Original Research Article

Comparative evaluation of nitrate reductase assay with conventional proportion method for isoniazid and rifampicin susceptibility testing in smear positive pulmonary tuberculosis patients

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ABSTRACT

Introduction : With the surge of multidrug-resistant tuberculosis (MDR-TB) strains there is an escalating need for precise and cost-effective methods for a precipitous diagnostic and drug susceptibility testing (DST), chiefly in resource limited countries where tuberculosis is endemic.

Aim : To find out the susceptibility pattern of Isoniazid (INH) and Rifampicin (RMP) in smear positive cases of pulmonary TB at MMIMSR Mullana and to evaluate the sensitivity and specificity of nitrate reductase assay (NRA) with standard proportion method for INH and RMP susceptibility testing.

Material and Methods: A cross sectional test validation study was carried out at Mycobacteriology division in the Department of Microbiology on 100 smear positive sputum samples from pulmonary tuberculosis patients.

Results: In comparison with L.J proportion method, NRA showed sensitivity, specificity, PPV, NPV, accuracy of 100%, 98.64%, 87.50%, 100%, and 98.76% respectively for INH. The sensitivity, specificity, PPV, NPV, accuracy of NRA compared to PM for RMP was 100%, 97.33%, 75.00%, 100%, 97.53% respectively.

Conclusion: Direct NRA is simple, easy to perform, prompt, reasonably less expensive, without requisite of expensive reagents and advanced instrumentation. It is highly specific and sensitive technique in the detection of drug resistant TB when tested in parallel with the proportion method. Therefore, NRA may be routinely employed in TB laboratories in developing countries for drug susceptibility testing of *M. tuberculosis*.

1. Introduction

Tuberculosis (TB) is a multi-systemic disease with a mutable presentation. indication of TB has been reported in human remains dated thousands of years. For a human pathogen with no known environmental reservoir, *Mycobacterium tuberculosis* has perfected the art of survival and has persisted in human populations from antiquity through modern time.[1] A total of 1.5 million people died from TB in 2018. Worldwide, TB is one of the top 10 causes of death and the leading cause from a single infectious agent. In 2018, an estimated 10 million people fell ill with TB worldwide. In 2018, the 30 high TB burden countries accounted for 87% of new TB cases. Eight countries account for two thirds of the total, with India leading the count.[2] The World Health Organisation (WHO) TB statistics for India for 2018 give an estimated incidence of 2.69 million cases. It is estimated that about 40% of the Indian population is infected with TB bacteria.[3]

MDR and extensive drug resistant (XDR) in TB is a matter of great concern for TB control programs since there is no cure for some MDR- TB strains of Mycobacterium TB.[4] Regrettably, most of the available drug susceptibility testing (DST) methods are either very time-consuming or too expensive to be widely adopted in low and

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middle-income countries. The proportion method (PM), the most commonly used method for determining drug susceptibility, requires up to four to six weeks for the results to be achieved.[5] In the last decade, fast, reproducible and low-cost phenotypic methods for determining the susceptibility to drugs have been described, such as the Microscopic Observation Broth-Drug Susceptibility assay[6], the colorimetric redox-indicator[7] and the nitrate reductase assay (NRA).[8] Among the low-cost techniques, the NRA appears to be feasible as a rapid, specific, sensitive and easily implemented DST method, especially for low and middle-income countries. The procedure relies on the ability of metabolically active Mycobacterium TB to reduce nitrate to nitrite. The presence of nitrite is used as a growth indicator that can be visually detected by change in color after adding chemical reagents that are generally available in any Mycobacteriology laboratory for the routine identification of M. TB isolates. With this back ground we aimed to find out the susceptibility pattern of Isoniazid (INH) and Rifampicin (RMP) in smear positive cases of pulmonary TB at M.M.I.M.S.R. Mullana and to evaluate the sensitivity and specificity of NRA with standard proportion method for INH and RMP susceptibility testing.

