Detection of Resistant-Associated Mutations and Genetic Profile of Resistance to Rifampicin and Isoniazid among Multi-Drug Resistant *Mycobacterium tuberculosis* Isolates from Plateau State.

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ABSTRACT
INTRODUCTION: This study was carried out to detect the most common resistant-associated mutations and genetic profile of resistance to rifampicin and isoniazid among MDR. *M. tuberculosis* isolates. METHODS: A total of 20 MDR *M. tuberculosis* isolates obtained from presumptive MDRTB cases among new and previously treated case TB in Plateau State by conventional Mycobacteriological techniques. *Mycobacterium Tuberculosis* Drug Resistant plus line probe assay (MTBDR plus) was used to analyzed the isolates.
RESULTS: Findings from this analysis indicated that the most frequent mutation at the rpoB gene occurred at codon (H526Y), that of Kat G mutation occurred at codon (S315T1) while that of inh A gene at codon (C15T). Heterogenic genetic profile of resistance (GPR) rate of 31.9% in the rpoB gene of RIF resistant isolates and homogenic (GPR) of 70% in katG gene of INH resistant isolates were reported in this study. Also, One mutation that occurred as a result of loss of WT8 (S531t) band which is an uncommon mutation was discovered. The result suggests a high degree of genetic variability and frequency of resistant conferring mutation involving codon H526Y of the rpoB gene and codon S531T in Kat G gene.
CONCLUSIONS: This study found a high level of heterogeneity and homogeneity in the genetic profile of resistance in rifampicin (RIF) and isoniazid (INH) respectively, which probably may have been responsible for high –level of resistance to RIF than INH by the *Mycobacterium tuberculosis* Isolates in the study population.

Key words: Multiple Drug Resistance, Mutation, Gene, Isolates, *Mycobacterium Tuberculosis*

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INTRODUCTION

The emergence of multi-drug resistant *Mycobacterium tuberculosis* strains in Nigeria has threatened concerted efforts towards tuberculosis treatment, prevention and control (1), The Prevalence of MDR *Mycobacterium tuberculosis* in Nigeria varies from region to region. It also depends on the study population and method used (2). The estimated MDR-TB prevalence in Nigeria is 2.2% and 9.4% among new and retreatment cases respectively (2). Survey on prevalence of drug resistance TB reported 4.8% MDR-TB in Nigeria, 2.9% MDR-TB among new cases and 14.3% MDR-TB in retreatment TB cases (3). A prevalence of 7.8% MDR-TB among TB cases in Jos University Teaching Hospital has been reported (4). Some private care facilities reported 4% in new cases and 18% retreatment cases of MDR-TB in Jos, Nigeria (5)

The development of drug resistance in *M. tuberculosis* isolates is the result of random genetic mutations in particular genes conferring resistance (6). The rifampin (RMP)-resistant *M. tuberculosis* isolates have mutations located in 81-base pair core region of the rpoBgene (7). On the other hand, the mutations causing isoniazid (INH) -resistance are located in several genes and regions (8). Also, about 50to 95% of INH-resistant strains have been found to contain mutations in codon 315 of the katG gene and an additional 10 to 15% have mutations in the ahpC-oxyRintergenic region often in conjunction with katG mutations outside of codon 315 (9).

The utilization of genotype MTBDR-plus technique in the rapid detection of MTB complex and resistance determination of rifampicin and isoniazid systemic has provided the necessary impetus for the treatment of tuberculosis (10) The genotype MTBDR-plus method has an advantage over the conventional drug susceptibility test (DST) which is cumbersome and time-consuming. The conventional DST may delay the administration of first anti-MTBdrugs and could therefore increase the infectiousness of individuals with the disease leading to transmission. The MTBDR-plus was designed to enhance TB treatment;unfortunately this assay is not routinely applied in most TB treatment centers especially in the developing countries. Therefore, the gap in the proper diagnosis and treatment of MTB complex still exist.

The detection of mutations conferring resistance to RMP and INH in MDR. *M. tuberculosis*strain is significant in determining the treatment course of suspected drug-resistant cases of tuberculosis patients. The rapid determination of drug resistance in clinical isolates of Mycobacterium tuberculosis is the pre-requisite for the initiation of effective chemotherapy, ensuring successful treatment of the patient and preventing further spread of drug-resistant isolates (11). This study is to determine the frequency of resistant mutations associated with RIF and INH, and its genetic profile of resistance.

