ISSN 2449-8955 European Journal of Biological Research *Research Article*

DOI: http://dx.doi.org/10.5281/zenodo.4295881

Direct detection of *Mycobacterium tuberculosis* with nitrate reductase assay and microscopic observation drug susceptibility

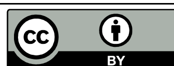
Olutayo Israel Falodun[1]*, Idowu Simeon Cadmus[2], Obasola Ezekiel Fagade[1]

[1] Department of Microbiology, University of Ibadan, Nigeria

[2] Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Nigeria

* Corresponding author: E-mail: falod2013@gmail.com; oi.falodun@ui.edu.ng

Received: 23 August 2020; **Revised submission:** 09 November 2020; **Accepted:** 28 November 2020



http://www.journals.tmkarpinski.com/index.php/ejbr

Copyright: © The Author(s) 2021. Licensee Joanna Bródka, Poland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/)

ABSTRACT: The global increase in tuberculosis drug resistant which is a threat to its control, require low cost method of diagnosis and detection. Available conventional and molecular methods consume time, and are expensive for countries with high disease burden. Nitrate Reductase Assay (NRA) and Microscopic Observation Drug Susceptibility (MODS) performance to directly detect tuberculosis resistance to four drugs was evaluated. The NRA (liquid and solid) and MODS performance of smear-positive sputum samples were evaluated; Sensitivities and specificities were compared with Proportion Method (PM). Sensitivity and specificity of liquid NRA (LNRA) were 90% and 98% (rifampicin), 81.8% and 100% (isoniazid), 88.9% and 98.1% (streptomycin), and 57.1% and 94.4% (ethambuthol). Also, the sensitivity and specificity for solid NRA (SNRA) were 69.2% and 98.3% (rifampicin); 100% and 100% (isoniazid); 88.9% and 95.2% (streptomycin); 70% and 80.6% (ethambuthol). Moreover, For MODS, rifampicin and isoniazid sensitivity and specificity was 100%, it was 100% and 98.1% for streptomycin, and 71.4% and 98.2% for ethambuthol. At day 14, the results available for LNRA, SNRA and MODS were 93%, 68.5% and 100% respectively. The agreement between LNRA and PM was 97% (RIF, INH and SM) and 90% (EMB). For SNRA, it was 93% (RIF), 100% (INH), 94% (SM) and 89% (EMB). While for MODS, it was 100% (RIF and INH), 98% (SM) and 95% (EMB). Direct NRA and MODS are sensitive, reliable and fast for antituberculosis drug susceptibility; they have potential to effectively and reliably detect drug resistant tuberculosis in the low resource countries.

Keywords: Tuberculosis drug resistance; *Mycobacterium* detection NRA; MODS; Diagnosis.

1. INTRODUCTION

Tuberculosis (TB) is an ancient infectious disease, a public health concern and a typical infection of the lungs [1]. The increase in TB and resistance to rifampicin and isoniazid, which are vital anti-tuberculosis drug, is a global challenge to TB infections control efforts [2]. This is because TB treatment regimens remain ineffective, second line therapies which remain limited by economic challenges are required for treatment while, the resistant strains are transmissible [3]. In 2017, the WHO estimated that incident cases was 10

million while, death cases was 1.3 million. This was a 1.8% decline from 2016. Moreover, in 2018, the notified new cases was 7.0 million, an increase from the 2017 which was reported to be 6.4 million and a wide increase from the annual notified cases of 5.7-5.8 million in the period 2009-2012. Also, a detection of 186772 Multidrug Resistant/Rifampicin Resistant-TB (MDR/RR-TB) cases was notified in 2018, an increase from the 160684 notified in 2017 [4-6].

In Nigeria, while the W.H.O. bacteriologically confirmed estimated cases of TB that were tested for rifampicin resistance was 65% (new cases) and 88% (retreatment cases); the MDR/RR-TB cases was 4.3% (new) and 15% (previous) in 2018 [6]. The treatment of MDR-TB cases could take as long as 24 months using expensive second line anti-tuberculosis drugs, some of which are administer by injection. More so, the cure rate is much lower (about 60%) compared to the susceptible strains of TB [7]. However, in most low income Sub-Saharan African countries, it is only the first line drugs that are available for the treatment of TB infections. Thus, MDR-TB prevalence is a concern in the region as its magnitude is largely unknown but the W.H.O. estimated cases in the region increased from 2.4% (new cases) and 13% (retreatment cases) in 2013 to 2.7% (new cases) and 14% (retreatment cases) in 2017 [6, 8]. The cases of MDR-TB that is reported to be on the increase necessitate a timely TB diagnosis to effectively manage patients, as well as putting measures for effective control and further spread of the infection in place.

