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Molecular diagnosis of MDR-TB using GenoType MTBDR*plus* 96 assay in Ibadan, Nigeria

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Summary: Multidrug resistant *Mycobacterium tuberculosis* (MDR-TB) is of great public health importance worldwide. This three month laboratory- based study (1st September-30th November, 2011) was carried out at the TB laboratories of the University College Hospital, Ibadan, Nigeria to determine the magnitude of MDR-TB using molecular based GenoType MTBDR*plus* 96 assay. Two sputum samples were collected from each subject. These were processed using Ziehl -Neelsen (ZN) reagents. The sputa were cultured on Loewenstein-Jensen egg –based medium and incubated at 37^0C for eight weeks. Mycobacterium tuberculosis complex (MTBC) was confirmed by colonial morphology and repeat ZN staining. All the Acid-fast bacill (AFB) positive smears and culture positive isolates were tested for genetic identification and drug susceptibility testing (DST) using PCR- based GenoType MTBDR*plus* 96 assay (HAINs Lifesciences, GmbH, Nehren, Germany) according to manufacturers' instructions. Of the 68 samples processed, 11 (16.2%) were AFB positive while six (8.8%) were positive for culture. Eleven (64.7%) out of the 17 samples tested for genetic identification were MTBC while six were Non Tuberculosis Mycobacteria (NTM). All NTM were from AFB positive sputum while none was from culture positive isolates. Of the six culture isolates tested for DST, three (33.3%) were susceptible to isoniazid and rifampicin; one (16.7%) showed mono-resistance to isoniazid while two (30.0%) were resistant to the two drugs. This study shows that MDR-TB is present in Ibadan. There is a need to make DST diagnostic facilities more available and accessible in Nigeria.

Keywords*:* Molecular diagnosis, MDR-TB, GenoType MTBDR*plus* 96assay, Ibadan, Nigeria

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INTRODUCTION

Tuberculosis (TB) is a chronic granulomatous infectious disease caused by members of the *Mycobacterium tuberculosis* complex (MTBC). According to the World Health Organization (WHO) report, one third of the current world population is infected with TB (WHO, 2010). The organism – *Mycobacterium tuberculosis* is one of the most successful pathogens in human history exerting enormous toll on public health. The resurgence of the disease globally has renewed interest in understanding its' epidemiology and pathogenesis (Rastogi and Sola, 2007, Stein, 2011).

Only a small fraction of the estimated 500,000 patients who have multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) and 1.37 million patients who have TB/HIV co-infections worldwide have access to drug susceptibility testing (DST) (WHO, 2009). MDR-TB is defined as TB that is caused by strain of *M. tuberculosis* that is resistant to both isoniazid and rifampicin - the two most important anti-TB drugs. Conventional methods such as smear microscopy and culture often lead to delay in diagnosis of infectious cases . This may be aggravated by disproportionate frequency of smear negative disease in HIV- associated TB (Perkins and Cunningham, 2007). The failure to quickly recognize and treat affected patients often lead to increased morbidity and mortality, secondary resistance including extensively drug resistant TB and on-going transmission within the community (Farmer *et al*, 1998; Van Rie and Enarson, 2006; Boehme *et al*, 2010). One of the most important advances in the field of TB research is the advent of molecular techniques that allow rapid diagnosis of both susceptible and MDR-TB strains.

GenoType MTBDR*plus*96 assay (HAINs Lifescience, Nehren, Germany) is a molecular based line probe technique that allows simultaneous detection of MTBC and MDR-TB. Resistance to rifampicin is achieved by detecting most common

mutations in the *rpo* gene while resistance to isoniazid is detected by presence of the most common mutations in the *katG* and *inhA* genes.

Unfortunately, this method is not readily available in many of the high burden countries including Nigeria. The burden of MDR-TB in Nigeria is largely unknown (WHO, 2010). This is mainly as a result of inadequate laboratory capacity to perform DST. Thus, this study was carried out to determine the magnitude of MDR-TB in Ibadan, Nigeria.