MATERIALS AND METHODS

The study population consists of new cases and previously treated cases of tuberculosis with or without history of resistance. For the new cases, there was no report of resistance while the previously treated cases were included based on history of resistance. Multidrug resistant *M. tuberculosis* strains were obtained from new cases and previously treated tuberculosis patients in Plateau State (2015-2017) for this analysis.

Sputum samples of previouslytreated and new case patients were cultured on Lowenstein Jensen medium and identified according to standard procedures. *Mycobacterium tuberculosis* isolates were subjected to first-line anti-MTB drug susceptibility test which was performed using the conventional agar proportion method accurately described by the manufacturer (Becton Dickson Microbiology Systems, Cockeysville, Md) instruction, with those resistant to both INH and RIF being MDR *M. tuberculosis*isolates.

Genomic extraction: DNA preparation was performed using the genolyzekit on strains isolated from Lowenstein Jensen medium slants in accordance with standardized protocol described by Genolyze Company. This procedure was carried out using the genolyse (R) DNA extraction kit version 1.0 (Hain Life science GmbH).

The Genotype MTBDR assay (Hain life science, Nehren, Germany) was performed according to the manufacturer instruction. The test is based on DNA strip technology and has three steps; DNA extraction, multiplex polymerase chain reaction (PCR)and reverse hybridization. In brief, for one PCR, 10ml amplification mix A containing 10x buffer, nucleotides, and DNA polymerase was mixed with 35ml of amplification mix B containing MgCl2, the primers, and dye. The 5ml of the MDR *M. tuberculosis* DNA was added to the mixture, making the final volume of PCR mix to be 50ml. The PCR consisted of 15mins of denaturing at 95, followed by 10cycles of 30s at 95 and 120s at 58, followed by 20 additional cycles of 25s at 95, 40s at 53, and 40s at 70, with a final extension at 70 for 8mins.

For hybridization, 20ml of the amplification products were mixed with 20ml of the denaturing reagent (provided with the kit) and denaturing was performed for 5mins in each of the plastic wells. Thereafter, 1ml of pre-warmed hybridization buffer was added into each well and one strip was placed in each well. The hybridization was performed at 45℃ for 30mins, followed by two washing steps. For colorimetric detection of hybridized amplicons, streptavidin conjugated with alkaline phosphate was added after which a substrate buffer was added. After final washing, strips were air-dried and fixed on paper provided by the manufacturer. The DNA of the standard strain H37RV and molecular-grade water was used as positive and negative controls respectively.

RESULTS

The results of the analysis in Table 1 showed that the most prevalent wild type of rpo B band occurred at loci 510-513. It was present in all RIF resistant isolates. Mutation band (MUT gene-2A -H526Y) was the highest (with eight) mutation bands that appeared at the rpoB gene. It occurred in 8(42.1%) of RIF resistant isolates. All the mutant genes of the rpoB were demonstrated including MUT1 (D516V), MUT2a (H526Y), MUT2B (H526D), and MUT3 (S531L). Among the INH resistant isolates, katG mutation band of MUT1 (S315T1) was the highest band that appeared. It was identified in 9(90%) of INH isolates occurring at the kat G. Also, 2(20%) INH resistant isolates had a mutation band on the INH A identified as MUT1 (C15T). MUT2 (A16G) band was present in 1 isolate on INH A gene. Nine isolates had high level mutation bands (kat G gene) and 3 isolates had low-level mutation (INH A) demonstrated using the MTBDR plus line probe assay.

The results of Table 2 showed that RIF resistant occurred in 19(95%) and RIF susceptible was found in 1 (5%) isolate among the 20 MDRTB isolates tested. Mutation gene (H526Y) of the rpoB gene was the most frequent with a 42.1%. Also, 5 unknown mutations were

identified on the rpoB gene. INH resistant occurred in 10 (50%) while INH susceptibility also identified in 10 (50%) of the MDR M. tuberculosis isolates. Resistant mutations at katG at the S315T1 gene occurred in 9(90%) of INH resistant isolates while that at INHA occurred at gene C15T in 2 (20%) of the isolates.

