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Detection of *Mycobacterium tuberculosis* by rapid molecular methods augments acid fast bacilli (AFB) smear microscopy in a non-culture tuberculosis laboratory

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Laboratory diagnosis of tuberculosis (TB) has evolved into simplified molecular procedures that are adaptable in routine settings. We compared the diagnostic performance of smear microscopy by Ziehl-Neelsen (ZN) for detection of acid fast bacilli (AFB) with two different rapid *Mycobacterium tuberculosis* (MTB) specific molecular methods; Xpert MTB/RIF (Xpert) and Geno Type MTBDR*plus* (MTBDR), using a total of 194 consecutive sputum specimens from cases of pulmonary tuberculosis (PTB) in Jos, Nigeria. AFB was detected in 20% of cases by SM; while MTB was detected in 21 and 24% cases by Xpert and MTBDR, respectively. Fifty two (27%) of 194 specimens tested, were positive for AFB, MTB or both. One of 52 (2.0%) AFB positive result was MTB negative while 11/52 (21%) MTB positive cases were AFB negative. There was concordance in 65% of positive results detected by the three methods. MTB drug resistance by the molecular methods occurred in 26 MTB positive cases, of which 8/26 (31%) were MDR-TB. The simultaneous detection of MTB and the associated drug resistance, using Xpert and MTBDR improved TB diagnosis in Jos, Nigeria. We suggest the use of parallel testing of sputum specimens by SM and Xpert, with the retesting of rifampicin (RIF) resistant presumptive MDR-TB cases by MTBDR, as algorithm for TB diagnosis in high TB burden countries with limited TB culture laboratories.

Key words: *Mycobacterium tuberculosis* acid fast bacilli, Ziehl-Neelsen, GeneXpert MTB/RIF, Geno Type MTBDR*plus*.

INTRODUCTION

Tuberculosis (TB) is one of the leading infectious diseases prevalent in low income countries where impoverished persons are at high risk of infection. The World Health Organization (WHO) estimates that about 3 million people living with TB are unreached annually while 450,000 infected persons mainly in Eastern Europe

and Asia develop multidrug resistance tuberculosis (MDR-TB) globally (WHO, 2013). Strategic interventions by global health organizations aimed at reducing TB mortality and improving methods of laboratory diagnosis resulted in the endorsement of two rapid molecular methods; GenoType MTBDR*plus* (MTBDR; Hain Lifescience GmbH, Nehren, Germany) and GeneXpert MTB/RIF (Xpert; Cepheid; Sunnyvale, CA), that simultaneously detect the presence of *Mycobacterium tuberculosis* (MTB) and the associated drug resistance from clinical specimens and culture isolates (WHO, 2008, 2011). Several studies have validated the diagnostic performances and suitability of the methods (Hillemann et al., 2005; Scott et al., 2011; Crudu et al., 2012; Caws et al., 2006; Barnard et al., 2012; Sekadde et al., 2013; Ocheretina et al., 2014). Consequently, Xpert has been recommended for use in HIV high-incidence, low-income settings (Steingart et al., 2013; Balcha et al., 2014).

Nigeria ranks 11th among the 22 high TB burden countries that account for 80% of global TB (WHO global TB report, 2013). With high burdens of TB, HIV and MDR-TB, the nation is scaling up implementation of Xpert at different tiers of diagnostic laboratories to serve the nation's population of about 170 million and TB incidence of 108/1000000 people (WHO, 2012). In Jos, North central region of Nigeria, implementation of Xpert commenced in February 2013 while MTBDR was in July 2010. Xpert serves only for routine diagnosis of TB in HIV positive patients, presumptive drug resistant cases and other risk groups while MTBDR is reserved for further testing of rifampicin (RIF) resistant cases detected by Xpert, presumptive MDR and clinical specimens that do not comply with Xpert technology.

Only few laboratories in Nigeria have capacity for TB culture. Thus, the national TB Directly Observed Treatment Short Course (DOTS) algorithm stipulates the commencement of TB treatment with positive AFB SM results despite the known limitations.

This study compared the detection of acid fast bacilli (AFB) using smear microscopy (SM) by Ziehl-Neelsen with two different MTB specific molecular methods: Xpert MTB/RIF and MTBDR in a non TB culture laboratory.

