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Evaluation of Direct Nitrate Reductase Assay for Drug Susceptibility Testing of *Mycobacterium tuberculosis* in Resource - Poor Settings

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Abstract: ***Direct Nitrate Reductase Assay (DNRA) was used for susceptibility testing of Mycobacterium tuberculosis to two front line anti-tuberculosis drugs (Rifampicin and Isoniacid) from 100 Acid fast bacilli smear-positive sputum samples. The DNRA results were compared with the gold standard conventional proportion method (PM). Ninety eight (98) sputum specimens result were comparable with the gold standard. DNRA results were obtained at day 10 for 72 specimens, results for 17 specimens were obtained at day 14, and the results for 9 specimens were obtained at day 18. Thus, 74% of DNRA results were obtained in 10 days. The sensitivities and specificities of DNRA were 100%, 97% and 100%, 99% for Rifampicin and Isoniacid respectively. Positive predictive values were 100% and 93% for Rifampicin and INH respectively while negative predictive values were 97% and 100% for Rifampicin and Isoniacid respectively. NRA has been demonstrated as rapid, accurate, and cost-effective method for drug susceptibility testing of Mycobacterium tuberculosis. Therefore, NRA constitutes a useful tool for detection of tuberculosis drug resistance in low-resource countries.***

Keywords: *Mycobacterium tuberculosis*, Drug testing, Nitrate Reductase Assay

1. Introduction

Tuberculosis (TB), an infectious bacterial disease with significant morbidity and mortality, is the leading cause of death in the world from a bacterial infections. The aetiological agent of TB is the group of *Mycobacterium tuberculosis* complex comprising of *Mycobacterium tuberculosis, M. bovis, M. africanum, M.microti, M. canettii, M. caprae, M. pinnipedii, M. suricattae and M. mung* [1].

TB was declared a global health emergency by the World Health Organization (WHO) in 1993 and claims approximately 1.7 million lives per annum, more than can be attributed to any other bacterial infection. It is estimated that one-third of the world's population is infected with causative agent, obligate human pathogen *M. tuberculosis*, with around 9 to 10 million new cases of TB being reported each year [2].

Transmission of TB usually occurs by an airborne or aerosol but can also occur through the gastrointestinal tract and approximately 5 to 10 percent of cases overall are thought to result from spontaneous reactivation of latent TB infection [3].

Improved living standards and better sanitation led to a significant decrease in TB incident even before advent of effective TB chemotherapy. The introduction of TB chemotherapy in the mid-twentieth century made TB curable for the first time and further reduced TB incidence in industrialized countries. Despite the availability of the BCG (Bacilli Calmette - Guerin) vaccine and TB chemotherapy, the disease is on the increase in recent years largely due to HIV infection especially in poverty stricken regions and also to immigration, increased trade and globalization. The alarming increase of drug resistant TB, especially multi-drug resistant – TB (MDR - TB), (defined as resistant to at least two frontline TB drugs – isoniazid and rifampicin) and extensively drug-resistant TB (XDR-TB) (additional resistance to fluoroquinolone and one of injectable antibiotics) has caused a great deal of concern and becoming an increasing threat to the control of the disease in recent years. Early detection of drug resistance is key to the eradication of tuberculosis.

The current methods for drug susceptibility testing (DST) of *Mycobacterium tuberculosis* are either costly or slow. As the prevalence of multidrug-resistant strains increases, there is need for fast, reliable, and inexpensive DST methods as against the traditional solid culture methods that can be applied in settings with scarce resources.

Several approaches have been made for DST of TB. The relatively cheap traditional solid culture method is sensitive and specific but very slow (not less than 4weeks). The Microscopic Observation Drug Susceptibility (MODS) and Molecular methods are rapid, sensitive and specific but needs sophisticated equipment which are not readily available in resource poor settings [1]. Other susceptibility methods are still under evaluation and not yet available for commercial use.

Therefore, the aforementioned short comings calls for the evaluation of an inexpensive Nitrate Reductase Assay (NRA)

which needs no sophisticated equipment for efficient and rapid (less than 14 days) susceptibility testing of TB in resource-poor settings.

The aim of this study was to evaluate the performance of direct NRA as an inexpensive and rapid Method (using Culture Method as the gold standard) in determining the susceptibility of *Mycobacterium tuberculosis* to rifampicin (RIF) and isoniazid (INH) in microscopy-positive clinical samples from patients with pulmonary tuberculosis in South Western Nigeria.

