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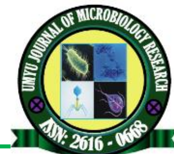

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Phenotypic and Genotypic Characterization of Multi-Drug Resistant *Mycobacterium tuberculosis*

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Abstract

This study characterized multi-drug resistant M. tuberculosis phenotypically by LJ-proportion method and genotypically by Geno Type MTBDR plus LPA. Out of the forty M. tuberculosis isolates tested, two (5.0%) were found to be multi-drug resistant by LJ proportion method and one (2.5%) was MDR by LPA. None was found to be mono-resistant to any of the drugs by LJ however, one isolate was mono resistant to RIF and one was mono resistant to INH by LPA. Comparison of Geno Type MTBDRplus LPA and phenotypic LJ-proportion methods showed that one isolate was mono resistant to RIF and one was mono resistant to INH by LPA, one and two MDR-TB isolates respectively were characterized by genotypic and phenotypic methods. The remaining isolates were found to be pan susceptible by both methods. One isolate was characterized as MDR with bands at rpoβ MUT2A region and ihnA MUT2 corresponding to H526Y and A16G mutations respectively. Rifampicin mono resistance with band at rpoβ MUT3 corresponding to S531L was found in one isolate. Also, isoniazid mono resistance was observed in one isolate with ihnA MUT2 band corresponding to A16G mutation. This study has shown an overall high prevalence of MDR-TB in the study area which needs to be urgently addressed. Laboratory facilities for rapid drug resistance detection are needed across the country for early and accurate diagnosis of TB and drug resistant cases. This remains an important step in managing TB drug resistance in Nigeria.

Key words: Multi-drug resistance, *M. tuberculosis*, phenotypic, genotypic, LJ-proportion, Geno Type MTBDR*plus* LPA, RIF, INH

INTRODUCTION

Multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* have emerged worldwide. In many countries and regions, these resistant strains constitute a serious threat to the efficacy of tuberculosis control programs. An important element in gaining control of this epidemic is developing an understanding of the molecular basis of resistance to the most important antituberculosis drugs; isoniazid and rifampicin. On the basis of this information, more exacting laboratory testing, and ultimately more appropriate and timely treatment regimens, can be developed (Somoskovi *et al.*, 2001).

Drug resistance in *M. tuberculosis* occurs as a result of random spontaneous chromosomal mutations during natural cell replication. These mutations are not drug induced and are not linked. The probability of a drug-resistant mutant occurring is directly proportional to the size of the bacterial population. The frequency of primary resistant organisms varies for each drug; however, it is usually 1 in 10^6 to 1 in 10^8 (Iseman, 1993).

Spontaneous resistance to isoniazid is estimated to occur once in every 10^6 organisms, and to rifampicin once in every 10^8 organisms. The probability of spontaneous mutants being simultaneously resistant to two or more drugs is the product of the individual mutants. The development of drug resistance is a man made amplification of a naturally occurring phenomenon. Previous treatment for tuberculosis predisposes to the selection of multi drug resistant organisms. Non compliance is a major factor in allowing the resistant organisms to survive (Iseman, 1993). Multi drug therapy is used to prevent the emergence of drug resistant mutants during the long duration of treatment.

Resistance can be classified as single-drug, multi-drug, or poly-drug resistance depending

on the number of drugs and/or which drugs are involved (Rieder, 1999).

Although an unequal global distribution of drug resistance exists between poor and rich countries, the problem is global. The regions where drug-resistant TB is more prevalent lack the resources to implement adequate measures to control even the susceptible types of the disease (Espinal *et al.,* 2001; Cohen *et al.,* 2003).

Molecular methods for MDR-TB detect the common mutations conferring resistance to RIF and INH, rather than the resistance phenotype. The commercially available line probe assays involve DNA extraction, polymerase chain reaction (PCR), and solid phase reverse hybridization of amplified DNA to probes covering the core region of the target gene, immobilized on a nitrocellulose strip. These tests can be applied on MTB isolates or on smear positive sputum (De Beenhouwer *et al.,* 1995; Rossau *et al.,* 1997).

