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Occurrence and Frequency of Gene Mutations Associated with Rifampicin (RIF) and Isoniazid (INH) Resistance from Multidrug Resistant *Mycobacterium Tuberculosis* (MDRTB) in Lagos, Nigeria

Raheem TY[1]*, Iwalokun BA[1], Fowora MA[1], Osuolale KA[2] and Adesesan AA[3]

[1]*Molecular Biology and Biotechnology Department, Nigerian Institute of Medical Research Yaba Lagos.*

[2]*Grant, Monitoring and Evaluation Unit (Biostatistics), Nigerian Institute of Medical Research.*

[3]*Centre for TB Research, Nigerian Institute of Medical Research Yaba, Lagos.*

***Correspondence:**

Raheem TY, Molecular Biology and Biotechnology Department, Nigerian Institute of Medical Research Yaba Lagos.

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ABSTRACT

Background: *Despite the tremendous improvements in the diagnosis and treatment of tuberculosis, pulmonary tuberculosis (PTB) remains a leading cause of morbidity and mortality in adults and children worldwide. The World Health Organization (WHO) recommends detection of MDR TB using bacteriological confirmation of TB and testing for drug resistance using rapid molecular tests, culture methods or sequencing technologies. M. tuberculosis strains resistant to multiple anti-TB drugs are becoming increasingly common. Different independent mutations of rpoB, katG and InhA genes encoding either the drug target or the enzymes involved in drug activation have been found to be one of the strategies responsible for resistance to Rifampicin and Isoniazid.*

Methods: *This cross-sectional, multicentre study was conducted on MDRTB isolates collected from pulmonary TB patients in Lagos, Nigeria from May 2012 to October 2016. After informed consent, structured questionnaires were administered to obtain sociodemographic data. Sputum samples were collected and processed for microscopy and culture using Lowenstein-Jensen medium. Isolates were identified by biochemical and molecular methods and drug susceptibility testing was performed using MIC, proportion and line probe Assay methods.*

Objectives: *The study investigated the occurrence and frequency of gene mutations associated with rifampicin (RIF) and isoniazid (INH) resistance from the isolated MDRTB and assessed whether all the M. tuberculosis which elicited MDRTB phenotype had mutation markers.*

Results: *Of the 48 M. tuberculosis that elicited MDR phenotype by the proportion and MIC methods, 2 (4.2%) isolates lacked any of the inhA, katG and rpoBgene mutations associated with RIF and isoniazid resistance in the line probe assay method. The highest occurring rpoB mutation conferring RIF resistance among the MDR M. tuberculosis tested was 97.3% with mutation point S531L at a frequency of 60.4% followed by H526D mutation (22.9%), H536Y (8.3%) and D516V (6.3%). The S315T mutation of the katG gene was responsible for 50% of isoniazid resistance among the MDR isolates. This was followed by C-15T mutation (14.6%), S94A mutation (12.5%) and 1194A (8.3%) of inhA mutation points.*

Conclusions: *Different mutations of genes encoding either drug target or the enzymes involved in drug activation such as S531L, H526D, H536Y, D516V, S315T, C-15T and S94A were detected in MDRTB isolates. The study also showed that lack of inhA, katG and rpoB resistance defining mutations may not be sufficient as markers of susceptibility to anti-TB drugs. There is need for a nation-wide study of the pattern of mutations of drug/enzyme gene targets in MDRTB in Nigeria.*

Keywords
Pulmonary tuberculosis, *M. tuberculosis*, Gene Mutations.

Introduction

Tremendous improvement in the diagnosis and treatment of tuberculosis had been made globally. However, pulmonary tuberculosis (PTB) remains one of the major causes of death in adults and children. According to the World Health Organisation [1], a fewer number of people who presented for MDRTB diagnosis were detected and Nigeria is one of the five countries in the world with underreported cases of TB cases (including MDRTB). In countries with underreported cases, intensified efforts are required to reduce underreporting and improve access to diagnosis and treatment. World Health Organization further emphasised some of the strategies for detecting more MDRTB and recommends detection of MDR TB using bacteriological confirmation of TB and testing for drug resistance using rapid molecular tests, culture methods or sequencing technologies [1]. The *M. tuberculosis* strains resistant to multiple anti-TB drugs are becoming increasingly common. These include improvement in the detection of TB and increasing bacteriological confirmation of TB cases, increasing coverage of testing for MDRTB among those bacteriologically confirmed TB cases.

