Genotypic and Phenotypic Detection of Multidrug-Resistant *Mycobacterium* Tuberculosis among HIV Patients Attending Tuberculosis Reference Centres in Northwest, Nigeria

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ABSTRACT

In light of the escalating issue of tuberculosis resistance, it is imperative to consistently evaluate and examine therapeutic strategies. This study focus on genotypic and phenotypic detection of multidrug-resistant *Mycobacterium* tuberculosis among HIV patients attending tuberculosis reference centres in Northwest, Nigeria. A total of 503 sputum samples were collected aseptically among three referral facilities in Northwest using random sampling method. Molecular drug susceptibility testing was performed using Geno Type® MTBD Rplus assay. The overall prevalence was 4.3 %. The highest prevalence in relation to state was observed from Katsina (22.2 %) and the lowest was from Sokoto (3.6 %). The highest prevalence was observed among male patients (4.8%) while the female patients (3.4%). Age 66-70 yrs recorded the highest prevalence (100 %) and the lowest was 36-40 yrs and 41-45yrs (4.4 %). The highest resistance patterns were isolates from Kebbi were resistance to INH+ RIF+ FLQ (50.0 %) and the lowest was from Kaduna INH+ AMG (20.0 %). Routine TB testing among HIV patients must be improved to guide co-management

Keywords: Multidrug-resistant, *Mycobacterium* tuberculosis, HIV patents, prevalence, sputum

INTRODUCTION

Tuberculosis (TB) is a disease of mankind usually caused by the *Mycobacterium tuberculosis* complex (MTBC) [1]. The disease mainly affects the lungs (pulmonary TB) and other parts of the body (extra-pulmonary TB). The existence of TB over the past millennia is not uncommon and outranks human immunodeficiency virus (HIV) infection/acquired immunodeficiency syndrome (AIDS) among the 10 prominent deadliest infectious diseases worldwide. Globally, an estimated 10 million individuals suffered from TB (90% adults) in 2017 [2] and this however remained a public health challenge with high mortality rate in developing countries [3].

The increasing trend toward globalization, transnational migration, inadequate treatment of active pulmonary TB, injudicious prescriptions among physicians accompanied by uninformed drug selection as well as default among patients exposing *M. tuberculosis* (MTB) to sub-lethal doses for shorter durations are potential targets for outbreaks of drug resistant (DR) TB [2].

Nigeria ranks not only among the 30 countries with a significant burden of TB, TB/HIV, and drug-resistant TB (DR-TB) but equally among the 14 countries accounting for more than 64% of the estimated number of incident TB cases worldwide in 2017 [2]. Nigeria also holds a record of several reports on the occurrence of DR-TB among patients across different settings. In Nigeria, there is an estimated 4.3% and 25% of patients with Multi Drug Resistance (MDR) among new cases and previously treated cases, respectively.

The effective treatment regimens for TB are first-line (streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide) and second-line (fluoroquinolones, amikacin, kanamycin, capreopmycin, ethionamide,

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prothionamide, cycloserine and para-aminosalicyclic acid) which may be challenged by development of drug resistant strains, adverse effects of the drug and prolonged treatment procedure [4,5].

The emerging of multidrug resistant tuberculosis (MDR-TB) is a global public health problem (Agumas *et al*., 2020) and has change the global out looked of the disease resulting in a setback made towards the global control of TB. The MDR-TB is defined as resistance of the MTB to at least to isoniazid and rifampicin [7].

The MDR-TB and extensively drug resistant TB (XDR-TB) arises due to spontaneous chromosomal mutation at a low frequency in anti-TB drugs target site [8]. The resistance to first and second line anti-TB drugs has been linked to mutations of genes namely:*kat*G and *inh*(for isoniazid); *rpo*B (for rifampicin); *gyr*A and *gyr*B (for fluoroquinolones);*rrs* and *eis* promoter region (foramikacin/kanamycin); and *rrs* and *tly*A (for capreomycin). The underlying risk factors for anti-TB drug resistance is due to treatment failure, acquired drug resistant *M. tuberculosis*, limited drug supply, indiscriminate use of anti-TB drugs as well as cost of drug susceptibility testing [2].

Studies by Daniel *et al*. [8] and Suchindra *et al*. [9] showed that the prevalence of anti-TB resistance was between 20-43% in the total population with higher prevalence seen in patients previously treated with anti-TB drugs. Recent data on meta-genomics of anti-TB resistance genes shows that the burden of anti-TB is 5 to 7 times higher in treated patients as opposed to the newly diagnosed patients [9]. This study focused on phenotypic drug resistance of mycobacterium isolated from HIV patents attending tuberculosis reference centres in northwest, Nigeria

MATERIALS AND METHODS

Study Area

The study was carried out in 6 states from the Northwest geopolitical zone of Nigeria, the National TB reference laboratory located at the National TB Training Centre, Saye, Zaria, Kaduna State. Broadly, the study population were drawn from the TB DOTS centers both in the public and private health facilities.

