EVALUATION OF NITRATE REDUCTASE ASSAY (NRA) FOR RAPID DETECTION OF DRUG RESISTANT TUBERCULOSIS AT NATIONAL TUBERCULOSIS CENTRE, NEPAL

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ABSTRACT

Background: Treatment of drug-resistant tuberculosis is often based on drug susceptibility testing results. Thus simple, rapid and economic test is very important for diagnosis of drug resistant tuberculosis and such method aids in TB control effectively. One such method is a Nitrate Reductase Assay (NRA).

Objective: To evaluate feasibility and performance of Nitrate Reductase Assay in the screening of drug-resistant tuberculosis.

Setting: National Tuberculosis Centre and SAARC TB and HIV/AIDS Centre, Thimi, Bhaktapur, Nepal from April 2008 to March 2009.

Methods: A prospective study comparing the sensitivity and specificity of the Nitrate Reductase Assay with the gold standard Lowenstein Jensen proportion method in determining drug susceptibility pattern to four primary anti-tubercular drugs i.e. isoniazid, rifampicin, streptomycin and ethambutal among clinical isolates.

Results: Among 121 specimens , the sensitivity and specificity of the Nitrate Reductase Assay for detection of Isoniazid resistance was 100% and 91%, for rifampicin was 100% and 98.95%, for streptomycin was 96% and 91.66% and for ethambutal was 100% and 98% respectively.

Conclusions: The Nitrate Reductase Assay is sensitive and specific enough for the detection of drug resistant tuberculosis. It is rapid, easy to use and inexpensive, making it suitable for developing countries. Its usefulness for national drug resistance surveys should be assessed.

Keywords: diagnosis, drug resistance, sensitivity, specificity, NRA,

INTRODUCTION

Tuberculosis (TB) is a serious public health problem in many developing countries. The drug resistant tuberculosis (DR-TB) and particularly multi-drug resistant tuberculosis (MDR-TB), defined as resistance to at least the two major anti-tuberculosis drugs isoniazid (INH) and rifampicin (RFP), has emerged in many countries in different regions of the world (WHO, 2009).

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In Nepal, tuberculosis is a major public health problem and recognized by the Government as a priority number one (P_1) program. About 45% of the total population is infected with TB, out of which 60% are adult. Every year about 40,000 people develop TB, of whom 20,000 have infectious pulmonary disease. Introduction of treatment by DOTS has already reduced the number of death, however it is estimated 5000-7000 people still die per year from TB (NTC, 2008). A national prevalence of MDR-TB is 2.9% among new TB cases and 11.7% among previously treated TB cases (STAC, 2008)

Rapid and precise diagnosis of drug resistant tuberculosis is necessary for effective patient treatment and to prevent transmission of the disease. Isolation, identification and susceptibility testing are essential procedures that should be performed as quickly as possible, so that adequate treatment can be prescribed. The drug susceptibility test currently available is the conventional proportion method on Lowenstein-Jensen (LJ) medium, which takes 4-6 weeks to yield culture result plus another 4-6 weeks for drug susceptibility test results. More rapid tests using liquid media, such as the BACTEC 460TB radiometric method and the MGIT, require specific equipment and consumables, and are expensive. Likewise, the new rapid tests based on molecular tools are generally not easy to use and need specialized staff. Rapid diagnosis of MDR patients is nevertheless necessary to avoid the spread of MDR strains. For developing countries, like Nepal the test should be specific, simple and applicable that can rapidly detect MDR Mycobacterium tuberculosis strains.

One rapid drug susceptibility testing method that could potentially satisfy these criteria is a Nitrate Reductase Assay, a colorimetric assay also known as Griess method (Golyshevskaia et al. 1996). This assay was initially developed at the Central Tuberculosis Research Institute in Moscow, Russia, where it was called the Griess method, after J.P. Griess, who discovered the chemistry of the detection method used (Griess and Benerkungen, 1879). It is a low-cost DST method that can be employed in areas of limited resources and low technical capacity (Canetti et al., 1969). It 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 reagents, which produce a color change (Kent and Kubica, 1985).

METHODOLOGY

Specimen processing: Sputum specimens were initially processed by modified petroff's method and ZN acid-fast bacilli (AFB) staining of the sediment was done according to standard protocols (WHO, 1998).

Conventional Lowenstein-Jensen method: Processed specimen was cultured on two slopes of LJ-medium and drug susceptibility test of isolate was performed by 1% proportion method according to standard protocols (WHO, 1998).