The danger of *M. tuberculosis* strains resistant to multiple anti-TB drugs was since reported as becoming increasingly common since 2003 [2]. Multidrug-resistant tuberculosis (MDR-TB) is the main indicator of previous treatment in tuberculosis (TB) patients and MDR-TB among treatment-naïve patients indicates infection with drug-resistant Mycobacterium tuberculosis strains, and such cases are considered primary drug-resistant cases [3].

Mutations associated with changes in the primary targets of activated INH are enzymes peroxidases involved in the biosynthesis of cell wall mycolic acids have been shown to contribute to INH resistance [4]. Resistance has been linked to mutation of rpoB, kat G and inh A genes for resistance to rifampicin and isoniazid respectively [5].

MDR *M. tuberculosis* strains seem to have accumulated independent mutations in genes encoding either the drug target or the enzymes involved in drug activation. In a few reference laboratories in Nigeria, MDR-TB is diagnosed phenotypically by the use of the drug sensitivity testing based on proportion method using LJ medium supplemented with isoniazid at 0.2 ug.mL concentration and rifampicin at 40 ug/mL. Although this method has been found to be very reliable in diagnosing MDR-TB, it is time consuming, laborious and lacks information on the mechanism of resistance [6,7]. To improve on MDR-TB diagnostic turnaround time, genotypic testing based on the use of line probe assay (LPA) is also performed. The LPA detects MDR-TB based on the detection of mutations in genes targeted by isoniazid and rifampicin from the DNA sample of sonicated Mycobacterial cells. They include katG, a catalase peroxidase gene involved in the activation of isoniazid. KatG gene is for producing hydrogen peroxide that will convert INH from inactive form to active form thereby causing a lethal effect on the *M. tuberculosis*; *i*f there is a mutation of the KatG gene, the drug will not be converted and the organism appears resistant to the drug [5].

RpoB gene is the RNA polymerase B gene responsible for the binding of Rifampicin antibiotic to the RNA of organism thereby causing a lethal effect in the organism. If there is resistance, the drug will not bind to the mRNA of the organism and the organism appears resistant to the Rifampicin drug.

The absence of mutations does not preclude resistance to antimycobacterial drugs because other mechanisms of resistance may be involved [8,9]. *Mycobacterium tuberculosis* and other members of the *M. tuberculosis* complex use several strategies to resist the action of antimicrobial agents and these are: mycobacterial cell is surrounded by a specialized, highly hydrophobic cell wall that results in decreased permeability to many compounds, active drug efflux systems and degrading or inactivating enzymes and mutation of the genes responsible for drug targets. The genes that are associated with these functions, have since been found in *M. tuberculosis* [9,10]. Considerable work has been done on the characterization of drug-resistant mycobacteria. Structural or metabolic genes encoding either the enzymes that activate antimycobacterial drugs or the protein targets of drug action that lead to a high level of resistance to a single drug when the genes are altered by mutation have been identified. In most cases, multidrug-resistant isolates have accumulated independent mutations in several genes [10].

The designated mutant katG in LPA is S315T. The second target gene in the isoniazid pathway is inhA, which encodes an enoyl ACP synthase involved in the biosynthesis of mycolic acid, an important lipid component in the cell wall of *M. tuberculosis*. The designated mutant inhA in LPA are C15T, A16G, T8C and T8A. For rifampicin, resistance is mediated by mutations in rpoB gene that encoded the beta subunit of *M. tuberculosis* RNA polymerase enzyme.