2. Materials and Methods

2.1. Study design

A cross sectional test validation study was carried out at Mycobacteriology division in the Department of Microbiology, MM Institute of Medical Sciences and Research Mullana, Ambala, Haryana. The study was conducted on 100 smear positive sputum samples from pulmonary tuberculosis patients according to RNTCP (Revised National Tuberculosis Control Programme)**,**[9] the samples selected by random sampling method. The Patient inclusion criteria were all the smear positive sputum samples from pulmonary TB patients. All Smear negative cases of suspected pulmonary tuberculosis and Patients on anti-tuberculosis treatment with HIV-TB, co-infection, and other immune-compromised patients as evident from history were excluded. After taking written consent and noting the demographic details, The sputum specimens were digested, decontaminated by the standard NaOH–NALC procedure[10] and concentrated by centrifugation, the sputum samples of the patient were subjected to ZN (Ziehl–Neelsen) stain and the slides were examined under 100_X oil immersion (Nikon E100) and interpreted as per RNTCP guidelines**.**The sediment obtained after NaOH–NALC method was re-suspended in 3 ml of sterile distilled water. Out of 3 ml suspension, 1.5 ml was used for direct NRA and remaining 1.5 ml for the direct proportion method. The processed sample (0.2 ml) was inoculated on LJ media (LJ media was prepared in our laboratory) taking necessary aseptic precautions. The

slopes were incubated at 37°C for a maximum period of 8 weeks and inspected once a week for growth,[9] From the growth, a bacillary suspension was prepared by diluting the growth with sterile distilled water (adjusted the turbidity with McFarland standard number 1). The suspension was then inoculated on LJ media with INH (0.2μg/ml) and RMP (2μg/ml) and the slants were incubated at 37ºC. Another LJ slant with H37Rv (ATCC 27294) strain was inoculated with INH and RMP with same critical concentration (served as an internal quality control). The first reading was taken after 28 days and second on 40th days of incubation respectively. The percentage resistance was calculated as the ratio of the number of colonies on the drug containing media to those on control media. If the ratio was greater than 1% then isolate was taken as resistant to these antibiotics.[11] For each specimen 0.5 ml of the undiluted decontaminated suspension was inoculated into 4.6 ml of 7H9-N medium (Middle Brook) containing RMP (Ranbaxy, Mumbai 2.0μg/ml) and another tube containing 4.6ml of 7H9-N medium with INH (Hi-media, Mumbai 0.2μg/ml). 0.5ml of the 1:10 dilution of sample in phosphate buffer saline (PBS) was inoculated into 4.6ml of drug free 7H9-N medium, which served as growth control. The control strain i.e. H37Rv was similarly inoculated on to the 7H9N medium with incorporation of the same antibiotics and potassium nitrate. All the tubes were then incubated at 37^{0}C. After five days of incubation, 0.2ml of freshly prepared reagent mixture Griess reagent was added to 1 ml of drug free medium. The results were classified as negative (no color change) or positive (pink color) (Figure 1). If there was a change in color in the drug free tube, then the tubes with antibiotic (containing patient's sample) and the tube containing control strain were also tested. If there was no color change, then the tubes were re- incubated and the procedure repeated on the 7th, 10th, 14th, 18th and 28th day. For INH, results were interpreted as described by Angeby et al[6] An isolate was considered resistant if the color change in the INH tube was equal to or greater than that in 1:10 diluted growth control tube and was considered susceptible if there was no color change, or if the color change was less than that in 1:10 diluted growth control. For Rifampicin, an isolate was considered resistant if there was any color change in the RMP tube, and was considered susceptible if there was no color change in the RMP tube. Culture results were confirmed after examination of ZN smear prepared from the growth To rule out bacterial contamination two drops of each positive suspension was inoculated on to a blood agar plate, incubated at 37ºC, and examined after 24 hrs.[12] Internal quality control was done using the fully susceptible M.tuberculosis H37Rv (ATCC 27294) (Figures 2, 3 and 4)

