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Timely Detection of Drug-resistant Tuberculosis Using Thin Layer Agar in Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author NOU designed the study, wrote the protocol, carried out the laboratory analyses and wrote the first draft of the manuscript. Author BDTP performed the statistical analysis. Author ANU contributed to the literature searches. Author AEA co-designed the study, managed the analyses and vetted the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: The global prevalence of drug-resistant tuberculosis poses serious public health challenges which have heightened the need for evaluation and utilization of new tools for the disease diagnosis, especially in developing countries. In order to evaluate the rapidity and accuracy of Thin Layer Agar (TLA) in the diagnosis of drug resistant TB; a comparative study was carried out in Nigeria.

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Methods: Concentrated sputum specimens were inoculated onto Lowenstein Jensen (LJ) slopes and quadrant TLA petri-plates containing 0.2 µg/ml of isoniazid, 0.5 µg/ml of rifampicin, 0.5 mg/ml of para-nitrobenzoic acid (PNB), and growth medium without drug served as control. Inoculated TLA plates were sealed, incubated, and examined microscopically at regular intervals for the appearance of microcolonies; and compared with the conventional LJ-proportion method.
Results: The mean time-to–detection of rifampicin-resistant strains of *Mycobacterium tuberculosis* (MTB) was 13 days and 56 days using TLA and LJ-proportion method respectively. The sensitivity, specificity, overall accuracy, positive and negative predictive values for detection of rifampicin resistance on TLA were 95.2%, 100%, 99.4%, 100% and 99.3% respectively. Using LJ-proportion method as the gold standard, 98.2% of rifampicin-resistant TB was detected on TLA.
Conclusion: TLA method may provide a viable alternative tool for early and accurate detection of TB drug-resistance, and consequent initiation of appropriate treatment for the disease control, particularly at the peripheral level of health services in limited-resource settings.

Keywords: Thin layer agar; detection; rapidity; rifampicin-resistant tuberculosis; resource-limited setting.

1. INTRODUCTION

Alarming increases in the prevalence of multidrug-resistant tuberculosis (MDR-TB) [1] coupled with the emergence of extensively drug-resistant tuberculosis (XDR-TB) [2] poses serious challenges to clinical management and control of the disease, especially in low-resource settings where conventional diagnostic approaches with major setbacks are still in use. Although multidrug-resistant tuberculosis (MDR-TB) constitutes only 5% of the global TB burden, less than 5% of the existing MDR-TB patients are currently being diagnosed due to severe laboratory constraints [3]. It is estimated that 90% of rifampicin-resistant strains of MTB are also resistant to isoniazid [4]. The WHO surveys on anti-TB drugs resistance have also shown that, globally, rifampicin mono-resistance occur at a very low prevalence [3]. In most settings, therefore, rifampicin resistance has been reliably used as a surrogate marker for MDR-TB. In Nigeria, despite a recent adoption of Xpert MTB/RIF test (Cepheid, Sunnyvale, CA, USA) into the Country's TB diagnostic algorithm, diagnosis of tuberculosis (TB) continues to rely heavily on sputum smear microscopy for acid-fast bacilli (AFB) - an insensitive method [5,6] that is particularly not suitable for detection of drug-resistance. Currently rated the fourth highest TB-burden country in the world and number one in Africa [7], Nigeria has an estimated MDR-TB burden of 2.9% for new TB cases and 14% for retreatment cases [7]. Yet, drug-resistant strains of *Mycobacterium tuberculosis* (MTB) in Nigeria are most frequently detected by treatment failures in patients receiving anti-tuberculosis therapy; laboratory detection of drug resistance is only attempted following treatment failures and clinical suspicion of drug resistance. Regrettably, the loss of several months of an inappropriate tuberculosis therapy may result in negative outcomes including escalation of the drug resistance, and complications that may lead to the patient's death while receiving the ineffective treatment, in addition to continued transmission of the disease with consequent setback on the disease control.

Efforts aimed at addressing the challenges of drug-resistant tuberculosis in resource-limited countries would certainly require enhancing the capacity of clinical laboratories for accurate disease diagnosis. In addition to AFB microscopy, there is need for introduction of simple culture tools that would facilitate accurate detection of the disease, especially smear-negative and drug-resistant TB. A novel micro-colony detection method, the Thin Layer Agar (TLA) culture, has been described with promising potentials of simplicity, relative speed and accuracy for diagnosis of all TB cases [8,9,10]. The TLA tool is a simple solid culture adaptation that utilizes ordinary light microscopy to detect microcolonies of *Mycobacterium tuberculosis* (MTB) complex long before macroscopic detection of the isolates could be achieved. In recent years, this technique has been scaled-up with remarkable success for simultaneous detection of TB and drug-resistance directly from primary cultures [11]. For implementation of this method, a simple laboratory with basic infrastructure for TB diagnostic testing may be adequate without need for expensive and sophisticated instrumentation. However the TLA method, like any new tool, may not be successfully implemented unless critical questions relating to its operation are answered from a number of studies in diverse geographical and epidemiological settings. This operational

study evaluates the accuracy of this method for detection of rifampicin-resistant TB in Nigeria.

