Diagnostic Performance of Line Probe Assay for the Diagnosis of Rifampicin and Isoniazid Resistant Tuberculosis in a Resource-Poor Country

Laura Madukaji[1]; Francis Enenche Ejeh[2]; Eke Ofuche[1]; Jay Osi Samuel[1]; Femi Owolagba[1]; Ughweroghene Omo-Emmanuel[3] , Prosper Okonkwo[1]
[1] *APIN, Public Health Initiative, Abuja, Nigeria .*
[2] *Department of Veterinary Microbiology, University of Maiduguri, Nigeria..*
[3] *USAID, Abuja, Nigeria.*

Corresponding Author
Francis Enenche Ejeh

Mobile:
+2348057243878

E mail:
enenchefrancis@yahoo.com;
enenchefrancis@unimaid.edu.ng

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Background and study aim: The use of LPA is still new in Nigeria and only available in TB reference laboratories. In this study, the performance of LPA version 2.0 was evaluated for the detection of resistant to first-line anti-TB drugs.

Patients and Methods: We evaluated the performance of LPA version 2.0 for the detection of rifampicin (RIF) and isoniazid (INH) resistance. Sputum samples from 223 participants were subjected to phenotypic drug susceptibility testing (PDST) and LPA. Statistical analyses included calculation of sensitivity, specificity, positive and negative predictive values. Cross tabulation was done along the kappa test to measure the degree of agreement between PDST and LPA. P-Value > 0.05 was considered significant.

Results: The overall sensitivity and specificity of 89.6% (95% C.I 82.5-94.5%) and 65.4% (95% C.I 44.3-82.7%) for detection of RIF resistance; for INH they were 76.6 (95% C.I 67.5-84.5%) and 76.7% (95% C.I 49.5-82.6%); and for MDR-TB, they were 67.0% (95% C.I 56.4-76.5%) and 72.0% (95% C.I 57.6-83.7%). The kappa values were 0.53 (0.001), 0.38 (p = 0.000) and 0.36 (p = 0.000) for the detection of RIF, INH and MDR-TB. There was moderate agreement between PDST and LPA for detection of RIF (κ = 0.57; P = 0.0001), INH (κ = 0.44; P = 0.0001), MDR-TB (κ = 0.43; P = 0.001).

Conclusion: The Line probe assay has good sensitivity and specificity for detecting rifampicin and isoniazid. However, the overall performance is moderate; this should be considered when interpreting the assay's results.

INTRODUCTION

Tuberculosis is a significant public health challenge. The world health organization (WHO) reported that over 10.4 million cases and 1.7 million deaths occur globally on an annual basis due to tuberculosis **[1]**. Nigeria and five other countries account for 60% of the global tuberculosis burden **[2]**. The incidence of tuberculosis cases in Nigeria was estimated at 345000 – 890000, with a potential of zoonotic tuberculosis grossly under-reported **[3]**. The End-TB program's significant challenges include rapid and accurate diagnosis of tuberculosis, development of drug resistance, and timely detection of drug-resistant tuberculosis. Other challenges include poverty and HIV **[4]**.

The diagnosis of tuberculosis in Nigeria is made mainly by acid-fast microscopy and culture and chest X-ray. These methods of tuberculosis diagnosis are faced with numerous problems. Acid-fast has the following challenges: low turnaround time but the technique has reduced sensitivity and low specificity; it is unable to differentiate between non-tuberculous mycobacteria (NTM) and *Mycobacterium tuberculosis* complex (MTBC) **[5, 6]**. Culture is the gold standard for the diagnosis of tuberculosis. However, the method requires 1-12 weeks before results are available. Also, contamination limits the use of culture for the diagnosis of

tuberculosis. The utilization of a chest X-ray (CXR) is limited because of radiation exposure, and the result is often subjective **[7]**. It is not suitable for TB diagnosis as it is known that CXR is an essential tool for early detection of tuberculosis (TB), CXR has high sensitivity, but limited specificity for detecting pulmonary TB as many CXR abnormalities that are consistent with pulmonary TB are also seen in several other lung pathologies **[8]**.

The conventional method for diagnosing drug-resistant tuberculosis was based on phenotypic drug susceptibility testing (PDST), agar or broth-based media. This method, as with culture, is time-consuming and prone to contamination. The World Health Organization endorsed using a line probe assay for diagnosing tuberculosis and detecting resistance to rifampicin and isoniazid **[9]**.