Nitrate Reductase Assay method :

Media preparation : Complete LJ-media was prepared and then 1g/1000 ml $NaN0_3$ was added to LJ medium and completely dissolved by stirring. The drugs were then added to the modified medium to prepare the drug containing medium and the medium was a liquated and inspissated. Six tubes with modified LJ medium are needed for each specimen: one containing INH at critical concentration (0.2 µg/ml), one containing RFP at critical concentration (40 µg/ml), one containing SM at critical concentration (4 µg/ml), one containing EMB at critical concentration (2 µg/ml) and two control tubes without any drugs added.

Procedure : The test was performed only on specimens with an AFB smear positive result (1+and above). Each of the six tubes described above was inoculated with 0.2 ml of the processed sputum sediment. After 28 days of incubation at 37°C, 0.5 ml of freshly prepared Griess reagent was transferred into one of the growth control tubes, and development of color was observed. If the color intensity was sufficient, the same amount of Griess reagent was pipetted into each of the drug containing tube and other control tube. The color intensity in the drug-containing tube was then compared to the control tube.

Interpretation of results : The results were classified as negative if there were no color changes or a very pale pink color was observed. Positive results varied from pink to deep red or violet. The results were thus, interpreted as follows:

Resistant (R) - An isolate was considered resistant to a certain drug if there was a positive color change in the drug tube in question and in the drug-free control tube.

Sensitive (S) - An isolate was considered sensitive to a drug if there was no color change in the drug tube in question and positive color change in the drug-free control tube.

If no color changes or pale pink color were observed in the control tube, the test was considered to be invalid.

Preparation of Griess reagent : Shortly before use, one part of 50% (vol/vol) concentrated hydrochloric acid was mixed with two parts of 0.2% (wt/vol) sulfanilamide and two parts of 0.1% (wt/vol) n-1-naphthylenediamine dihydrochloride.

Preparation of inoculum : 1ml sterile distilled water was added to the sediment obtained after processing the sputum specimen.

RESULTS

The test was completed on 121 specimens of 144 smear-positive (31 1+, 31 2+ and 82 3+) specimens. Of the remaining 23 specimen for which the test was not completed, 12 were culture-negative by both gold standard and Nitrate reductase assay method and 11 were contaminated on culture by either one or both methods.

Comparison of the Nitrate Reductase Assay results with conventional drug susceptibility test result for 121 specimens is depicted in the Tables 1a and 1b. Among 28 strains that were resistant to INH by the conventional method, 28 (100%) were also found to be resistant by the nitrate reductase assay method. Not a single isolate was falsely identified as being INH susceptible. Among 93 strains that were susceptible to INH by the conventional method, 85(91.39%) were also found to be susceptible by the direct nitrate reductase assay method.Similarly, 26 of 26 (100%) isolates that were resistant to RFP by the conventional DST were also found to be resistant by the nitrate reductase assay method. Among 95 strains that were susceptible to RFP by the conventional method, 94 (98.9%) were found to be susceptible by the nitrate reductase assay method. 24 of 25 (96%) isolates that were resistant to SM by the conventional DST were also found to be resistant by the nitrate reductase assay method, with Only one isolate falsely identified as susceptible. Among 96 strains that were susceptible to SM by the conventional method, 88 (91.66%) were found to be susceptible by the nitrate reductase assay method. 21 0f 21 (100%) isolates that were resistant to EMB by the conventional DST were also found to be resistant by the nitrate reductase assay method. Among 100 strains that were susceptible to EMB by the conventional method, 98 (98%) were found to be susceptible by the nitrate reductase assay method. Considering proportion method as standard, the sensitivity and specificity of direct NRA was determined to be 100% and 91% for INH; 100% and 98.95% for

Table 1a: Drug Susceptibility Pattern of M. tuberculosis (n=121) determined by the proportion method and Nitrate Reductase Assay method

Drugs	Proportion Method		NRA Method	
	Resistant	Sensitive	Resistant	Sensitive
INH	28	93	36	85
RFP	26	95	27	94
SM	25	96	32	89
EMB	21	100	23	98

Table 1b: Comparison of Nitrate Reductase Assay results with Conventional DST

Drugs	Conventional method	Direct Nitrate Reductase Assay method					
		Resistant	Sensitive	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
INH	Resistant = 28	28	0	100	91	78	100
	Sensitive = 93	8	85				
RFP	Resistant = 26	26	0	100	98.95	96.30	100
	Sensitive = 95	1	94				
SM	Resistant = 25	24	1	96	91.66	75	98.88
	Sensitive = 96	8	88				
EMB	Resistant = 21	21	0	100	98	91.30	100
	Sensitive=100	2	98				

RFP; 96% and 91.96% for SM; and 100% and 98% for EMB. Similarly, the positive predictive value (PPV) was found to be 78%, 96.30% , 75% and 91.30% with Negative predictive value (NPV) being 100% , 100% , 98.88% and 100% respectively.