Detection of drug resistant TB using conventional methods on Lowenstein-Jensen (LJ) medium is cheap but, it is cumbersome and takes a long time [7]. Commercial liquid automated systems like the BACTEC MGIT 960 and line probe assays are fast; nevertheless, the equipment required are expensive, the running costs are high and are technically complex. All these may make them difficult for implementation in low resource countries. In addition, the low speed of liquid-based indirect susceptibility prolongs taking decisions to manage MDR-TB patients [7, 9]. The fast molecular methods [10-12]; are expensive and require manpower that are well-trained [13, 14]. They may therefore be unaffordable for the developing countries and may not be practicable for routine use. This therefore necessitates a need for a fast and affordable method that can easy detect drug resistant TB especially in low resource nations.

First description of Nitrate Reductase Assay (NRA) was in 2002 [15]. It was performed on solid medium as indirect assay just like the proportion method on L-J media; the liquid based assay has also been studied [16, 17]. The principle on which this technique is based is nitrate being utilized and converted by *Mycobacterium tuberculosis* to nitrite that can be detected by adding Griess reagent leading to pink-purple colour production [15]. While in some studies, the method has been evaluated [18, 19], the only study in Nigeria was by Ani et al. [20] in Jos a city in northern part of the country.

Microscopic observation drug susceptibility is a low-cost technology based on liquid culture method that detects TB resistance [21, 22]. This technique relies on the observation of the characteristic cord-like structure of a tissue culture plates with the use of an inverted microscope. The principle upon which the method relies include: faster growth of *M. tuberculosis* in broth culture than on solid media; characteristics growth of tubercle bacilli that makes it detected visually using and inverted microscope much earlier than when naked eye could view mycobacterial growth on solid media and with incorporation of drugs in the medium enable direct susceptibility testing [23]. Elsewhere, this technique was evaluated [9, 24] but no record of its evaluation in Nigeria. In this study, the sensitivity of NRA (solid and liquid media) and MODS with PM as ‘gold standard’ on Lowenstein Jensen medium for DST of MTB using four first-line anti-tuberculosis drugs was evaluated.

2. MATERIALS AND METHODS

2.1. Study area and sample processing

The study was a cross-sectional, laboratory-based comparative study carried out in Ibadan, Nigeria. Processing of the samples (sputum) was done using the N-acetyl-L-cysteine–NAOH–sodium citrate (NALC-NAOH) decontamination technique. Briefly, in a 15 mL centrifuge tube, equal volume (2 mL) of the sample and NALC-NAOH (Mycopep) solution were added. It was tightly capped, voretxed for about 20 seconds and left to stand for betewwen 15 and 20 minutes. Phosphate buffer (pH 6.8) was added to 14 mL mark and centrifuged for 15 minutes at 3000 x g. The pellet was retained after the supernatant has been carefully decanted; and was reconsistituted by mixing with phosphate buffer and was used as the inoculum.

2.2. Ethical approval

Ethical approval for this study was obtained from the University of Ibadan/University College Hospital Ethical committee with approval number NHREC/05/01/2008a.

2.3. Nitrate Reductase Assay in liquid media

Nitrate reducatse assay also called Griess method is based on the principle that *Mycobacterium tuberculosis* are capable of reducing nitrate to nitrite and this is used for biochemical identification of mycobacterial species. Nitrite presence can be detected by addition of Griess reagent. The technique was done as previouly described [25]. Briefly, in 4.6 mL of 7H9-N medium of which RIF, INH, SM and EMB at concentration of 40 µg/mL, 0.2 µg/mL, 8.0 µg/mL and 2.0 µg/mL respectively was incoporated, undiluted sample (0.5 mL) was added. Also, 0.5 mL of diluted (1:10 dilution) sample was used to inoculate 4.6 mL of 7H9-N medium without drug. The inoculated media were incubated at 37°C for 5 days after which an aliquote of 1mL of the media without antimicrobial was withdrawn and developed with 0.2 mL fresh griess reagent. The mixture was observed for a colour cahnge, and if there was a colour change (strong or weak pink), the process was repeated for the culture that contain antibiotics. If colour change was not observed in the tubes without antimicrobial, the incubation was continued and process repetaed for 7, 10, 14 and 18 days.