2. Materials and Methods

2.1 Study Sites and Sample Collection

All clinical samples were drawn from those submitted to the Department of Medical Microbiology of Lagos University Teaching Hospital (LUTH), Lagos, Osun State Hospitals Management Board Health Care Facilities and University College Hospital (UCH), Ibadan which serves as the tuberculosis reference laboratory for the entire South - western geopolitical zone of Nigeria. Hundred Microscopy positive samples from One hundred patients were included in the study. Ethical approval for this study was sought from Ministry of Health, Osun State.

2.2 Sample Processing

About 10ml of sputum specimen was collected from patients suspected of having tuberculosis based on clinical symptoms. All specimens were digested and decontaminated of other bacteria by the standard N-acetyl- L- cysteine-NaOH-Na citrate method [4].

2.2.1 Smear Test

Decontaminated samples were placed on microscope slides, heat fixed and stained with Ziehl- Neelsen standard procedure. Dried stained slides were examined microscopically at X 100 magnification and graded according to WHO guidelines.

2.2.2 Direct NRA Drug Sensitivity Testing

The NRA uses two well-known properties of *Mycobacterium tuberculosis*:

1) The rate of growth in liquid Medium is considerably quicker than that on solid medium.
2) Reduces nitrate to nitrite, which is revealed as a colour change of the culture media, using the Griess method [5].

Mycobacterial growth can thus be detected long before it would be visible to the naked eye [6]. Incorporation of anti –TB drugs and setting up of controls at the outset enables direct susceptibility testing with clinical specimen.

The specimens were processed by digestion-decontamination method and thereafter concentrated by centrifugation at 3,200 *g* for 20 min. The supernatants were discarded, and a small portion of the sediment were used for preparation of microscopic slides, which were then stained with the Ziehl-Neelsen technique [7]. Of these, microcopy positive samples were selected. The rest of the remaining sediment were re-suspended in 3 ml of sterile distilled water and then used to inoculate the culture medium used in both drug susceptibility tests (1.5 ml for direct NRA and 1.5 ml for the proportion method).

Lowenstein-Jensen (L-J) medium, with 1,000 µg of potassium nitrate (KNO_3)/ml and with or without antimicrobial agents incorporated was used as the culture medium. The following critical concentrations were used: 0.2 µg/ml for Isoniacid (INH), 20 µ g/ml for Rifampicin (RIF). The medium was prepared in 7-ml portions in 15.5mm glass tubes with screw plugs. Before NRA testing, part of the suspension was diluted 1:10 in sterile distilled water. For each strain, 0.2 ml of the undiluted suspension was inoculated into the tubes containing L-J medium with KNO_3 and the antibiotics, and 0.2 ml of the 1:10 dilution was inoculated into three drug-free tubes containing L-J medium with KNO_3 incorporated. The latter tubes served as growth controls.

The tubes were incubated at 37°C and 0.5 ml of Griess reagents was added to one drug-free control tube after 10 days. If any colour change (strong or weak pink/violet) could be seen, the corresponding antibiotic-containing tubes was also tested and the susceptibility results read. If no colour change was seen in the growth control tube, the remaining control tubes and the antibiotic tubes were re-incubated. The procedure was repeated at day 14 and, if needed, at day 18, using the last growth control tube. The Griess reagents were prepared as follows: reagent A consisting of 800 mg of sulfanilic acid plus 5 N acetic acid (100ml), and reagent B consisting of 500 mg of naphthylamine plus 5 N acetic acid (100 ml). Equal parts of reagents A and B were mixed shortly before use.

2.2.2.1 Controls

In order to prevent false negative results (if the produced nitrite have further reduced to nitric oxide), a small amount of powdered zinc (Sigma, USA) was added to each negative tube and tested for generated nitrite in the medium by Griess reagent as described above. Bacterial contamination may cause false positive results. Therefore, to ensure that the media were free of contaminants the inoculated NRA broths were streaked on blood agar plates to detect any non-specific bacterial growth before testing for colour changing.

For medium batch quality control, the following *M. tuberculosis* reference strains were used: fully susceptible H37Rv (ATCC 27294) and INH resistant H37Rv (ATCC 35822).

