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Validity of Ziehl-Neelsen and LED Fluorescence Microscopy Technique in the Diagnosis of Mycobacterium Tuberculosis Infection among HIV Patients in Lafia, Nigeria, 2017

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Abstract

Tuberculosis (TB) is a public health problem caused by the Mycobacterium tuberculosis complex. An estimated 70% of TB patients living with HIV are from sub-Saharan Africa largely depends on direct smear microscopy light microscopy for TB diagnosis, which has low Sensitivity, especially among HIV patients. Hence, we conducted this study to evaluate the Performance of Auramine O LED Fluorescence microscopy and the Ziehl-Neelsen technique in Tuberculosis among HIV-positive patients. The study was conducted using a cross-sectional design among 107 consecutively selected HIV/AIDS patients with presumptive Tuberculosis attending ART clinic in Dalhatu Araf Specialist Hospital (DASH), Lafia, Nasarawa State. Three samples were collected from each patient in two visits. Each sample was examined using ZN, LED FM, and Gene-Xpert. Data on socio-demographic characteristics was collected from the subject participant. Univariate and bivariate analyses were done using MS Excel and Epi Info. The Sensitivity and Specificity were compared using McNemar's chi-square. Seventy-three (68%) patients were female, 33 (30.8%) were in the age group 28-32 years, and 61 (56%) were employed. Ziehl-Neelsen and LED fluorescent microscopy respectively yielded 11 (10.3%) and 15 (14.0%) positive results, while Gene-Xpert gave 18 (16.3%). The Sensitivity for direct ZN and LED FM were 61.1% and 83.3 %, respectively (P-value 0.01) with 100% specificity for both methods. LED microscopy has a much higher sensitivity than ZN microscopy and would be a better alternative in the diagnosis of Tuberculosis in high HIV settings where the use and expertise with culture and Gene-Xpert are limited.

Keywords: *HIV, LED Fluorescence, Sensitivity, Specificity, Ziehl-Neelsen.*

Introduction

Tuberculosis (TB) is a public health problem caused by infection with the *Mycobacterium tuberculosis* complex. It is more common in developing countries and is associated with poverty and poor housing conditions [1, 2]. Large household size, overcrowding and consumption of animal products like unpasteurized milk and milk products have been documented as risk factors for Tuberculosis [3, 4]. Tuberculosis affects the lungs and other organs of the body which are known as pulmonary and extra-pulmonary Tuberculosis, respectively. Pulmonary Tuberculosis (PTB) accounts for 80% of all TB cases [5, 6]. TB is spread from person to person through the air containing the bacilli from the cough, sneeze, or spit of an infected person. The most important source of infection is an untreated PTB patient – people ill with TB can infect up to 10-15 other people through close contact [6]. The case

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definition of PTB is any person presenting with cough lasting 2 weeks or more, accompanied with one or more of the following symptoms: weight loss, tiredness, fever, night sweats, chest pain, shortness of breath, loss of appetite, coughing without or without blood [7]. HIV increases the risk of acquiring TB with a 10% chance of developing active Tuberculosis yearly. Tuberculosis causes a third of global HIV/AIDS deaths. Without treatment, TB mortality rates are higher than HIV; and about 70% of pulmonary TB cases died within 10 years of infection [7]. However, according to WHO reports, only 2.4% of all people living with HIV or AIDS have been tested for TB [8]. In 2013, it was estimated that TB–HIV deaths were about 360,000 and 400,000 in 2014, and about 41% of the deaths occurred in Africa, where 38% (170,000) of the deaths was recorded in Nigeria [7].

The most important control strategy for TB is the early detection and appropriate treatment of TB cases. Globally the Case Detection Rate (CDR) of TB has been estimated at only 64% against the 70% target set by the World Health Assembly in 2005. This means that about 36% of the incident TB cases are not detected [9]. “This leaves a gap of approximately 3.3 million people worldwide with TB who were “missed,” either because they were not diagnosed or because they were diagnosed but not reported” [7]. Tuberculosis diagnosis in Nigeria is largely dependent on direct smear microscopy, and hence the TB case detection rate in Nigeria was about 15% in 2014 [7], very low when compared to other nations in the world.