2.2. Ethical considerations

The present study was approved by the Ethical Committee of Maharishi Markandeshwar Institute of Medical Sciences

and Research. The approval was on the agreement that patient anonymity must be maintained, good laboratory practice, quality control ensured, and that every finding would be treated with utmost confidentiality and for the purpose of this research only. All work was performed according to the International Guidelines for Human Experimentation in Biomedical Research.Approval was obtained from the subjects by taking the informed consent.

2.3. Data management and statistical analysis

The data entry was carried using Microsoft Office Excel worksheet and then exported to statistical software and analyzed using appropriate statistical tests by using Statistical Package for Social Services (SPSS vs 21 for Mac IBM Inc Chicago).

3. Results

A total of 100 smear-positive sputum samples were included in the study. The mean age of patients was 35 years (range 20–75years). Out of 100 patients 76 were male and 24 females. Out of 100 smear-positive sputum samples, Mycobacteria were isolated in 85 cases, 81 (95.29%) strains of MTB were isolated on both LJ medium and MB7H9. Thus 4 samples were excluded from data analysis and the 81 samples constituted the sample size. By PM method as the grading of the smear increases to 3+ (51.21%), the rate of isolates appeared earlier i.e. 4 weeks (43.90%) followed by 2+ (35.36%), 1+ (12.19%), scanty which showed least growth (1.21%) and took around 8 weeks. While with respect to growth on NRA and Grading of smear maximum results were obtained on the tenth day (38.09 %) and with 3+ grade (50.00%).Table 1

Table 2 Shows the comparison of results of drug susceptibility testing of 81 strains of *Mycobacterium tuberculosis* by PM and NRA. For INH, proportion method detected 7 resistant strains and 74 susceptible strains. While NRA detected 8 resistant strains and 73 susceptible strains. 1 false-negative result was obtained with NRA. For RIF, 6 and 75 strains were detected to be resistant and susceptible respectively by means of proportion method, whereas 8 and 73 were resistant and susceptible respectively by means of NRA method. Two (2) false-negative results were produced with the NRA. The sensitivity, specificity, PPV, NPV, accuracy of NRA compared to PM for INH was 100%, 98.64%, 87.50%, 100%, 98.76% respectively. The sensitivity, specificity, PPV, NPV, accuracy of NRA compared to PM for RMP was 100%, 97.33%, 75.00%, 100%, 97.53% respectively.

4. Discussion

The "captain of all these men of death", TB has been a scourge of the human kind from time immemorial. Till date, no other disease in history matches the sheer magnitude of
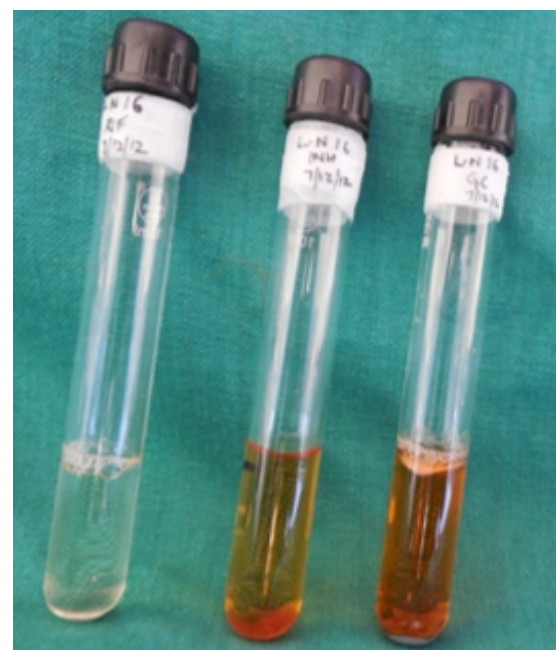