2. MATERIALS AND METHODS

2.1 Collection and Processing of Samples

Subjects for this study were recruited from patients attending TB/HIV clinic in Nigeria Institute of Medical Research (NIMR), a national infectious disease reference centre located in Lagos, South-West of Nigeria. Specifically, 524 sputum specimens were collected from 181 subjects of all age groups, who attended the clinic between September, 2009 and August, 2010. Three sputum specimens were collected from each patient over two days (spot-morning-spot) and preserved by refrigeration at 4℃ prior to processing within 24 hours. However, 19 subjects failed to submit their third sputum specimens. All specimens were processed for AFB smear microscopy as well as culture. Ethical clearance for the study was duly obtained from the University of Calabar Teaching Hospital Ethical Committee, Calabar, and validated in NIMR, Lagos. Also, all the study participants received and signed a letter of informed consent.

Macroscopic examination of each sputum specimen was done to determine its consistency. All salivary specimens were excluded. Sputum specimens were processed using the N-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) method as described by Kent & Kubica [8] and the sputum concentrate resuspended in 2 ml Middlebrook 7H9 broth. Smears made from the concentrate were stained using Ziehl Neelsen (ZN) method and the slides examined using x 1000 magnification. A smear was considered positive if it contained at least 3 bacilli per 300 fields [12].

Lowenstein Jensen slopes were inoculated with approximately 0.1 ml of decontaminated specimens, incubated at 37℃ and examined for growth of mycobacteria after 2 weeks in the first instance and twice weekly for up to 8 weeks. Confirmed isolates of MTB were subjected to drug susceptibility testing using proportion method as described by National Committee for Clinical Laboratory Standards [13].

2.2 Direct DST on Thin Layer Agar

Quadrant TLA plates were prepared with 5 ml of 7H10 Middlebrook agar per compartment containing the following: one with no drug addition (growth control); one with 0.5 mg/ml of para-nitrobenzoic acid (PNB)*;* one with 0.2 µg/ml of isoniazid (INH); and one with 1 µg/ml of rifampicin (RMP). Dilutions of concentrated sputum specimens were made according to AFB load on the direct smear as described by Robledo et al. [11]: >250 AFB/field = 10^{-3} dilution; 25–250 AFB/field = 10^{-2} dilution; <25 AFB/field = no dilution, from which 0.1ml was inoculated onto each compartment. The plates were sealed in transparent polyethene bags and incubated for six weeks at 37℃ in an atmosphere of 5 - 10% CO_2. Plates were examined microscopically for growth, while still sealed using x40 objective of a standard light microscope, once during the first week; then 2 - 3 times weekly for six weeks. Each batch included a known drug-susceptible MTB strain as positive control*,* inoculated onto a separate TLA plate. All suspected MTB complex microcolonies were confirmed by AFB microscopy using ZN-technique. Drug resistance was identified by the detection of MTB complex microcolonies in a drug medium.

MTB complex microcolonies were identified by their cording morphology, speed of growth, AFB positivity, and absence of growth in *para*-nitrobenzoic acid medium. The isolates were further confirmed with conventional biochemical tests including, nitrate reduction test, niacin test, and catalase activity at neutral pH and 68℃.

Results were statistically analyzed with Wilcoxin Signed-rank test and Chi–square test at 95% confidence level using SPSS 15.0 statistical software. Sensitivity was calculated as the number of positive cultures on LJ or TLA divided by the total number of positive cultures on any culture medium. Similarly, the specificity, overall accuracy, positive predictive value (PPV), and negative predictive value (NPV) of TLA and LJ were calculated using standard procedures as described by Martin et al. [14] and Parikh et al. [15].