The GenoType® MTBDR assay (Hain Life sciences, Nehren, Germany) simultaneously detects the common mutations in the *rpoB* and *katG* gene (Hillemann *et al.,* 2005). The GenoType® MTBDR*plus*, a newer version of the genotype MTBDR detects more of the common mutations in the *rpoB* and *katG* genes, and also mutations in the *inhA* promoter region, making it the most sensitive line probe assay for detection of resistance (Hillemann *et al.,* 2007).

Evaluation studies of these assays have reported sensitivity and specificity of 98-100% for rifampicin, and of 70-100% for isoniazid, with results in 1-3 days (Ling *et al.,* 2008).

Most of these studies were performed in developed countries and there is limited data on the performance of the tests in developing countries (Ling *et al.,* 2008). A major limitation of these assays in developing countries could be the expertise in molecular biology required to perform them correctly, the unidirectional work flow laboratory infrastructure and the cost of molecular assays.

This study therefore, was aimed at characterizing multi-drug resistant *M. tuberculosis* by phenotypic and genotypic techniques in Zaria, Kaduna State.

MATERIALS AND METHODS

Mycobacterial Isolates

The test isolates *M. tuberculosis* were obtained from National Tuberculosis and Leprosy Training Centre, Saye, Zaria, Nigeria.

The isolates were confirmed by standard Microbiological techniques. They were examined for morphology by making a smear and staining by the Ziehl-Neelsen procedure. Typical acid fast bacilli were further subjected to test according to the manufacturer's instructions by SD BIOLINE TB Ag MPT64 Rapid; a rapid immuno chromatographic identification test for the *M. tuberculosis* complex (MTBC) that uses mouse monoclonal anti-MPT64. The kit has sensitivity and specificity of 98.6% and 100% respectively. The test cassette consists of a sample pad, a gold conjugate pad, a nitrocellulose membrane and an absorbent pad. Mouse monoclonal anti-MPT64 was immobilized on the nitrocellulose membrane as the capture material (test line). Another antibody which recognized another epitope of MPT64, conjugated with colloidal gold particles was used for antigen capture and detection in a sandwich type assay.

Four colonies were suspended in 200 µl of the extraction buffer prior to the test. The cassette was removed from the foil pouch and placed on a flat dry surface. One hundred micro litres (100 µl) of the suspended colonies in buffer was added into the sample well. As the test began to run, a purple colour moved across the result window in the centre of the device. After 15 minutes of sample application, the appearance of two colour bands ("T" test band and "C" control band) within the result window was considered a positive result. Confirmed MTBC isolates were stored at -20ºC for further use.

Drug susceptibility assays

Susceptibility to isoniazid (INH) and rifampicin (RIF) was determined by the proportion method on Lowenstein Jensen egg based slopes containing different concentrations of INH and RIF (0.2 µg/ml and 40 µg/ml respectively) (Canetti *et al.,* 1969). Standard antibiotic powders (INH and RIF) were obtained from Sigma-Aldrich (Lot. No. SLBC 3024V and SLBD respectively).

The inoculum was prepared by directly suspending colonies grown for approximately three weeks on Lowenstein Jensen drug free slopes to a turbidity equivalent to a 1.0 MacFarland standard. The 1.0 MacFarland standardized suspension was further diluted to 10^{-1}, 10^{-2} and 10^{-3}. The 10^{-1} suspension was subsequently inoculated on the drug-containing medium. Three drug-free LJ slopes were inoculated with 1:10, 1:100 and 1:1000 diluted suspensions of a 1.0 MacFarland standardized inoculum. This was done for each sample tested. The drug-susceptible MTB reference strain ATCC 27294 (H37Rv) was used as a susceptible control and known resistant strains (ATCC 35825 H37Rv for INH and ATCC35838

H37Rv for RIF) were used as resistant controls. The slopes were incubated at 37°C and read after 4 and 6 weeks. An isolate was considered resistant if the proportion of bacilli resistant to the critical concentration of a drug exceeded 1% (Canetti *et al.*, 1963; Canetti *et al.*, 1969).