2.4. Interpretation of LNRA

If colour change (strong or weak pink) was observed, it was classified as positive and the tubes with antibiotics were tested with the griess reagent. If there is no colour cahnge, the tubes were re-incubated and the procedure repeated at day 7, 10, 14 and 18 if the need be (Fig. 1). An isolate was considered resistant with a colour change in the antibiotic tube greater than 1:10-diluted growth control on the same day [25].

2.5. Nitrate Reductase Assay on solid media and microscopic observation drug susceptibility

The NRA method on solid media was carried out as previouly described [18] with some modifications regarding critical concentration of rifampicin antibiotics; while the MODS assay was done as described previously [18, 25].

2.6. Interpretation of SNRA

After seven days of incubation, 0.5 mL of Griess reagents was added to one drug-free control tube. If any colour change (strong or weak pink) was noticed, the corresponding antibiotic-containing tubes were also tested and the susceptibility results read. If no colour change was seen in the control tube, the remaining

control tubes and the antibiotics tubes were re-incubated. The procedure was then repeated at day 10 and, if needed, at day 14 and day 18, using the last growth control tube (Fig. 2).

2.7. Proportion method (PM) and quality control

This was the reference method and was done using Lowestein-Jensen (L-J) medium as previously described [27, 28]; while strains of H37Rv (ATCC 27294) and MDR (ATCC 35838) were used as control reference strain. Before use, they were freshly subcultured on LJ medium.

3. RESULTS

3.1. Performance of liquid NRA

Among the samples processed, LNRA detected growth in 61 and was compared with PM. An excellent agreement (96.7%) for rifampicin, isoniazid and streptomycin was obtained, while the agreement observed for ethambuthol was 90.2% (Fig. 1). The sensitivity and specificity of growth detection by LNRA and PM of rifampicin resistance was 90% and 98% respectively, whereas, for isoniazid, it was 81.8% and 100% while, it was 88.9% and 98.1% for streptomycin, and 57.1% and 89.2% for ethambuthol, respectively (Table 1).

Table 1. Comparison of PM and LNRA susceptibility (%).

Drug NRA result	Isolates with the proportion results		Sensitivity	Specificity	Predictive values	
	Resistant	Susceptible			Positive	Negative
RIF resistant	9	1				
RIF susceptible	1	50	90.0	98.0	90.0	98.0
INH resistant	9	0				
INH susceptible	2	50	81.8	100	100	96.1
SM resistant	8	1				
SM susceptible	1	51	88.9	98.1	88.9	98.1
EMB resistant	4	3				
EMB susceptible	3	51	57.1	94.4	57.1	94.4

RIF - Rifampicin, INH - Isoniazid, SM - Streptomycin, EMB - Ethambuthol, PM - Proportion method, LNRA - Liquid nitrate reductase assay.

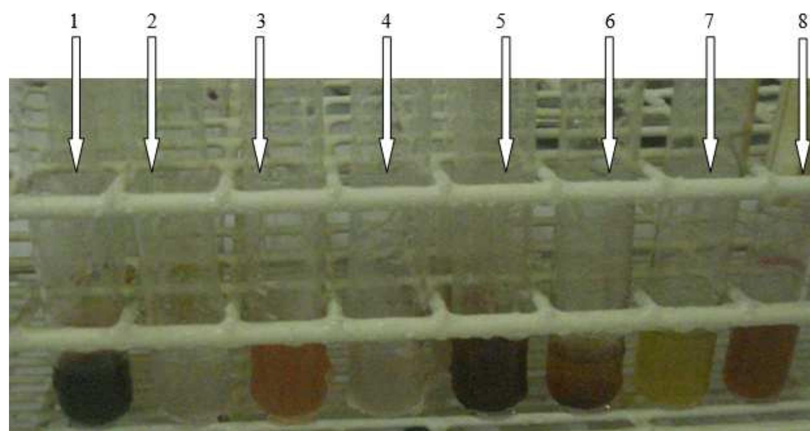


Figure 1. Nitrate reductase assay in liquid medium showing positive (growth) and negative (no growth) samples (1 and 5 positive; 2, 4, 7 negative; 3, 6 and 8 intermediate).

