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Original Research Article

EVALUATION OF THE PERFORMANCE OF NITRATE REDUCTASE ASSAY FOR RAPID DETECTION OF ISONIAZID AND RIFAMPICIN RESISTANCE IN SUSPECTED MDR- PULMONARY TUBERCULOSIS PATIENTS

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ABSTRACT

Background: Tuberculosis (TB) remains a major global health challenge, despite the availability of effective vaccines and drugs. Multidrug-resistant tuberculosis (MDR-TB) complicates treatment outcomes, and early detection is crucial for infection control. The nitrate reductase assay (NRA) has emerged as a promising alternative for rapid drug susceptibility testing (DST). The objective is to compare the efficacy of direct and indirect nitrate reductase assay (NRA) on Lowenstein-Jensen (LJ) for the detection of MDR-TB.

Materials and Methods: A comparative-validation study was conducted in the Department of Microbiology at Maulana Azad Medical College in collaboration with Lok Nayak Hospital and the New Delhi Tuberculosis (NDTB) Centre. A total of 32 suspected MDR-TB patients were enrolled based on Revised National Tuberculosis Control Programme (RNTCP) criteria. Sputum samples were processed for conventional microscopy, culture on LJ medium, and DST using direct and indirect NRA on LJ. Results were validated against the proportion method (PM), which was considered the gold standard. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and kappa statistics were used for comparison.

Results: The study included 22 males (68.8%) and 10 females (31.2%), with a mean age of 33.34 ± 2.34 years. Direct NRA on LJ medium showed a sensitivity of 94.1% and specificity of 100% for isoniazid resistance, while indirect NRA on LJ showed a sensitivity of 93.3% and specificity of 100%. For rifampicin, direct NRA on LJ had a sensitivity of 92.3% and specificity of 100%, whereas indirect NRA on LJ had a sensitivity of 93.8% and specificity of 100%. Kappa values indicated excellent agreement with the PM.

Conclusion: The NRA method demonstrated high concordance with the gold standard PM, offering a rapid, cost-effective, and reliable alternative for MDR-TB detection in resource-constrained settings. Direct NRA on LJ medium showed the highest diagnostic accuracy, making it a promising tool for early MDR-TB diagnosis and improved patient management.

Keywords: Tuberculosis, Multidrug-resistant TB, Nitrate reductase assay, Drug susceptibility testing, Lowenstein-Jensen medium, Proportion method, Rapid diagnosis.

INTRODUCTION

Tuberculosis was a massive scourge in the ancient world and during the 19th century it was among the leading causes of morbidity and mortality in developed countries. It was rightly referred to as the "Captain of all these men of death", by John Bunyan in the18th century and is still the biggest health challenge of the world.[1] Tuberculosis (TB) remains a worldwide public health problem despite the fact that the causative organism was discovered more than 100 years ago and highly effective vaccine and drugs are available making tuberculosis a preventable and curable disease.[2]

Global efforts to control the TB pandemic have been destabilized by the emergence and spread of strains that are resistant to the commonly used first line anti-TB drugs isoniazid (H), rifampicin (R), ethambutol (E), and pyrazinamide (Z). Strains resistant to at least H and R, the two most efficacious TB drugs are termed multidrug-resistant (MDR).[3] MDR-TB treatment is rather complicated as it requires second line drugs some of which are only injectables, are less efficacious, more toxic and more expensive than the first line agents.[4]

Multidrug- resistance occurring primarily as a consequence of poor treatment services could lead to emergence of extensively drug-resistant tuberculosis (XDR-TB) if MDR-TB is not managed properly. The recent emergence of XDR-TB defined as MDR-TB strains with resistance to a fluoroquinolone and to at least one injectable second line drug (kanamycin, amikacin or capreomycin) has further complicated the problem of MDR-TB.[3] Early detection of MDR-TB is of primary importance for both patient management and infection control.