Fig. 1: INH Sensitive RIF resistant

Fig. 2: Sensitive RIF INH resistant

Table 1: Distribution of *mycobacterium tuberculosis* strains to susceptibility profile

Drug profile (n=81)	PM	NRA method
Susceptible to both INH and RMP	70 (86.41%)	67(82.71%)
Susceptible to RMP and Resistance to INH	05 (6.17%)	06(7.40%)
Susceptible to INH and Resistance to RMP	04(4.93%)	06(7.40%)
Resistance to both INH and RMP	02 (2.46%)	02(2.46%)

Table 2: Comparison of diagnostic accuracy of conventional PM and NRA.

Drug profile+ NRA	*Drug susceptibility testing results		Nitrate reductase assay				
ISONIAZID Resistance	R	S	SE	SP	PPV	NPV	ACC
	7	1	100	98.64	87.50	100	98.76
Susceptible	0	73					
RIFAMPICIN Resistance	6	2	100	97.33	75.0	100	97.53
Susceptible	0	73					

R= Resistance, S= Susceptible, SE= Sensitivity (%), SP= Specificity (%), PPV= Positive predictive value (%), NPV= Negative predictive value (%), ACC= Accuracy (%)
For INH x^2= 69.92, df= 1, p< 0.0001 (Highly significant).
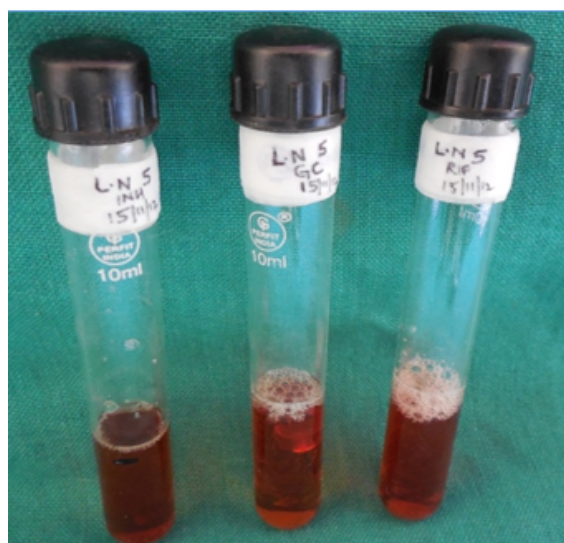For RMP x^2= 59.13, df= 1, p< 0.0001 (Highly significant).

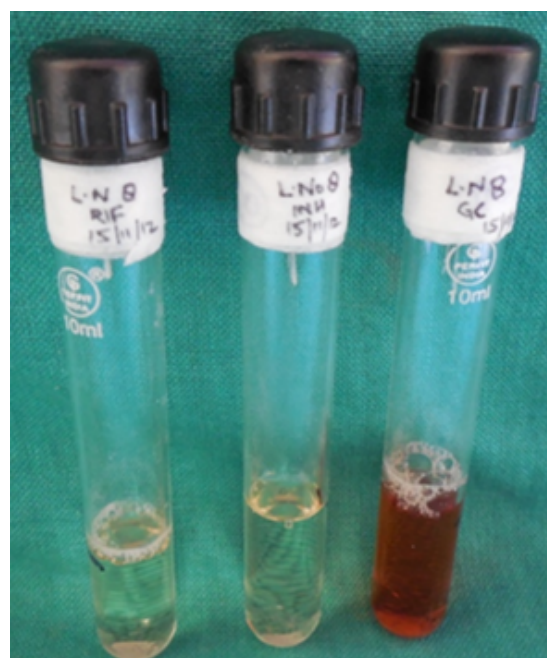**Fig. 3:** Resistant to both INH and RIF (MDR TB)