3. RESULTS

3.1 Detection of MDR-TB Using TLA and Proportion Method

Using the conventional LJ-Proportion method as the gold standard for detection of drug-resistant TB, only 21 of the total 164 MTB isolates tested were resistant to both rifampicin and isoniazid,

amounting to a 12.8% MDR-TB detection (Table 1). Whereas 4 strains resistant to INH were sensitive to RMP, all the rifampicin-resistant strains were also resistant to isoniazid. However, only 20 of the total 21 MDR-TB strains were detected on TLA; the sensitivity, specificity, overall accuracy and negative predictive value for detection of rifampicin-resistance on TLA were 95.2%, 100%, 99.4% and 99.3%, respectively. The corresponding values for detection of isoniazid-resistance were 100%, same as that of LJ proportion method (Table 2). The difference between the performance values of TLA and the standard method, for detection of drug resistant TB, was not statistically significant (P=0.132).

3.2 Time-to-detection of Drug-resistant MTB Strains Using TLA and LJ-Proportion methods

The mean time-to-detection of rifampicin-and isoniazid-resistance on TLA was 13 days, compared to 56 days using the standard LJ-proportion method. The detection time of drug-resistant TB strains on TLA varied in direct proportion to the bacillary load of sputum samples: ranging from an average of 10 days, with smear-positive specimens, to 15 days with smear-negative and paucibacillary specimens (Table 3). The difference in time-to-detection of drug-resistant MTB strains between TLA and the standard method was statistically significant (P = 0.005).

4. DISCUSSION

In spite of Nigeria's huge burden of TB and HIV co-infection, infrastructure for accurate detection of TB - particularly smear-negative and drug-resistant TB - is grossly inadequate. With the reported high rates (5.5%) of drug-resistant TB among HIV-infected patients in the country [16], accurate detection of the disease becomes more crucial for enhanced prognosis, increased survival of patients, prevention of acquisition of further drug resistance, and reduced spread of drug-resistant strains to vulnerable populations. In recent years, the WHO has endorsed the implementation of some newly developed

Table 1. Detection of MDR-TB using TLA and proportion methods

Method	No. (%) resistant to RMP	No. (%) resistant to INH	MDR % (n)
TLA	20 (12.2)	25 (15.2)	12.2 (20/164)
LJ	21 (12.8)	25 (15.2)	12.8 (21/164)
Total	21 (12.8)	25 (15.2)	12.8 (21/164)
		P = 0.030	

n= number of MDR strains detected per total number of MTB isolates tested

Table 2. Performance of TLA in drug susceptibility testing

	TLA INH	RMP	LJ INH	RMP
Sensitivity % (n)	100 (25/25)	95.2 (20/21)	100 (25/25)	100 (21/21)
Specificity % (n)	100 (139/139)	100 (143/143)	100 (139/139)	100 (143/143)
Overall accuracy- % (n)	100 (164/164)	99.4 (163/164)	100 (164/164)	100 (164/164)
PPV % (n)	100 (25/25)	100 (21/21)	100 (25/25)	100 (21/21)
NPV % (n)	100 (139)	99.3 (143/144)	100 (139/139)	100 (143/143)
		P = 0.132		

Table 3. Mean time-to-detection of drug resistance using TLA and LJ-proportion method

Sputum AFB-status	TLA days (n)	LJ proportion method days (n)
Negative	14 (816/55)	67 (3686/55)
±[a]	15 (222/15)	64 (970/15)
+[b]	13 (625/50)	55 (2139/50)
2+[c]	11 (200/19)	45 (857/19)
3+[d]	10 (250/25)	41 (1031/25)
Average	13 (2113/164)	53 (8683/164)
	P = 0.005	

n= the total number of days used for detection of drug resistant strains divided by number of specimens, [a] 1-2/300 fields, [b] 1-9/100 fields, [c] 1-9/10 fields, [d]1-9/field

molecular tools, particularly the Xpert MTB/RIF test (Cepheid, Sunnyvale, CA, USA) and the Genotype MTBDR*plus* (Hain LifeSciences, Nehren, Germany), for rapid detection of TB as well as TB drug resistance [7,17]. Despite their apparent simplicity, reliability and ease of producing rapid test results, these molecular assays have been associated with some limitations including, poor sensitivity profiles with paucibacillary specimens [18], occurrence of non-interpretable results [19], and inability to eliminate the need for TB culture and drug susceptibility testing [18]. Such limitations may be considerably addressed if these tests are used alongside a complimentary TB-culture tool like the novel TLA technique. In concordance with previous studies elsewhere on TLA [11,14], this study confirmed the accuracy of TLA method for timely detection of drug-resistant MTB strains in smear-positive as well as smear-negative patients. Using the conventional proportion method as the reference standard, 95.2% of rifampicin-resistant strains were detected on TLA, with an overall accuracy of 99.4%. Detection of rifampicin, as well as isoniazid, resistance on TLA was achieved in an average of 13 days, about a quarter of the time (53 days) used for the standard LJ-proportion method ($P = 0.005$). Although with slightly lower values, the performance of TLA in this study is comparable with that obtained by Robledo et al. [11] in a similar work done in Colombia. Particularly interesting was the high sensitivity value of TLA technique for detection of MTB strains in smear-negative sputum specimens, which may underscore an important advantage of the method over the rapid molecular assays. Unlike the TLA tool, many molecular assays do not work well with smear-negative specimens [20,21,22] which have presented additional diagnostic challenges in sub-Saharan Africa, where many HIV-infected patients with TB are smear negative. Since most of these assays are not indicated for direct use on smear-negative specimens, results may sometimes require several weeks of incubation using conventional culture methods thereby diminishing a very important attribute of these tests - speed. Moreover, even when there is a significant improvement in the rapid turn-around time of these molecular assays, it may be worthwhile to consider whether this time difference - just a matter of days in the case of TLA- has any important effect on the outcome of patient care; after all, test turnaround time has been identified as just one component in the overall evaluation and treatment of patients with TB [23].