Genotypic assays were carried out by the GenoType® MTBDR*plus* molecular line probe assay according to the manufacturer's specifications as follows:

DNA extraction

Sterile plastic loops (one per sample) were used to suspend colonies from the LJ slants into 2ml micro centrifuge tubes containing 100 µl molecular grade water and centrifuged at 10,000xg for 15 min. The supernatant was discarded and the pellet re-suspended in molecular grade water, to ensure that the suspension of extracted DNA was free of impurities that might inhibit the PCR. The re-suspended solutions were vortexed until they appeared slightly opaque (milky). The tubes were arranged in floating racks, placed in water bath and heated at 95 ºC for 20 min. This was to kill the bacilli and partially lyse the cells, thereby rendering the solution non infectious and to obtain a higher yield of DNA.

The tubes were placed in ultrasonic bath and incubated for 15 min. The powerful ultrasonic shockwaves created by the sonicator disrupted the cell walls of the tubercle bacilli, causing further cell lyses, and releasing the DNA and other cell debris into the molecular grade water. Finally, the tubes were centrifuged at maximum speed (10,000xg) to separate the impure cell debris (containing the cell wall, proteins and other macromolecules) from the DNA. The heavier debris formed the pellet and the lighter DNA (free from impurities) was suspended in the supernatant. The supernatants were transferred into clean labeled micro centrifuge tubes for further use.

PCR amplification of the extracted DNA

The master mix was prepared in clean DNA-free room. The master mix was made of five components with a total volume of 45µl for each PCR reaction (35µl of the primer nucleotide mix (PNM), 5µl of buffer, 2µl of $MgCl_2$, 3µl of molecular grade H_2O and 0.2 µl of *Taq* polymerase). The master mix was then well mixed and 45µl required for each specimen was transferred into the PCR tubes and 5µl of each sample was added to the corresponding tube containing 45 µl of master mix and then mixed by gently pipetting up and down a few times. The tubes were mixed slightly and spun down for 5 - 10 seconds in a mini-centrifuge before they were placed into the thermal cycler for amplification. A 30 cycle (10 + 20) thermal cycler program was used for the amplification. This involved ten cycles of denaturation at 95 ºC for 30 seconds and elongation at 58 ºC for 120 seconds; followed by an additional 20 cycles of denaturation at 95 ºC for 25 seconds, annealing at 53 ºC for 40 seconds, elongation at 70 ºC for 40 seconds and final extension at 70 ºC for 8 minutes. The amplicons were used for further analysis.

Denaturation of DNA

The TwinCubator® (shaking water bath) was pre-warmed to 45 ºC and 20µl of denaturation solution (NaOH) was added to each labeled well of the TwinCubator® tray followed by the addition of 20µl of the amplicons respectively. The mixture was mixed gently by pipetting up and down five times and then incubated at room temperature for 5mins.

Hybridization and detection

One (1) ml of the pre-warmed hybridization buffer (HYB) was carefully added to the wells using a pipette. The tray was then carefully tilted back and forth so that the purple denaturation solution and green hybridization buffer were thoroughly mixed. The tray was placed on the TwinCubator® and the labeled strips added to each well ensuring that the strips were completely covered by the liquid. This was incubated at 45ºC for 30mins. After incubation, the glass panel lid was opened and the condensate that formed during the incubation wiped off. The HYB buffer was aspirated completely from each well using a Pasteur pipette. One (1) ml of the pre-warmed red stringent wash buffer (STR) was dispensed into the tray using a multi-channel pipette avoiding the contact of the strips with the tips. After 15 minute incubation at 45 ºC in the TwinCubator®, STR buffer was aspirated and disposed of with a Pasteur pipette. The STR buffer was washed off with 1 ml of Rinse solution (RIN) for 1 minute. One (1) ml of the Conjugate (CON) solution was dispensed into each well and the glass panel lid was closed and incubated for 30 minutes on the TwinCubator®. The strips were washed twice with 1 ml of Rinse solution (RIN) for 1 minute in the TwinCubator® to wash off the excess CON solution. One (1) ml of sterile distilled water was added to each strip-containing well, and a 1 minute wash performed on the TwinCubator® to wash off the RIN solution after which the distilled water was completely decanted. One (1) ml of the Substrate solution was dispensed into each well and incubated for 15 minutes on the TwinCubator® after which the Substrate solution was aspirated and the strips washed twice with sterile distilled water. A pair of clean tweezers was used to remove the strips

from the TwinCubator® tray and placed onto absorbent paper. The developed strips were partially dried and transferred to the GenoType® MTBDR*plus* score sheet.