MATERIALS AND METHODS

The study was carried out at the TB laboratories of the Department of Medical Microbiology and Parasitology, University College Hospital (UCH), Ibadan, Nigeria. The TB laboratories at UCH consist of TB culture laboratory which has capacity for sputum smear microscopy and culture while molecular TB laboratory is equipped with facilities for rapid detection of MTBC and MDR-TB.

This was a three month (1st September- 30th November, 2011) laboratory based study in which sputum samples from new pulmonary TB patients were processed for microscopy, culture and DST. Information of the specimens that were sent to the laboratory was retrieved from request forms. Ethical approval was obtained from the University of Ibadan and UCH joint ethical committee.

Microscopy and culture

Each sputum sample was smeared, air-dried, fixed and stained with Ziehl -Neelsen (ZN) reagents. Samples containing saliva were discarded. A known positive Acid Fast Bacilli (AFB) and slide stained with egg albumin were used as positive and negative controls respectively. The results were read according to the grading system of the International Union Against TB and Lung Diseases (Enarson, 2000).

The sputum was decontaminated using N-Acetyl L-cysteine /Sodium Hydroxide (NALC/NaOH) method (Kent and Kubica,1985). The resulting solution was mixed by vortex mixer. About one ml from the mixture was inoculated into freshly prepared Loewenstein- Jensen (LJ) medium and incubated at 37^{o}C for eight weeks. *M. tuberculosis* strain H37Rv and sterile LJ medium were used as positive and negative controls respectively. Contamination on culture was determined by looking for visible growth before two weeks of incubation while suspicious isolates were confirmed by colonial morphology, ZN reaction and standard biochemical tests (Barrow and Feltham, 1995).

Drug susceptibility testing (DST)

All AFB smear positive sputum and culture positive isolates were subjected to DST using GenoType MTBDR*plus* 96 assay. The procedure was divided into three stages: namely DNA extraction, PCR amplification and hybridization using MTBDR*plus* dedicated twincubator.

DNA extraction from AFB positive sample and MTBC isolate on LJ slope were carried out using boiled lysate. Briefly, AFB positive samples were treated with NALC-NAOH thereafter, 500µL of decontaminated sample in glycerol storage solution was aliquot into labeled 2.0 ml screw cap tubes. A loopful of isolate on LJ medium was inoculated in 300µL of molecular grade water (MGW). The 2 samples were spinned for 15 minutes in a table –top centrifuge with aerosol tight rotor inside class 11 safety cabinet at 10,000rpm. Thereafter, supernatant were discarded while sediment was re-suspended in 100 µL of MGW by vortexing. The sediment was then incubated inside water bath at 99^{o}C for 20 minutes. The 2.0 ml screw tube containing the sediment with the bacteria was placed in the sonicator bath for 15 minutes. Thereafter, bacteria in the tubes were spinned down for 5 minutes at full speed. Five mls from the supernatant was used as DNA solution for PCR reaction.

Five µL of the DNA solution was added to already prepared 45 µL of master mix to give a final volume of 50µl. A control sample was prepared using 5 uL of water as against 5 µL of DNA. Detection was achieved through hybridization of the amplified DNA on MTBDR*plus* specific twincubator. The entire procedure was carried out according to the manufacturers' instructions (Hain Lifescience, Nehren, Germany,2012). Data was analyzed by using frequency tables and percentages.

RESULTS

A total of 68 sputum samples were processed during the study period. Out of this, 11 (16.2%) were AFB positive while the majority, 57 (83.3%) were negative. Six (8.8%) were culture positive while a greater percentage 62 (91.2%) were negative for culture.

Seventeen positive samples consisting of 11 AFB positive sputum (64.7%) and six culture positive isolates (35.3%) were subjected to DST, out of which 11 (64.7%) were MTBC (5 AFB positive and 6 culture positive) while six (35.3%) were Non Tuberculosis Mycobacteria (NTM). Figure 1 shows GenoType MTBDR*plus* 96 assay banding patterns of the MTBC.

Of the six culture isolates tested for DST, three (33.3%) were susceptible to isoniazid and rifampicin; one (16.7%) showed resistance to only isoniazid; two (30.0%) were resistant to the two antibiotics while none was NTM strain (Table 1), all the 5 confirmed AFB positive isolates were susceptible. The six NTM strains (54.5%) were all from AFB positive sputum.

