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Molecular Characterization of Drug-resistant *Mycobacterium tuberculosis* Isolated from HIV-Positive Patients in Rivers State, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Drug resistant tuberculosis is a major challenge in the global bid to control the disease burden and improve treatment outcomes of DRTB infected individuals.
Aim: To determine the molecular characteristics and pattern of drug resistance to first line anti-tuberculosis drugs in HIV sero-positive patients in River State using line probe assay.
Methods: The Line Probe Assay (LPA) was used to assess the drug resistance pattern and gene mutations in 260 sputum specimens collected consecutively from 260 adult, HIV sero-positive subjects presenting with symptoms suggestive of TB in clinical settings across Rivers State, Nigeria.
Results: The results showed a 61.2% (n = 159) prevalence of TB among all study subjects. LPA analysis showed that 16 (10.1%) were multidrug resistant strains, 17 (10.7%) Rifampicin (RIF) monoresistant strains, 24 (15.1%) Isonaizid (INH) monoresistant strains and 102 (64.2%) drug susceptible strains. Among all 33 RIF-resistant strains, 24 (75%) had a mutation in *rpoB* S531L at the MUT3 band. Another mutation was observed at *rpoB* H526D (1/17) in RIF-monoresistant strains but not in MDR-TB strains. In INH resistant strains, mutations were observed at the *katG*

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gene [32/39 (82.1%)] which constituted 13/15 (86.7%) in MDR-TB strains and 19/24 (79.2%) in INH-monoresistant strains (p =0.002). Overall frequency of *inhA* mutation was 6/39 (15.4%); MDR-TB strains [2/15 (13.3%)] and INH-monoresistant strains [4/24 (16.7%), p = 0.2]. Combined *KatG* and *inhA* mutation was found in 1/39 (2.6%) of INH-monoresistant strains and in none of MDR-TB strains. Of the single *inhA* mutation bands, only 1/39 (4.2%) MUT 3B (T8A mutation) was observed in INH-monoresistant strains.

Conclusion: The results showed that drug-resistant TB is prevalent in Rivers State with various gene mutations observed in the different TB strains isolated, making LPA analysis a necessity in the bid to improving prevention and control efforts in the region.

Keywords: Mycobacterium tuberculosis; HIV; drug resistance; MDRTB; rifampicin; isonaizid; line probe assay.

1. INTRODUCTION

Tuberculosis (TB) is a global health challenge affecting millions of people with a high morbidity and mortality rate [1]. It is caused by *Mycobacterium tuberculosis (M. tuberculosis)*an acid-fast bacillus, with chronic cough and weight loss being the most common symptoms[2,3]. About 9 million cases of TB are reported yearly, with Sub-Saharan Africa contributing to about 30% of the global TB burden [3,4]. The global epidemic of HIV has also exacerbated the prevalence of TB, with a significant proportion of TB infected persons, being immune compromised due to HIV coinfection [5]. The occurrence of drug resistance in TB patients has been a major obstacle in the global control of TB, making the standard treatment and management protocols ineffective, and increasing the global TB burden [6]. Studies show that about 1/3rd of HIV-infected persons diagnosed with TB die about a year after commencing treatment [7,8,3]. The estimated prevalence of HIV in Nigeria is 3.6% making it the country with the second largest number (3.3 million) of people living with HIV in Africa [9,10]. The WHO reports that 26% of persons with TB infection in Nigeria are coinfected with HIV and the country has been reported to have the tenth largest TB burden in the world [3]. Drug resistant TB (DRTB) is a growing global health problem, posing a challenge to the control of TB, as well as prolonging treatment time, limiting treatment options and increasing cost of treatment [11,12]. The estimates based on modeling predict MDR-TB prevalence in Nigeria to range from 1.8% (0.0 to 4.3%) for new cases up to 7.7% (0.0 to 18.0%) for previously treated patients [7,9,10]. Genetic variations have been observed among drug resistant microorganisms including *M. tuberculosis.* These genetic mutations/ differences have been reported to confer a considerable amount of drug resistance to infectious agents, rendering any therapy or regimen ineffective [13,14]. Studies suggest that resistance-conferring mutations in *M. tuberculosis* may play a role in the transmission of DRTB [15,16]. Knowledge of the molecular characteristics of *Mycobacterium tuberculosis* isolated in the clinical setting is important in the bid to controlling the spread of tuberculosis, whilst improving the management and treatment of infected persons.