Conventional methods for detection of MDR-TB involve primary culture of specimens and isolation of Mycobacterium tuberculosis, followed by drug susceptibility testing (DST). This process, referred to as indirect susceptibility testing has a long turn around time (TAT) of around 2-3 months. Recently, the focus has shifted to rapid direct tests in which decontaminated respiratory samples are directly inoculated in drug-free and drug-containing medium or amplified for detection of MDR-TB. Some of the direct tests being studied with prospects for applicability in developing countries include the nitrate reductase assay (NRA), microscopic observation drug susceptibility (MODS) assay, and more recently molecular assays such as the GenoType® MTBDR (Hain Life sciences, Nehren, Germany), and its newer version – the GenoType® MTBDRplus.[4]

The NRA test, initially introduced as an indirect assay is performed on solid medium as for the proportion method, though liquid-based assays have recently been studied.[5,6] The principle used for DST is where the drug containing media are tested and the colour change in these media indicate resistance to the particular drug being tested, while absence of any colour change indicates that the organism is susceptible to the drug. TAT is reduced as there is no need to wait for the growth of the Mycobacterium.

MATERIALS AND METHODS

Settings: This study was carried out in the Department of Microbiology, Maulana Azad Medical College in conjunction with the Chest Clinic of associated Lok Nayak Hospital and New Delhi Tuberculosis (NDTB) Centre.

The study was a comparative-validation study carried out on patients of age greater than 18 years. The study included thirty two such patients (n =32) from Chest Clinic and NDTB Centre, who were suspected to have multidrug-resistant (MDR) pulmonary tuberculosis, as per the RNTCP defintion[7]. After proper counselling and obtaining their informed consent, these patients were included in the study. Patients with negative sputum smear by direct microscopy and those not showing Mycobacterium tuberculosis in their culture were excluded from the study.

Sample Collection & Transport: Two sputum samples, five to ten ml each were collected in sterile leak proof container from each MDR- pulmonary TB suspect attending the Chest Clinic of Lok Nayak Hospital and NDTB Centre (Intermediate Reference Laboratory). These samples were then transported quickly to the Mycobacteriology laboratory, Maulana Azad Medical College.

Sample Processing: They were decontaminated by N-Acetyl L- Cysteine and Sodium hydroxide (NALC-NaOH) method using 1% Sodium hydroxide and then used for conventional laboratory techniques (sputum microscopy after staining with modified Ziehl- Neelson technique and culture on Lowenstein-Jensen media).

The LJ culture bottles which were incubated at 37ºC were examined daily for first 5 to 7 days for the detection of growth of rapidly growing mycobacteria and any contaminants. After that, all cultures were examined weekly for 8 weeks. Any growth seen on the medium was examined by ZN staining to confirm the acid fast bacilli (AFB). All the cultures showing growth were then examined for the rate of growth, pigmentation, colony morphology and other properties. The colonies were subcultured on two more slopes of LJ media for niacin and catalase tests for confirmation of Mycobacterium tuberculosis.

Nitrate Reductase Assay: Nitrate reductase assay 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 by adding Griess reagent, which produce a pink colour change in the LJ media. Nitrate reductase assay uses the detection of nitrite as indication of growth when it is used as a drug susceptibility test. The standard LJ medium was prepared with 1000 µg/ml of potassium nitrate (KNO3) added to it. Drug containing nitrate

LJ media were prepared by adding appropriate amount of drugs aseptically to LJ fluid before inspissation.

Drug Susceptibility Testing (DST): DST was done using the processed sputum sample using direct nitrate reductase assay (NRA) on LJ medium. The positive cultures were further processed for DST by proportion method (PM), indirect nitrate reductase assay (NRA) on LJ media.

Statistical analysis: Drug susceptibility by proportion method was taken as gold standard. NRA was validated against proportion method using sensitivity, specificity, positive predictive value and negative predictive value. Kappa value was calculated and a value of more than 0.75 represents excellent agreement beyond chance, a kappa below 0.40 represents poor agreement, and a kappa of 0.40 to 0.75 represents intermediate to good agreement.