RESULTS

Out of the forty *M. tuberculosis* isolates tested, two (5.0%) were found to be multi-drug resistant by LJ-proportion method and one (2.5%) was MDR by LPA. None was found to be mono-resistant to any of the drugs by LJ-proportion method, however, one isolate was mono resistant to RIF and one was mono resistant to INH by LPA (Figure I).

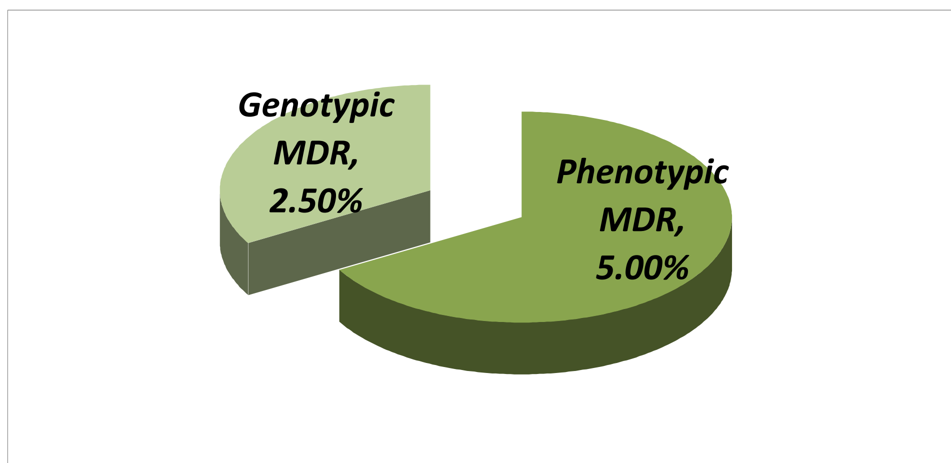

Figure I: Phenotypic and Genotypic Multi drug resistant *M. tuberculosis*

Table 1 shows the comparison of Geno Type MTBDR*plus* LPA and phenotypic LJ-proportion methods. The comparison showed that one isolate was mono resistant to RIF and one was mono resistant to INH by LPA only and none of such by the LJ Proportion method, one and two MDR-TB isolates respectively were characterized by genotypic and phenotypic methods. The remaining isolates were found to be pan susceptible by both methods.

Table 1: Comparison of Geno Type MTBDRplus LPA and phenotypic LJ-proportion methods

Susceptibility	Geno Type MTBDR*plus*	phenotypic LJ-proportion
RIF mono-resistance	1	0
INH mono-resistance	1	0
MDR-TB	1	2
Pan susceptible	37	38
Total	40	40

The banding patterns of mutations associated with rifampicin and isoniazid resistance in multidrug resistant and mono-resistant strains detected by MTBDR *plus* is shown in Table 2. One isolate was characterized as MDR with bands at *rpoB* MUT2A region and *ihn*A MUT2 corresponding to H526Yand A16G mutations respectively. Rifampicin mono resistance with band at *rpoB* MUT3 corresponding to S531L was found in one isolate. Also, isoniazid mono resistance was observed in one isolate with *ihn*A MUT2 band corresponding to A16G mutation.

Table 2: Patterns of gene mutations detected by Geno Type MTBDRplus assay in drug resistant *M. tuberculosis* isolates

Gene	Band	Gene region or mutation	MDR strains(n =1)	RIF mono resistant strains(n = 1)	INH mono resistant strains(n = 1)
rpoβ					
	WT1	506-509			
	WT2	510-513			
	WT3	513-517			
	WT4	516-519			
	WT5	518-522			
	WT6	521-525			
	WT7	526-529			
	WT8	530-533			
	MUT1	D516V			
	MUT2A	H526Y	1		
	MUT2B	H526D			
	MUT3	S531L		1	
katG					
	WT	315			
	MUT1	S315T1			
	MUT2	S315T2			
inhA					
	WT1	-15/-16			
	WT2	-8			
	MUT1	C15T			
	MUT2	A16G	1		1
	MUT3A	T8C			
	MUT3B	T8A			