Treatment of MDR-TB is cumbersome and involves the use of second line drugs (SLDs), which are more complex, toxic, costly, less effective, associated with a greater incidence of adverse reactions and require longer treatment duration than first-line drugs [17,18]. In some settings, drug resistance has been linked with treatment failure and death in 10-30% of TB cases. The results of a meta-analysis on MDRTB treatment outcomes of subgroups of MDRTB patients using the DOTS-Plus treatment strategy revealed that high proportions had poor treatment outcomes, with higher treatment success rates reported in the subgroup on individualized treatment regimens over those on standardized regimens [14]. The rise rates of drug resistance and TB treatment failure has led to the World Health Organization (WHO) declaring drug-resistant TB (DRTB) it a global a priority, vital to addressing and accelerating progress towards the elimination of TB [19]. Early detection of drug-resistant *M. tuberculosis* is key to rapid, effective patient treatment and reduction in ongoing transmission [16].

In order to tackle these challenges, the WHO has endorsed the use of rapid molecular techniques which have dramatically improved the speed and quality of diagnosis of DRTB, one of which is the line probe assay (LPA) [20]. This study determined the molecular characteristics pattern of drug resistance to first line anti-tuberculosis drugs in HIV sero-positive patients in River State, Nigeria, using line probe assay.

2. METHODS

2.1 Study Area

The study was conducted in the directly observed treatment short-course (DOTS) and HIV clinics, located at the University of Port Harcourt Teaching Hospital (UPTH), Central Chest Clinic, Rivers State Ministry of Health (CCH) and the Braithwaite Memorial Specialist Hospital (BMSH) in Rivers State, Nigeria.

2.2 Study Sample

Two hundred and sixty (260) subjects with symptoms suggestive of TB (chronic cough > 2 weeks) who presented at the HIV clinics, of the aforementioned TB/HIV care centers were consecutively recruited for the study.

2.3 Inclusion Criteria

- Patients >18 years of age who gave informed consent were recruited into the study.
- Individuals presenting with symptoms suggestive of TB (Cough more than two weeks)

2.4 Exclusion Criteria

- TB patients who were HIV sero-negative,
- HIV sero-positive patients who were unable to produce sputum.

2.5 Specimen Collection

Duplicate sputum samples (spot and early morning) were collected in sterile, wide-mouthed bottles from patients that were able to produce. The collected sputum samples were preserved in a refrigerator (4^{o}C) at site of collection and subsequently moved in a cooling box daily to the Medical Microbiology Laboratory of UPTH for initial processing for initial processing, following which they were stored in a refrigerator at -20ºC.

Stored sputum samples were eventually moved in cold chain in batches to the Nigeria Institute of Medical Research (NIMR) in Yaba, Lagos for further processing. For the purpose of transportation, the samples were packed according to international requirement for packaging infectious disease agents and shipped in dry ice to NIMR. Identification and drug susceptibility testing were carried out using a molecular based technique (GenoType®MTBDRplus LPA, HAIN Lifescience Germany).

2.6 Specimen Analysis

For molecular characterization and detection of drug resistance pattern, decontaminated sputum samples were subjected to molecular LPA (GenoType®MTBDRplus by HAIN Lifescience Germany) for the genotypic identification of MTB and its drug susceptibility testing.

2.7 Line Probe Assay

Five hundred microliters (500µl) of decontaminated sputum samples of were subjected to line Probe Assay [LPA]) with GenoType®MTBDRplus (HAIN Lifescience), following the manufacturer's instructions.