The results in Table 3 indicated that mutation at H526Y of the RIF was the most prevalent among the MDR, *M. tuberculosis* isolates. It occurred in 8 of the isolates. The most prevalent gene mutation of the INH resistant among MDR *M. tuberculosis* occurred at gene S315T1 in 9 isolates. The single rpoB with the highest percentage of mutated gene was H526Y with 21.1% while that of kat G was S315T1 with 70% and inhA mutation at C15T1 was 10%.

Table 4 showed that eleven isolates had mutation at a frequency of 57.9% as a result of loss of WT8 occurring at loci 530- 533 and the presence of corresponding MUT gene. The most frequently identified mutation on the rpoB gene was found at codon H526Y gene which had 26.3% (5/19). In the INH isolates, the most frequent mutation occurred at position S315T1 in 90% (9/10) of isolates at katG and -15 -16 position on the INH A with 40% (4/10) of isolates.

Table1: Band Patterns of Drug Resistant *Mycobacterium Tuberculosis* Strains Using Genotype MTBDR plus Assay in Plateau State, Nigeria.

Gene	Band	Gene region/mutation	Rif mono resistance (N=19) n (%)	Inh mono resistance (N =10) n (%)	MDR (N=10) n (%)
rpoB					
	WT 1	506 – 509	18 (94.7)	0 (0)	9 (90)
	WT 2	510 – 513	19 (100)	0 (0)	10 (100)
	WT 3	513 – 517	17(89.5)	0 (0)	8 (80)
	WT 4	516 – 519	18 (94.7)	0 (0)	9 (90)
	WT 5	518 – 522	17 (89.5)	0 (0)	8 (80)
	WT 6	521 – 525	18 (94.7)	0 (0)	9 (90)
	WT 7	526 – 529	15 (79.0)	0 (0)	8 (80)
	WT 8	530 – 533	8(42.1)	0 (0)	3 (30)
	MUT 1	D 516V	1 (5.3)	0 (0)	0 (0)
	MUT 2A	H526Y	8 (42.1)	0 (0)	3 (30)
	MUT 2B	H 526 D	6 (31.6)	0 (0)	1 (10)
	MUT 3	S531 L	6 (31.6)	0 (0)	3 (30)
kat G	WT1	315	0 (0)	4 (40)	3 (30)
	MUT I	S315T 1	0 (0)	9(90)	9 (90)
	MUT 2	S315T 2	0 (0)	0 (0)	1(0)
inh A	WT 1	- 15/ - 16	0 (0)	6 (60)	6 (60)
	WT 2	- 8	0 (0)	7 (70)	7 (70)
	MUT 1	C15T	0 (0)	2 (20)	2 (20)
	MUT 2	A16G	0 (0)	1 (10)	1 (10)
	MUT 3A	T8C	0 (0)	0 (0)	0 (0)
	MUT 3B	T8A	0 (0)	0 (0)	0 (0)

Key: Inh – isoniazid; MDR, Multi drug resistant; MUT, Mutation; Rif, Rifampicin; WT, Wild Type.

Table 2: Patterns and Frequency of Resistant Mutations Associated with RMP and INH ResistantDetected by the GenoType MTBDR plus Assay in Plateau State, Nigeria.

rpo B Mutations	Relative Frequency (no of mutant isolates) n %	kat G Mutations	Inh A Mutations	Relative Frequency (no of mutant isolates) n %
D516V	1(5.3)	S315T1	WT	9(70)
H526Y	8(42.1)	WT	C15T	2(20)
H526D	6(31.6)	WT	A16G	1(10)
S531L	6(31.6)	WT		
			UK(WT1,2)	1(10)
			UK(WT2)	1(10)
UK (WT8)	1(5.3)	-	INH Resistant	10(50)
UK (WT3, WT8)	2(10.5)			
UK (WT5, WT6)	1(5.3)	-	INH Susceptible	10(50)
UK(WT3,WT4,WT5,WT8	1(5.3)	-	-	-
Rif resistant	19(95)	-	-	-
Rif susceptible	1(5%)	-	-	-

INH, isoniazid; RIF, rifampicin; UK, unknown mutation characterized by no hybridization to one or more wild-type probes or to any of mutation probes; WT, wild type.