Another important concern in the use of many molecular methods is the requirement for sophisticated laboratory facilities, supply chain systems, technical expertise and a very stable electricity supply lacking in the very places where the assays may be needed. Consequently, the operational costs of these assays seem to be quite prohibitive for use in limited-resource settings except in those areas where governments or other donor agencies provide sufficient funding to develop and maintain necessary infrastructure. As a result, their application in low-income settings is remote. In contrast, the TLA offers a simple culture-based tool for detection of TB as well as TB-drug resistance, requiring ordinary light microscopy and BSL-2 facilities (for processing specimens and inoculating cultures only) which may be easily found in TB laboratories of most rural healthcare settings. The requirement for sophisticated biosafety systems lacking in such settings is not a serious impediment since manipulation and subsequent examinations of the direct sputum cultures for growth and drug susceptibility testing of MTB isolates are carried out in sealed petri-plates. Minimal training or exposure of inexperienced laboratory scientists and technicians is however required; but identification of MTB microcolonies by their very distinctive cording appearance would also not pose a challenge to experienced microscopists.

Yet, in spite of its apparent robustness, the novel TLA method has a limitation in high contamination rates of the cultures [14,24] often necessitating incorporation of antimicrobial agents into media to control bacterial and fungal overgrowth. Such additions are capable of interacting with the anti-TB drugs being tested to interfere with the accuracy of the final results. Thus there may still be need for progressive evaluation of the TLA method alongside any of the standard rapid TB-culture tools for proper standardization.

5. CONCLUSION

However, given its simplicity and accuracy for detection of TB-drug resistance in this study, the TLA technique is poised to emerge as an excellent alternative tool for accurate detection of rifampicin-resistant TB in resource-limited settings. Endorsement of this method for use at peripheral laboratories of resource-limited settings would greatly enhance the capacity for accurate diagnosis, appropriate treatment