Fig. 4: Sensitive to both INH and RIF BY NRA Method

the misery inflicted by TB on the human race in terms of morbidity and mortality. [13] The most worrisome drift during recent years is an upsurge in MDR-TB strains. An estimated 4.8 lakh cases of MDR-TB emerge annually worldwide with 1.3 lakh incident multi-drug resistant TB patient in India alone which includes 79000 MDR-TB Patients estimates among notified pulmonary cases. [3] This increase in MDR-TB is largely attributed to poor management of the DOTS Programme and the inability to rapidly diagnose drug resistance. Currently, drug-susceptibility testing is largely based on culture which is hampered by lengthy turn-around times, expensive equipment, high reagent costs and the need for skilled staff and extensive bio safety facilities. It is therefore indispensable that improved diagnostics are

developed which are both affordable and rapid. The present study which is the first of its kind in India as far as our knowledge is concerned where the application of NRA directly to sputum samples using a liquid medium for the rapid detection of *M. tuberculosis* resistance to RMP and INH was done. In the study the average time to detection of mycobacterial growth was found to be 10 days by NRA and 28 days by Proportion method. The present study is supported by Affolabi et al [12] for NRA where the average TTD was 10 days. The results of Angeby et al [8] and Gupta

et al[14] were available in most of the cases in 7 and 10 days by NRA and 28 days by Proportion method respectively which reflects that NRA using a liquid medium is less time consuming and gives faster results than Proportion method which is beneficial for the patient for early treatment. In a study by Sethi et al[11] the results were available in 7 – 14 days by NRA as compared to Proportion method which takes 4 – 6 weeks. Resistance to RIF is almost always associated with multidrug resistance and thus can serve as a marker of MDR-TB if resources are limited.[14] Hence it is important to know the resistant to RIF. Our study is in complete concordance with the studies using direct nitrate reductase assay for drug susceptibility testing of *M. tuberculosis* conducted by.Affolabi et al,[12] Mishra et al,[15] Thapa et al.[16]

In resource-constraint areas cost is an imperative aspect where most of the disease is prevalent. A cost by NRA as compared with PM and molecular assays accentuates the need of this colorimetric assay to be routinely used in laboratories in low-income countries. The supplementary equipment, media, and infrastructure required for NRA are routinely present in most microbiological laboratories. Moreover, NRA saves time and labour (avoids pre-isolation of MTB from sputum specimens) as this assay can be performed directly on smear positive sputum specimens. In the current study NRA was found to be a highly specific and sensitive technique in the detection of drug resistant MTB when tested in parallel with the PM. However, NRA posseses some limitations such as some strains (< 1%) of *M. Tuberculosis* lacks nitrate reductase activity rendering the test invalid. Considering this concern, the direct NRA DST could be performed in parallel with the reference standard (LJ PM) to confirm the presence of *M.tuberculosis* complex isolates.

5. Conclusion

NRA was found to be a highly specific and sensitive technique in the detection of drug resistant TB when tested in parallel with the PM. In addition, NRA was found to be rapid, inexpensive, easy to perform and does not require any special instrumentation. Therefore, NRA may be routinely employed in TB laboratories in developing countries for DST of *M.tuberculosis*. However, NRA possesses some limitations such as some strains (< 1%) of *M. Tuberculosis* lacks nitrate reductase activity rendering the test invalid. Considering this concern, the direct NRA DST could be performed in parallel with the reference standard (PM) to confirm the presence of *M.tuberculosis* complex isolates.

6. Conflicts of interest

All contributing authors declare no conflicts of interest.

7. Source of Funding

None.