Table 3

Pattern of Mutated Genes Associated with RIF and INH Resistance Among MDR. *M. Tuberculosis Isolates in Plateau State, Nigeria*

Drug	Gene Mutated	No. (%) of mutated gene
RIF (N=19)	**H526Y**	**4(21.1)**
	H526Y/H526D	**3(15.8)**
	H526Y/S531L	**1(5.3)**
	S531L	**3(15.8)**
	H526D	**2(10.5)**
	H526D/S531L	**1(5.3)**
	UK	**5(26.3)**
		7(17)
INH (N=10)	**S316T1**	**1(10)**
	S315T1/C15T	**1(10)**
	C315T1/A16G	
	C15T1	**1(10)**

RIF: Rifampicin; INH: Isoniazid

Table 4: Most Frequently Identified Mutations within INH and Rif Associated Loci Among Drug-Resistant *M. Tuberculosis* Isolates from Plateau State, Nigeria.

Drug	Locus	Mutated Position	Relative Frequency % (No. of Mutant Isolates/No. of DR Isolates)
RIF	rpoB	506-509	5.3(1/19)
		513- 517	10.5(2/19)
		516 -519	5.3(1/19)
		518 – 522	10.5(2/19)
		521- 525	5.3(1/19)
		526 -529	15.8(3/19)
		530-533	42.1(8/19)
		D516V	10.5(2/19)
		H526Y	26.3(5/19)
		H526D	21.1(4/19)
		S531L	21.1(4/19)
INH	Kat G	315	60(6/10)
		S315T1	90(9/10)
	Inh A	-15/-16	40(4/10)
		-8	30(3/10)
		C15T	20(2/10)
		A16G	10(1/10)

RIF: Rifampicin; INH: Isoniazid; DR: Drug Resistant; rpoB: RNA polymerase; Kat G: Catalase- Peroxidase enzyme; Inh A: isoniazid enoyl A Reductase.

DISCUSSION

The WT2 (510-513) was the most frequent wild type band that appeared in all rif resistant isolates which is an indication that WT2 was not mutated in RIF gene therefore not involved in any resistance in this study. The high level of the absence of wild type 8 (530-533) is an indication that this wild band was highly mutated and therefore deeply involved in RIF resistance and may likely be responsible for the high level of unknown mutations at rpoB gene in this study. Mutant gene band (H526Y) was the most frequent on the rpoB gene therefore, may be responsible for the RIF resistance. Six of the RIF resistant isolates, had WT8 with presence of the corresponding MUT3 while WT8 was missing with no gain in MUT3 in 4 isolates which may indicate the presence of unknown or rare mutation. Similarly, RIF resistant isolates with the missing of WT8 probe without any MUT

band were reported in other studies from New Delhi, France and Vietnam (11, 12, 13). The kat G wild type band was absent in most of the INH resistant isolates and therefore responsible for isoniazid resistance without any form of unknown mutation at kat G gene. There was high level of the presence of S315T1 band of kat G gene with resultant high level of resistance to INH and MDR reported in this study. On the inh A gene, wild type 1 and wild type 2 band were absent and therefore involved in low level INH resistance and two forms of unknown mutation at the inh A gene. The mutant gene 1 (C15T) and mutant gene 2(A16G) bands were present in few of inh A gene resulted in low percentage INH resistance. There was high presence kat G mutant gene bands and low presence of inh mutant gene bands responsible for high - level mutation and low-level mutation.

In this study, the Genotype MTBDR plus assay identified RIF resistance specific mutation by rpoB MUT probes, which was detected in 14(76.7%) of the 19 RIF resistant isolates.

Higher specific mutation on the rpoB gene was reported in another study in India (11) and in South Africa (14).

The result of this study showed that of the 14 specific mutations of the rpoB gene, 4 had mutations at codon H526Y only, 2 had mutation at codons H526Y and H526D and 1 had mutation at codon H526Y and S531L. The remaining 7 had 2 single mutations at codon H526D, 4 single mutations at codon S531L and 1 at 2 codons (H526D and S531L). A similar finding was reported by a study conducted in India (15). In contrast, Lacoma et al. (16) reported the mutation patterns in rpoB identified by MTBDR plus in 11 isolates to be different from what was observed in the present study. They demonstrated S531L in 6 strains, D526Y in 1 strain, H526D in 1 strain, D516V in 2 strains and S531t in the last strain. S531t mutation matched the lack of a band for the WT8 probe which was designated through DNA sequencing (16). The discovery of S531t by Lacoma et al (16) agrees with the finding of this study where mutation was found in 1 isolate as a result of missing rpoB WT8 wild-type band.