Line Probe Assay in diagnosing tuberculosis is still new in Nigeria and only available in reference laboratories. This study evaluates the performance of the line probe assay version 2.0 to detect rifampicin and isoniazid by using conventional 1% phenotypic drug susceptibility testing as the reference standard in Nigeria.

METHODS

Study participant:

This study was a prospective analysis of hypothetical Drug-Resistant TB cases screened by acid-fast bacilli (AFB) test. Study participants ranged from 15 to 80 years who were enrolled in DR-TB treatment and care. At the time of screening for TB, the HIV status of participants were documented by the DOT focal person. Sputum samples (clinical specimens) were collected from participants who gave their consent. These participants were recruited from DOT centres in Benue, Nasarawa, FCT, Niger and Kogi States. FCT being the federal capital territory, has more and well-structured DOT. Hence, we had more participants from FCT. We recruited 223 patients from the Directly Observed Treatment Short-Course (DOT) centres in 5 states in the North Central zone of Nigeria. One sputum sample was collected from each patient in 50-mL wide-mouthed sterile Falcon tubes and processed in Biosafety cabinet type II. Written informed consent was obtained from all patients at the time of enrolment into the study from October 2018 to August 2019.

Inclusion criteria:

- Availability of test results for LPA and PDST
- All ages
- Patients with HIV results
- Ability to produce sputum without an inducement

Exclusion criteria:

- Extra-pulmonary tuberculosis
- Invalid LPA results
- Inconclusive results
- Contamination of PDST culture
- Non-availability of corresponding results for both LPA and PDST

Study setting:

Every state in Nigeria has a Directly Observed Treatment Short-course (DOT) centre. However, there is still inadequate laboratory testing, as only nine culture laboratories are functional. To increase TB culture and LPA, the National TB and Leprosy Control Program (NTBLCP) strengthened the Specimen referral system through a national mapping of the states and zones and linking them to their respective testing sites (reference laboratories). Both old and newly enrolled patients on treatment are enlightened about TB and anti-TB therapy, including drug refill frequency and clinic days. Clinic days are for health talk and general drug pick-up, and this is when the DS-TB & DR-TB focal persons move to their assigned DOT centres to collect specimens for the first culture/LPA test & follow up. Specimens are collected, triple packaged and transported to the linked reference laboratories. Test results are conveyed to the DOT centres through the same DS-TB and DR-TB focal person. Samples were processed at the Zankli TB Reference Laboratory. Zankli TB reference Laboratory is one of the reference laboratories for the Northern States in Nigeria. The reference laboratory has the capacity of running AFB microscopy, Genexpert, first and second-line phenotypic culture and DST, first and second-line genotypic Line Probe Assay (LPA). This study's samples were processed using LPA Genotype MTBDRplus, and culture-Drug Susceptibility Testing Lowenstein Jensen proportion method (LJ-DST).

Culture:

We decontaminated and digested the sputum samples by the NALC-NaOH method (NALC 2.9% & NaOH 4%). Afterwards, the mixture was

centrifuged at 3000 g for 15 minutes. In each tube, the pellet was suspended in 2.5 ml of phosphate buffer pH 6.8, inoculated onto sterile Lowenstein Jensen agar slants and then incubated. We stored the remaining concentrates (pellet and buffer) at 4°C for future use. The slants were checked in the first week for contamination and or NTM to enable a prompt repeat of the culture process. On the eight weeks, cultures were read according to the National TB Standard Procedure.

Phenotypic Drug Susceptibility Testing (PDST):

We used conventional 1% proportion phenotypic drug susceptibility testing (DST) on Lowenstein Jensen (LJ) medium. The first-line drug's final concentration was isoniazid (0.2ug/ml) and rifampin (40ug/ml). The cultures with confirmed MTB were sub-cultured onto the drug-containing sterile LJ slants and non-drug containing slants (controls) and incubated for four weeks. Cultures were read after four weeks of incubation. All procedures were carried out according to the National Standard operating procedure (SOP) for LJ Culture/DST

Line Probe Assay Version 2.0 (LPA):

A chemical DNA extraction method (Genolyse kit from Hain Lifescience, Germany) was used to extract DNA from the concentrate. The manufacturer's instruction was strictly followed to get the DNA extracts. Polymerase chain reaction (PCR) was performed using 35µl of primer nucleotide mix, 10µl of Taq DNA polymerase-PCR buffer mix and 5µl of supernatant in a final volume of 50µl. Amplification was done in a thermal cycler. Twincubator was used to perform Reverse hybridization. Genotype MTBDRplus kit instruction was strictly followed to find any deletion in wild-type gene loci and mutations in rpoB (RNA polymerase B subunit), Kat G (catalase-peroxidase) and inhA (inoyl coenzyme A reductase) loci.