METHODOLOGY

A total of 194 consecutive sputum specimens were tested at the AIDS Prevention Initiative in Nigeria (APIN) laboratory Jos, Nigeria between February 2013 and April 2014. Specimens were registered for the study if patients were: referred from the DOTS-TB clinics in Jos, Nigeria, provisionally diagnosed for pulmonary TB and eligible for testing by Xpert. Of the total number of specimens tested, 151 were new or presumptive TB, 30 presumptive drug resistant TB and 13 treatment failed cases. Each of the specimens was tested using SM, Xpert and MTBDR methods.

Smear microscopy

Non homogenized sputum (direct) was used for smear microscopy as recommended by the National DOTS–TB (NTBLCP, 2009). Smears were prepared directly from un-concentrated sputum specimens and left to dry overnight in a class 11 biosafety cabinet, fixed with 5% phenol alcohol, stained by ZN method and examined for AFB.

MTBDR

DNA extraction with GenoLyse was performed by suspending 1 ml of 4% NaOH treated sputum specimen in 1.5 ml screw caped micro centrifuge tube and centrifuged for 15 min at 10,000 rcf. Pellet was re-suspended in 100 µl of lysate buffer, vortexed intermittently and incubated in a heat block at 95°C for 5 min and allowed to cool for 5 min after brief spinning. 100 µl of neutralization buffer was added to the lysate, vortexed and centrifuged for 5 min at full speed in a tabletop centrifuge. A positive control specimen (in-house verified positive specimen), was treated along with test specimens. Amplification mixture (43 µl) was dispensed into amplification tubes and 7 µl each of supernatant (DNA) of test and positive control specimens was added. Nuclease free water (7 µl) was used in place of DNA for negative control. PCR and detection were performed according to manufacturer's instruction (Hain Lifescience, 2014).

Xpert

Following the manufacturer's instruction, (Cepheid, 2010) reagent buffer containing NaOH and isopropanol was added in a 2:1 ratio to sputum to a final volume of at least 2 mL. After 15 min incubation at room temperature and intermittent hand mixing for liquefaction and inactivation, 2 ml of treated specimen was added to the cartridge containing DNA amplification mixture and fluorescent detection probes and then loaded onto the Xpert module. The semi nested real time amplification and detection in integrated reaction proceeded to completion in 2 h with results displayed as MTB negative or MTB positive (with semi-quantification) and RIF sensitive or resistant.

Statistical analysis

Statistical analysis of results was performed using the Statistical Package for Social Sciences (SPSS) version 17 for descriptives and Chi square distribution. P value < 0.05 was considered significant.

RESULTS

Detection of AFB and MTB

Fifty two of 194 (27%) specimens were positive by: SM; 40/194 (21%), Xpert; 41/194; (21%) and MTBDR; 46/194 (24%). A total of 34 (65%) positive results were concordantly detected by the three methods against18 (35%) discordance of which; 13/52 (25%) were AFB negative-

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Table 1. Acid fast bacilli and *M. tuberculosis* detected using smear microscopy, Xpert and MTBDR*plus*. N=52 (%).

Assay	Number positive	%
Concordant results (AFB, MTB)		
smear microscopy Xpert MTB/RIF GenoType MTBDRplus	34	65
Total positive cases	34	65
Discordant results (AFB, MTB)		
Smear microscopy Xpert MTB/RIF	3	6
Smear microscopy GenoType MTBDRplus	1	2
Smear microscopy	1	2
Total positive cases	5	10
Discordant results (MTB)		
Xpert MTB/RIF GenoType MTBDRplus	2	4
Xpert MTB/RIF	2	4
GenoType MTBDRplus	9	17
Total positive cases	13	25

MTB positive, 1/52 (2%), AFB positive- MTB negative and 5/52 (10) AFB positive-MTB positive but detected by either Xpert or MTBDR (Table 1). TBDR detected significantly higher positive results in each case than SM and Xpert (p=0.006) (Chi square analysis).

Drug susceptibility

Twenty six of the 51 (51%) MTB positive cases were resistant to drugs. MDR was detected in 8/26 (31%) cases and mono resistance detected in 6/26 (23%); RIF and 12/26 isoniazid (INH); (46%) (Table 2).