The result of this study also found high-level of resistance in 7 isolates and low-level resistance in 3 isolates. There were 10 specific mutations of the Kat G and inh A genes, 7 had single mutations at codon S315T1 only, 1 had mutation at codons S315T1 and C15T, 1 had mutation at codons S315T1 and A16G and the other had single mutation at codon C15T only. This signifies homogeneity in the pattern of mutation in katG gene and heterogeneity in the inh A gene. The mutation pattern obtained in this study contravened that reported by Feliciano et al. in 2015. Feliciano (17) reported that 5 isolates with high-level resistance to isoniazid drug due to mutation S315T1 (KatG gene) and 5 with low-level resistance patterns, and 4 of them were due to mutation C15T (inhA regulatory region) and no mutation at codon A16G.

The high number of isolates with high-level isoniazid resistance (kat G) is an indication that most M. tuberculosis isolates obtained from drug resistant TB cases should not be subjected to first- line anti-TB drugs, rather it should be second-line. Also, whenever M. tuberculosis is isolated from drug resistant cases in the study and where facilities are not available for DST for second- line drugs, an empirical treatment could be administered with second-line drugs.

This study found that 3(15.9%) of the 19 isolates that showed resistance to RIF in rapid molecular test that had the same genotypic profile of resistance (Loss of rpoB WT8 wild type band and appearance of rpoB MUT3 band), which infers mutation S531L. This finding contravened that of Feliciano and his coworkers in Brazil who found 75% of isolates having the same genotypic profile of resistance to RIF (17). A similar finding was made by Hillemann et al. (18), who found in their study in which the mutation S531L occurred in 73.6% of the isolates evaluated by Genotype MTBDR plus. Vijdea et al. (19) and Yadav et al. (20) found respectively, 86% and 72% of the S531L mutation in strains resistant to rifampicin that were subjected to the same genotypic testing.

This study found heterogeneity in the genotypic profile of resistance to rifampicin as 21.1% and 15.8% of isolates revealed loss of rpoB WT8 wild type band, appearance of rpoB MUT2B and MUT3 bands, which infers mutations H526D and S531L respectively. Others show different forms of genotypic profile of resistance to rifampicin involving loss of rpoB WT8 and appearance of rpoB MUT1, MUT2A, MUT2B, MUT2B, and MUT3. This report contradicted that of Feliciano et al. (17) who found the genotypic profile of resistance to rifampicin to have a high level of homogeneity. On the other hand, Feliciano and co-workers found heterogeneity in the genotypic profile of resistance to isoniazid (17), but this finding again contravened the report of this study which observed some level of homogeneity in the genotypic profile of isoniazid. Thisstudy found 9 isolates with high level resistance profile to the drug due to mutation S315T1 (Kat G gene) and 3 isolates with low level resistance patterns with 2 of them due to mutation C15T (inhA regulatory region). The genotypic profile of resistance to isoniazid obtained in this study contradicted the report of Feliciano et al. (17) who found that the genotypic profile of resistance to isoniazid was heterogenous in their study.

Also, Vijdea et al. (19) evaluated two distinct subgroups in relation to the resistance profile to isoniazid. In one of the subgroups, the S315T1 mutation was discovered in all the isolates tested, which agrees with the report of this present study. He also found in the other group the mutation C15T was the most frequent among the isolates with low- level resistance to the drug (Isoniazid). Lacoma et al (16) observed that in isolates with high-level resistance, the most frequent mutation was S315T in the Kat G gene. Both findings corroborated the report of this present study.