The hybridization strips consist of 27 reaction zones (6 control probes and 21 probes for mutation). All control probes must be present in a valid test, and the absence and the presence of any mutation band imply resistance to the particular antibiotic tested. The control probes include conjugate control, amplification control, Mycobacterium tuberculosis complex control (TUB), rpoB amplification control, inhA amplification control and katG amplification control. For detecting rifampicin resistance, the rpoB gene (coding for the β-subunit of the RNA polymerase) and high-level. For INH resistance, the katG gene (coding for the catalase-peroxidase) is examined. The promoter region of the inhA gene (coding for the NADH enoyl ACP reductase) was examined to detect low-level INH resistance.

LPA was compared with the gold standard (phenotypic culture/DST) to evaluate the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). All the results for the samples were analyzed to compare the diagnostic performance of the assays used. We used Graph pad prism version 3.5 to perform the statistical analyses.

RESULTS

Demographic characteristic of study participants:

A total of 223 suspected Patients with TB were recruited in the study. Of the 223 recruited in the study, 141 patients' specimens were included in the study analyses, while 82 (36.8%) were excluded from the study analyses based on the conditions described in figure 1. Among all the patients' specimens, 168 (72.1%) were culture positive, of which 27 (12.1%) were identified as NTM. Thirty-four (15.3%) were negative by culture on LJ, and 21 (9.4%) were contaminants. LPA had 14 (6.0%) invalid results, 68 (30.5%) specimens with valid LPA results were excluded from the study analyses because they do not have the corresponding result for phenotypic drug susceptibility testing on LJ slant. Other samples that were excluded from the study analyses are presented in figure 1.

The mean age of those included in the study was 39.2±15.2 years. Eighty-one (57.9%) were males, and 60 (42.7%) were females. Patients with new TB cases were 90 (70.2%), and retreatment cases were 42 (29.8%). Thirty-one (22.0%) were HIV positive 63 (44.4%) were from FCT (Table 1).

Diagnostic performance of LPA Using Phenotypic Drug Susceptibility Testing (Lowenstein-Jensen) as Gold Standard:

To assess the performance of LPA test in detecting RMP, INH, and MDR-TB, culture LJ drug susceptibility testing was used as the gold

standard. LPA reported sensitivity, specificity, positive and negative predictive values of 89.6% (95% C.I: 82.5 – 94.5%), 65.4 (95% C.I: 44.3 – 82.3%), 92.0% (95% C.I: 85.3 – 96.3%) and 58.6% (95% C.I: 38.9 – 76.5%) in detecting RMP. There was a moderate agreement (kappa = 0.53; P = 0.000) between LPA and culture LJ DST in detecting rifampicin resistant TB. The sensitivity and specificity of LPA in detecting INH resistance were 76.6% (95% C.I: 67.5 – 84.3%) and 67.7% (95% C.I: 49.5 – 82.6%). There was a moderate agreement (Kappa = 0.38; P = 0.000) between LPA and culture in detecting INH resistant TB. Also, LPA and culture had moderate agreement (Kappa = 0.36; P = 0.000) in detecting MDR-TB. LPA reported a sensitivity and specificity of 67.0 (95% C.I: 56.4 – 76.5%) and 72% (95% C.I: 57.6 – 83.7%) for detecting MDR-TB (table 2).