The Sensitivity of ZN microscopy is low, ranging from 52 to 70%. The conventional light microscopy sensitivity is grossly compromised in the diagnosis of TB among HIV patients and other cases where the bacterial load is less than 10,000 organisms/ml sputum sample such as children and extra-pulmonary TB cases [10, 11, 12]. Due to the low ZN sensitivity, many cases TB cases go undetected, thereby making TB control more challenging, and this has contributed to the challenges in TB control. Mercury fluorescence has been shown to improve tuberculosis diagnosis by 10% with a good turnaround time when compared with conventional bright-field microscopy [13, 14]. However, the cost of maintenance due to the short life span of mercury, equipment cost, the requirement for the dark room, and high skilled required have made the use of fluorescence microscopy challenging in developing countries like Nigeria [15]. Due to challenges associated with the use of mercury fluorescence microscopy, Light-emitting diodes (LEDs) have been identified as an alternative to conventional FM, and having been distributed across most Nigeria health centres, and its Performance has been limitedly studied among HIV patients; hence, our study was aimed to evaluate the validity of LED Microscopy in the diagnosis of tuberculosis infection among HIV patients attending Dalhatu Araf Specialist Hospital, Lafia.

Methods

Study Area and Site

Nasarawa State, with Lafia as its capital, is in the North-Central geopolitical zone of Nigeria. Its population is 2,523,400 according to the 2016 projection. The study was conducted at Dalhatu Araf Specialist Hospital, a 300-bed facility in Lafia. It serves clients within Lafia, local Government Area and the neighboring states. The facility has about 13,780 adult and pediatric HIV patients receiving HAART. DASH laboratory is an accredited laboratory by the Medical Laboratory Council of Nigeria and serves as a referral centre for the neighboring laboratories.

The DASH laboratory has different departments like the Hematology, Microbiology, ART Laboratory, Gene-Xpert services, Clinical Chemistry and Histopathology.

Study Design and Population

This was a cross-sectional study among HIV patients with presumptive pulmonary TB visiting Dalhatu Araf Specialist Hospital.

Inclusion Criteria

All HIV patients with presumptive TB who are able to give sputum and are not on any anti-tuberculosis drugs.

Exclusion Criteria

HIV patients with presumptive TB who are critically ill or admitted for other illnesses.

Sample Size Determination

The sample size was calculated using the formula for sample size determination [16, 17].

$$\textit{Sample size } (n) \textit{ based on Specificity } n = \frac{Z^2_{\alpha/2} \times S_P\,(1 - S_P)}{L^2 \times P}$$

Description:

n = required sample size for this study.

P = Estimated prevalence of pulmonary TB in TB suspects attending health facility =0.5.

S_P = anticipated specificity = (0.965) [18].

α = size of the critical region ($1-\alpha$ is the confidence level) = 0.05.

$Z_{\alpha/2}$ = standard normal deviate corresponding to the specified size of the critical region (α) =1.96, and

L = absolute precision desired on either side (half-width of the confidence interval) of Sensitivity or specificity=0.05.

$$n = \frac{1.96^2 \times 0.965\,(1 - 0.965)}{0.05^2 \times 0.5} = \frac{0.12975}{0.00125} = 103.8 \sim 107$$

Sampling Technique

Samples were collected consecutively during clinic days. Monday and Thursday–from every HIV-positive subject with a suspected TB infection until the sample size was reached.

Data Collection Technique

Socio-demographic Characteristics

A proforma was developed to abstract data on the study subjects from source documents. Clinicians diagnosed patients presenting at the HIV clinic to be presumptive tuberculosis patients after a clinical examination. A maximum of 10 patients were recruited on a daily basis over a period of 6 weeks after informed consent was received from them to participate in the study.

Specimen Collection

Three early morning sputum samples were collected for testing from 107 participants using wide-mouthed sterile containers. Two early morning sputum samples were collected during the patient's first visit, and the remaining sample was collected at the second visit. The subject was briefed on how and when to collect the samples. The sputum was labeled with an identification number corresponding to the identifier on the proforma. The physical appearance of the sample was examined and documented as salivary, mucoid, muco-purulent, purulent, or blood-stained.

Smear Preparation

A small portion of purulent or muco-purulent material was collected with the stick/loop and transferred to the slide. A spread was made in an area equal to about 2-3 x 1-2 cm using repeated circular movements and allowed to air dry at room temperature. The flame was passed under the slides slowly in about three consecutive times without overheating the slides.

Gene-Xpert Procedure

Sputum was liquefied and inactivated with 2:1 sample reagent dilution, then 2ml of the liquefied material was transferred into test cartridge and then inserted into MTB-RIF platform. The sample was automatically filtered

and washed. Ultrasonic lysis filtered captured organisms released DNA molecules. The DNA molecule released mixed with the dry PCR reagents. Hemi-nested real-time amplification and detection occurred in an integrated reaction tube and result.

Data Management

Slides were reading, recording, and reporting: Slides were blindly examined by two trained laboratorians who had worked for more than five years on the TB bench for both Ziehl-Neelsen microscopy and fluorescence microscopy. The slides were scanned and systematically ready from one side to the other and back again using 100 objectives for ZN microscopy, while 40 x objectives was used for confirmation of AFB for fluorescence microscopy.