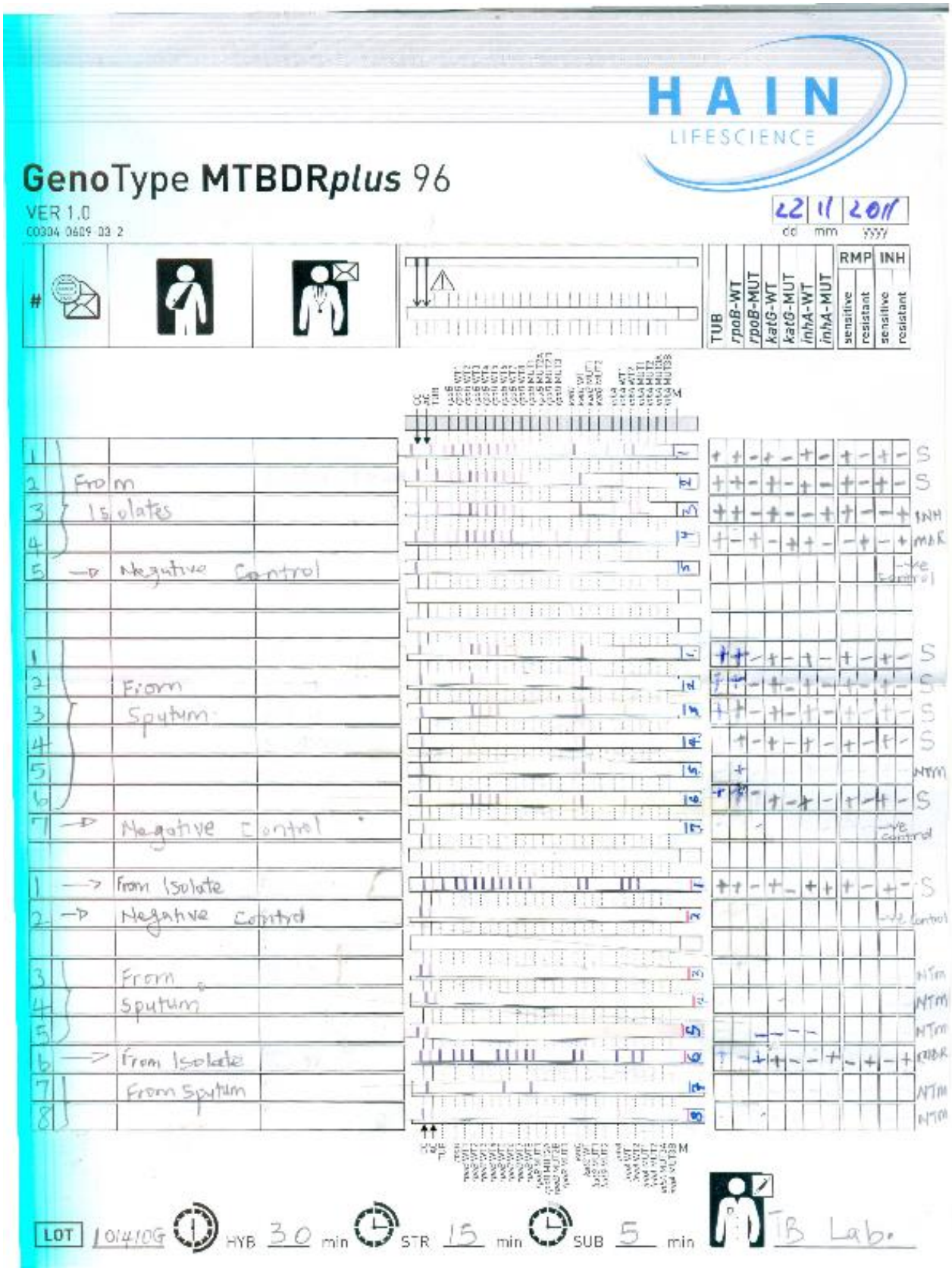

Fig 1. Banding pattern of MTBC

Table1. Drug susceptibility results by specimen

Specimen	Drug Susceptibility Result			
	Susceptible	INH Resistance	MDR-TB	NTM
	No (%)	No (%)	No (%)	No (%)
AFB positive Sputum (n=11)	05 (45.5%)	0 (0.0%)	0 (0.0%)	06 (54.5%)
Clinical isolates (n=06)	03 (50.0%)	01 (16.7%)	02 (33.3%)	0 (0.0%)
Total (n=17)	08 (47.1%)	01 (5.8%)	02 (11.8%)	06 (35.3%)