As has been previously been described by several authors, this study established that S315T substitution in kat G is the most common mutation involved in INH resistance, which has also been related to high levels of INH resistance. In contrast, mutations causing low levels of INH resistance are not as clearly elucidated, as they are much more complex and involve different genes; however, a firm relationship has been found between mutations in the inh A regulatory region and low or intermediate levels of resistance in this study. This study revealed that among the INH resistant Isolates S315T1 mutation of Kat G gene was the most frequent while S315T2 was completely absent in all the INH resistant isolates. This present study also found that in the inh A (Low-level resistance) -15/-16 and -8 mutations were the most common and C15T and A16G were the only mutant genes demonstrated.

This study found low-level resistance with mutation detected in 40% and 30% in

the inhA regulatory regions -15/-16 and -8 only respectively and 90% in S315T1 of kat G. The report of this study therefore contradicted the findings of Lacoma et al (16) who found low-level resistance with mutation detected in 59% in inh A regulatory region and only 3.7% were in Kat G (S315T1). In strain with high-level resistance, they noticed the opposite distribution: 80.9% of mutation corresponded to the S315T1 mutation in Kat G and only 4.7% were located in inh A. This discrepancy in the percentages of mutations detected in inh A and Kat G region is normal and expected, as inh A mutation is higher in low-level resistance while Kat G mutation is higher in high-level resistance.

In the Genotype MTBDR- plus assay, resistance to INH is detected by probes of the Kat G and inh A gene. The higher frequency of resistance to INH occurred due to mutation of the Kat G gene, whereas lower frequency of resistance was caused by the mutations in the promoter region of the inh A gene (21). Of the 10 INH resistant isolates, Kat G mutation occurred in 90% (9/10) of the isolates. In all of these 10 isolates, specifics mutations were found at codon S315T1 of the Kat G gene, which was also reported by other studies conducted in North-west Ethiopia (22) and India (23). Some studies reported lower frequency of mutation in the Kat G gene at codon S315T1 from Uganda, France and South Africa (11,12,13).

Mutations in the inh A gene occurred in only four of the 10 INH resistant isolates, which is similar to the frequency of inh A mutation reported from the North-West part of Ethiopia (22). Compared with these findings, a study from North India reported the occurrence of low frequency of INH resistance mutation in the inh A gene (23). In contrast to these findings, higher inh A gene mutation was reported from Tunisia (24) and Canada (25). Specific inh A mutations were found in 3 of the 10 inh-resistant isolates, which had mutation in codon C15T in 2 of the 3
isolates and mutation in codon A16G in 1 of the isolates, but in the remaining 1 isolate, the inh A WT1, and WT2 genes were missing without the presence of a specific mutation band.

The study highlighted a high prevalence of MDR-TB among the study population. As MDR-TB is a highly growing health problem worldwide and usually follow a much longer treatment course involving more expensive, less effective and more toxic second or third-line anti-TB drugs, effort should be made to develop new and affordable drugs with better efficacy and fewer side effects. Clinically, more effort should be made to minimize the number of defaulted patients. Greater measures should be taken to provide adequate treatment for vulnerable populations such as those who have serious underlying diseases such as chronic debilitating illnesses.

In conclusion, despite consistent advances in the control of tuberculosis, the challenge of increasing resistance to anti TB drugs persists. Considering the limited number of recent national data about the epidemiology of *M. tuberculosis* resistance, it becomes urgent to build an epidemiological profile of the bacillary resistance in the country. To achieve this goal, it would be critical to optimize and increase access to proper and timely diagnosis including sensitivity testing especially with line probe assay (LPA) which could be an effective and affordable option.

This study found five unknown mutations detected by MTBDR plus assay

which portend a serious danger in the treatment, prevention and control of MDRTB. Also, a new mutation type (Loss of wild Type 8) was found which has not been described in the study area. The result obtained from Genotype MTBDR Plus assay could be used, especially mutation patterns, genotypic profile of resistance mutation and specific mutation type of difference MDR. M. tuberculosis could be used to determine molecular genotypic cluster to establish transmission in the absence of spoligotyping and MIRU-VNR typing.

Once there is a high level of specific gene mutation at the rpoB or Kat G gene, heterogenic genotypic profile of resistance (GPR)should be expected while low level of specific rpoB or Kat G mutations has been found to be associated with GPR homogeneity. This study found a high-level of heterogeneity and homogeneity in the genotypic profile of resistance to RIF and INH respectively which probably may have been responsible for high level of resistance RIF than INH by the *M. tuberculosis* isolates in the tested population

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