DISCUSSION

In this study, high sensitivity and specificity of the nitrate reductase assay method, relative to conventional methods were demonstrated in the identification of resistance of M. tuberculosis to four primary anti-tubercular drugs i.e. INH, RFP, SM and EMB. The sensitivity and specificity of the direct nitrate reductase assay for detection of isoniazid resistance was 100% and 91%, for rifampicin was 100% and 98.95%, for streptomycin was 96% and 91.66% and for ethambutal was 100% and 98% respectively.

In a similar study by sloutsky et al. (2005) in Lima, Peru showed the sensitivity and specificity of the method for INH resistance was 99.1% and 100%, and for RMP resistance was 93.5% and 100% respectively. Study by Angeby et al. (2005) showed sensitivity and specificity of the direct NRA using the direct proportion method as reference for INH, RFP, SM, and EMB were 100 and 100% , 93 and 100% , 76 and 100% and 55 and 100% respectively. In another report by Angeby et al. (2002), sensitivities and specificities for drugs as determined by the NRA method compared to those determined by the BACTEC 460 method were 100% and 100% for rifampicin, 97% and 96% for isoniazid, 95% and 83% for streptomycin, and 75% and 98% for ethambutol, respectively. Other study of the performance of the indirect NRA method by Golyshevskaia et al (1996) in Sweden, sensitivity and specificity for INH were 97% and 96% respectively, and 100% for RMP.

The spread of multidrug-resistant (MDR) strains of Mycobacterium tuberculosis is an increasing public health concern in many parts of the world, especially in low-income countries, where most cases occur. The time lag to diagnose this is a significant threat to the patient, the community, and health care workers. So, earlier identification of MDR-TB cases is important as this would minimize the risk of disease progression and amplification of drug resistance due to optimal therapy. Conventional DST requires on an average 20-40 days for initial culture growth, plus an additional 28-42 days for DST itself. In contrast, the turnaround time for the direct NRA in this study was uniformly 28 days, a time saving of 4-6 weeks. Further reductions in turnaround time are possible if the initial colorimetric readings are taken on the 21st day, as suggested in the original protocol (safonova et al., 2001).

Other low-cost methods have been proposed, such as the MTT or resazurin assays (Palomino et al., 2004). They have been shown to be comparable to the NRA, at least for RFP when performed indirectly (Palomino et al., 2004). However, they make use of liquid medium in a microplate format and that makes the techniques more complex and might also constitute a biohazard. Instead, the NRA utilizes standard solid L-J medium, although with $NaNO_3$ incorporated and (apart from being safer), it could therefore be easily adopted in any culture laboratory.

The ability to reduce nitrate is typical for M. tuberculosis, although some other mycobacterial species, like Mycobacterium kansasii, and most rapid growers share this characteristic (Kent and Kubica, 1985). Nitrate reductase-negative strains of M. tuberculosis are rare (1%) (Kent and Kubica, 1985) and would create no false results since the control would be negative and the test would therefore be invalid. No such strains were encountered in this study. Strains of Mycobacterium bovis do not reduce nitrate, for which reason the NRA technique is not applicable to DST of them. Another possible limitation is that nitrite might be further reduced to nitric oxide, which cannot be detected by the reagents used. When the nitrate reduction test is performed for the purpose of species identification, zinc powder is added to all negative tubes (Kent and Kubica, 1985). Zinc reduces nitrate rapidly, and a true negative test will directly turn red, while there will be no color change in a tube where reduction has passed beyond nitrite. Since the result was always confirmed, this step was omitted in this study. However, further studies will be needed to clarify the role of zinc powder in the NRA.

This study demonstrates the potential usefulness of the direct NRA as a rapid, susceptible and specific screening tool for MDR-TB. Even though more studies are needed to further assess the accuracy and applicability of the method, the direct nitrate reductase assay has the potential to become an

inexpensive alternative for DST where resources are scarce, especially for RFP, the most important anti-tuberculosis drugs. It might then be used either as a rapid screening tool alone or in combination with other methods. In addition to its rapidicity, the direct NRA has further obvious benefits that would facilitate its institution in resource poor setting. Furthermore, the test uses only simple reagents that are inexpensive and easily obtained, does not require maintenance of any specialized equipment, and requires minimal laboratory space and staffing.

CONCLUSION

In this study, direct NRA test for culture and susceptibility testing was evaluated in comparison to gold standard proportion method. The method was found to be having high sensitivity and specificity. This study revealed that the direct nitrate reductase assay method has capability of accurate and rapid drug susceptibility testing for primary four anti-tuberculosis drugs viz, INH, RFP, SM, and EMB. More importantly, the test will provide patients and clinicians with the benefits of greater access to fast and accurate drug susceptibility testing result for these first-line drugs.

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