DISCUSSION

Multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) is defined as TB caused by mycobacteria isolate that is resistant to the two first-line anti-TB drugs - rifampicin and isoniazid. The disease poses a serious challenge to TB control due to its complex diagnostic and treatment obstacles (WHO, 2010). We present report of a study on molecular diagnosis of MDR-TB using GenoType MTBDR*plus* 96assay.

This study shows PTB prevalence of 16.2% by AFB positivity and 8.8% by culture. This does not agree with 1.5% reported for smear microscopy in Ilorin, Nigeria (Salami and Oluboyo, 2008) and 3.3% to 2.2% for microscopy and culture at the same center years back (Kehinde *et al*, 2010). The higher rate obtained in the present study may be due to the fact that the sputum samples were from symptomatic clinically confirmed PTB patients.

Six (35.3%) of the 17 samples submitted for genetic identification were found to be NTM while 11 (64.7%) were MTBC (Fig 1). This may suggest that diagnostic tools used in this study especially smear microscopy have low specificity. The fact that all the six NTM strains were from AFB positive sputum (Table 1) emphasizes the need to isolate the causative organism in pure culture. More so, as smear microscopy does not distinguish MTBC which causes human disease from non-pathogenic NTM. All the tested culture isolates were MTBC (Table 1). Information on AFB negative but culture positive samples was not documented. This kind of laboratory report may be seen in patients with TB/HIV co-infections (Boehme *et al*, 2010). The inability to accurately diagnose and treat such patients may lead to increased morbidity, mortality and ongoing transmission in the community (Van Rie and Enarson, 2007; Boehme *et al*, 2010).This is one of the limitations of the study.

Two (30.0%) of the six tested culture isolates were MDR-TB strains while none was from AFB positive sputum (Table 1). This implies that culture isolation is more reliable for detection of DST than AFB positive sputum specimen. This may be due to the fact that pure isolate on culture medium provides more quality and quantifiable DNA than AFB positive clinical specimen. This is demonstrated by the fact that banding patterns are more pronounced in samples containing culture isolates than those of AFB positive sputum (Fig 1). The fact that 6-8 weeks incubation period is required to cultivate the organism on solid culture may limit its use for DNA extraction as this may cause diagnostic delay. More studies are needed to address poor DNA yield from AFB positive clinical sample in order to improve its suitability for DST testing.

The GenoType MTBDR*plus* 96 assay (HAIN Lifescience, Nehren, Germany) is a DNAstrip technology that allows for genetic identification of the MTBC and its resistance to rifampicin and/or isoniazid from cultivated samples or pulmonary smear-positive clinical specimens. Identification of rifampicin resistance is enabled by the detection of the most significant mutations of the *rpoB* gene (coding for the B-subunit of the RNA polymerase). Detection of high level isoniazid resistance is shown by the presence of the *katG* gene (coding for catalase peroxidase) while low level isoniazid resistance is demonstrated by the presence of promoter region of the *inhA gene* which codes for the NADH enoy ACP reductase.

In this study, one culture isolate (16.7%) showed mono resistance to isoniazid. This isolate shows the presence of i*nhA* mut1 which signifies low level isoniazid resistance. The clinical implication of the isolated low level isoniazid resistance needs further investigations, unlike rifampicin resistance which has been proved as an indicator for detection of MDR-TB in about 90% of cases (Piatek *et al*, 1998; El-Hajj *et al*, 2001, Klopper et al, 2013).

In conclusion, we found that MDR-TB is present in Ibadan and that DNA extraction from isolates on solid culture gives better result than those from AFB positive sputum. More studies are needed to improve suitability of clinical specimens for DST testing.

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