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Evaluation of Nitrate Reductase Assay for Detection of Multi-drug Resistant *Mycobacterium tuberculosis* among Patients at National Tuberculosis Reference Laboratory Zaria Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author KM designed the study. Authors KM and EN managed the literature searches. Authors EN and KM wrote the protocol. Author KM performed the statistical analysis. Authors OSO, EDJ and EN managed the analysis of the study. Authors KM and OSO wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate nitrate reductase assay for detection of multi-drug resistant *Mycobacterium tuberculosis* among patients at National Tuberculosis Reference Laboratory Zaria Nigeria.
Study Design: Hospital based cross sectional study
Place and Duration of Study: National Tuberculosis Reference Laboratory Zaria Nigeria from December 2015 to June 2016.
Methodology: A total of 437 re-treatment patients' samples were screened for Acid Fast Bacilli (AFB), 72 were smear positive. Out of 72 smears positive, 62 were culture positive, using

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Lowenstein Jensen medium, 57 were found to be *Mycobacterium tuberculosis* complex (MTBC) using immunochromatographic test. In this study the susceptibility of 57 MTBC isolates to isoniazid (INH), rifampicin (RIF), streptomycin (STR) and Ethambutol (EMB) was determined by Lowenstein Jensen proportion method (LJPM) and Nitrate Reductase Assay (NRA)

Results: The sensitivity and specificity of NRA compared to that of LJPM were observed to be 98% and 89%, 98% and 92%, 64% and 80%, 68% and 77% for RIF, INH, STR, and EMB respectively. Positive predictive values were 91%, 93%, 87% and 83% for RIF, INH, STR and EMB respectively. Negative predictive values were 80%, 92%, 67% and 90% for RIF, INH, STR and EMB respectively .Overall, the sensitivity, specificity ,positive predictive value and negative predictive value of NRA in detecting MDR-TB were 90%,82%,85%and 73% respectively. Good agreement was found in all the tests with κ values of 0.63, 0.61, 0.61 and 0.62 for RIF, INH, EMB and MDR-TB respectively only STR shows moderate agreements with k value of 0.59.

Conclusion: In the 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. On the basis of our findings, NRA has the potential to be a useful tool for accurate detection of MDR-TB in the study area.

Keywords: Multidrug resistant TB; nitrate reductase assay; LJ proportion method; drug susceptibility testing.

1. INTRODUCTION

Tuberculosis (TB) is causing more deaths worldwide than any other infectious disease. The World Health Organization (WHO) estimated that in 2011, there were 8.7 million cases of TB, with nearly 1 million deaths among HIV-negative cases and 0.43 million deaths associated with HIV infection [1]. Case detection of all forms stood at 51%. The mortality rate for all forms of TB remains 27 per 100,000 populations (46,000 deaths per year) [1]. Based on 2012 survey Nigeria ranked 4th among the highest tuberculosis burden countries in the world and first in Africa. The point estimates of TB prevalence rates were 318 and 524 per 100,000 population (15 years and above) respectively [2].

Multidrug-resistant TB (MDR-TB) is TB that is resistant to atleast, rifampicin (RIF) and isoniazid (INH) the two most important first-line drugs with or without other first line drugs. In Nigeria, the national MDR-TB survey showed a prevalence of 2.9% among new cases and 14.3% for retreatment cases [1]. Currently there are 2 national Tuberculosis reference laboratories and 6 zonal Tuberculosis reference laboratories for culture which generally use Lowenstein-Jensen (LJ) medium with limited use of liquid cultures.

Indirect susceptibility testing on LJ medium is the most common method for detection of TB drug resistance in Africa. There is an urgent demand for early and proper detection of MDR and XDR-TB cases for the effective management and control of TB. In recent years, a multitude of techniques for rapid DST has been designed and evaluated .However, the investment and recurrent costs is an obstacle for the broad implementation of these techniques in resource-limited settings (RLSs) of Africa [3].