3.2. Solid NRA performance

For SNRA, growth detection was in 72 samples and was compared with the PM. The comparison showed that there was an excellent agreement between SRNA and PM for rifampicin (93.1%), isoniazid (100%) and streptomycin (94.4%); while a very good agreement (84.7%) was observed for ethambuthol (Fig. 2). Sensitivity and specificity of growth detection for rifampicin resistance was 69.2% and 98.3% respectively, but was 100% and 100% for isoniazid. Moreover, that of streptomycin was 88.9% and 95.2%, but was 70% and 98.1% respectively for ethambuthol (Table 2).

Table 2. Comparison of PM and SNRA susceptibility (%).

Drug NRA result	Isolates with the proportion results		Sensitivity	Specificity	Predictive values	
	Resistant	Susceptible			Positive	Negative
RIF resistant	9	1	69.2	98.3	90.0	93.5
RIF susceptible	4	58				
INH resistant	16	0				
INH susceptible	0	56	100	100	100	100
SM resistant	8	3				
SM susceptible	1	60	88.9	95.2	72.7	98.4
EMB resistant	7	4				
EMB susceptible	3	58	70	80.6	63.6	95.1

RIF - Rifampicin, INH - Isoniazid, SM - Streptomycin, EMB - Ethambuthol, PM - Proportion method, SNRA - Solid nitrate reductase assay.

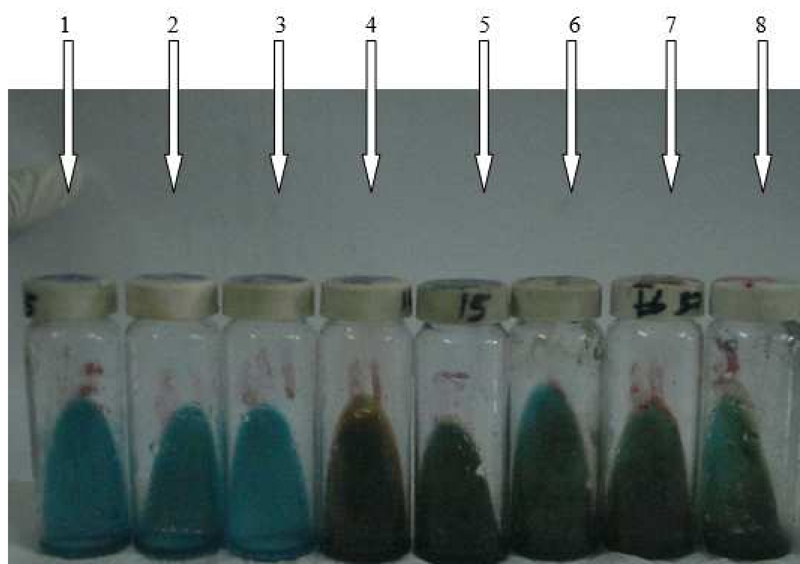


Figure 2. Nitrate reductase assay tubes showing positive (growth) and negative (no growth) samples (1-3 = no growth; 4-8 = growth). The bluish color indicates that there was no mycobaterial growth while the pinkish color indicates presence of nitrite from nitrate due to presence of mycobacterial growth.

3.3. Performance of MODS

For MODS, detection of growth was in 62 samples and was compared with PM. The comparison showed excellent agreement for rifampicin (100%), isoniazid (100%), and ethambuthol (93.5%), while a very

good agreement (88.4%) was also obtained for streptomycin (Fig. 1). The sensitivity and specificity of growth detection for both rifampicin and isoniazid resistance was 100% and 100% respectively, while it was 100% and 98.1% for streptomycin, it was 71.4% and 98.2% for ethambuthol (Table 3).

Table 3. Comparison of PM and MODS susceptibility (%).

Drug NRA result	Isolates with the proportion results		Sensitivity	Specificity	Predictive values	
	Resistant	Susceptible			Positive	Negative
RIF resistant	12	0	100	100	100	100
RIF susceptible	0	50				
INH resistant	11	0				
INH susceptible	0	51	100	100	100	100
STR resistant	10	1				
STR susceptible	0	51	100	98.1	90.9	100
EMB resistant	5	1				
EMB susceptible	2	54	71.4	98.2	83.3	96.4

RIF - Rifampicin, INH - Isoniazid, SM - Streptomycin, EMB - Ethambuthol, PM - Proportion method, MODS - Microscopic drug susceptibility.