RESULTS

Sociodemographic and clinical findings: The study included 32 participants, with a mean age of 33.34 years (± 2.34). The male-to-female ratio was [11:5]. Common complaints by the patients in decreasing frequency were cough (n=26; 81.2%) and anorexia (n=26; 81.2%), followed by weight loss (n=25; 78.1%) and fever (n=25; 78.1%), breathlessness (n=16; 50%). Crepitation on examination was present in 8 cases (25%).

A little more than half of the patients (53.1%) belonged to Category II under RNTCP, while the rest (46.9%) belonged to Category I. Family history of tuberculosis was found in 12.5% of cases (n=4). All 4 who had a positive family history also had history of anti-tubercular therapy (ATT) intake in the family. Out of the total patients, X-ray chest was suggestive of pulmonary tuberculosis in 23 cases (71.9%).

Microbiological data: Of all the sputum smear positive patients included, half of them showed 2+ RNTCP grading, while a quarter showed 3+ grading. About 15.6% of patients (n=5) had a 1+ grade, while 9.4% (n=3) had scanty bacilli per 100 oil immersion fields. All patients (n=32; 100%) had positive Lowenstein-Jensen medium growth of rough, tough and buff colonies that were AFB positive, showed canary yellow colour when tested for Niacin and produced less than 45 mm bubbles indicating weak catalase activity confirming Mycobacterium tuberculosis strain.

Drug Susceptibilty Testing:

Table 1: Comparison of drug susceptibility testing (DST) results by direct nitrate reductase assay (NRA) on LJ medium Vs proportion method (PM) (n=29).

Antitubercular drugs		RES	SUS	Sensitivity	Specificity	PPV	NPV	Percentage (%)	Kappa
Isoniazid	RES	11	2	88.2%	91.7%	93.8%	84.6%	89.7%	0.79
	SUS	1	15						
Rifampicin	RES	12	1	94.1%	100%	100%	92.3%	96.6%	0.93
	SUS	0	16						

[Table 1] provides an overview of parameters like sensitivity and specificity of direct NRA to detect resistance for isoniazid was 88.2% and 91.7% respectively while it was 94.1% and 100% respectively for rifampicin. It had an exemplary positive predictive value of 93.8% and 100% for INH and RIF respectively. An excellent agreement between the results of direct NRA and proportion method for isoniazid and rifampicin resistance was found with kappa value of 0.79 and 0.93 correspondingly. Comparison of DST results was done for 29 samples excluding the three samples that were contaminated and could not be recovered for DST by direct NRA on LJ medium.

Table 2: Comparison of results of drug susceptibility testing (DST) by indirect nitrate reductase assay (NRA) on LJ medium and proportion method (PM) on LJ medium (n=32).

Antitubercular drugs		RES	SUS	Sensitivity	Specificity	PPV	NPV	Percentage (%)	Kappa
Isoniazid	RES	14	1	94.1%	93.3%	94.1%	93.3%	93.8%	0.88
	SUS	1	16						
Rifampicin	RES	14	1	94.4%	100%	100%	93.3%	96.9%	0.94
	SUS	0	17						

[Table 2] implies an excellent agreement beyond chance for detecting drug resistance, by the indirect NRA on LJ medium which showed >90% sensitivity and specificity in detecting isoniazid and rifampicin resitance, with a noteworthy positive predictive value of 94.1% and 100%, respectively for both drugs on comparison with the PM.

Table 3: Time taken for drug susceptibility results by direct nitrate reductase assay (NRA) on LJ medium

Day of testing	Number of positive specimen by Direct NRA (n=29)	Number of positive specimen by Indirect NRA (n=32)
Day 7	8 (27.6%)	16 (50%)
Day 14	16 (55.2%)	12 (37.5%)
Day 21	5 (17.2%)	4 (12.5%)

[Table 3] implies that by the direct NRA on LJ medium, results were available for most of the samples (82.8%) in 14 days. While indirect NRA gave results for 50% sample in 7 days and 87.5% by 2 weeks.