Rationale for the study

Mutations of drug target genes in *M. tuberculosis* remain very important in the emergence of MDRTB. Mutation patterns in *M. tuberculosis* vary from strains to strains and with geographical and environmental differences. The Line Probe Assay detects MDR-TB based on the detection of mutations in genes targeted by isoniazid and rifampicin [7]. They include katG, a catalase peroxidase gene involved in the activation of isoniazid. The *rpoB* mutations included in the LPA DNA strip technology are D516 V, H526Y, H526D and S531L. These markers are strongly associated with MDR-TB in *M. tuberculosis* by eliciting MDR phenotype [10]. There is therefore the need to understand the mutation types and frequency in the MDR-TB among circulating strains of *M. tuberculosis* in the study area, which is presently having limited information regarding the most frequent mutations in the katG and inhA pathways for isoniazid resistance and the rpoB gene for rifampicin resistance.

Objectives of the study

This study is to (i) determine the prevalence of MDR-TB isolated in Lagos State, Nigeria (ii) assess the occurrence and frequency of gene mutations associated with rifampicin (RIF) and isoniazid (INH) resistance from the isolated *MDRTB*, (iii) compare different methods of detecting MDRTB and (iv) investigate whether all the *M. tuberculosis* which elicited MDRTB phenotype had mutation markers.

Study site

This study was carried out at the Nigerian Institute of Medical Research Yaba, Lagos, Nigeria.

Study design

This was a cross-sectional study conducted on stocked MDRTB Isolated from TB patients in Lagos State between May 2012 and October 2016.

Ethical considerations

The study was approved by the Institutional Review Board of the Nigerian Institute of Medical Research, Yaba, Lagos.

Sample size

A total of 306 *M. tuberculosis* were studied out of which 48 MDRTB were confirmed by proportion method, Resazzurin Microtitre Assay (REMA) and Line Probe Assay (LPA) for drug susceptibility and resistance testing.

Data Analyses

Data obtained after questionnaire administration were double-entered into Microsoft excel 2007 version and Epi INFO version 6.1. They were cleaned and validated for completeness and error before export to Statistical Package for Social Science (SPSS version 26) where analyses were done. Demographic variables such as age, sex, education, occupation and clinical data such as the presence of fever, cough, hemoptysis, night sweat, diabetes, and HIV were used as covariates and summarized as frequency and percentages (%) as well as mean ± standard deviation (SD). Chi - square(χ^2)and Fisher Exact (when expected frequency (e) < 5) test was used to evaluate the relationship between proportion method of DST and LPA as diagnostic tools for MDR TB infections.

Drug Susceptibility Testing (DST)

Inoculum preparation - Freshly grown colonies (> 50) of *M. tuberculosis* isolates from LJ medium were transferred to a tube containing 3-4 ml phosphate-buffered saline and 6 to 9 sterile glass beads. Tubes were vigorously agitated on a vortex mixer and clumps were allowed to settle for 30 min. The suspensions were transferred to sterile tubes and adjusted with phosphate buffer saline to equal the density of 0.5 McFarland standard for use as the standard inoculum in the Broth microdilution method (BMM) and reazurin microtitre method (REMA) [11].

REMA: Resazzurin Microtitre Assay (REMA) technique was performed using Middlebrook 7H9- liquid medium in 96-well microtitre plates with U-shaped wells as described by Leite et al., 2000 [12]. The wells of the microtitre plates were filled with 0.1 ml amounts of Middlebrook 7H9 broth, supplemented with oleic acid, albumin, dextrose and, catalase (OADC) enrichment. The stock suspensions of drugs were diluted in Middlebrook 7H9 broth and seven serial dilutions for each drug and the microtitre plates were stored at –25°C until use. The antibiotics gradient was 3.2-0.05 µg/ml for INH, 16-0.25 µg/ml for RIF and, 32-0.5 µg/ml for STR and ETM. Each well was inoculated with 5 µl of 0.5 McFarland standard bacterial suspension. A well without anti-TB drugs was also inoculated with 10^{-2} dilution of 0.5 McFarland standard as growth control. The microtitre plates were sealed, placed in plastic bags and incubated at 37°C for 14 days in a moisturized incubator. The Minimum Inhibitory Concentration (MIC) was defined as the lowest drug concentration that exhibited no growth by visual reading, and the strains were considered susceptible for each drug if MICs were below or equal to the critical concentration as described by Heifets and Iseman [13]. The results were evaluated after 7, 10 and 14 days and they were compared with those obtained by the proportion method using the critical concentration of drugs.