initiation, and control of drug-resistant tuberculosis.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Wilson ML. Recent advances in the laboratory detection of *Mycobacterium tuberculosis* complex and drug resistance. Clinical Infectious Diseases. 2011;52(11): 1350–1355.
2. Gandhi NR, Moll A, Sturm AW. Extensively drug-resistant tuberculosis as cause of deaths in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 2006;368:1575–1580.
3. World Health Organization. The Global MDR-TB & XDR-TB Response Plan 2007–2008; 2008.
   Available:http://www.whqlibdoc.who.int/hq/2007/WHO_HTM_TB_2007.387_eng.pdf
   (Accessed 05 May 2008)
4. Gillespie SH. Evolution of drug resistance in *Mycobacterium tuberculosis*: Clinical and molecular perspective. Antimicrobial Agent and Chemotherapy. 2002;46(2): 267-274.
5. Apers L, Mutsvangwa J, Magwenzi J, Chigara N, Butterworth A, Mason P, Van der Stuyft P. A comparison of direct microscopy, the concentration method and the mycobacteria growth indicator tube for the examination of sputum for acid-fast bacilli. International Journal of Tuber-culosis and Lung Disease. 2003;7:376–38.
6. Toman K. Tuberculosis case-finding and chemotherapy. Case Detection, Treatment and Monitoring – Questions and Answers, 2nd Edition. Geneva: World Health Organization; 2004.
7. World Health Organization. Global tuber-culosis report, WHO/HTM/TB/2014.08, Geneva, Switzerland; 2015.
8. Mejia GI, Castrillon L, Trujillo H, Robledo JA. Microcolony detection in 7H10 thin layer culture is an alternative for rapid diagnosis of *Mycobacterium tuberculosis* infection. International Journal of Tuberculosis and Lung Disease. 1999;3(2): 138-142.
9. Irfan S, Alasan R, Kanji A, Hassan Q, Azam I. Evaluation of a microcolony detection method and phage assay for rapid detection of *Mycobacterium tuberculosis* in sputum samples. South East Asian Journal of Tropical Medicine and Public Health. 2006;37(6):1187–1195.
10. Robledo JA, Mejia GI, Morcillo N, Camacho M, Luna J, Zurita J, Bodom A, Velasco M, Palomino JC, Martin A, Portaels F. Evaluation of a rapid culture method for tuberculosis diagnosis: A Latin American multi-centre study. International Journal of Tuberculosis and Lung Diseases. 2006;10(6):613-619.
11. Robledo JA, Mejia GI, Paniagua L, Martin A, Guzman A. Rapid detection of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* by the direct thin-layer agar method. International Journal for Tuberculosis and Lung Disease. 2008;12(12):1482-1484.
12. Kent PT, Kubica GP. Public health micobacteriology. A guide for level III laboratory. Atlanta: Center for Disease Control; 1985.
13. National Committee for Clinical Laboratory Standards. Susceptibility testing of *Mycobacteria, Nocardia*, and other aerobic *Actinomycetes*, Tentative Standard – Second Edition. Washington, DC: NCCLS; 2000.
14. Martin A, Waweru MP, Okatch BU, Ouma AN, Bonte L, Varaine F, Portaels F. Implementation of the thin layer agar for the diagnosis of smear-negative pulmonary tuberculosis in a high HIV prevalence setting in Homa Bay, Kenya. Journal of Clinical Microbiology; 2008.
    DOI: 10.1128/JCM.00264-09
15. Parikh R, Mathai A, Parikh S, Shekar GC, Thomas R. Understanding and using sensitivity, specificity, and predictive values. Indian Journal of Opthalmology. 2008;56(1):45–50.
16. Dinic L, Akande P, Idigbe EO, Ani A, Onwujekwe D, Agbaji O, Akanbi M, Nwosu R, Adeniyi R, Wahab M, Lekuk C, Kunle-Ope C, Nwokoye N, Kanki R. Genetic determinants of drug-resistant tuberculosis

among HIV-infected patients in Nigeria. Journal of Clinical Microbiology. 2012; 50(9):2905–2909.

17. World Health Organization. WHO endorses new rapid tuberculosis test. WHO, Geneva; 2010.
Available:www.who.int/mediacentre/news/releases/2010/
(Accessed 8 December 2010)
18. Nikam C, Patel R, Sadani M, Ajbani K, Kazi M, Soman R, Shetty A, Georghiou SB, Rodwell TC, Catanzaro A, Rodrigues C. Redefining MTBDR*plus* tests results: What do indeterminate results actually mean. International Journal of Tuberculosis and Lung Disease. 2016;20(2):154–159.
19. Miotto P, Piana F, Penati V, Canducci F, Migliori GB. Genotype MTBDRplus: A further step toward rapid identification of drug-resistant *Mycobacterium tuberculosis*. Journal of Clinical Microbiology. 2008;46: 393-394.
20. Morgan M, Kalantri S, Flores L, Pai M. A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: A systematic review and meta-analysis. BMC Infectious Disease. 2005;5:62-70.
21. Brossier F, Veziris N, Truffot-Pernot C, Jarlier V, Sougakoff W. Performance of the Genotype MTBDR line probe assay for detection of resistance to rifampin and isoniazid in strains of *Mycobacterium tuberculosis* with low- and high-level resistance. Journal of Clinical Microbiology. 2006;44(10):3659-3664.
22. Yagui M, Perales MT, Asencios L, Vergara L, Suarez C, Yale G, et al. Timely diagnosis of MDR-TB under program conditions: Is rapid drug susceptibility testing sufficient? International Journal of Tuberculosis and Lung Diseases. 2006;10: 838.
23. Zeka AN, Tasbakan S, Cavusoglo C. Evaluation of GeneXpert MTB/RIF assay for rapid diagnosis of tuberculosis and detection of rifampin resistance in pulmonary and extrapulmonary specimens. Journal of Clinical Microbiology. 2011;49(12):4137–4141.
24. Umoh NO, Asuquo AE, Abia-Bassey LN, Asibong UE, Pokam BT. Evaluation of thin layer agar for rapid and accurate diagnosis of tuberculosis in a resource-limited setting. Mary Slessor Journal of Medicine. 2013;12(2):4–10.

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