The result was considered positive or negative when confirmed by the supervisor and are quantified using the WHO/IUATLD scale. Data Entering, Storage, and Confidentiality: The test results were recorded in the data form and entered in Microsoft Excel, followed by data cleaning. Socio-demographic characteristics such as age, sex, address, educational status, and employment status were collected. Data collected were kept in lockable cabinets and database pass-worded to maintain limited access and confidentiality. Study identification numbers identified all records to maintain confidentiality.

Statistical Analysis

All data were analyzed using open Epi Info version 7 at a 5% level of significance and 95% confidence level. Frequencies and proportions were calculated. The Sensitivity and Specificity of smear microscopy were calculated using the positive Gene-Xpert as the gold standard. The Sensitivity and Specificity were compared using the McNemar Chi-square test.

The following formula was used to calculate Sensitivity (Se), Specificity (SP), positive and Negative predictive valve, and the 95% confidence interval.

$$Se = \frac{\text{TP}}{\text{TP} + \text{FN}}$$

$$SP = \frac{\text{TN}}{\text{TN} + \text{FP}}$$

$$NPV = \frac{\text{TN}}{\text{TN} + \text{FN}}$$

$$PPV = \frac{\text{TP}}{\text{TP} + \text{FP}}$$

95%,

$$\text{C.L } 1.96\sqrt{\frac{P(1-P)}{n}} \leq \pi \gg p + 1.96\sqrt{\frac{P(1-P)}{n}}$$

Where Se: Sensitivity, Sp: Specificity, TP: True positive results; TN: True negative results; FN: False-negative results; FP: False-positive results.

Ethical Consideration

Ethical approval was obtained from the Dalhatu Araf Specialist Hospital Research Ethical Committee. Verbal informed consent was obtained from these participants of age >17 years and emancipated minors. Assent was also obtained from subjects less than 17 years.

Limitation

Gene-Xpert was used in this study as a gold standard in place of culture due to the cost, time constraint, and also the challenges of transporting viable samples to the National Tuberculosis Reference Laboratory. Challenge of missing data since data abstraction was used in the study. Challenges were resolved by triangulation from the different source documents.

Result

Three sputum samples were collected from 107 study subjects in two consecutive days, giving a total of 321 sputum specimens collected from 107 subjects with a mean age of 34±11.9. Thirty-eight percent of the participants were within the age group 28-32 years.

There were 73 (65%) females, 53 (50%) with secondary education, and 60 (56%) urban dwellers (Table 1).

Each of the 107 sputum specimens was tested with Ziehl-Neelsen, LED fluorescence

microscopy, and Gene-Xpert. Out of this, ZN direct smear yielded 11 (10.3%) positive MTB results, while 15 (14.0%) were positive for LED FM direct smear. Out of the 96 (98.7%) of ZN AFB-negative, 7 (7.3%) were positive with Gene-Xpert. The Sensitivity for direct ZN was 61.1% and Specificity of 100% with the percent agreement Cohen's kappa 0.72 and (p-value: 0.01) (Table 2). Out of the 92 (86.0%) LED FM AFB Negative, 3 (3.3%) were positive with Gene-Xpert. The Sensitivity for direct LED FM was 83.3 % and Specificity of 100%. The percent agreement Cohen's kappa was 0.89 (p-value: 0.13) (Table 3). The incremental Sensitivity between ZN and LED FM was 22.2% (Table 4).

Table 1. Socio-demographic Characteristics of HIV Patients with Presumptive Tuberculosis Infections

Variables	**Frequency**	**Percent**
Age (Years)		
≤17	6	5.6
18-22	5	4.7
23-27	14	13.1
28-32	33	30.8
33-37	15	14.0
38-42	17	15.9
43-47	6	5.6
48-52	4	3.7
53-57	2	1.9
≥58	5	4.7
Occupation		
Employed	61	57
Unemployed	46	43
Location		
Urban	60	56
Rural	47	44
Educational Level		
No Formal Education	24	22
Primary Education	18	17
Secondary Education	53	50
Tertiary Education	12	11
Gender		
Male	34	32
Female	73	68

Table 2. Validity of Ziehl-Neelsen Technique against Gene-xpert in the Diagnosis of Tuberculosis Infection among HIV-Positive Patients

ZN	**Gene-xpert**		**Total**	**Sensitivity/CL**	**Specificity/CL**	**Kappa**	**P-value**
	Positive	**Negative**		**61.1%**	**100%**	**0.72**	**0.01**
				(60.8-94.2)	**(95.9-100)**		
Positive	11	0	11	-	-	-	-
Negative	7	89	96	-	-	-	-
Total	18	89	107	-	-	-	-

Table 3. Validity of LED Fluorescence Technique against Gene-Xpert in the Diagnosis of Tuberculosis Infection among HIV-Positive Patients.