2.8 DNA Extraction

DNA extraction was carried out by transferring 500µl of decontaminated sputum sample into a labeled 1.5 ml screw cap tube and centrifuged for 15 minutes at a speed of 10,000 x g in a centrifuge with an aerosol tight rotor. The supernatant was discarded and the resulting residue was resuspended in 100 µl of Lysis Buffer (A- LYS) by vortexing, to begin lysis of DNA from the sputum sample. The suspension was incubated in a water bath at 95ºC for 15 minutes, and 100µl Neutralization Buffer (A-NB) was added to each cell lysate and sample shaken for 5 seconds to end the DNA lysis reaction. The suspension was vortexed and centrifuged for another 5 minutes at a speed of 13,000 x g. The resulting supernatant obtained was the DNA, 5 µl of the DNA supernatant was used for PCR while the remainder was transferred into a new tube and stored at -20□C.

2.9 Amplification of Primers

The master mix was prepared by mixing 10µl of Amplification mix A (AM-A) and 35µl of Amplification mix B (AM-B), to make up 45µl master mix per PCR tube. To each 45µl amplification (master) mix, 5µl of DNA supernatant was added while 5 µl DNA of control strain H37Rv ATCC 25618 and 5 µl of molecular grade water were used as positive and negative controls respectively to make up a final volume of 50µl. the master mix were then subjected to Polymerase Chain Reaction (PCR) as previously documented.

2.9.1 Reverse hybridization of amplicons to immobilized gene probes and detection

Hybridization and stringent washing buffers were preheated to 45°C. Twenty (20) µl of denaturation buffer was mixed thoroughly in a plastic 12–well tray with 20 µl of amplified sample and incubated at room temperature for 5min. Subsequently, 1ml of hybridization buffer was added to each well and mixed. One pre-labeled test strip was added into each well, and the wells were incubated for 30min at 45°C. All solutions were completely aspirated following incubation. One (1) ml stringent buffer was then added to each strip and incubated for 15min at 45°C. After the aspiration of the solution, 1ml rinse buffer was added to each strip and incubated at room temperature for 1min. The following incubation steps took place at room temperature. The rinse buffer was completely removed, 1ml of diluted conjugate buffer was added to each strip, and they were incubated for 30min. After incubation, all solutions were removed and the test strips were rinsed twice with rinse buffer for 1min, followed by a washing step with distilled water for 1min. One (1) ml substrate buffer was added to each strip, and they were incubated for 5 to 8 min. All solutions were removed, and the reaction was stopped by two rinses with distilled water. The test strips were dried and then taped to the MTBDRplus 2.0 assay worksheet for interpretation.

2.10 Data Analysis

The data collected was analyzed with the Epi Infor v7 software (CDC, USA). The proportion of the phenotypic characteristics of each drug resistant strain were analyzed with the Chi-square statistics at a 95% confidence interval, with a p-value of less than 0.05 considered significant.

3. RESULTS

Fig. 1 shows that 159 (61.2%) clinical specimens tested positive for *Mycobacterium tuberculosis* and 101 (38.8) were negative.

Table 1 shows the pattern of drug resistance as determined by LPA. There were 16 (10.1%) multidrug resistant M. tuberculosis strains, 17 (10.7%) Rifampicin (RIF) monoresistant strains, 24 (15.1%) Isonaizid (INH) monoresistant strains and 102 (64.2%) drug susceptible strains.

Table 2 shows the genetic mutation patterns as shown by LPA. Among all 33 RIF-resistant strains, 24 (75%) (10 of MDR-TB strains and 14 of RIF-monoresistant strains had a mutation in *rpoB* S531L at the MUT3 band (p=0.907). Another mutation was observed at *rpoB* H526D (1/17) in RIF-monoresistant strains but not in MDR-TB strains. In INH resistant strains, mutations were observed at the *katG* gene [32/39 (82.1%)] which constituted 13/15 (86.7%) in MDR-TB strains and 19/24 (79.2%) in INH-monoresistant strains (p =0.002). Overall frequency of *inhA* mutation was 6/39 (15.4%); MDR-TB strains [2/15 (13.3%)] and INH-monoresistant strains [4/24 (16.7%), p = 0.2]. Combined *KatG* and *inhA* mutation was found in 1/39 (2.6%) of INH-monoresistantstrains and in none of MDR-TB strains. Of the single *inhA* mutation bands, only 1/39 (4.2%) MUT 3B (T8A mutation) was observed in INH-monoresistant strains. MUT2 (A16G mutation) and *inhA* MUT3A (T8C mutation) bands were not seen.