LPA reported sensitivity and specificity of 83.3% (95% C.I:65.3-94.4%) and 75.0% (95% C.I: 19.4-99.4%) respectively in detecting rifampicin resistant TB among Patients with HIV. The agreement between both tests for detection of RMP-resistant TB was moderate (Kappa = 0.40; P = 0.016), the sensitivity and specificity of LPA to detect isoniazid-resistant TB among patients with HIV were 73.9 (95% C.I: 51.6 - 89.8%) and 50.0 (15.7 - 84.3%). Also, LPA reported sensitivity and specificity of 57.1% (95% C.I: 34.1 - 78.2%) and 60.0% (95% C.I: 26.3 - 87.8%) to detect INH resistant TB among HIV patient. The agreement between both tests for the detection of INH resistance among Patients with HIV was fair (Kappa = 0.22; P = 0.213). Although, LPA performance in detecting INH and MDR –TB were fair compared with culture, the results for HIV negative patient indicated better performance by LPA (Table 3 and 4). The sensitivity and specificity of LPA to detect rifampicin resistant TB among HIV negative patient were 92.1% (95% C.I: 84.3 - 96.7%) and 63.6% (95% C.I: 40.7 - 82.2%). There was moderate agreement (Kappa = 0.57; P = 0.000) between LPA and culture to detect RMP resistant TB among HIV negative patients. The sensitivity, positive and negative predictive values of LPA to detect INH-resistant TB among HIV-infected patients was 77.4% (95% C.I: 66.9 - 85.8%), 73.1% (95% C.I: 52.2 - 88.4%), 90.3% (95% C.I: 81.0 - 96.0%) and 50.0% (95%C.I: 33.4 - 66.7%) respectively. The sensitivity, positive and negative predictive values of LPA to detect MDR TB among HIV negative patients were 70.0% (95% C.I: 57.9 - 80.4%), 75.0% (95% C.I: 58.8 - 87.3%), 83.1% (95% C.I: 71.0 – 91.6%) and 58.9% (95%C.I: 44.2-72.4%); the Kappa agreement was 0.43. (Table 4).

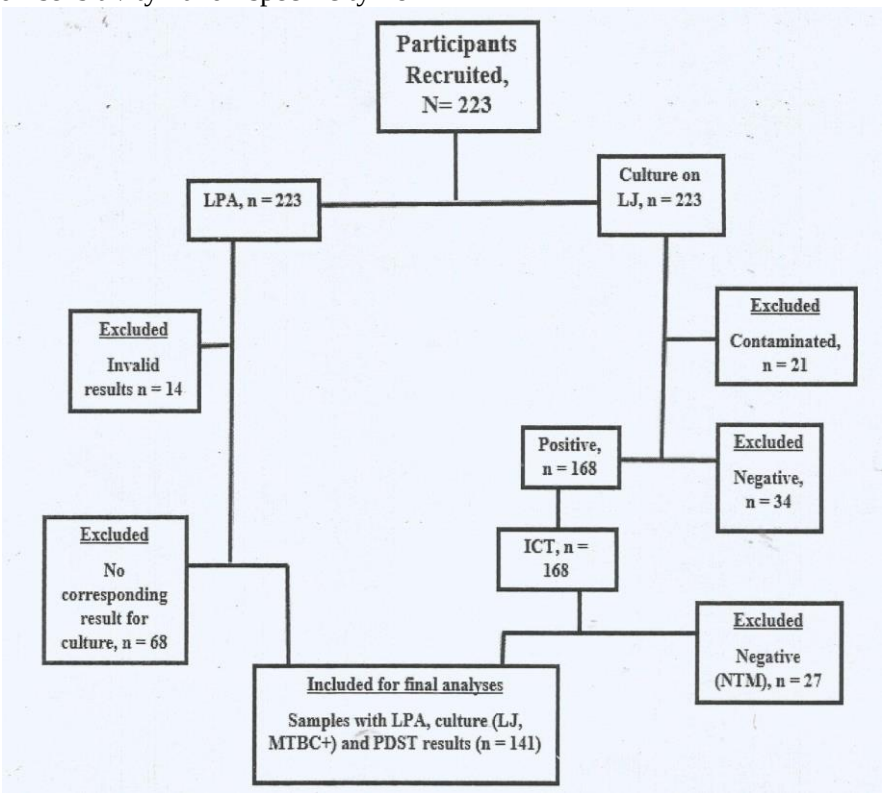

Figure (1): Schematic Representation of the Workflow.

Table (1): Demographic and Clinical Characteristics of Study Participants.

Parameters	Frequency	Percentages (%)
Age (Mean ± SD)	39.17 ± 15.4	
Sex		
Male	81	57.4
Female	60	42.7
HIV Status		
Positive	31	22.0
Negative	110	78.0
State		
Benue	10	7.1
FCT	63	44.4
Kogi	25	17.7
Nasarawa	28	19.9
Niger	15	10.6

HIV = Human Immunodeficiency Syndrome; FCT = Federal Capital Teritory.

Table (2): The overall Performance of LPA test as compared to LJ DST in detecting resistance to rifampicin, isoniazid, and MDR-TB.

