The Role of MTBDR *plus* ver 2.0 in the Detection of Drug Resistant-Tuberculosis in Smear-Negative Pulmonary Tuberculosis in Kano, Nigeria

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Abstract: The development of multidrug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis XDR-TB coupled with Human Immunodeficiency Virus Co-infection with TB has brought a major setback in the tuberculosis control strategies worldwide including Nigeria. The Genotype MTBDR *plus* VER 2.0 is widely recognized for its excellent performance due to its rapid detection of drug resistant tuberculosis especially from acid fast bacilli (AFB) smear-positive pulmonary TB cases which has brought much hope towards reversing the spread of all forms of tuberculosis. The study aimed at evaluating Genotype MTBDR *plus* VER 2.0 for detection of Drug Resistant-TB from sputum samples of smear-negative pulmonary TB cases. A total of 175 smear-positive and 278 AFB smear-negative sputum samples were purposely selected from sputum samples that were referred to North West Zonal Tuberculosis Reference laboratory. All the samples were rifampicin resistant cases (confirmed by Genxpert) from their respective health centers across North-Western Zone of Nigeria and were then subjected to Line Probe Assay (LPA) using Genotype MTBDR *plus*ver 2.0. The result shows that 87% of the AFB smear-positive samples were identified as positive for *Mycobacterium tuberculosis* complex (MTBC) by the LPA and 13.7% were negative. Whereas 79%of the AFB smear-negative samples were also detected as negative for MTBC by the LPA and 21% were positive for MTBC. The study further revealed that 44.7% of the AFB smear-positive and 46.5% of AFB smear-negative samples were found to be rifampicin resistant by LPA. Also 36.4% of AFB smear-positive and 34.5% of the AFB smear-negative sample were found to be MDR-TB by the LPA. Most importantly, 15.1% of the smear-positive samples and 17.2% of AFB smear-negative samples were found to be susceptible to both rifampicin and isoniazid by the LPA. The study demonstrated that the Genotype MTBDR *plus*Ver 2.0 detects 87% of the positive smears and 79% of the negative smears as positive and negative for MTBC respectively. Most importantly, it detects 21% of the AFB smear negative samples as positive for MTBC with some of them identified as rifampicin resistant and MDR-TB respectively. This therefore reveals the excellent performance of Genotype MTBDR*plus*Ver 2.0 in the diagnosis of DR-TB both in smear positive samples and smear-negative samples within a short turnaround time.

Key words: Acid Fast Bacilli, Line Probe Assay, Multidrug resistant Tuberculosis, Smears.

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INTRODUCTION

The World Health Organization (2017) reveals that an estimated 10.4million incident TB occurred worldwide in 2016. Studies by Meaza *et al*. (2017) revealed that TB ranks as the second leading cause of death from an infectious disease worldwide, after the Human Immunodeficiency Virus (HIV).The development of multidrug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) further poses greater challenges to the tuberculosis control strategies worldwide including Nigeria. In this regard, Nigeria was documented as 1st in Africa and 4th among 6 countries that accounted for 60% of worldwide TB burden with an estimated 4.3% and 25% MDR/RR-TB in New& previous TB cases (WHO, 2017). So far, accurate and rapid detection of TB including all forms of drug resistant TB has been identified as crucial for timely initiation of TB treatment and a successful TB control. Among the detection methods, smear microscopy has been reported to have low sensitivity, and culture based tests for identification of tubercle bacilli and drug susceptibility testing take weeks to give results (Chaudhary, 2018). Thus, the WHO has recommended the implementation of rapid diagnostic tests such as molecular tests to detect and help combat M/XDR tuberculosis (TB) (Tukvadze, *et al*., 2012).

Molecular tests that allow rapid detection of *Mycobacterium tuberculosis* Complex (MTBC) and drug resistance in 4-8 hours include Real-time-PCR based GeneXpert which provides only rifampicin (RIF) susceptibility andthe Line probe assays (LPA) which give sensitivity of both the first line anti-tubercular drugs (Chaudhary, 2018). Line probe assays such as the Genotype MTBDR*plus* assay uses DNA amplification followed by reverse hybridization to detect the presence of *Mycobacterium tuberculosis* DNA and the most common genetic mutations conferring resistance to rifampicin (RIF) (in *rpoB* gene) and isoniazid (INH) (in *katG* and *inhA* genes) and the test can be performed within 8 hours.

The previous version of LPA (GenoType MTBDR*plus* version 1.0) introduced in 2008 has been evaluated only for high-grade smear-positive specimens and to detect the level of drug resistance in culture isolates (Tukvadze *et al*., 2012; WHO, 2008). Yadav *et al*. (2013) and Hillemann *et al*. (2005) demonstrated that the assay, can only be used for smear-negative sputum samples and scanty positives samples (i.e. those with bacillary loads less than 10 bacilli in 100 fields of the smear) only after isolation becomes culture. Consequently, the latest version of LPA (GenoType MTBDRplus version 2.0, Hain Lifescience, Nehren, Germany) was introduced to overcome these limitations and has been widely recognized for its excellent performance due to its ability to detect MTB and their Drug Susceptibility Test (DST) patterns for the two first-line drugs RMP and INH in both smear-positive (including scanty) and smear-negative, and also culture-positive samples (Singh, *et al*., 2017). This success has brought much hope towards reversing the spread of all forms of tuberculosis. The study therefore, aimed at evaluating Genotype MTBDR *plus* VER 2.0 for detection of DR-TB from sputum samples of smear-negative pulmonary TB cases.