3.4. Total performance of the three diagnostic methods

The total performance of LNRA, SNRA and MODS showed that for LNRA, it was 81.1% (sensitivity), 97.6% (specificity), 85.7% (positive predictive value - PPV) and 96.7% (negative predictive value - NPV) while, for SNRA, sensitivity was 83.3%, and specificity was 96.6%, while it was 83.3% (PPV) and 96.6% (NPV). Also for MODS, it was 95.0% (sensitivity), 99.0% (specificity), 95.0% (PPV) and 99.0% (NPV) (Table 4 and Fig. 3).

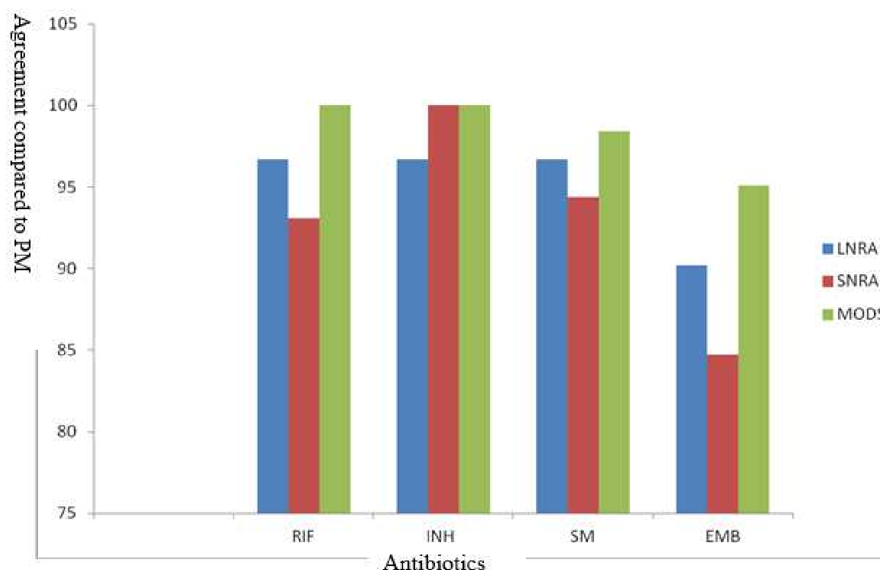


Figure 3. Agreement of the three methods compared to PM.

PM - Proportion method, LNRA - Liquid nitrate reductase assay, SNRA - Solid nitrate reductase assay, MODS - Microscopic observation drug susceptibility.

Table 4. Total performance of the techniques (%).

Drug NRA result	Isolates with the proportion results		Sensitivity	Specificity	Predictive values	
	Resistant	Susceptible			Positive	Negative
NRA (Broth)						
resistant	30	5	81.1	97.6	85.7	96.7
susceptible	7	302				
NRA (Solid)						
resistant	40	0				
susceptible	8	228	83.3	97.6	85.7	96.7
MODS						
resistant	38	2				
susceptible	2	205	95.0	99.0	95.0	99.0

NRA - Nitrate reductase assay, MODS - Microscopic observation drug susceptibility.

3.5. Turnaround time (TAT) of the methods

The time between the date of the sample processing (sample inoculation) and when the positive result for both mycobacteria detection and susceptibility result was obtained for the three methods are shown in Table 5. For LNRA, the TAT was from 5-18 days (mean of 8.7 ± 3.9 days); for SNRA, it was 7-18 days (mean of 11.7 ± 4.4 days) while, it ranged from 5-14 days (mean of 7.3 ± 3 days) for MODS. The available result at day 14 was 93.0% (LNRA), 68.5% (SNRA) and 100.0% (MODS). However, the TAT of the methods was not statistically significant ($p = 0.176$).

Table 5. The TAT for culture positive samples for the three methods.

TAT (no of days)	LNRA			SNRA			MODS		
	Frequency	%	Cumulative %	Frequency	%	Cumulative %	Frequency	%	Cumulative %
5	14	24.6	24.6	-	0	0	20	32.8	32.8
7	22	38.6	63.2	12	21.1	21.1	22	36.1	68.9
10	9	15.8	79	20	35.1	56.2	18	29.5	98.4
14	8	14	93	7	12.3	68.5	1	1.6	100
18	4	7	100	18	31.6	100	-	100	100
Total	57	100		57	100		61		

TAT - Turnaround time, LNRA - Liquid nitrate reductase assay, SNRA - Solid nitrate reductase assay, MODS - Microscopic observation drug susceptibility.