Global efforts for TB control, especially in resource limited settings, are being challenged by the lack of rapid, reliable and inexpensive techniques for the detection of *M. tuberculosis.* Results from the conventional culture detection methods come too late to influence a timely decision on patient management. Early detection is the key in both patient management and controlling transmission of *M. tuberculosis.* Liquid media systems such as the Mycobacterial Growth Indicator Tube (MGIT), are more rapid and detect more mycobacterial isolates than LJ [4]. The use of liquid systems the MGIT 960, has improved TAT to about 25–45 days. Liquid culture systems require expensive equipment and high biosafety level structures with constant electricity [5]. The traditional method for detection of MDR TB with indirect susceptibility testing, involving isolation of the bacterium followed by drug susceptibility testing (DST), has a long turnaround time (TAT) of 10 to 12 weeks [6]. Molecular tools such as Line Probe Assay (LPA) have been in use in Nigeria, but are expensive to run and require constant electricity. Therefore, faster, inexpensive and reliable tests are urgently needed [7].

Nitrate Reductase Assay (NRA) is a simple colorimetric method that requires basic TB culture equipment with short turnaround

time.NRA is simple, inexpensive, reliable and reproducible test. Therefore, rapid, affordable, accurate and easy to use test for MDR-TB in Nigeria remains a priority. The aim of this study was to evaluate nitrate Reductase assay for detection of multi-drug resistant Tuberculosis compared to LJ proportion method, in Nigeria.

2. MATERIALS AND METHODS

2.1 Settings

The NTBLTC is the largest TB referral center in northern Nigeria; NTBLTC serves as the National Training Center for community health workers and laboratory health personnel's involved with Diagnosis of tuberculosis and treatment at the Peripheral, State and Zonal levels. This center is one of the two National TB reference laboratories that is equipped with TB biosafety level 3 (BSL-3) and TB molecular diagnostic laboratories.

2.2 Sample Collection

A total of 437 re-treatment patients samples were collected and screened for Acid Fast Bacilli (AFB).Positive smears were culture on Lowenstein Jensen Media while culture positive samples were first identified for MTBC using immunochromatographic test kits.

2.3 Preparation of Lowenstein- Jensen Medium with Drugs

Isoniazid (INH), Streptomycin (STR), Rifampicin (RIF) and Ethambutol (EMB) were obtained as powder from Sigma Aldrich (Bornem, Belgium). Each drug was prepared at a concentration of 10 mg/ml in sterile distilled water with the exception of RIF, which was dissolved in dimethylsulphuroxide (DMSO). Stock solutions were filtered (0.45 µm) sterilized and stored at -70℃ and used within six months. Different concentration of 0.2 µg/ml INH, 2 µg/ml EMB, 40 µg/ml RIF and 4 µg/ml STR, were incorporated into LJ medium and then inspissated at 85℃ for 45 minutes. After preparation, the media were incubated for 48 hours at room temperature for sterility check before use [8].

2.4 Preparation of Lowenstein- Jensen Medium with KNO_3 and Drugs

Two grams of potassium nitrate were dissolved in 10 mls of sterile distilled water. And 1ml of prepared KNO_3 was added to 200 ml of the drug containing and drug free LJ medium, dissolved by stirring, aliquoted and then inspissated for 45 minutes at 85℃. After inspissations, the media was incubated for 48 hours at 37℃ for sterility check before use. For NRA, the tubes containing LJ with antibiotics and KNO_3 were used for the test while LJ with KNO_3 were used as a control [8].

2.5 Drug Susceptibility Testing by Proportion Method

The Indirect PM (IPM) was performed on LJ medium with the same recommended critical concentrations of antibiotics as mentioned previously. The culture of *M. tuberculosis* strain was harvested and suspended in tube containing sterile distilled water with 5-7 sterile glass beads then vortexed for about 30 seconds to homogenise the bacterial suspension and then allowed to stand for 15-30minutes for large aggrevates of bacteria to settle and turbidity of bacterial suspension was adjusted to match the McFarland turbidity No. 1. The original bacterial suspension was further diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. The tubes were arranged in order of C_1, C_2, C_3, S, I, R and E representing 10^{-2}, 10^{-3} 10^{-4}, streptomycin, Isoniazid, Rifampicin and Ethambutol respectively.