METHODS

Study area

The study was conducted at the North West Zonal Tuberculosis reference laboratory located at Aminu Kano Teaching Hospital, Kano, which serves as a referral center for the diagnosis of tuberculosis in North western region of Nigeria and beyond.

Ethical approval

Ethical clearance for the study was obtained from the Ethical Review Committee of Aminu Kano Teaching Hospital, Kano with reference No; NHREC/21/SUB/2008/AKTH/EC/2194

Inclusion and exclusion criteria

All sputum samples that were confirmed as rifampicin resistant TB by GeneXpert were included in the study and all those that were not rifampicin resistant were excluded.

Specimen collection and processing

Sputum samples (in duplicate) that were submitted to TB reference laboratory for analysis were used in the study. A total of 175 smear-positive and 278 AFB smear-negative sputum samples were purposely selected. The samples were confirmed as rifampicin resistant TB using GeneXpert assay. The samples were collected in 50 ml wide-mouthed sterile falcon tubes according to NTBLCP SOP Manual (2011) and NCCLS (2000) and were then subjected to pretreatment involving digestion, homogenization, decontamination and concentration in

Biosafety Cabinet Level II (BSL II) using NALC-NaOH solution, 0.067M phosphate buffer (pH 6.8) and buffered Saline (PBS) according to NTBLCP SOP Manual (2011) and NCCLS (2000). The deposit of decontaminated sputum samples were also Ziehl-Neelsen stained and acid fast bacilli were detected. All sputum samples collected, reagents and equipment used were handled according to standard mycobacteriological procedures as described by NTBLCP SOP Manual (2011) and NCCLS (2000). The decontaminated sputum samples were then used for DNA Extraction.

DNA Extraction

DNA Extraction was done using chemical method with the aid of GenoLyse kit VER (1.0) LOT JD00051A according to manufacturer's instruction (HainLifescienceGmbH, Nehren, Germany, 2015) and WHO (2008). Using a sterile graduated pipette 0.5 ml of the decontaminated sputum sample was transferred into microcentrifuge tube. This was done for all the samples, after which the tubes were closed and centrifuged for 15 minutes at 10000xg. The supernatant was discarded and 100ml lysis buffer (A-LYS) was added and re-suspended by vortexing gently for 30 seconds. The tubes were arranged in a floater inside the BSC II and incubated for 5minutes in a water bath at 95^{o}C. Then, 100ml Neutralisation buffer (A-NB) was added and vortexed for 30 seconds and the tubes were centrifuged at maximum speed (10,000xg). The heavier debris formed the pellet and the lighter DNA (free from impurities) was suspended in the supernatant which was transferred into clean labeled micro-centrifuge tubes for further use NTBLCP SOP Manual (2011) and WHO (2008). .

DNA Master Mix Preparation and Amplification

The master mix was made up of 10µl of the AM-A and 35µl of AM-B Reagent which was placed in a PCR tube and mixed very well. This was prepared inside dead air box in a clean DNA free room. Then 5µl of each sample (containing the extracted DNA from above) was added to the corresponding tube containing the master mix and then mixed gently by pipetting up and down a few times. The PCR tubes were then placed in a 30 cycle (10 + 20) thermal cycler program for amplification (Hain Lifescience 2013 manual).

Hybridization and Detection

Hybridization was performed using an automated GT Blot 48 which allows the automated hybridization of the DNA and detection using STRIP technology was done according to the operator's manual (Hain Lifescience, Nehren, Germany). The results were interpreted based on the operating manual provided by the manufacturer (HAIN Lifescience GT-Blot 48 Operator manual, 2013).

RESULTS

The result of the study shows that 87% of the AFB smear positive samples were also identified as positive for *Mycobacterium tuberculosis* complex (MTBC) by the LPA. Whereas 79% of the AFB smear negative samples were detected as negative for MTBC by the LPA. However 13% of the AFB smear-positive samples were found to be MTBC negative with the LPA and 21% of AFB smear-negative samples were positive for MTBC using the LPA.

The study further revealed that 44.7% of the AFB smear-positive and 46.5% of AFB smear-negative samples were found to be rifampicin resistant only by LPA. Also, 36.8% of AFB smear-positive and 34.5% of the AFB smear-negative sample were found to be MDR-TB by the LPA. Most importantly, 15.1% of the smear-positive samples and 17.2% of AFB smear-negative samples were found to be susceptible to both rifampicin and isoniazid by the LPA.