2.2.3 Conventional Culture Technique

The conventional culture technique serves as growth control for direct NRA. Two hundred fifty microliters of decontaminated sputum was used to inoculate one Lowenstein-Jensen (LJ) slant. LJ slant medium was incubated at 37^0C and examined twice a week, from week 2 to 8 following inoculation. Slants were interpreted as gold standard according to criteria developed by the National Centers for Disease Control and Prevention [7]. The isolated cultures from processed samples were then used for indirect

DST by PM, the gold standard for DST of *Mycobacterium tuberculosis* (MTB)

2.2.4 DST by Proportional Method (PM)

The PM on LJ medium without nitrate was performed with the same recommended critical concentrations of first-line anti-tubercular drugs as previously described [8]. For each strain, part of the suspension was diluted 1:100, and 200 µl of the dilution was inoculated on two tubes of LJ medium without antibiotics and 200 µl of the undiluted suspension was inoculated into two LJ medium tubes with antibiotics incorporated. All tubes were incubated at 37°C. Preliminary results could be reported earlier for resistant strains, sometimes as early as after 20 days. Final susceptibility results were reported only after 40 days following the standard procedure [9]. An isolate was reported as resistant if the number of colonies growing on the antibiotic containing medium was 1% or more of the number of colonies developing on the drug-free control. The results obtained by the proportion method were used as the gold standard to compare to the results of NRA for susceptibility testing.

3. Results

Direct drug sensitivity testing for two of the first-line anti-tubercular drugs, rifampicin (RIF), and isoniacid (INH) was performed on 100 Acid fast bacilli (AFB) positive clinical sputum specimens using direct NRA and conventional Proportion Method (PM) testing. Of these 100 specimens, 27 specimens had 1+ acid-fast bacilli positivity; 59 had 2+ positivity; 9 had 3+ positivity; and 5 had scanty positivity with 7, 5, 4, 3, 3 bacilli load/100 oil immersion field). Ninety eight (98%) samples were culture positive by conventional method; the remaining 2(2%) samples were culture negative, identified as non-tubercle mycobacteria (1) and contaminated (1).

The results of 72 (74%), 17 (17%) and 9 (9%) isolates were obtained after 10, 14 and 18 days respectively with DNRA and were compared with the results produced using PM that were obtained after 28 days for 8 (8.16%) isolates and 40 days for 90 (91.83%) isolates. For RIF, 21(21.4%) and 23(23.5%) strains were detected as resistant while 77(78.6%) and 75(76.5%) strains were detected susceptible by DNRA and PM methods respectively.

For INH, 14(14.3%) and 13(13.3%) strains were detected as resistant while 84(85.7%) and 85(86.7%) strains were detected susceptible by DNRA and PM methods respectively; therefore, 1 false positive results was obtained with NRA.

A total of 11(11.2%) strains of 98 strains available for comparison with gold standard PM were detected by both methods to be resistant (MDR) to both RIF and INH; 9 of these MDR strains are from patients from MDR ward.

The age range of the patient was 17 to 83years comprising of 59 females and 41 males.

The sensitivities and specificities of DNRA compared to those of conventional PM were observed to be 100%, 97% and100%, 99% for RIF and INH respectively. Positive predictive values were 100 and 93% for RIF and INH respectively. Negative values were 97% and 100% for RIF and INH respectively

4. Discussion

The contribution of TB laboratories to the management of TB in the world is important for the rapid determination of drug susceptibility, especially in low-resource countries where most cases of MDR TB occur [10]. Numerous alternative methods have been proposed for the detection of TB drug resistance. Most have shown high sensitivity and specificity values for INH and RIF, the most important drugs in TB treatment [5] [11] [12].

Current methods for DST of MTB are either expensive or have a long a turnaround time (TAT); therefore, a cost-effective and rapid drug susceptibility method is required to guide tuberculosis treatment. This study focuses on RIF and INH being two of the four first-line anti-TB drugs, *i.e*. RIF (Rifampicin), INH (Isoniacid), STR (Streptomycin) and EMB (Ethambutol).

In this study, direct DST was performed for 100 AFB positive clinical sputum specimens with a positivity score of 1+, 2+ and 3+. In addition, five scanty positive specimens were also used to check the sensitivity of the test and good results was achieved. All of five scanty positive specimens were available for the tests (culture positive). An interesting finding about these five specimens was that they had scanty positivity with 7, 5, 4, 3, and 3 bacilli load. So it could be assume that if the specimens were carefully processed, then it is capable of achieving good results even with a lesser number of bacterial loads.

DNRA and Conventional PM were performed for the same specimens and high level of agreement was achieved. Of 100 AFB positive samples, 98 specimens could be used for the comparison between DNRA and PM, while one was identified as non-tubercle mycobacteria (NTM), and one contaminated. NTM were identified initially when readings of DNRA (absence of color) and DPM (growth on PNB containing LJ-slopes) were taken and further confirmed by growth characteristics, pigmentations, and biochemical tests. The results for the two used drugs were similar to those obtained previously from other authors [8] [14] [15] [18]. It is also in agreement with indirect NRA evaluation of Cobal *et al* [13] and comparable with direct NRA of Shikama *et al* [14], as well as the internationally accepted gold standard PM.