DISCUSSION

The increasing trend in multidrug-resistant tuberculosis (MDR-TB) along with other drug resistant tuberculosis poses a significant challenge to public health. To contain their spread within the population, rapid detection of MDR-TB strains is crucial. Since current methods for drug-susceptibility testing (DST) of Mycobacterium tuberculosis (MTB) are often either expensive or time-consuming, there is need for a cost-effective and rapid alternative techniques.

A meta-analysis by Martin et al,[8] indicates that the nitrate reductase assay (NRA) is highly sensitive and specific for detecting RIF- and INH-resistant TB in culture isolates and clinical sputum specimens. Most studies report a sensitivity of 95% or higher, with nearly all achieving 100% specificity and high accuracy. The average turnaround time (TAT) for results is 5-12 days with indirect NRA and 14-21 days with direct NRA. NRA is also compatible with existing laboratory infrastructure, as it utilizes the classical Lowenstein-Jensen (LJ) medium with added potassium nitrate (KNO3) and just require observation of colour change to determine the results. And hence does not require sophisticated equipment or expensive reagents, making it accessible for widespread use.

The current study demonstrated a strong concordance between the NRA and the proportion method (PM) for drug-susceptibility testing of Mycobacterium tuberculosis. The κ values were 0.93 for rifampicin (RIF), 0.79 for isoniazid (INH) by the direct NRA and 0.94 for RIF, 0.88 for INH by the indirect one. Indirect NRA exhibited high sensitivity and specificity for RIF (94.4% and 100%) and INH (94.1% and 93.3%). These findings are consistent with other studies that have reported high sensitivity and specificity for INH and RIF.[9,10] Resistance to RIF almost always indicates MDR-strain and hence the high sensitivity and specificity for RIF by NRA can be useful to identify MDR-TB in resource limited settings. The turnaround time (TAT) for indirect NRA in majority of samples in the present study was 7-10 days. Not to forget to add another 3 to 6 weeks it takes to obtain the culture isolate.

To reduce the TAT of drug-susceptibility testing (DST) by eliminating the pre-isolation step, the nitrate reductase assay (NRA) has been directly applied to clinical sputum specimens. Full agreement was observed for the detection of rifampicin (RIF) resistance, although some discordant results were noted for other drugs.[11] Visalakshi et al,[12] reported sensitivity and specificity of the direct NRA and indirect proportion method (PM) to be 94% and 98%, and 100% and 98% for RIF and isoniazid (INH) respectively, demonstrating excellent agreement between the two tests. Additionally, Shikama et al. found 100% sensitivity and specificity of NRA for RIF.[13] Closely resembling sensitivity and specificity data was obtained in our study. However there was 9% contamination rate in our study comparable to a previous study by Bwanga F,[14] for the direct NRA. The reason could be as the directly added sputum sample has other commensal flora and that LJ media is not a selective media without any added antibiotics. While the Nitrate Reductase Assay (NRA) offers several advantages, it also has certain limitations. A very small proportion (<1%) of Mycobacterium tuberculosis (MTB) strains do not produce nitrate reductase,[15] which can render the test ineffective. However, in our study, routine biochemical analyses, including the nitrate reductase test, did not identify any such strains. Additionally, NRA may produce positive results in some non-tuberculous mycobacteria, such as M. kansasii, M. szulgai, M. flavescens, M. terrae complex, and certain rapid-growing species, [15] whereas M. bovis remains nitrate-negative. In our setting, LJ media were periodically observed for rapid growers till day 7 and all 32 samples were negative for their growth.

Another potential issue is that nitrate can be further reduced beyond nitrite to nitric oxide, making it undetectable by the Griess reagent. To address this, zinc dust was added to all tubes that initially tested negative.[16] Zinc facilitates the rapid reduction of nitrate, causing a red color change in true-negative samples, while tubes where reduction has proceeded beyond nitrite remain unchanged.