Gold Standard: Culture on Lowenstein-Jensen Agar Slant							
LPA Assay		**Rifampicin**		**Isoniazid**		**MDR**	
		Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible
		(n = 115)	(n = 26)	(n = 107)	(n = 34)	(n = 67)	(n = 18)
LPA Assay	Resistant	103	9	82	11	61	14
	Susceptible	12	17	25	23	6	4
Sensitivity (95% C.I)		89.6 (82.5 – 94.5)		76.6 (67.5 – 84.3)		67.0 (56.4 – 76.5)	
Specificity (95% CI)		65.4 (44.3 – 82.7)		67.7 (49.5 – 82.6)		72.0 (57.6 – 83.7)	
Positive Predictive Value (95% CI)		92.0 (85.3 – 96.3)		88.2 (79.8 – 94.0)		81.3 (70.6 – 89.4)	
Negative Predictive Value (95% CI)		58.6 (38.9 – 76.5)		47.9 (33.3 – 62.8)		54.6 (41.9 – 66.9)	
Kappa (P Value)l		**0.53 (0.000)**		**0.38 (0.000)**		**0.36 (0.000)**	

LPA = Line Probe Assay; MDR = Multi-drug Resistance; C.I = Confidence Interval; MTBDR = *Mycobacterium tuberculosis* drug resistance, LJDST = Lowenstein-Jensen Drug Susceptibility Testing.

Table (3): Performance of LPA test as compared to LJ DST in detecting resistance to rifampicin, isoniazid, and MDR-TB among HIV Patients.

Gold Standard: Culture on Lowenstein-Jensen Agar Slant							
LPA Assay		**Rifampicin**		**Isoniazid**		**MDR**	
		Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible
		(n = 30	(n = 4)	(n = 23)	(n = 8)	(n = 21)	(n = 10)
MTBDR-PLUS	Resistant	25	1	17	4	12	4
	Susceptible	5	3	6	4	9	6
Sensitivity (95% C.I)		83.3 (65.3 – 94.4)		73.9 (51.6 – 89.8)		57.1 (34.1 – 78.2)	
Specificity (95% CI)		75.0 (19.4 – 99.4)		50.0 (15.7 – 84.3)		60.0 (26.3 – 87.8)	
Positive Predictive Value (95% CI)		96.2 (80.4 – 100.0)		81.0 (58.1 – 94.6)		75.0 (47.7 – 92.7)	
Negative Predictive Value (95% CI)		37.5 (8.5 – 75.6)		40.0 (12.2 – 73.6)		40.0 (16.3 – 67.8)	
Kappa (P Value)		**0.40 (0.016)**		**0.22 (0.213)**		**0.15 (0.372)**	

LPA = Line Probe Assay; MDR = Multi-drug Resistance; C.I = Confidence Interval; MTBDR = *Mycobacterium tuberculosis* drug resistance, LJDST = Lowenstein-Jensen Drug Susceptibility Testing

Table (4): Performance of LPA test as compared to LJ DST in detecting resistance to rifampicin, isoniazid, and MDR-TB among HIV Negative patients.

Gold Standard: Culture on Lowenstein-Jensen Agar Slant							
LPA Assay		**Rifampicin**		**Isoniazid**		**MDR**	
		Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible
		(n = 88)	(n = 22)	(n = 84)	(n = 26)	(n = 70)	(n = 40)
MTBDR-PLUS	Resistant	81	8	65	7	49	10
	Susceptible	7	14	19	19	21	30
Sensitivity (95% C.I)		92.1 (84.3 – 96.7)		77.4 (66.9 – 85.8)		70.0 (57.9 – 80.4)	
Specificity (95% CI)		63.6 (40.7 – 82.8)		73.1 (52.2 – 88.4)		75.0 (58.8 – 87.3)	
Positive Predictive Value (95% CI)		91.0 (83.0 – 96.0)		90.3 (81.0 – 96.0)		83.1 (71.0 – 91.6)	
Negative Predictive Value (95% CI)		66.7 (43.0 – 85.4)		50.0 (33.4 – 66.7)		58.9 (44.2 – 72.4)	
Kappa (P Value)		**0.57 (0.000)**		**0.44 (0.000)**		**0.43 (0.000)**	

LPA = Line Probe Assay; MDR = Multi-drug Resistance; C.I = Confidence Interval; MTBDR = *Mycobacterium tuberculosis* drug resistance.