Table 2. Rifampicin and isoniazid resistant *M. tuberculosis* detected using XPert MTB/RIFand GenoType MTBDR*plus* N=26(%).

Resistant *M. tuberculosis*	XPert	MTBDR*
Rifampicin (mono- resistance)	3	6
Isoniazid (mono-resistance) *InhA* =11, *KatG* =1	N/A	12
MDR	3	8
Total	6	26

*Three additional cases of rifampicin mono resistant and 5 MDR were detected by MTBDR. N/A= Not applicable.

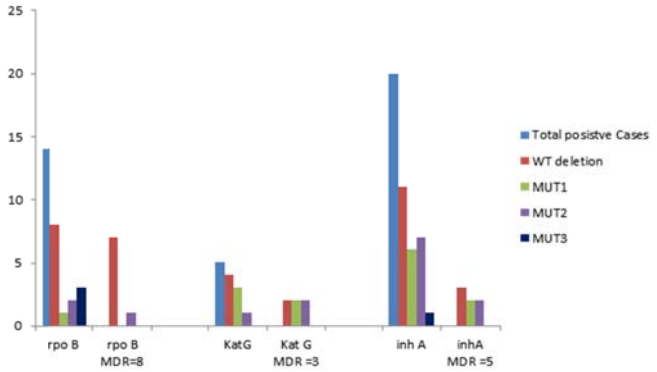

Figure 1. Resistance profiles of Rifampicin and isoniazid by GenoType MTBDRplus (N=26).

Mutation types detected by MTBDR

Mutation (MUT) type 3 on *rpoB* gene region conferring RIF resistance occurred in 3/14 (21%) followed by MUT2B 2/14 (14%) cases (Figure 1). Other RIF resistance types on the *rpoB* wild type regions occurred with no noticeable corresponding mutations in 8/14 (57%) cases (Figure 2, Lanes 2, 3, 4 & 5). INH mono resistance was more frequent on the i*nhA* gene region; 11/20 (55%) than on *katG* gene; 1/20 (5%). Three of 5 (60%) *katG* gene associated INH resistance were MDR (Table 2, Figure 1). MTBDR test strips of some results showing RIF and INH resistance profiles are shown in Figure 2.