Sample Source

The samples were drawn from the DOTS clinics which have diagnosed at least one new bacteriologically confirmed TB between May - September 2024.

Ethical Consideration and Informed Consent

The ethical clearance were sourced from National TB reference laboratory Research Ethics Committee for this research work in compliance with the code of ethics for biomedical research involving human subjects with good Clinical Practice, preserving confidentiality and anonymity.

Sample Size

Tadesse *et al.* [10] developed the following equation for determining sample size. Tadesse *et al.,* [10], utilized a prevalence of 35% from a study conducted at the TB clinic of the National TB Reference Laboratory Zaria (NTRL).

To calculate for n:

$\frac{Z^2Pq}{d^2}$ Where n = the desired size when the population is greater than 10000.

Z= the standard normal deviation usually set at 1.96 (95% confidence level).

P= 0.5 i.e. 50% proportion in the normal population expected to have a particular characteristic.

q= the degree of accuracy required usually set at 0.5 (50% proportion).

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$$\mathbf{n} = \frac{(1.96)^2 \times 0.5 \times 0.5}{(0.05)^2}$$

From the study test data collated at the National TB Reference Laboratory Zaria, the prevalence of TB positive patients was estimated to be about 50 percent. So n = 503

The calculated sample size collected for this study was 453 samples. The total overall samples collected were 503 inclusive of the 10% attrition ratio added to adjust the sample size.

Sample Collection

A total of 503 sputum samples were collected aseptically among three referral facilities in northwest using simple random sampling. After expectorating, sputum of good quality that is purulent and mucoid, particularly early morning sputum samples of at least 2mls, were collected from each patient coming from different states using wide mouthed sputum containers.

The samples were transported to the National TB Reference Laboratory Saye, Zaria at least 6 hours after collection for microscopy, culturing, DNA extraction, genotypic GeneXpert and Line Probe assay using cold ice packs in cooler boxes ensuring the specimens are triple packaged at each of the transportation systems.

Phenotypic detection of the Mycobacteria

Decontamination, Digestion and Culturing

Five (5) mL of sputum were placed into a 50 mL sterile conical screw cap centrifuge tube that had been labelled. For thick specimens, an equal amount of PBS buffer were added, stirred, and then put to tubes. Buffer were employed as a blank or negative control; an equal amount of NALC-NaOH were introduced to the specimen in the tube and tightly capped for 20 seconds of vortexing at a moderate pace. The tubes were inverted 5 times to ensure that the NALC/NaOH solution were evenly distributed throughout the whole surface of the tube. After allowing the tubes to stand for 15 minutes, 35ml of sterile 0.067M phosphate buffer (pH 6.8) were added to the specimen in the tube to the 45ml mark in order to limit the ongoing effect of NaOH and lower the viscosity of the combination (NTBLCP, 2013). Tightly closed tubes were inverted many times before being put into centrifuge buckets and spun for 15 minutes at 3,000 x g at 4°C. The supernatant were carefully emptied into a trash container, and the sediment were resuspended in 2ml of phosphate buffer for inoculation into Lowenstein-Jensen (LJ) medium containing glycerol and cultured for 8 weeks at 37° C. Tubes were checked after four to seven days and then weekly, growth were recorded, and negatives were removed after eight weeks.

To continue Acid Fast Bacilli, any growth suspected of being Mycobacteria were smeared and discoloured with ZN (AFB). A rapid diagnostic test (Standard Diagnostics Bioline) were utilized to establish whether the isolates were MTBC or NTM.

Acid Fast Bacilli (AFB) Smear Microscopy with Zeihl Neelson (ZN) Staining

Slides were fixed (including positive and negative controls) to be stained and put in a staining rack to ensure that they did not touch each other. Each slide were saturated with 1% carbol fuchsin working solution and heated to steaming temperatures. Slides were stained for five (5) minutes before being gently rinsed with running tap water to remove excess carbol fuchsin (until no more color ran off). After rinsing, slides were allowed to drip off excess water. To decolorize the slides, they were soaked in 3% acid alcohol for 3 minutes. Slides were rinsed for 1 minute with running tap water and then totally decant the water. For one minute, the slides were counterstained with 0.1% methylene blue and then rinsed with tap water. Slides were allowed to drain, underside cleaned and allowed to dry completely before being examined under a bright field fluorescence microscope for 15 minutes (NTBLCP, 2013).