After 7 days, 30 µl of 0.02 per cent resazurin sodium salt solution was added to each well and again incubated for a further 24 h at 37°C. A change in colour of the resazurin dye from blue to pink was considered as positive growth and MIC was determined as corresponding to the concentration in the last blue colour in a row. All the experiments were repeated in duplicates. Isolates with MICs of INH <0.25 µg/mL and RIF ≤1 µg/mL were defined as being sensitive to INH and RIF, respectively. Reporting recommendations have been addressed by the Clinical and Laboratory Standards Institute [11].

DNA extraction for molecular assays

DNA extraction was carried out by sonication on the cultured organisms, followed by heat killing at 80°C for 30 minutes, followed by multiplex amplification with biotinylated primers and reverse hybridization. Identification of MTBC and NTM species were carried out by using specific sets of primers designed to amplify a species-specific 23S rRNA gene sequence of *Mycobacterium* species. One ml of MGIT 960 culture of reference or clinical *M. tuberculosis* isolate was heated with 40 mg Chelex-100 (Sigma-Aldrich, St. Louis, MO, USA) at 95°C for 20 min and then centrifuged at 12,000 × g for 15 min. For a PCR, 2 µl of supernatant was used as a source of DNA.

This technique involved DNA amplification targeting the 23s rRNA region of NTM isolates, followed by reverse hybridisation to specific oligonucleotide probe immobilised on membrane strips. The kit for common Mycobacteria (CM), which identifies 15 Mycobacterium species, including *M. tuberculosis* complex, was used [14].

Amplification mixture (45 µl) was prepared in DNA free room, including 5 µl extracted DNA (20-100 ng DNA) in the reaction

mixture contained 35 µl primer nucleotide mix, 5 µl 10 × polymerase incubation buffer for HotStarTaq (QIAGEN, Hilden, Germany), 2 µl 25 mM $MgCl_2$ solution, 0.2 µl Hot StarTaq and 3 µl water (biology grade water). Amplification was carried out in a thermal cycler (MJ Research, PTC-100 Thermal Cycler, GMI, Inc, USA), which involved 01 cycles of denaturation solution (DEN) at 95°C for 15 min, annealing of primers at 95°C for 30s, 2 min at 58°C for 10 cycles, then 20 cycles at 95°C for 25 s, 53°C for 40 s and 70°C for 40 s and final primer extension at 70°C, 8 min for 01 cycle. The amplified products were stored at +8 to −20°C until hybridization was done in a hybridization machine (Profiblot; Tekan, Maennedorf, Switzerland). The hybridization included the chemical denaturation of the amplification products, hybridization of a single-stranded, biotin-labelled amplicon to membrane-bound probes, stringent washing, the addition of a streptavidin/alkaline phosphatase conjugate, and an alkaline phosphatase mediated staining reaction. Here, 20 µl of DEN (blue) was dispensed in the corner of each well and then 20 µl of amplified product was added and incubated for 5 min at room temperature. Then 1 ml of pre-warmed hybridization buffer (HYB, Green) was added, followed by gentle shaking until a homogenous colour was developed. Now strip was placed in a manner to make sure complete flooding of solution over strips. Then tray was placed in TwinCubator and was incubated for 30 min at 45°C, followed by complete aspiration of HYB. Washing was done by 1 ml of stringent wash solution to each strip and incubated for 15 min at 45°C in TwinCubator. Again, strips were washed once with 1 ml of ringer solution for 1 min on TwinCubator. Then 1 ml of diluted conjugate was added to each strip and incubated for 30 min on TwinCubator. Strips were washed again with 1 ml of ringer solution for 1 min, after that 1 ml of diluted substrate were added to each strip and incubated for 3-20 min in the absence of light without shaking. Rinsing was done twice with distilled water to stop the reaction. Strips were removed and dried between two layers of absorbent paper. Evaluation and interpretation of results were done based on the presence and absence of different bands and compared with reference band as provided in the kit [14].

Standard strain of *M. tuberculosis* complex H37 Rv, *Mycobacterium fortuitum*, *Mycobacterium intracellulare* and *M. abscessus* obtained from National TB Reference Laboratory, Nigerian Institute of Medical Research, Yaba, Lagos were used as control in this study.