4. DISCUSSION

In other to initiate effective anti-TB treatment, rapid drug susceptibility result is pivotal. Such rapid methods which are also low cost are required in Nigeria and other low resource countries where the disease is endemic. Two diagnostic methods RNA (LRNA and SRNA) and MODS compared to PM (gold standard) were evaluated. An excellent agreement (97.7%) obtained in the comparison of LNRA with PM for RIF, INH and SM as well as the 90.2% agreement for EMB agrees with the report of another study in India [16]. In addition, while excellent agreement (96.2%) was observed between LNRA and PM in this study, a good

agreement (86.0%) was observed in another study carried out in Sri Lanka, a low TB prevalent country [29]. The sensitivity and specificity of RIF and INH obtained in this study were comparably similar to the report of some previous studies [16, 30]. In addition, the sensitivity and specificity obtained for RIF in this study is also similar compared to the report from another study in Sri Lanka [29].

The TAT for LNRA (5-18 days) with 93.0% of the results that were obtained at day 14 did not agree with the 3-9 days previously reported, and the 93.0% results obtained at day 7 from a similar study [30]. However, the mean TAT of 8.7 days in this study is shorter compared to the 10 days previously reported [29]. Also, the full agreement of SNRA and PM obtained for INH and excellent agreement for RIF is important, because the combination of both drugs is the most valuable drug against TB infection. This is also in agreement with the report of a recent study in Nepal [31].

Except for RIF, the sensitivity obtained for INH, SM and EMB were better compared to the report from other studies in Sweden [18] and Nepal [32]. However, the specificity obtained from the present study is similar to the latter studies. Also, while total sensitivity and specificity of SNRA obtained in this study agrees with the report of Musa et al. [18], there were little discrepancies in the percentage agreement obtained in this study for all the antibiotics except for INH that was similar as previously reported by Sethi et al. [32]. Furthermore, a lower sensitivity for RIF was obtained in this study compared to the sensitivities reported from similar studies in Benin Republic, India and Nepal [19, 25, 31]. However, the percentage agreement obtained in this study is similar to the latter studies.

Moreover, the sensitivities of SNRA for all the antibiotics in this study is similar compared to the reported sensitivities from another study in Jos, Nigeria [20]. Apart from the similar sensitivity, specificity, PPV and NPV compared to the report of Martin et al. [33], the value for RIF in this study was lower. Also, in another study carried out in Tunisia [34], a similar specificity was observed for all the drugs. Furthermore, the obtained SNRA results of samples in 10 days for 56.2%, 14 days for 68.5% and 18 days for 100% is similar to the 16% samples obtained in 10 days, 64% (14 days) and 100% (18 days) reported by Musa et al. [18], 96% in 18 days by Affolabi et al. [25] and 93% in 18 days by Boum et al. [17]. However, this observation differs from those reported by Bwanga et al. [9], Kammou et al. and recently by Halwai et al. [31].

The agreement, sensitivities and specificities obtained for all the antibiotics in this present study is a good pointer for MODS as a tool for diagnosis of TB and drug resistant detection. The observed agreement in this study is comparably similar to the reported agreement for RIF and INH in a related study from Peru and Ethiopia [22, 24]. While the sensitivity, specificity, PPV and NPV obtained in this study is similar to that of a recent study in India [35], a lower value of the respective parameters was reported for both rifampicin and isoniazid in Uganda [9]. Similarly, the total performance of MODS in this study in terms of sensitivity and NPV are better compared to the report of Kirwan et al. [36]. The reason for the disparity might be due to the studied samples. While the present study was on pulmonary tuberculosis, the latter study was on lymph node tuberculosis. The MODS TAT was the shortest compared to LNRA and SNRA and was also better than the MODS evaluation in Uganda [9] but similar to the TAT previously reported in Peru [22]. Moreover, the median TAT (7 days) observed in the present study for MODS was the same with that of Bwanga et al. [9] but lower than the 9 days by Shiferaw et al. [24].

In line with the challenges that are common to local laboratories especially developing countries, about 40 minutes is required to process one sample using LNRA, about 75 minutes for SNRA and 60 minutes for MODS. Using the methods to detect *Mycobacterium* resistant strains, is fast and easy. For both LNRA and SNRA, special equipment is not required, however, MODS requires the use of inverted microscope. Although,

training of personnel to use the methods is easy; in order to avoid aerosol generation, sample processing should be with care in a biosafety cabinet. Preparation of culture media requires about 40, 75 and 50 minutes for LNRA, SNRA and MODS respectively. For LNRA and MODS, cross contamination is possible and to some extent with SNRA. The MODS technique has added advantage of good biosafety because once MODS plate is sealed it is never opened. The methods are suitable for local laboratories.