4. DISCUSSION

The 10.1% prevalence rate of MDR-TB found in this study is higher than 5.5% - 6.0% reported in similar studies carried out in Lagos and Calabar, Nigeria [11,21]. The rate in this study is similar to 13% reported in Abuja [22], while the 2017 WHO estimate of TB prevalence is 2.9% in new cases and 14% (95% CI: 10-19) for Nigeria in previously treated cases.3 This study result reflects the major gene mutations and resistant patterns associated with MDR-TB in *M. tuberculosis* strains. Mutation for RIF resistance was identified predominantly by one of four *rpoB* mutant probes in 88.2% (15/17) strains of RIF-monoresistant cases, occurring in codons 530-533 (∆WT8). This finding is similar to the 88.6% reported by Barnard et al., in South Africa [23]. Mutation in codon S531L was detected in 70.6% (12/17) of RIF- monoreistant resistant strains, while other studies found it varying from 47 to 70.5% [24,25]. In similar studies S531L mutation occurred more frequently in MDR-TB isolates in comparison to RIF mono-resistant strains [26]. However, in this study, frequency of S531L mutation in MDR-TB was 66.7% (10/15). Less frequent mutations (5.9%) were observed in codons 526-529 (∆WT7) and H526D.

Specific mutation in codon S315T1 of *katG* was found in 79.2% (19/24) of INH mono-resistant strains. Variations to this finding have been reported, *katG* mutations account for the most

Table 1. Pattern of drug resistance according to patients' treatment status

Resistant type	Resistant drug	Frequency (n =159), (%)
Multi-Drug Resistant	INH and RIF	16 (10.1)
Mono- resistant	RIF	17 (10.7)
	INH	24 (15.1)
Drug Susceptible	SS	102 (64.2)

Key: INH: Isoniazid; RIF: Rifampicin; SS: Susceptible

Table 2. Band patterns of drug resistant *Mycobacterium tuberculosis* strains using line probe assay

Gene	Band	Gene Region/ mutation	RIF Monoresistant strains (n = 17)	INH Monoresistant strains (n = 24)	MDR-TB strains (n = 15)	*P value* Mono-RIF versus MDR-TB
rpoB						
	WT1	506-509	17(100)	24(100)	15(100)	1.00
	WT2	510-513	17(100)	24(100)	15(100)	1.00
	WT3	513-517	17(100)	24(100)	14(93.3)	0.907
	WT4	516-519	17(100)	24(100)	14(93.3)	0.907
	WT5	518-522	17(100)	24(100)	15(100)	1.00
	WT6	521-525	17(100)	24(100)	15(100)	1.00
	WT7	526-529	16(94.1)	24(100)	14(93.3)	0.812
	WT8	530-533	1(5.9)	24(100)	2(13.3)	0.478
	MUT1	D516V	0(0)	0(0)	0(0)	-
	MUT2A	H526Y	0(0)	0(0)	0(0)	-
	MUT2B	H526D	1(5.9)	0(0)	0(0)	0.545
	MUT3	S531L	12(70.6)	0(0)	10(66.7)	0.907
katG						
	WT	315	17(100)	4(16.7)	2(13.3)	0.01*
	MUT1	S315T1	0(0)	19(79.2)	10(66.7)	0.002*
	MUT2	S315T2	0(0)	0(0)	3(20.0)	0.12
inhA						
	WT1	- 15/ -16	17(100)	20(83.3)	13(86.7)	0.980
	WT2	-8	17(100)	23(95.8)	15(100)	1.00
	MUT1	C15T	0(0)	3(12.5)	2(13.3)	0.242
	MUT2	A16G	0(0)	0(0)	0 (0)	-
	MUT3A	T8C	0(0)	0(0)	0 (0)	-
	MUT3B	T8A	0(0)	1(4.17)	0(0)	-

