterium tuberculosis* have traditionally relied on slow and cumbersome procedures requiring a minimum of 3 - 4 weeks to produce results [2]. Other methods, such as the BACTEC 460 TB System [3], and oxidation-reduction dyes, e.g. tetrazolium [4], and Microplate Alamar Blue assay (MABA) [5], are faster but have the drawback of requiring either radioactive or expensive substrates, and are consequently not feasible in most resource-poor settings. A cost effective and rapid drug susceptibility method is required to guide TB treatment.

With similar characteristics to colorimetric methods, the nitrate reductase assay (NRA) has been described, which is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite after growth in the presence or absence of antibiotics. The use of specific reagents produces a change of color in the presence of nitrites indicating a positive result [6]. Since it is described around the globe as simpler, cheaper and rapid, evaluation of NRA as an alternative method for determining *M. tuberculosis* susceptibility to rifampicin (RIF), isoniazid (INH), streptomycin (STR) and ethambutol (EMB) in our laboratory setting which is technically insufficient is necessary.

2. Material and Methods

The NRA was performed on a total of 113 isolates of *M. tuberculosis*. Eighty three isolates were from cases reported for pulmonary TB at National Tuberculosis Center (NTC), Thimi, Bhaktapur, Nepal, between November 2009 and May 2010; and thirty isolates were of proficiency testing strain from Supranational Reference Laboratory, Germany.

The PM was carried out on Lowenstein and Jenson (LJ) medium according to the standard procedures with the recommended critical concentrations of 40 μg/ml for rifampicin, 0.2 μg/ml for isoniazid, 2 μg/ml for ethambutol and 4 μg/ml for streptomycin [2]. NRA was performed according to the already described protocol [6].

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The following critical concentrations were used: 0.2 µg/ml for INH, 40 µg/ml for RIF, 4 µg/ml for STR and 2.0 µg/ml for EMB. Briefly, fresh subculture (1 µl loops of bacteria) from isolates of *M. tuberculosis* grown on LJ medium was taken and vortexed in small amount of sterile distilled water and turbidity was adjusted according to McFarland standard No.1. Part of the suspension was diluted 1:10 in sterile distilled water. For each isolate, 0.2 ml of suspension was inoculated into the tubes containing LJ medium with potassium nitrate (KNO_3) and the antitubercular drugs; 0.2 ml of the 1:10 dilution was inoculated into drug free media (LJ media) containing KNO_3 which served as growth controls. Tubes in triplicate were incubated at 37˚C for 14 days and 0.5 ml of a mixture of three reagents (25 µl of concentrated HCl, 50 µl of 2% sulphanilamide and 50 µl of 1% n-1-napthylethylenediamine dihydrochloride) was added to one drug free control tube after 7 days of incubation. If its colour changed to pink then tubes with drugs were tested. An isolate was considered resistant if there was colour change (pink or deep red to violet) in the drug tube in question greater than in the 1:10 diluted growth control on the same day. If the tubes did not show any colour change and remains the same, these were further incubated for 10 days and for 14 days as described [6]. Statistical analysis of data was carried out using χ^2 test at 5% level of significance using Statistical Package for Social Science (SPSS version 17.0).

3. Results

Altogether 113 *M. tuberculosis* isolates were tested and the results were available in 7 days for 63.3% of the strains, in 10 days for 82.3% of the strains, and in 14 days for 100% of the strains by NRA method (**Table 1**). Of the total isolates, isolates showing resistance to INH, RIF, STR and EMB were 46.0%, 36.3%, 30.9% and 12.4% respectively, by the proportion method while resistance to respective drugs was 45.1%, 35.4%, 31.8%

and 11.5% by NRA. The sensitivity and specificity for NRA were 98.1% and 100.0%, 95.1% and 98.6%, 91.4% and 94.9%, and 78.6% and 97.9% for INH, RIF, STR and EMB respectively (**Table 2**). The results showed that NRA and proportion method do not differ significantly ($P > 0.05$ for all the drugs). There was high agreement between both methods (**Table 3**) when tested against INH, RIF, STR and EMB with kappa, k = 0.98, 0.93, 0.86 and 0.81 respectively.