One in hundred dilutions (10^{-2}) was inoculated into control (C_1) and four (4) drugs (S I R E), 10^{-3} dilution was inoculated into C_2 and 10^{-4} was inoculated into C_3 and then all tubes were incubated at 37℃ for six weeks. Growth was recorded at 28 days and at 42 days as follows: 3+ for confluent growth, 2+ for more than 100 colonies, and 1-100 actual numbers of colonies. Susceptibility or resistance was recorded when the proportion of bacteria in drug-containing medium to that of drug free medium is < 1 or ≥ 1 respectively. *M. tuberculosis* reference strains H37Rv (ATCC 27294) sensitive to first-line anti-tuberculosis drugs, RIF-resistant (ATCC 35838), INH-resistant (ATCC 35822), EMB-resistant (ATCC 35837) and STR-resistant (ATCC 35820) were used as susceptible and resistant controls. All strains were sub cultured in Lowenstein–Jensen (LJ) medium for 4 weeks before being studied [8].

2.6 Drug Susceptibility Testing by Nitrate Reductase Assay

Bacterial suspensions was made from a culture of MTB Isolates by dispensing a loopful of bacteria in 0.5 mL of sterile distilled water in

15mL falcon tube containing a few 3-mm diameter glass beads and vortexed to obtain a uniform solution., approximately 2.5 mL of sterile distilled water was added. The inoculum turbidity was adjusted to a McFarland tube no. 1 and diluted 1: 10 in sterile distilled water. Undiluted suspension (200 µ l) was inoculated into tubes of LJ medium with KNO_3 (1 mg/ml) containing each of the drugs at the concentrations described above and 200 µ l of the 1 : 10 dilution was inoculated into three control tubes without antibiotics. The assay was developed with reagent mix (50% conc. HCl, 0.2% sulfanilamide and 0.1% N-(1-naphthyl) ethylene diamine dihydrochloride at ratio of 1: 2: 2) after 7 days of incubation at 37℃ , a 0.5 ml of reagent mix was added to one control tube. When the clear reagent mixture turned pink, the drug-containing tubes were subsequently developed. When there was no colour change, the tubes were re-incubated and the procedure repeated on days 10 and 14. An isolate was considered resistant if the drug-containing tube produced a colour change that was more intense than the drug-free tube. *M. tuberculosis* reference strains H37Rv (ATCC 27294) sensitive to first-line anti-tuberculosis drugs, RIF-resistant (ATCC 35838), INH-resistant (ATCC 35822), EMB-resistant (ATCC 35837) and STR-resistant (ATCC 35820) were used as susceptible and resistant controls. All strains were sub cultured in Lowenstein–Jensen (LJ) medium for 4 weeks before being studied [9].

3. RESULTS AND DISCUSSION

Drug susceptibility testing was performed on NRA to first line drugs on which fifty seven (57) confirmed isolates, showed highest resistance as against rifampicin (82.5%) followed by isoniazid resistance (77.2%). Streptomycin and ethambutol resistances were 65% and 51% respectively. Whereas ethambutol had highest number of susceptible isolates of 49%, streptomycin 35%, isoniazid 22.8% and rifampicin (17.5%) with lowest susceptible was recorded as shown in Table 1.

Multi-drug resistant tuberculosis is most difficult and expensive for management and treatment during the recent years is an increasing. Rapid detection of MDR strains is therefore needed to stop their spread in the population. Current methods for DST of MTB are either costly or very slow. So, a cost-effective and rapid drug-susceptibility method is required to guide the treatment of TB. NRA result of Bwanga et al. [10] in Uganda NRA was suggest that NRA is the most sensitive, specific, and cheapest test for MDR-TB in low income settings. Rapid detection of MDR-TB and thereby rapid drug susceptibility testing (DST) to first line drugs, are critically important to the successful control of TB [11].

The results of drug susceptibility testing by NRA indicate that the resistance rate was higher as against of isoniazid and rifampicin, which are the most important first line drugs used for the treatment of tuberculosis. This could be as a result of overuse of these drugs for the treatment of TB. This is in agreement with the work of Mahadev et al*.* [12] and Iqbal et al*.* [13] who reported higher resistance to rifampicin and isoniazid.