Key: WT= Wild Type, MUT= Mutant, D=Aspatate, V=Valine, H=Histadine, Y=Tyrosine, S=Serine, L=Leucine, T=Threonine, C=Cysteine, A=Alanine, G=Glycine

DISCUSSION

The most important measure of TB drug resistance is the number of new cases that are MDR-TB (Dye *et al.*, 2002). This study showed a prevalence rate of 5.0% (2/40). The development of drug resistance is a man made amplification of a naturally occurring phenomenon. Previous treatment for tuberculosis predisposes to the selection of multi drug resistant organisms. Non compliance is a major factor in allowing the resistant organisms to survive. The availability of drugs in the open market and a private sector that delivers drugs to the population in an unregulated fashion in Nigeria could also be factors that might favour development of MDR-TB.

The genotypic drug resistance assay revealed that all the resistant isolates were hetero-resistant; a phenomenon where some cells within a population may remain susceptible to the antibiotic, whereas other cells display varying degrees of drug resistance. This was determined by the simultaneous detection of the wild type and mutant molecular susceptibility (Foundation for innovative new diagnosis, 2012).

In patients infected with a fully susceptible strain, drug resistance can develop gradually during inadequate treatment due to selection of cells with random mutations in sites associated with drug resistance (i.e. secondary resistance). In this case, as the proportion of susceptible cells decrease and resistant cells increase, a hetero-resistant population of cells will be present. These cells are primarily identical throughout the genome but a proportion of the population differs in sites associated with drug resistance (Foundation for innovative new diagnosis, 2012).

In contrast, patients infected with fully susceptible strains may develop mixed infections if they are co-infected with another strain that is drug resistant, resulting in a mixed population of two genetically distinct strains, one drug susceptible, and the other drug resistant.

Also, hetero-resistance likely represents a natural variation in the population of cells of *M. tuberculosis* (Foundation for innovative new diagnosis, 2012). Prescription of inadequate treatment regimen, irregular drug supply, poor drug quality with low bioavailability, and poor compliance among the study population could

be attributed to the development of hetero-Discordance between genotypic and phenotypic assays was observed in one isolate with RIF mono resistance. The isolate was classified as MDR by the phenotypic assay. This could be due to silent mutation or synonymous single nucleotide polymorphism (sSNP) at the target site. Silent mutations do not result in structural changes in the *inhA* and so do not interfere with its inhibition by INH. Moreover, findings of silent mutations in *inhA* are not surprising as SNPs occur every 3 kb of MTB genome (Comas *et al.*, 2011). Ando, *et al.* (2014) reported a silent mutation in a significant number of INH resistant *M. tuberculosis* clinical isolates. Mutations conferring INH resistance in other genes such as *mabA* (G609A) (Ando *et al.*, 2014) *aphC* (alkyl hydroperoxide reductase), *kasA* (ß-ketoacyl-ACP synthase) and *nadh* (NADH dehydrogenase) (Cohn *et al.*, 1997; Balasubramanian *et al.*, 2012) not included in Geno Type MTBDR*plus* have also been reported.

The critical concentration of INH on LJ medium which is being used for over 50 years is 0.2µg/ml (Jamieson *et al.*, 2014). Lower critical concentrations of 0.0312µg/ml for low-resistance observed in this study. level resistance and 0.125µg/ml for high-level resistance were reported by Gumbo, (2010).

CONCLUSION AND RECOMMENDATION

The findings showed that one isolate each was mono resistant to RIF and INH by LPA, one and two MDR-TB isolates respectively were characterized by genotypic and phenotypic methods. The remaining isolates were found to be pan susceptible by both methods. One isolate was characterized as MDR with bands at rpoß MUT2A region and ihnA MUT2 corresponding to H526Yand A16G mutations respectively. Rifampicin mono resistance with band at rpoß MUT3 corresponding to S531L was found in one isolate. Also, isoniazid mono resistance was observed in one isolate with ihnA MUT2 band corresponding to A16G mutation.

The use of robust molecular techniques such as DNA sequencing employed for the detection of occult cases with low-level resistance and other resistance not included in the Geno Type MTBDRplus kit is recommended.

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