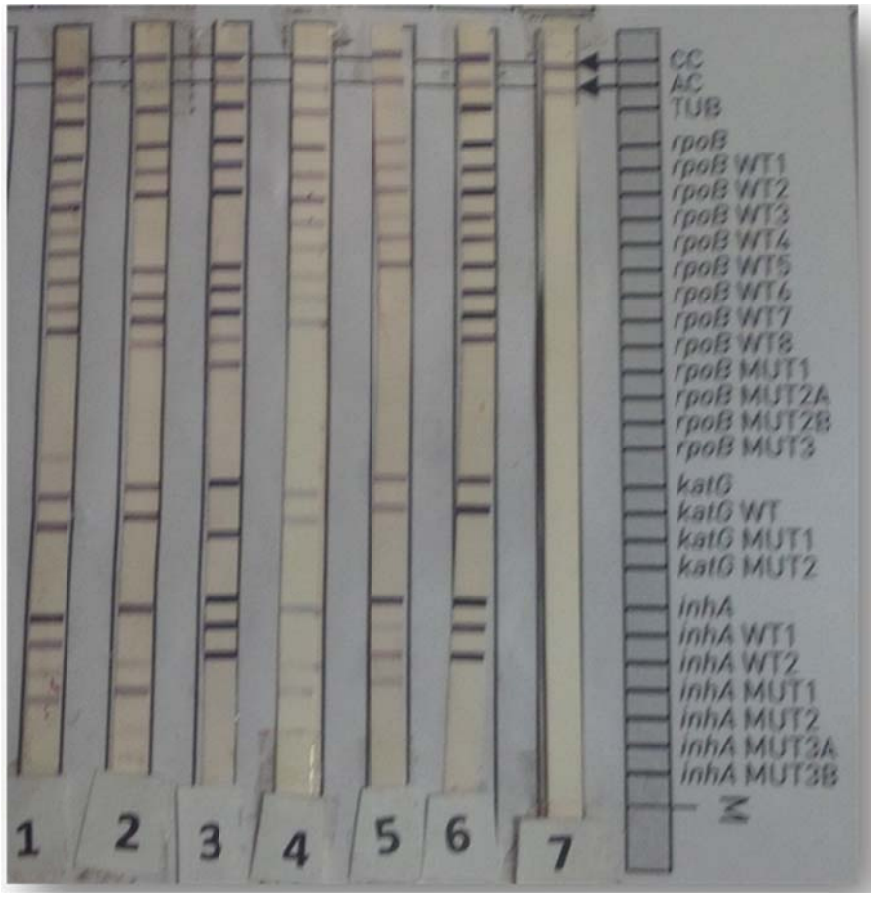

Figure 2. Test strips showing some results of Geno Type MTBDR*plus*. M = Results evaluation template, CC = Conjugate control, AC = Amplification control, TUB = *Mycobacterium tuberculosis* complex, WT = Wild type probe, MTU = Mutation; Lane 1. *rpoB* WT 8 deletion, *rpoB* MUTs 1 and 3 insertion = MDR. Lane 2. *rpoB* WTs 3 and 4 deletion, *inhA* WT 1 deletion, *inhA* MUT 1 insertion = MDR. Lane 3. *rpoB* WTs 3 and 4 deletion, *rpoB* MUT 1 insertion, *katG* WT deletion, *katG* MUT 1 insertion = MDR. Lane 4. *rpoB* WT 8 deletion, *inhA* WT 1 missing, *inhA* MUT 1 insertion= MDR. Lane 5 *rpoB* WTs 6, 7 and 8, deletion, i*nhA* MUT 1 = MDR. Lane 6. Positive control; RIF sensitive, INH sensitive. Lane 7. Negative control; NIL DNA.

DISCUSSION

Direct SM is generally used for TB diagnosis in most low income high TB burden countries due to the limited availability of the gold standard TB culture methods. This study compared SM for detection of AFB with Xpert and MTBDR for MTB. Our results indicated that 25% of the positive results were AFB negative while 2% AFB were MTB negative. Due to limitations of this study, further identification tests were not performed to ascertain the specific identities of “positive” and “negative” results detected. The results however suggested low prevalence of AFB positive non *M. tuberculosis* (NTM) infection in Jos, Nigeria. A previous study on the genetic diversity of Mycobacterium species in Jos Nigeria reported *M. tuberculosis* as the dominant genotype (Ani et al., 2010).

There was agreement in the detection of RIF resistance by Xpert and MTBDR (6/14; 43%) though MTBDR detected more MTB and RIF resistant cases. The finding was significant in the study setting considering the fact that decisions on treatment were based on the drug resistant profiles of either or both assays.

MTBDR also detected genetic profiles on respective gene regions of RIF and INH. Mutations (MUT 3 and MUT 2B) on *rpoB* gene region detected in this study have been reported in other geographical regions (Hirano et al., 1999; Mokrousov et al., 2004; Aparna et al., 2010). RIF resistance on the *rpoB* gene region with no observable corresponding mutation (Figure 2, Lanes 2, 3, 4 and 5) has been associated with mutations that may have occurred outside the region of analysis. (Ramasamy and Musser 1998; Huong et al., 2005; Hillmann et al., 2007) (Figure 2, Lanes 2 to 5).

The results also show that INH mono resistance

occurred more frequently on the *inhA* gene 10/20 (50%) regions than *katG* region; 1/20 (5%). Complete *katG* deletion is associated with a high level of INH resistance (MIC >5 µg/mL) (Tomasz et al., 2013). A total of 3/5 *kat G* related INH resistance in this study were MDR.

The use of the Xpert and MTBDR enhanced TB diagnosis in Jos, Nigeria by providing prompt TB diagnosis, especially in AFB negative cases, HIV patients and presumptive MDR-TB. The simultaneous detection of MTB and drug resistance was relevant for early and improved patients' management in the absence of MTB culture and drug susceptibility test (DST).

In conclusion, the molecular methods used in this study are simple to perform and adaptable in routine settings. We recommend that the assays be considered for routine use in the absence of TB culture laboratories. However due to the high cost of the equipment for molecular assays and issues of sustainability, the assays may not be readily affordable in most of the low income countries. These limitations underscore the need for increased funding to support the implementation and sustainability of the methods.

Conflict of interests

The authors did not declare any conflict of interest.

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