Table 1: Status of Rifampicin Resistant TB (RR-TB) Sputum Samples using Acid fast Bacilli Smear and the Line Probe Assay

AFB SMEAR/LPA STATUS	DRUG-RESISTANCE TB STATUS			
	RMP-R only	**INH-R Only**	**MDR-TB**	**Susceptible to RMP and INH**
AFB Smear-positive/LPA Positive (n = 152)	68 (44.7%)	05 (3.3%)	56 (36.8%)	23 (15.1%)
AFB Smear-Negative/LPA Positive (n = 58)	27 (46.5%)	1 (1.8%)	20 (34.5%)	10 (17.2%)

Table 2: Drug-Resistance TB Status of Acid Fast Bacilli (AFB) Smear Positive and Smear Negative sputum samples using Line Probe Assay (LPA) (MTBDR *plus* VER 2.0)

ACID FAST BACILLI (AFB) SMEARS TATUS		LINE PROBE ASSAY (LPA) STATUS	
		LPA Positive (%)	LPA Negative (%)
AFB Smear Positive (n = 175)		152 (87%)	23 (13%)
AFB Smear Negative (n = 278)		58 (21%)	220 (79%)
TOTAL	**453**	**210 (46.4%)**	**243 (53.4%)**

DISCUSSION

Rapid detection of TB as well as all forms of drug resistant TB becomes necessary to interrupt the chain of transmission and acquisition of new TB as well as initiating prompt and accurate treatment. According to Sighn *et al*. (2017) the previous version of LPA (GenoType MTBDR*plus* version 1.0) has been evaluated only for high-grade smear-positive specimens and detection of drug resistance in culture isolates. This has led to the introduction of GenoType MTBDR*plus* version 2.0 which detects MTB and their DST patterns both in smear-positive, smear-negative and culture-positive samples.

The findings of this study indicated that the Genotype MTBDR *plus* Ver 2.0 detects more than 80% of the positive smears and 70% of the negative smears as positive and negative for MTBC respectively, which is closely related to the previous study conducted by Meaza *et al*. (2017) using direct smear negative sputum sample revealed that the sensitivity of Genotype MTBDR *plus* VER 2.0 LPA was found to be 77.8% for the detection of *M. tuberculosis* among smear negative samples. However, compared to the observation of this study which detected 21% of the smear negative samples as positive to MTBC by the LPA, Singh *et al*. (2017) in their study revealed that LPA was able to diagnose MTBC in 38.2% of Ziehl-Neelsen sputum smear-negative specimens and concluded that on comparing with composite reference standard, the LPA assay had 71.5% sensitivity and 100% specificity in the diagnosis of tuberculosis.

The study further revealed that 44.7% of the AFB smear-positive and 46.5% of AFB smear-negative samples were found to be rifampicin resistant only by LPA. In contrast to the observations of this study, Yadav *et al*. (2013) and Singh *et al*. (2017) in their studies reported that 1% of smear-positive and 27.6% of smear-negative strains were RIF-resistant.

The observations that 46.5 9% and 34.5% of the smear negative samples were identified as RIF-resistant and MDR-TB implies that although the samples were smear negative they could be a dangerous source of resistant TB. As such there is the need to also screen all smear negative TB samples using the LPA prior to discarding the samples. Also, compared to the observation of this study which reported RIF resistance of 46.5% among AFB smear negative samples, Singh *et al.* (2017) reported lower rates of RIF resistance of 27.6% among AFB smear negative samples in their study.

The study also indicated that 36.8% of AFB smear positive samples were found to be MDR-TB by the LPA and this are higher than the observations by Yadav *et al*. (2013) who revealed that 28% of smear positive strains in their studies were MDR-TB.

Singh *et al*. (2017) reported higher rates of INH-resistance of 24% among the smear negative strains compared to 1.8% reported in this study. Likewise this study (Table 2) reported lower rate of 3.3% of INH mono-resistance among the smear positive samples compared to 10% INH mono-resistance reported by Yadav *et al*. (2013). Most importantly, 15.1% of the smear positive samples were found to be susceptible to both rifampicin and isoniazid by the LPA. This reemphasizes the need to confirm all AFB smear positive samples using the LPA so as to avoid inappropriate administration of TB drugs which could be detrimental to the health of the patients and to avoid the risk of possible resistance development.

CONCLUSION

The present study demonstrated that the LPA can be used to detect the drug resistant status of MTBC of smear negative samples as 79% of them were detected as negative. The study however, reemphasizes the need to screen all smear-negative TB samples using the LPA prior to discarding the samples as some of the smear-negative TB samples were identified as RIF-resistant and MDR-TB in the study. The study also, recommends that all rifampicin resistant TB samples identified by genexpert assay should further be confirmed using LPA as some of them were found to be susceptible in this study, so as to avoid inappropriate administration of TB drugs which could be detrimental to the health of the patients.

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Competing interests:

The authors have declared that no competing interests exist.

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