DISCUSSION

This study's findings showed that the line probe assay has high sensitivity (89.6%, 95% CI; 182.5-94.5%) and moderate specificity (65.4%, 95% CI; 44.3 - 82.7%) in the detection of rifampicin. The kappa agreement between LPA and phenotypic drug susceptibility testing (PDST) was 0.53. The overall performance of LPA for the detections of rifampicin in this study was higher than previously reported by Scott et al. **[10]**. Scott and co reported 40% (95% CI 5.27 - 85.34%) sensitivity of LPA to detect rifampicin in a study to compare the performance of

GeneXpert with other nucleic acid assays. A sensitivity of 60.00% (95% CI; 14.66 - 94.73% was reported in a study to evaluate genotype MTBDR plus assay as a tool for drug resistance survey **[11]**. In another study, Chryssanthou et al. **[12]** report a sensitivity of 75.00% (95% CI; 19.41 - 99.37%). While Maschmann et al **[13]** reported 82.14% (95% C. I; 63.11 - 93.94%). The specificities reported by previous authors were higher than those reported in this study **[11–13]**. Also, Lower specificities (66.67%, 95% C.I; 9.43-99.5% and 50.00%, 95% C. I; 1.20-98.72%) were reported by Sangsayunh *et al* **[14]** and Miolt *et al* **[15, 16]**. The overall performance of LPA for the detection of INH in this study was moderate, and the sensitivity was 65.4%, 95% C. I; 44.3- 82.7%. This result was higher than the previous finding by Rigouts *et al.* **[11]** (54.90%), Maschmann Rde *et al.* **[13]** (60.42%), Dorman *et al.* **[17]** 62.07% and Scott *et al.* **[10]** (66.67%). However, the specificity of LPA for the detection of isoniazid resistance was lower in this study than the previously reported **[10, 11, 13, 17]**.

The overall sensitivity of LPA to detect MDR-TB in this study was higher than previous studies in Brazil, where the sensitivity was 59.2%) **[13];** 60.00% in Tanzania **[11]**. Our finding is similar to 69.64% in China **[18]**. The performance of LPA in this study was low compare to the most recent studies. Meaza *et al.* **[19]** reported higher sensitivity (96.4%) to detect MDR-TB by LPA. He *et al.* **[20],** in another study that uses LPA version 1.0 reported lower performance for detecting rifampicin, isoniazid and multi-drug resistance TB. The low performance reported by He and co maybe because of the difference in DNA isolation used in the current LPA version and those used in the earlier version. It was also noted that LPA version 2.0 has a lower chance of cross-contamination than LPA version 1.0 **[21]**.

The low performance of LPA version 2 in this study compared with the previous study uses the same versions maybe because of the participant's sputum status, study design, and technical know-how.

Our finding revealed that the performance of LPA among HIV negative patients was better than among HIV positive patients. Also, the Kappa agreement between LPA and cultures for the detections of rifampicin among HIV negative patient was higher than in patients with HIV (0.57 vs 0.40). The difference in the diagnostic performance of LPA among patient with HIV and patients without HIV in this study is perhaps because of the low bacillary load among HIV positive patients with TB. It was observed that the limit of detection of MTB by LPA is 10, 000 CFU/ml while that of culture is 10-100 CFU/ml. LPA likely missed specimens with low bacillary load **[5, 22]**.

The sensitivity and specificity of LPA for detection of INH resistance were higher among patients without HIV than patient with HIV (77.4% and 63.6% vs 73.9% and 50.0%). Similar results were obtained for the detection of multi-drug resistant tuberculosis.

CONCLUSION

Analyzing the overall performance of LPA in this study gave moderate sensitivities among patients with HIV and patients without HIV. This test's performance for detecting INH, RMP mono resistance, and MDR agreed with the gold standard. There is a need for comparing the performance of LPA against phenotypic DST and genomic sequencing in countries that started implementing the use of LPA in less than 3 to 5 years.

This study is limited by: the performance of LPA requires caution for interpretation and is dependent on the power of observation; there is also a need for interlaboratory competency assessment to assess the performance of workers at private and zonal reference laboratories. In addition, there was a high rate of contaminations which may affect the interpretation of the results presented in this study.

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Competing interest: None declared.

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Ethical considerations

The Health Research Ethics Committee of the Benue State University Teaching Hospital, Makurdi, Nigeria gave the permission (BSUTH/MKD/HREC2013B/2017/0011) for the conduct this study

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