Genotype MTBDR assay

The Genotype MTBDR assay (Hain Lifescience, Nehren, Germany) was performed as recommended by the manufacturer. The amplification mixture contained 35 µl of primer-nucleotide mix provided in the kit, 5 µl of 10× *Taq* polymerase incubation buffer containing 2 mM of $MgCl_2$, 1 to 2 unit(s) of thermostable *Taq* DNA polymerase, and 5 µl of extracted chromosomal DNA solution in a final volume of 50 µl. The following amplification parameters were used: 5 min of denaturation at 95°C, followed by 10 cycles of 30 s at 95°C and 2 min at 58°C, followed by 20 additional cycles of 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C, ending with a final extension step of 8 min at 70°C. Hybridization and detection were performed with a TwinCubator semi-automated washing and shaking device according to the manufacturer's instructions and using the reagents provided with the kit. Briefly, 20 µl of denaturation solution was mixed to 20 µl of amplified sample and incubated at room temperature for 5 min. One milliliter of prewarmed hybridization buffer was added before the membrane strips were placed and shaken in the hybridization solution for 30 min at 45°C. After two washing steps, a colorimetric detection of the hybridized amplicons was obtained by the addition of the streptavidin alkaline phosphatase conjugate. All LPA runs adhered to ISO 15189 standards, which require the use of an ATCC *M. tuberculosis* H37Rv laboratory strain for positive control. Two negative controls were used to test for area-specific contamination [15].

Results

Of the 306 *M. tuberculosis* isolates, 48 MDR phenotype by the proportion method showing a prevalence of 15.7%. Tables 2 and 3 showed four (4) distinct resistance patterns were observed among the MDR isolates with INH RIF occurring the most (48/306) and INH RIF EMB occurring the least (2/306). Table 2 showed different resistance types which were polyresistance in 30/306 isolates and monoresistance for INH (16/306) and RIF (4/306). On the whole, 208 (68%) isolates were found to be pan susceptible. Of the 48 MDR *M. tuberculosis* isolated tested, two (2) isolates did not harbour any of the four (4) rpoB mutations associated with RIF resistance and lack of mutations associated with INH in katG or inhA genes were detected in nine (9) isolates. Table 3 showed that the highest occurring *rpoB* mutation conferring RIF resistance among the 48 MDR *M. tuberculosis* tested was 97.3% with mutation point S531L at a frequency of 60.4% followed by H526D mutation (22.9%), H536Y (8.3%) and D516V (6.3%). The S315T mutation of the katG gene (Table 3) was responsible for 50% of isoniazid resistance among the MDR isolates. This was followed by C-15T mutation (14.6%), S94A mutation (12.5%) and 1194A (8.3%) of inhA mutation points.

Table 4 showed the pattern of the detection of MDRTB Isolates by MIC, Proportion and LPA methods. Of the 48 MDRTB isolates detected by proportion method, 95.8% and 81.25% were detected by MIC and LPA methods, respectively.

Table 1: Age, Gender and Treatment History of the Participants with MDRTB Isolates.

Age distribution	No with MDRTB	Gender		Treatment History	
		Male	Female	New treatment	Retreatment
18-35	22	2	19	3 (37.5)	2 (25)
≥ 36	26	27	3	6 (54.5)	5 (45.5)

Mean=34.3 years

Table 2: Susceptibility Pattern of the *M. Tuberculosis* Isolated from the Study Participants.

Resistance Types	No of M. Tuberculosis	%
Mono Resistance	20	6.5
(INH OR RIF)		
INH and RIF Resistance (MDRTB)	48	15.7
Polyresistance (INH, RIF, EMB)	0	0.0
PAN Susceptible	238	77.8
TOTAL	306	100

Table 3: Occurrence and Frequency of rpoB, Kat G and InhA Mutation Pattern in the MDR *M. Tuberculosis*.

Drug Target	Mutation Point	No of MDRTB Showing the Mutation (N= 48)	Frequency (%)
rpoB	S531L	29	60.4
	H526D	11	22.9
	H536Y	4	8.3
	D516V	3	6.3
kat G	S315T	24	50.0
	S315N	3	6.3
inhA	S94A	6	12.5
	C-15T	7	14.6
	1194A	4	8.3
	121TN	1.0	2.1

Table 4: Detection of MDRTB Isolates by Minimum Inhibitory Concentration (MIC), Proportion and LPA methods. N= 48.