References

1. Adigun R, Singh R, Tuberculosis. Tuberculosis. [Updated 2020 Jun 7]. In: StatPearls [Internet]. Treasure Island (FL): Stat Pearls Publishing; 2020. Available from: https://www.ncbi.nlm.nih.gov/books/NBK441916/accessed.
2. Global tuberculosis report 2019. WHO.INT accessed on 01.11.2020.
3. India TB Report ; 2020. Available from: https://tbcindia.gov.in/accessedon05.11.2020.
4. Prasad R, Gupta N, Singh A, Gupta P. Multidrug-resistant and extensively drug-resistant tuberculosis (M/XDR-TB): management in special situations. *Int J Med Sci Public Health*. 2015;4(12):1626–33. doi:10.5455/ijmsph.2015.01082015364.
5. Canetti G, Froman S, Grosset J, Hauduroy P, Langerova M, Mahler HT, et al. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. *Bull World Health Organ*. 1963;29:565–78.
6. Caviedes L, Lee TS, Gilman RH, Sheen P, Spellman E, Lee EH, et al. Tuberculosis Working Group in Peru. Rapid, efficient detection and drug susceptibility testing of Mycobacterium tuberculosis in sputum by microscopic observation of broth cultures. *J Clin Microbiol*. 2002;38:1203–8.
7. Reis RS, Neves I, Lourenco SLS, Fonseca LS, Lourenco MCS. Comparison of Flow Cytometric and Alamar Blue Tests with the Proportional Method for Testing Susceptibility of Mycobacterium tuberculosis to Rifampin and Isoniazid. *J Clin Microbiol*. 2004;42(5):2247–8. doi:10.1128/jcm.42.5.2247-2248.2004.
8. Angeby KAK, Klintz L, Hoffner SE. Rapid and Inexpensive Drug Susceptibility Testing of Mycobacterium tuberculosis with a Nitrate Reductase Assay. *J Clin Microbiol*. 2002;40(2):553–5. doi:10.1128/jcm.40.2.553-555.2002.
9. Revised National Tuberculosis Control Programme. Manual for laboratory technicians, smear microscopy for detection of acid-fast bacilli. Central TB Division, Directorate General of Health Services Ministry of Health and Family Welfare, Nirman Bhavan; 2009.
10. Kent PT, Kubica GP. A guide for the level III laboratory. In: Public health mycobacteriology; 1985.
11. Sethi S, Sharma S, Sharma SK, Meharwal SK, Jindal SK, Sharma M, et al. Drug susceptibility of Mycobacterium tuberculosis to primary antitubercular drugs by nitrate reductase assay. *Indian J Med Res*. 2004;120(5):468–71.
12. Affolabi D, Odoun M, Sanoussi N, Martin A, Palomino JC, Kestens L, et al. Rapid and Inexpensive Detection of Multidrug-Resistant Mycobacterium tuberculosis with the Nitrate Reductase Assay Using Liquid Medium and Direct Application to Sputum Samples. *J Clin Microbiol* . 2008;46(10):3243–5. doi:10.1128/jcm.00083-08.
13. Sharma SK, Mohan A. Tuberculosis: From an incurable scourge to a curable disease journey over a millennium. *Indian J Med Res*;137:455–93.
14. Gupta A, Sen MR, Mohapatra TM, Anupurba S. Evaluation of the Performance of Nitrate Reductase Assay for Rapid Drug-susceptibility Testing of <i>Mycobacterium tuberculosis</i> in North India. *J Health, Popul Nutr*. 2011;29(1):20–5. doi:10.3329/jhpn.v29i1.7563.
15. Mishra B, Muralidharan S, Srinivasa H. Direct drug susceptibility testing ofMycobacterium tuberculosisto primary anti-tubercular drugs by nitrate reductase assay. *Indian J Pathol Microbiol*. 2009;52(3):343–4. doi:10.4103/0377-4929.54989.
16. Lamsal A, Thapa B, Timilshina M, Verma SC, Ghimire GR, Gautam A, et al. Direct Nitrate Reductase Assay for detection of drug resistance in Mycobacterium tuberculosis: rapid, simple and inexpensive method for low resource laboratories. *Int J Infect Microbiol*. 2013;2(2):34–8. doi:10.3126/ijim.v2i2.8319.

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