In conclusion, the observation from this study showed that direct NRA (liquid and solid) and MODS on sputum smear positive samples are highly sensitive, accurate, reliable, easy and fast methods for tuberculosis and drug resistant tuberculosis detection and can be implemented in low resource countries.
Limitation of the study: The limitation of the study is the small sample size.

Authors' Contributions: The study was designed by OIF and SIC. OIF managed literature search and data acquisition/analysis and wrote the first draft. OEF and SIBC supervised the work. All authors read and approved the final manuscript.

Conflict of Interest: The author has no conflict of interest to declare.

Acknowledgement: The study was supported by the [APHRC/IDRC] under grant [No. ADDRF Award - 2010 ADF 005] to OIF; [Fogarty International/NIH] under grant [No. D43TW007995) to ISC.

REFERENCES

1. Barberis I, Bragazzi N, Galluzzo I, Martini M. The history of tuberculosis: from the first historical records to the isolation of Koch's Bacillus. J Prev Med Hyg. 2017; 58: 9-12.
2. Prasad R, Gupta N, Banka A. Multidrug-resistant tuberculosis/rifampicin-resistant tuberculosis: Pricinple of management. Lung India. 2018; 35: 78-81.
3. Kendall EA, Cohen T, Mitnick CD, Dowdy DW. Second line drug susceptibility testing to inform the treatment of rifampin-resistant tuberculosis: a quantitative perspective. Int J Infect Dis. 2017; 56: 185-189.
4. WHO. Global Tuberculosis Report - Multidrug-/rifampicin-resistant TB (MDR/RR-TB) Update, 2018.
5. MacNeil A, Glaziou P, Sismanidis C, Maloney S, Floyd K. Global epidemiology of tuberculosis and progress toward achieving global targets. Morbid Mortal Week Rep. 2019; 68: 11-263.
6. WHO. Global tuberculosis report. Geneva: World Health Organization, 2019.
7. Bwanga F, Hoffner S, Haile M, Joloba ML. Direct susceptibility testing for multi drug resistant tuberculosis: A meta-analysis. BMC Infect Dis. 2009; 9: 67.
8. WHO. Global tuberculosis report. World Health Organisation, 2014.
9. Bwanga F, Haile M, Joloba ML, Ochom E, Hoffner S. Direct Nitrate Reductase Assay versus Microscopic Observation Drug Susceptibility test for rapid detection of MDR-TB in Uganda. PLoS ONE. 2011; 6(5): e19565.
10. Goloubeva VM, Lecocq P, Lassowsky F, Matthys F, Bastian I. Evaluation of Mycobacteria Growth Indicator Tube for direct and indirect susceptibility testing of *Mycobacterium tuberculosis* from respiratory specimens in a Siberian prison hospital. J Clin Microbiol. 2001; 39: 1501-1505.
11. Johansen IS, Lundgren BA, Thomsen VØ. Direct detection of multidrug-resistant *Mycobacterium tuberculosis* in clinical specimens in low- and high-incidence countries by line probe assay. J Clin Microbiol. 2003; 41: 4454-4456.
12. Mayta HR, Gilman HF, Arenas T, Valencia L, Caviedes SH, Montenegro E, et al. Evaluation of PCR based universal heteroduplex generator assay as a tool for rapid detection of multidrug-resistant *Mycobacterium tuberculosis* in Peru. J Clin Microbiol. 2003; 41: 5774-5777.