CONCLUSION

In our experience, NRA assay (direct NRA and indirect NRA on LJ medium) had an excellent agreement with the proportion method for DST of isoniazid and rifampicin, indirect showing slightly better parameters than direct. Overall, it's a useful diagnostic tool for rapid testing for DST in large scale samples in resource limited settings. The direct NRA on LJ medium had the least turnaround time. However, the contamination rates were higher by this method with comparatively low overall sensitivity and specificity. The validity of this test should be further evaluated on a larger sample and in cases of suspected tuberculosis.

REFERENCES

1. Jha BC, Dass A, Nagarkar NM, Gupta R, Singhal S. Cervical tubercular lymphadenopathy: changing clinical pattern and concept in management. Postgrad Med J. 2001;77:185-7.
2. K. Park. Tuberculosis. In: Park's Textbook of Preventive & Social Medicine. 23rd ed. Banarsidas Bhanot Publishers; 2013. p 166-83.
3. CDC. Notice to readers: revised definition of extensively drug-resistant tuberculosis. MMWR. 2006;55(43):1176.

4. WHO. Guidelines for the programmatic management of drug-resistant tuberculosis. Geneva: World Health Organization; 2006.
5. 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.
6. Syre H, Phyu S, Sandven P, Bjorvatn B, Grewal HM. Rapid Colorimetric Method for Testing Susceptibility of Mycobacterium tuberculosis to Isoniazid and Rifampin in Liquid Cultures. J Clin Microbiol. 2003;41(11):5173-7.
7. TBC India. Revised National Tuberculosis Control Programme. DOTS-Plus Guidelines. New Delhi: Central TB Division; 2010.
8. Martin A, Panaiotov S, Portaels F, Hoffner S, Palomino JC, Angeby K. The nitrate reductase assay for the rapid detection of isoniazid and rifampicin resistance in Mycobacterium tuberculosis: a systematic review and metaanalysis. J Antimicrob Chemother. 2008;62(1):56-64.
9. Gupta A, Sen MR, Mohapatra TM, Anupurba S. Evaluation of the performance of nitrate reductase assay for rapid drug-susceptibility testing of mycobacterium tuberculosis in north India. J Health Popul Nutr. 2011;29(1):20-5.
10. Sethi S, Sharma S, Sharma SK, Meharwal SK, Jindal SK, Sharma M. Drug susceptibility of Mycobacterium tuberculosis to primary antitubercular drugs by nitrate reductase assay. Indian J Med Res. 2004;120(5):468-71.
11. Musa HR, Ambroggi M, Souto A, Angeby KA. Drug Susceptibility Testing of Mycobacterium tuberculosis by a Nitrate Reductase Assay Applied Directly on Microscopy-Positive Sputum Samples. J Clin Microbiol. 2005;43(7):3159-61.
12. Visalakshi P, Meharwal SK, Myneedu VP, Behera D.Evaluation of direct method of drug susceptibilitytesting of Mycobacterium tuberculosis to rifampicin and isoniazid by nitrate reductase assay in a national reference laboratory. Diagn Microbiol Infect Dis 2010;66:148-52.
13. Shikama ML, Ferro e Silva R, Villela G, Sato DN, Martins MC, Giampaglia CM et al. Multicentre study of nitrate reductase assay for rapid detection of rifampicin-resistant M. tuberculosis. Int J Tuberc Lung Dis 2009;13:377-80.
14. Bwanga F. Rapid tests for multidrug resistant tuberculosis in low income settings [PhD thesis]. Stockholm and Kampala; Karolinska Institute and Makerere University: 2010.
15. Kent PT, Kubica GP. Public Health Mycobacteriology: a guide for the level III laboratory. Atlanta, Georgia: CDC, US Department of Health and Human Services; 1985.
16. Centro Panamericano de Zoonosis. Tuberculosis bacteriology. Technical note 11. Buenos Aires: Centro Panamericano de Zoonosis, 1988 [Spanish].