LED FM	Gene-xpert		Total	Sensitivity/CL	Specificity/CL	Kappa	P-value
	Positive	Negative		83.3% (60.8-94.2)	100% (95.9-100)	0.89	0.13
Positive	15	0	15	-	-	-	-
Negative	3	89	92	-	-	-	-
Total	18	89	107	-	-	-	-

Table 4. Comparison of Validity of ZN and LED FM Technique in the Diagnosis of Tuberculosis Infection among HIV-Positive Patients at DASH, Lafia, 2017

Validity	ZN Technique (Percent)	LED FM Technique (Percent)	Incremental Yield	P-value
Sensitivity	61.1	83.3	22.2%	0.01
Specificity	100	100		

Discussion

Our study has shown the Performance of FM Microscopy to be better than ZN Microscopy with an incremental sensitivity of 21.2%. FM Microscopy has also yielded more positives than ZN Microscopy among gender, with the male having a higher yield than females. There is also more AFB Positive among the employed, those residing in the rural setting, and participants within the age group 28-30 years. The study shows a similar sensitivity of ZN Microscopy with the previous studies conducted among HIV patients with suspected pulmonary TB in Kampala, Uganda, with a Sensitivity of 51% [19] and also among pulmonary and extra-pulmonary TB patients in South India with a Sensitivity of 61.1% [20]. The results of our study differ from other studies that recorded a high sensitivity of 82.6%, 71%, and 95.6% [21, 22]. The difference observed in other studies may be because the studies were conducted among non-HIV patients' presumptive TB as the study population. Although other factors like study design, operational design, and gold standard may have also contributed to this discrepancy observed in this study. Our results showed that ZN has a lower sensitivity in the diagnosis of TB amongst HIV patients. This result agrees with a study conducted among HIV subjects in Thailand which shows ZN microscopy has a low sensitivity in the diagnosis of TB among HIV patients [23]. ZN low sensitivity also applied in extrapulmonary TB and in the use of other specimen likes cerebrospinal fluid or other body fluids.

Our findings also revealed that LED FM has a high sensitivity of 83.3%. This may be due to the large area seen by the objectives Len and less time required by the microscopes to read a slide. The use of a trained microscopic with the previous experience in using LED microscopy in their routine diagnosis of Tuberculosis, may have also contributed to this high Sensitivity of LED FM. Studies have shown that LED fluorescence microscopy performs better than mercury fluorescence microscopy. Hence, the use of LED Technology, which may have also contributed to the increase in Sensitivity in fluorescence microscopy, has been demonstrated among HIV-positive subjects in various studies [20, 21]. The result of our study was similar with other studies, which reported 95.2%, 83.1% among non-HIV positive, however, high Sensitivity of fluorescence microscopy had been demonstrated among HIV positive subjects in many studies [20, 21]. Our study showed a significant difference between ZN microscopy and LED FM with an increase in

Sensitivity of 22.2% (P<0.01). This is similar to other studies conducted in Kenya, were the Sensitivity of FM methods, and ZN were statistically significant (FM-80%, ZN-65%) [24]. Our findings differ from the study among non-HIV patients, which reported no statistically significant difference between LED Fluorescence Microscopy and ZN in the diagnosis of pulmonary tuberculosis [25]. There was a perfect concordance agreement (unweighted kappa= 0.89%) between ZN and LED FM. Contrarily to our study, many have shown a good concordance agreement between ZN and LED microscopy [25]. Fluorescence microscopy offers well-described benefits and improves the diagnostic value of the sputum smear, especially among those with a low density of bacilli missed by ZN microscopy. Moreover, fluorescence microscopy allows more rapid screening of sputum specimens than ZN.

The findings from our study showed a similar specificity of 100% for ZN and LED FM. The result may be due to the choice of gold standard other than culture used by other studies. Gene-Xpert has high sensitivity and able to detect even fragments of RNA virus. Studies have shown that gene-Xpert has a similar sensitivity with the culture [26, 27], and due to the limitations involved in the use of culture, gene-Xpert was used as a gold standard. Another reason would be the use of experienced microscopists. They examined the slides independently of the value of the Gene-Xpert result. Moreover, it may also be likely that high magnification was used for both methods, which led to the increase in Specificity for both methods. The result was similar to Mohammad, who evaluated ZN's performance and found the Specificity to be 100% for the ZN [22]. However, the study results were different from the Specificity obtained from other studies that reported 80-90% [24, 28, 29].

Conclusion

The Sensitivity of ZN microscopy is low, and LED microscopy has a higher sensitivity than ZN and would serve as a better replacement for ZN in the diagnosis of TB in high HIV setting where there are limited use of culture Gene-Xpert.

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Conflict of Interests

The authors have not declared any conflict of interest.

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