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Rapid Diagnostic Test for Sputum Positive Isolate Detection

Colonies from solid cultures were selected using a conventional sterile loop after AFB was confirmed by ZN staining. The SD Bioline device were taken out of the refrigerator and allowed to attain room temperature. Before testing, the device were withdrawn from the foil pouch and roughly 3-4 colonies were suspended in 200 g of extraction buffer dispensed in a sterile screw capped tube or condensation fluid from the culture slant agar tubes. A 100 g suspension of the specimen were removed from the fluid and transferred directly to the sample well (S) before being placed on a flat, dry surface. Allow fifteen (15) minutes for the sample to run through the window. A colour band appears on the left side of the result window to indicate that the test is ran properly (control band). The test results are indicated by the illuminated section of the result pane. The test band was revealed by the emergence of a colour band in the result window.

Identification of Mycobacterium tuberculosis Complex

After 8 weeks on LJ base glycerol media, cultures with positive growth (a rough, buffy, pale-yellow appearance) were smeared in a clean grease free slide, stained using the ZN staining method, and then compared for the presence of AFB by Bright field microscopy from their primary smear clinical concentrates. AFB-positive cultures were submitted to SD Bioline, a fast test for TB antigen MPT64, which can identify antigen specific for Mycobacterium tuberculosis complex (MTB) group. MTB positive after SD Bioline confirmation indicates Mycobacterium tuberculosis complex.

Identification of Non-Tuberculous Mycobacteria (NTM)

After 8 weeks, cultures exhibiting positive development showed a smooth, buffy, and yellow or red appearance on LJ medium. The isolates were smeared on a clean grease-free slide, stained with ZN staining procedure, and then analysed for the presence of AFB using Bright field microscopy. The SD BlOLlNE fast assay identification tests were performed on cultures with positive AFB smears. Those found to be MTB complex negative using the SD Bioline assay were subcultured in Lowenstein Jensen (LJ) medium. Those that developed on LJ base glycerol medium were thought to be NTMs.

Antimicrobial Susceptibility Test (AST)

The Biological Safety Cabinet 2 plus was used to put up 10 specimens to work with at once. A 3mm sterile loop was utilized to collect loopful primary culture, which was then placed on the side wall of a Bijou bottle holding 1 milliliter of sterile distilled water and 6 glass beads of various sizes (5 mm diameter). The bacterial inoculums were emulsified on the side wall of the Bijou bottle with inoculation loop in round rotating movements until the bacterial mass was completely emulsified and the suspension was thoroughly dissolved in 1 ml of sterile distilled water.

Then, for 20-30 seconds, vortex the bottle to further break up clumps. After around 15 minutes, 2-3 cc of distilled water was slowly added to the suspension to allow the coarse particles to settle. The supernatant was carefully transferred to another sterile bottle with dimensions comparable to those of the McFarland Standard No. l using a sterile transfer pipette. The opacity/turbidity of the suspension was matched with McFarland Standard No 1 against a background to avoid clumping. This is a clean bacterial suspension that has been standardized at 1 mg/ml, which is equivalent to 10^6-10^8 cfu/ml without clumping. The turbidity of the bacterial suspension was adjusted as needed to meet the McFarland criterion N01.

Where the suspension was too turbid, sterile distilled water was added, and where the suspension was insufficiently turbid, more cells were added to the suspension, instead of allowing the suspension to settle and discarding some of the supernatant to concentrate cells, and adjusting the turbidity with a few drops of sterile distilled water. The calibrated bacterial suspension was diluted with an automated pipette by transferring 10m of neat into 0.99ml (100l/9.9ml sterile distilled water; this is the 10-2 dilution). l0l was transferred from the 10-2 dilution into 0.99ml sterile distilled water to generate the 10-4 dilution (O'Reilly and Daborn, 1995; NTBLC, 2013). the following medication concentrations were added to LJ Medium and Critical proportion for proportion method interpretation.

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The LJ slant was taken out of the fridge and dried. Before inoculation, the slants were carefully labelled and the condensed moisture was removed. l0l (one loopful) of suspension dilutions were inoculated on the drug-free and drug-containing slants for culture using a conventional inoculating loop with an internal diameter of 3mm. Drug-free slants are inoculated for growth control (GC). Two tubes labelled GC1 were inoculated with suspension S1 (dilution 10-2), and two tubes labelled GC3 were injected with suspension S3 (dilution 10-2). (dilution 10-4). S1 (dilution 10-2) was inoculated into tubes containing test media containing INH, RMP, and EMB (one tube per drug) and cultured at 37°C for 4-6 weeks.