13. Jureen PJ, Werngren D, Hoffner, SE. Evaluation of the line probe assay (LiPA) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*. Tubercul. 2004; 84: 311-316.
14. Skendersn G, Fry M, Prokopovica I, Greckoseja S, Broka L, Metchock B, et al. Multidrug-resistant tuberculosis detection, Latvia. Emerg Infect Dis. 2005; 11: 1461-1463.
15. Angeby KK, Lisbeth K, Sven EH.. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with nitrate reductase assay. J Clin Microbiol. 2002; 40: 553-555.
16. Poojary A, Nataraj G, Kanade S, et al. Rapid antibiotic susceptibility testing of *Mycobacterium tuberculosis*: it's utility in resource poor settings. Ind J Med Microbiol. 2006; 24: 268-272.
17. Boum Y, Orikiriza P, Rojas-Ponce G, et al. Use of colorimetric culture methods for detection of *Mycobacterium tuberculosis* complex isolates from sputum samples in resource-limited settings. J Clin Microbiol. 2013; 51: 2273-2279.
18. Musa HR, Ambroggi M, Sonto A, Angeby KAK. Drug susceptibility testing of *Mycobacterium tuberculosis* by a nitrate reductase assay applied directly on microscopy positive sputum samples. J Clin Microbiol. 2005; 43: 3159-3161.
19. Gupta A, Sen MR, Mohapatra TM, et al. 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: 20-25.
20. Ani AE, Dalyop YB, Agbaji, O, et al. Drug susceptibility test of *Mycobacterium tuberculosis* by nitrate reductase assay. J Infect Dev Count. 2009; 3: 16-19.
21. Moore DA, Mendoza JD, Gilman RH, et al. Microscopic observation drug susceptibility assay, a rapid, reliable diagnostic test for multidrug-resistant tuberculosis suitable for use in resource-poor settings. J Clin Microbiol. 2004; 42: 4432-4437.
22. Moore DA, Evans CA, Gilman W, et al. Microscopic-observation drug-susceptibility assay for the diagnosis of TB. N Engl J Med. 2006; 355: 1539-1550.
23. Caviedes L, Moore DA. Introducing MODS: a low-cost, low-tech tool for high-performance detection of tuberculosis and multidrug resistant tuberculosis. Ind J Med Microbiol. 2007; 25: 87-88.
24. Shiferaw G, Woldeamanuel Y, Gebeyhu M, Evaluation of microscopic observation drug susceptibility assay for detection of multidrug resistant *Mycobacterium tuberculosis*. J Clin Microbiol. 2007; 45: 1093-1097.
25. Affolabi D, Odoun M, Sanoussi N, 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: 3243-3245.
26. Universidad Peruana Cayetano Heredia. MODS. A user guide. Microscopic observation drug susceptibility assay. Laboratory de investigacion y Desarrollo (LID) Laboratorio de investgacion en Enfermedadaes infecciosas Area de Mycobacterium. 2008.
27. Canetti G, Froman S, Grosset J, et al. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull World Health Org. 1963; 29: 565-578.
28. Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. Centers for Disease Control, Atlanta, GA. 1985.
29. Adikaram CP, Perera J, Wijesundera SS. The manual mycobacteria growth indicator tube and the nitrate reductase assay for the rapid detection of rifampicin resistance of *M. tuberculosis* in low resource settings. BMC Infect Dis. 2012; 12: 326.
30. Syre H, Phyu S, Sandven P, et al. Rapid colorimetric method for testing susceptibility of *Mycobacterium tuberculosis* to isoniazid and rifampicin in liquid cultures. J Clin Microbiol. 2003; 41: 5173-5177.

31. Halwai D, Gurung R, Poudyal N, et al. Evaluation of nitrate reductase assay in 7H11 agar for diagnosis of multi-drug resistant tuberculosis in eastern Nepal. Trop Med Health. 2018; 46: 26.
32. Sethi S, Sharma S, Sharma SK, et al. Drug susceptibility of *Mycobacterium tuberculosis* to primary antitubercular drugs by nitrate reductase assay. Indian J Med Res. 2004; 120: 468-471.
33. Martin A, Imperiale B, Ravolonandriana P, et al. Prospective multicentre evaluation of the direct nitrate reductase assay for the rapid detection of extensively drug-resistant tuberculosis. J Antib Chemoter. 2013; 353: 1-4.
34. Kammoun S, Smaoui S, Marouane C, et al. Drug susceptibility testing of *Mycobacterium tuberculosis* by a nitrate reductase assay applied directly on microscopy-positive sputum samples. Int J Mycobacteriol. 2015; 4: 2002-2006.
35. Agarwal A, Katoch C, Dhole TN, et al. Evaluation of Microscopic observation drug susceptibility (MODS) assay as a rapid, sensitive and inexpensive test for detection of tuberculosis and multidrug resistant tuberculosis. Indian Med J. 2019; 75: 58-64.
36. Kirwan DE, Ugarte-Gil, C, Gilman RH. Microscopic observation drug susceptibility assay for rapid diagnosis of lymph node tuberculosis and detection of drug resistance. J Clin Microbiol. 2016; 54(1): 185-189.