Method	Susceptible	Percentage (%)	P Value
MIC	46	95.8	0.01
Proportion	48	100	
LPA	39	81.25	

P = 0.01. This showed that there was significant difference in the results for MDRTB recorded when minimum inhibitory concentration (MIC), proportion and LPA methods were used.

Results and Discussions

Table 2 showed the prevalence of the resistant or susceptibility patterns of all the *M. tuberculosis* isolates with 6.5%, 15.7% and 77.8% mono resistance, MDR and pan susceptibility respectively. Mutations associated with changes in the primary INH (kat G and InhA) and Rifampicin (rpoB) drug targets were detected in this study. The MDRTB prevalence of 15.7 % recorded in this study was similar to the study of Lana et al., [7] in a study carried out in Lagos. However, INH MDRTB detected by LPA in this study was 39 out of 48 (81.25%) unlike the 48/48 (100%) by proportion method. Compared to the proportion method, the MIC and LPA methods of drug susceptibility testing correctly diagnosed 95.8% and 81.25% cases, respectively. The study, therefore, showed discordance in resistant results between LPA and Proportion methods. This finding is similar to the study conducted by Lana et al., [7] where only 95 % detection of resistance in MDRTB was detected using LPA method. This study showed that phenotypic methods using proportion and /or MIC methods appeared to be better options for detection of MDRTB and may be required for all LPA negative MDRTB test.

The designated mutant of katG in LPA was S315T while the designated mutant of inhA in LPA are C15T, A16G, T8C and T8A while that of rpoB of is S531L. The results of this study showed that 50% and 60.4% of the isolated MDRTB had S315T and S531L mutants of katG and rpoB respectively while 14.6% C15T mutants of inhA responsible for INH resistance (Table 3). For rifampicin, resistance is mediated by mutations in rpoB gene encoded the beta subunit of M. tuberculosis RNA polymerase enzyme.

Occurrence and frequency of the different mutants in different environments may cause strains to strains variations and may in turn, influence the diagnostic value of the LPA method.

Table 4 showed the percentage detection of MDRTB with minimum inhibitory concentration (MIC), Proportion and LPA methods (p = 0.01). This showed that there was significant difference in the results for MDRTB recorded when minimum inhibitory concentration (MIC), proportion and LPA methods were used. Nine (18.75%) of the isolates were false-negative by LPA which could be due lack of *inhA*, *katG and rpoB* resistance defining mutations This difference implied that drug resistance could be due to combined effect of mutation and other mechanisms of drug resistance or it could be due to the absence of the mutation points among MDRTB circulating in Lagos. Louw et al., [5]; Lana et al.,[7] and Raheem et al.,[9] had earlier suggested that other biological mechanisms such as efflux pumps could be a factor that could be responsible for resistance even where mutation of the rpoB gene and other drug target genes did not occur in the M. tuberculosis. Variations of mutations by regions and environment could be responsible for failure to detect MDRTB by LPA in addition to other factors.

Conclusions

Though 84.3% of the TB isolates were drug susceptible, 15.7% of the isolates were Multidrug Resistant TB (MDRTB) in this study. Different gene mutation markers such as *inhA*, katG*, rpoB* S531L, H526D, H536Y, D516V, S315T, C-15T and S94A were detected in the MDRTB isolates. 81.25% of the MDRTB were detected by line probe assay indicating rpoB, katG and inhA mutations, which are determinants of resistance to Rifampicin and Isoniazid. However, 18.75% of the resistance detected by proportion method (which indicated 100% of the MDRTB) were not detectable by LPA method. MDRTB were detected more with phenotypic (proportion method) than those based on drug target gene mutations alone. The study showed that the absence of *inhA*, katG *and rpoB* resistance defining mutations may not be enough as markers of susceptibility to anti-TB drugs. Phenotypic methods using proportion and or MIC methods should be performed on all LPA negative MDRTB samples to reduce false-negative results and increase detection of MDRTB cases.

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