4. Discussion

Traditional drug susceptibility testing such as the PM on LJ or agar medium is time consuming. For developing countries, it would be helpful to have a simple and inexpensive test that can rapidly detect resistant *M. tuberculosis* strains. The reporting time of NRA was between 7 - 14 days with majority in 10 days as against 28 - 42 days for the conventional PM. This is very much comparable with the results of MGIT and BACTEC 460 TB, which require the use of expensive instruments and high running cost. The rapidity with which test could be performed has also been reported with comparable accuracy to that of NRA such as the MTT or resazurin assays [7]. However, they make use of liquid medium in a microplate format and that makes the technique more complex and might also constitute a biohazard. Instead, the NRA utilizes standard solid LJ-medium, although with KNO_3

Table 1. Number of days required for result by NRA method.

No. of days	No. of specimens reported	Cumulative %
7	56	63.3
10	37	82.3
14	20	100.0

No: Number.

Table 2. Comparison of indirect nitrate reductase assay results with conventional proportion method.

Drugs	Conventional proportion method	Nitrate reductase assay method					
		Resistant	Sensitive	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
INH	Resistant = 52	51	1	98.1	100	100	98.4
	Sensitive = 61	0	61				
RIF	Resistant = 41	39	2	95.1	98.6	97.5	97.3
	Sensitive = 72	1	71				
STR	Resistant = 35	32	3	91.4	94.9	88.9	96.1
	Sensitive = 78	4	74				
EMB	Resistant = 14	11	3	78.6	97.9	84.6	97
	Sensitive = 99	2	97				

PPV: Positive Predictive Value; NPV: Negative Predictive Value.

Table 3. Percentage agreement between the proportion and the NRA methods for susceptibility testing of *M. tuberculosis* to each drug tested.

Drugs	No. of isolates with the following results		Percent agreement
	PR method-susceptible NRA method-susceptible	PR method-resistant NRA method-resistant	
INH	61	51	99.1
RIF	71	39	97.3
STR	74	32	93.8
EMB	97	11	95.6

incorporated and it could therefore be easily adopted in any culture laboratory.

Nitrate reductase-negative strains of *M. tuberculosis* are very unusual [8] and on the other hand, false susceptible results would in this case be detected by the lack of a positive reaction also in drug free growth. *M. bovis* does not reduce nitrate, therefore the NRA technique is not applicable. Disadvantages of NRA are; the culture is killed by the mix reagent used to develop the assay, requiring that multiple cultures be prepared if comparative testing will be performed and only fresh cultures must be used (<14 days).

In present study, a high level of agreement between NRA and PM was determined for isoniazid and rifampicin. However, despite a higher agreement between NRA and PM for streptomycin (93.8%) and ethambutol (95.6%), low sensitivity of streptomycin (91.4%) and ethambutol (78.6%) was found. For isoniazid and rifampicin, sensitivity and specificity was found to be high; 98.1% and 100.0%, and 95.1% and 98.6% respectively. These results are very important since rifampicin and isoniazid are the two most important drugs used in the treatment of TB [9]. Higher accuracy results for INH, RIF and EMB and lower accuracy results for STR were shown in a similar multicenter study [10]. The percentage agreement of 98.3%, 98.3%, 90.8% and 93.3% by NRA for INH, RIF, STR and EMB respectively, were observed in Brazilian study [11]. However, marginally lower sensitivity and specificity for STR and EMB have also been reported [7,12]. For STR and EMB testing, performance differences were seen among different laboratories rather than drug sensitivity testing method which is argued as inherent difficulties of testing with these two drugs stressing the need for continuing participation in proficiency testing [13].

5. Conclusion

In the context of emergence of MDR-TB, the NRA may be of great importance due to its higher sensitivity and specificity for the rapid detection of rifampicin and isoniazid resistance, the two most important drugs for tuberculosis treatment

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