The NRA method utilized nitrite as the indicator of growth and results were observed much earlier than using visible growth as an indicator. Since the assay used clinical specimens, TAT can be decrease to 10days from the six to eight weeks normally required for the isolation of the bacilli. Affolabi *et al* [15] found that a liquid medium-based NRA further reduced the TAT with clinical sputum specimens as

56% of their results were obtained in 10 days, the TAT agrees with this study.

In this study, the sensitivities and specificities of DNRA compared to those of conventional PM were observed to be 100%, 97% and100%, 99% for RIF and INH respectively. Positive predictive values were 100 and 93% for RIF and INH respectively. Negative values were 97% and 100% for RIF and INH respectively (Table 1). Therefore, making the results to be in agreement with previous authors.

In a meta-analysis of direct susceptibility testing for MDR-TB by Brosch *et al* [16], the sensitivity, specificity, and time to results of four direct DST tests were compared with the conventional indirect testing for detection of resistance to RIF and INH in MTB. NRA was one of the four direct tests used and showed sensitivity and specificity to RIF of 99% and 100% and to INH of 94% and 100% respectively.

Furthermore, Martin *et al* [17] reported the evaluation of NRA for ofloxacin, a second-line drug, and found complete agreement with the agar PM. Therefore, NRA also has the capability to be used for the evaluation of second-line drugs.

Visalakshi *et al* [18] observed sensitivity and specificity of the DNRA and IPM to be 94% and 98%, and 100% and 98% for RIF and INH respectively. Additionally, Shikama *et al* [14] stated 100% sensitivity and specificity of NRA for RIF with a TAT of 15 days. In another study, Shikama *et al* [14] found the reproducibility of NRA was 100% for INH and 97% for RIF. The meta-analysis by Martin *et al* of NRA suggests that the NRA is highly sensitive and specific for determining RIF- and INH-resistant TB in both culture isolates and directly on clinical sputum specimens. Most of the studies had a sensitivity and specificity of 95% or greater with high degree of accuracy. Therefore, the findings of this study agree with the previous researches from the above authors. The average TAT was between 5 and12 days with indirect NRA, and 14 and 21 days with direct NRA.

One of the biggest asset of NRA is cost effectiveness as there is no need to change laboratory infrastructure as it is performed in classical LJ or Middlesbrook medium, routinely used in TB laboratories, with the addition of KNO_3. There is no need for any sophisticated equipment or expensive reagents, making it widely accessible method for resource poor countries.
The NRA Results are easy to observe by a colour change of the medium. The basis of the NRA test is the reduction of measurable nitrate by metabolic activity of MTB cells. The isolates, resistant to certain anti-TB drugs, may have lower metabolic activity [5] (for example, *rpo*B and *kat*G mutations), leading to RIF and INH resistance, which might affect the expression levels of nitrate reductase enzymes. These strains could have a low level of resistance that could not be detected by the NRA. By keeping in mind the results of various studies, it is concluded that the direct NRA has the potential to be an inexpensive alternative method for DST of MTB. As this assay is applied directly, it may also be useful to reduce the burden of the laboratories.

Being advantageous, NRA has some limitations, such as some strains (<1%) of MTB lack nitrate reductase [7]. In addition, nitrate might be reduced to nitric oxide beyond nitrite which cannot be detected by Griess reagent. Hence, zinc-dust was added to all negative tubes in accordance to Centro Panamericano protocol [19]. Zinc reduces nitrate rapidly, and a true-negative test will directly turn red while there will be no change in colour in a tube where reduction has passed beyond nitrite. Another possible limitation of NRA is that it can give positive results with atypical mycobacteria such as *M. kansasii*, *M. szulgai*, *M. flavescens*, *M. terrae* complex, and some rapid growers [7] while *M. bovis* is nitrate-negative.

5. Conclusion

The results of this study indicate that NRA is suitable for relative early determination of isoniacid and rifampicin susceptibility pattern. On the basis of the findings, it is concluded that NRA has the potential to be a useful tool for rapid drug susceptibility testing of *Mycobacterium tuberculosis* in resource-poor countries with limited laboratory facilities because of its low-cost, relative rapidness, reproducibility of results, simplicity and lack of requirement of expensive reagents and sophisticated equipment.

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