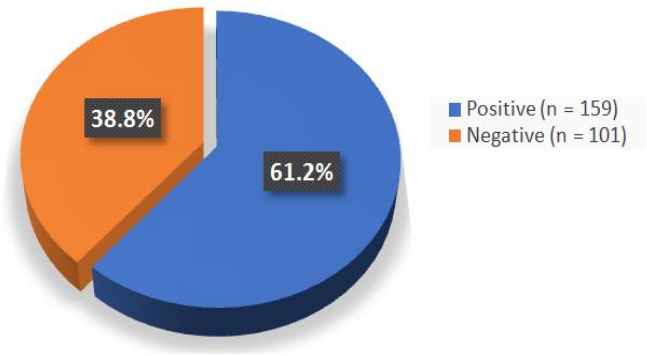

Fig. 1. Prevalence of Mycobacterium tuberculosis in study subjects

common mechanism for INH resistance and is associated with high level INH resistance [15,27]. This has been reported in high TB burden countries.3Mutation in *inhA* gene, conferring low-level resistance, was found to be 16.7% in this study. Other studies have a similar results.[5,6,28] However, Barnard et al., reported high prevalence of *inhA* mutations (41.7%) and also found significant difference in prevalence of mutations in MDR-TB strains as compared to mono-INH resistant strains [17].

Mutational analysis of RIF is less complex, involving only the *rpoB* gene locus while that of INH resistance is more complex because more genes are evaluated, the *katG* and the *inhA*. Although the genotypic analysis of *rpoB* for RIF resistance is thought to be sufficient for evaluating the public health threat of drug-resistant TB, there appears to be some disagreement by some researchers [29]. In this study, an overall prevalence of 15.1% INH-resistance and 10.7% RIF resistance were observed, highlighting the importance of evaluating both drug susceptibilities. This is particularly important in settings that are HIV-prevalent, in which INH prophylaxis is being considered. Such preventive measures might not be effective and may increase the rates of INH resistance, thereby, exacerbating the diagnostic challenges for MDR-TB. Furthermore, misdiagnosing patients as MDR-TB when they are only RIF-resistant would lead to inappropriate second-line treatment, when such treatment in resource-limited settings is already limited. Another advantage of determining the susceptibility of INH is its importance as an enrolment criterion for clinical trials of new TB treatment regimens for drug-sensitive as well as MDR-TB protocols [30,31].

5. CONCLUSION

The study showed that the major gene mutations and resistant patterns associated with MDR-TB in the *M. tuberculosis* strains isolated in the local setting occurred in codons 530-533 (ΔWT8) and S531L (MUT3) of the *rpoB*, S315T1 (ΔWT) of the *katG* and -15/-16 (ΔWT1) of *inhA* genes respectively. Gene mutations and resistance patterns associated with MDR-TB in *M. tuberculosis* strains detected by LPA in this study may contribute to the prevention and control efforts of MDRTB in Rivers State and the South south region of Nigeria.

DISCLAIMER

The products used for this research are the predominantly used products in our area of research and country and are only being mentioned here for the advancement of knowledge. There is no conflict of interest between the authors of this work and the manufacturers of the product. The research was not funded by the manufacturers of the product.

CONSENT AND ETHICAL APPROVAL

Ethical approval for the study was obtained from the Ethics Committee of all centers involved in the study, prior to commencement of the study. Willing signed informed consent was obtained from all participants before they were included in the study and personal information of all subjects were kept confidential. Individuals diagnosed with tuberculosis were referred to the attending physicians for appropriate treatment and management.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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