The sensitivity and specificity of NRA compared to that of LJPM were observed to be 98% and 89%, 98% and 92%, 64% and 80%, 68% and 77% for RIF, INH, STR, and EMB respectively. Positive predictive values were 91%, 93%, 87% and 83% for RIF, INH, STR and EMB respectively. Negative predictive values were 80%, 92%, 67% and 90% for RIF, INH, STR and EMB respectively. Overall, the sensitivity, specificity, positive predictive value and negative predictive value of NRA in detecting MDR-TB were 90%, 82%, 85%and 73% respectively. Good agreement was found in all the tests with κ values of 0.63, 0.61, 0.61 and 0.62 for RIF, INH, EMB and MDR-TB respectively only STR shows moderate agreements with k value of 0.59 (Table 2).

When the results of NRA and LJ PM were compared, higher agreement was observed for RIF and INH, however there were disagreements in relation to the antibiotics EMB and STR. The factors which led to lower specificity and sensitivity values of LJ could be as a result of degradation of the antibiotics in LJ medium. The higher rate of detection of RIF resistance by NRA method is the reason for the use of NRA for the screening of MDR strains, as resistance to RIF is a marker for MDR detection. Most studies have shown high sensitivity and specificity values for INH and RIF, the two most important drugs used for TB treatment [9,14,15].

In a multicentre study, the NRA was evaluated in different settings to determine the susceptibility of *M. tuberculosis* to first-line anti-tuberculosis drugs. Higher accuracy of 96.6% was observed for INH and RIF, while that for STR and EMB were lower (85.3%) [16]. Percentage agreement of 98.3%, 98.3%, 90.8% and 93.3% by NRA

Table 1. Drug susceptibility of *Mycobacterium tuberculosis* complex by nitrate reductase assay

Drugs	Streptomycin	Isoniazid	Rifampicin	Ethambutol
Resistance	37(65%)	44(77.2%)	47(82.5%)	29(59%)
Susceptible	20(35%)	13(22.8%)	10(17.5%)	28(49%)

Key=MDR-TB = multidrug resistant tuberculosis, NRA = nitrate reductase assay, LJPM = Lowenstein Jensen proportion Method

Table 2. Evaluation of drug susceptibility testing of *Mycobacterium tuberculosis* using nitrate reductase assay and LJ proportion method

Drugs	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	κ-statistic
Streptomycin	64	80	87	67	0.59
Isoniazid	98	92	93	92	0.61
Rifampicin	98	89	91	80	0.63
Ethambutol	68	77	83	90	0.61
MDR-TB	90	82	85	73	0.62

Key: PPV = positive predictive value, NPV = negative predictive value, κ = kappa

were observed for INH, RIF, STR and EMB respectively by Shikama et al. [17]. However, similar lower sensitivity and specificity for STR and EMB have also been reported by Lemus et al. [18] and Montoro et al. [19] as also reported in this study.

Lemus et al. [18] in India also observed sensitivity of 91.7%, 96.5%, 88.0% and 93.9% for INH, STR, EMB and RIF, respectively, and then specificity was higher than 99.1% for all of the drugs. While in this study higher sensitivity for INH and RIF was observed to be 98% and this could be as the result of the patients categories used for the study. Visalakshi et al. [20] observed sensitivity and specificity of the direct NRA and indirect LJPM to be 94% and 98%, and 100% and 98% for RIF and INH respectively which is similar to the result obtained in the study. Moreover, Bwanga et al. [10] in Uganda stated 98% sensitivity and specificity of NRA for RIF and 98% sensitivity and 94% specificity of NRA for INH. In another study of Gupta et al., [21] sensitivity of NRA was 98.4% ,97% 88.5% and 94.5% for RIF, INH, STR and EMB with 99 % agreement between the results of NRA and LJPM for INH and RIF while this study have reported lower sensitivity for STR and EMB.

The higher detection rate of MDR-TB by NRA could be as a result of borderline that exhibit color change lower than that obtained in the control tube and therefore this borderline isolate are frequently associated with problems of interpretation of DST by NRA. Bacterial contamination may cause false positive results and low inoculation in the media could also result in false positive result [22]. More than half of MDR-TB isolates were resistant to all four first-line anti tuberculosis drugs tested. Re-treatment cases were significantly more likely to have MDR-TB than non-MDR-TB. Previous studies have shown that resistance to rifampicin is a significant predictor of resistance to isoniazid and streptomycin; isolates resistant to rifampicin were also resistant to isoniazid and streptomycin [23].

4. 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. On the basis of the findings, NRA has the potential to be a useful tool for accurate detection of MDR-TB in the study area.

CONSENT

All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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