

Drug susceptibility of *Mycobacterium tuberculosis* to primary antitubercular drugs by nitrate reductase assay

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Background & objectives: Rapid susceptibility testing of *Mycobacterium tuberculosis* strains is imperative for therapy selection but traditional drug susceptibility tests take weeks or are expensive. In this study we evaluated nitrate reductase assay which utilizes the detection of nitrate reduction as an indication of growth and therefore results can be obtained faster than by visual detection of colonies.

Methods: One hundred 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. The bacteria were inoculated on Lowenstein-Jensen (LJ) medium with primary antitubercular drugs and potassium nitrate was incorporated. After incubation for 7-14 days, nitrate reduction indicating growth could be detected by colour change when reagents were added.

Results: Resistance of isolates as determined by both methods for isoniazid, rifampicin, streptomycin and ethambutol was 32, 35, 62 and 15 per cent respectively. Agreement between NRA and proportion method was 99 per cent for isoniazid and ethambutol. Complete agreement (100%) was found for rifampicin and streptomycin. Results were available in 7-14 days by NRA as compared to proportion method which takes 4-6 wk.

Interpretation & conclusion: Nitrate reductase assay is a rapid and inexpensive method for susceptibility testing of *M. tuberculosis* for primary antitubercular drugs and could be an appropriate alternative to existing methods, particularly in resource-poor settings.

Key words Drug susceptibility - *Mycobacterium tuberculosis* - nitrate reductase assay

The spread of multiple drug resistant strains of *Mycobacterium tuberculosis* has become a major public health concern in both developed and developing countries¹⁻⁴. Factors contributing to recent outbreak and continued spread of multi drug resistant tuberculosis (MDR-TB) include upsurgence of human immunodeficiency virus (HIV) infection/acquired immuno deficiency syndrome (AIDS), insufficient

control procedures and laboratory delays in identification and susceptibility testing of *M. tuberculosis* isolates^{5,6}. Thus rapid susceptibility testing of *M. tuberculosis* isolates obtained from MDR-TB patients would be of help in the management of individual patients. The standard methods for drug susceptibility testing of *M. tuberculosis* such as proportion method, absolute

concentration method and the resistance ratio method are used globally but depend upon culture and therefore time consuming⁷. The BACTEC method⁸ though quicker requires specialized instrumentation and is not feasible in most resource poor settings¹. Thus, there is need for a fast, reliable and inexpensive method for antimicrobial susceptibility testing of *M. tuberculosis*. Therefore, in the present study, we evaluated a new nitrate reductase assay (NRA) which is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, the reduction can be detected by using specific reagents which produce a colour change⁹.

Material & Methods

Strains: One hundred consecutive clinical isolates of *M. tuberculosis* obtained from patients of pulmonary tuberculosis over a three year period (January 2000 - January 2003) in the Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh formed the study material. *M. tuberculosis* H₃₇Rv served as control. All the isolates were stored at -70°C and cultured on standard Lowenstein-Jensen (LJ) medium before use and were identified by standard biochemical tests¹⁰.

Proportion method: Drug susceptibility of all 100 isolates to isoniazid (INH), rifampicin (RIF), streptomycin (STR) and ethambutol (EMB) was performed by standard method¹⁰. Briefly, LJ media with drug incorporated in various concentrations and plain LJ medium for control were prepared. The growth from a 3-4 wk old culture was scraped with a loop and bacterial suspension was made in sterile distilled water, vortexed and matched with McFarland opacity tube No.1. Dilutions of 10⁻² and 10⁻³ were made and inoculated on both the control and drug containing media and incubated at 37°C. The first reading was taken after 28 days of incubation and the second on 40th day. The percentage resistance (R) was calculated as the ratio of the number of colonies on the drug containing media to those on the control medium.

$$R (\%) = \frac{\text{No. of colonies on drug media}}{\text{No. of colonies on control medium}} \times 100$$

If R = >1 per cent, the isolate was taken as resistant

Nitrate reductase assay for drug susceptibility: NRA was performed as described by Golyshvskaya *et al*¹¹ and Angeby *et al*¹². 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 3ml of phosphate buffer saline (PBS, pH 7.4) and turbidity was adjusted according to McFarland standard no.1. Part of the suspension was diluted 1:10 in PBS. For each isolate, 0.2 ml of suspension was inoculated into the tubes containing LJ medium with potassium nitrate (KNO₃) and the antitubercular drugs; 0.2 ml of the 1:10 dilution was inoculated into drug free media (LJ media) containing KNO₃ 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-naphthyl-ethylenediamine dihydrochloride) was added to one drug-free control tube after 7 days of incubation. If its colour changes 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 by Angeby *et al*¹².

Statistical analysis of data was carried out using Mc Nemar's test.

Results & Discussion

Taking proportion method as Gold standard for susceptibility testing, we compared nitrate reductase assay with it (Table). The resistance as seen by NRA for INH, RIF, STR and EMB, was 32, 35, 62 and 15 per cent respectively. Comparable values by proportion method were 33, 35, 62 and 16 per cent respectively (Table). The results showed that NRA and proportion method do not differ significantly ($P > 0.05$ for all the drugs). Thus an excellent agreement between the results of NRA and proportion method was found for all the primary antitubercular drugs *i.e.*, 100 per cent for rifampicin and

Table. Sensitivity of antitubercular drugs by nitrate reductase assay and proportion method

Antitubercular drugs	Susceptible by both methods	Resistant by both methods	Susceptible by NRA only method	Susceptible by proportion method	Agreement (%)
Isoniazid	67	32	1	0	99
Rifampicin	65	35	0	0	100
Ethambutol	84	15	1	0	99
Streptomycin	38	62	0	0	100

No significant difference between two methods ($P > 0.05$)
 NRA, nitrate reductase assay

streptomycin, 99 per cent for isoniazid and ethambutol. Paniotov *et al*¹³ have found 100 per cent agreement of susceptible strains between nitrate reductase assay and Canetti's proportion method as recommended by WHO¹⁴. Angeby *et al*¹² have also shown good concordance between BACTEC 460 and NRA *i.e.*, 100 per cent for rifampicin and overall agreement of 94 per cent. The NRA method utilizes the standard detection of nitrate reduction as an indication of growth and therefore results can be obtained much faster than visual detection of colonies. In majority of the isolates, results could be obtained within 7 to 14 days compared to the proportion method which takes about 4 to 6 wk. The NRA was easy to read. Although BACTEC 460 or mycobacterial growth indicator tube (MGIT) also requires 7-10 days, but is expensive and requires instruments. E test though can produce results within 7-10 days but again this is not cost-effective. Genetic methods such as line probe assay (Innogenetics, Belgium)¹⁵, are fast but are too expensive to be used in resource poor settings and have been developed mainly for rifampicin susceptibility testing. More than 99 per cent of *M. tuberculosis*⁹ strains possess nitrate reductase enzyme and are capable of reducing nitrate to nitrite. Some other *Mycobacterium* species like *M. kansasii*, *M. smegmatis*, also possess this enzyme. However, these strains are not frequently encountered in human infections and they can be identified by morphological and biochemical tests.

M. bovis does not reduce nitrate, therefore the NRA technique is not applicable.

In conclusion, nitrate reductase assay, as observed in the present study was found to be rapid, inexpensive and easy to perform. As it does not require much instrumentation, it could be used routinely in laboratories in developing countries for drug susceptibility testing of *M. tuberculosis*. It might be possible to apply the nitrate reductase test directly to microscopy positive sputa, thus drastically reducing the time needed for detection of drug resistant *M. tuberculosis*.

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