The first reading was taken on the 28th day, and any drug-resistant strains was reported at that time. Strains that were responsive to a medication on the 28th day needed to be incubated until the 42nd day for a clear interpretation. Format for reading, 50 colonies = Actual count, 50-100 colonies = 1+, 100-200 colonies = 2+, 200500 colonies = 3+, >500 colonies (confluent growth) = 4+. To allow for result interpretation, growth on the GC3 tube is predicted to be between 30 and 100 colonies. When there is no growth after 6 weeks on drug-free media, the test cannot be interpreted and must be redone.

The number of colonies on drug medium injected with S1 was compared to the number of colonies on GC3, (controls) inoculated with a 1% dilution of S1. Similarly, the number of colonies injected with S2 on drug medium was compared to the number of colonies inoculated with 1% dilution of S2 on GC4 (controls).

Amplified DNA using Hain Line Probe Assay Genotype MTBDRplus

The twin cubator and shaking water bath were pre-warmed to 45°C. Before use, the reagents Hybridization (HYB), Rinse (R), and Stringent (STR) were pre-warmed in a water bath to 37 - 45°C. Sample numbers were written on the tray wells. 20µL denaturation solution and 20µL amplified sample were added to each well and incubated at room temperature for 5 minutes. Strips for genotypic medications (MTBDRplus) were removed with tweezers and placed in the appropriate trays for each drug. Each well received one ml of pre-warmed hybridization buffer, and the strips were inserted in the wells away from the reagents. The tray were gently shook to obtain a uniform colour before being placed in the shaking twin incubator and incubated at 45°C for 30 minutes.

After completely aspirating the hybridization buffer, 1 ml of stringent solution were applied to each well and incubated for 15 minutes at 45 C in the shaking twin incubator. By decanting and turning the tray upside down, the stringent solution were fully eliminated. Each strip were rinsed with 1 ml rinse solution for 1 minute on the shaking twin cubator before being incubated for 30 minutes on the shaking twin incubator with 1 ml of diluted conjugate. On the Twin incubator, the solution were removed, and the strip washed twice for l minutes with 1 ml rinse solution and rinsed with 1 ml distilled water. After rinsing, each strip received 1000 cc of diluted substrate and were incubated in the dark without shaking.

Rinsing twice with 1 mL pure water stopped the reaction. Strips were retrieved from the tray with tweezers, dried between papers, and attached to the worksheet. The results were interpreted using charts. During the hybridization processes, probes are placed in the strips that, if present in the amplicons, will complement the right DNA sequence. When the amplicons are subjected to all of the conditions specified in the protocol, these complementary sequences are visible as bands due to the biotinylation of the primers employed in the amplification process.

RESULTS AND DISCUSSIONS

Prevalence of *Mycobacteria Tubercles* (MTB) among HIV Patients

Table 1 shows the prevalence of MTB among HIV patients in relation to state. The highest prevalence was recorded from Katsina (22.2 %) followed by Zamfara (5.5%), Kano (4.7 (3.6%), Kebbi (4.3%), Kaduna (4.2%) and the lowest was recorded from Sokoto (3.6 %)

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The prevalence of MTB among HIV patients in relation to gender is as given in Table 2. Out of 503 samples collected the overall prevalence was 4.3 %. The highest prevalence was observed among male patients (4.8%) while the female patients (3.4%).

The prevalence of MTB among HIV patients in relation to age groups is as given in Table 3. Age 66-70 yrs recorded the highest prevalence (100 %) followed by 56-60 yrs (8.3 %), 21-25 yrs (7.8 %), 26-30 yrs. (6.7 %), 31-35 yrs (4.7 %) and the lowest was 36-40 yrs and 41-45yrs (4.4 %)

Table 1: Prevalence of MTB among HIV patents attending tuberculosis reference centres in northwest, Nigeria in relation to state

States	**No. of samples**	**No. (%) MTB positive**
Kebbi	46	2 (4.3)
Kaduna	118	5(4.2)
Kano	63	3(4.7)
Katsina	9	2(22.2)
Zamfara	36	2(5.5)
Sokoto	222	8(3.6)

Table 2: Prevalence of MTB HIV patents attending tuberculosis reference centres in northwest, Nigeria in relation to gender

Sex	**No. of samples**	**No. (%) MTB positive**
Male	327	16 (4.8)
Female	176	6(3.4)
Total	503	22(4.3)

Table 3: Prevalence of MTB HIV patents attending tuberculosis reference centres in northwest, Nigeria in relation to Age Group

Age Group	**No. of samples**	**No. (%) MTB positive**
≥-20	87	0(0.0)
21-25	89	7(7.8)
26-30	89	6(6.7)
31-35	42	2(4.7)
36-40	71	3(4.4)
41-45	45	2(4.4)
46-50	36	0(0.0)

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51-55	24	0(0.0)
56-60	12	1(8.3)
61-65	7	0(0.0)
66-70	1	1(100)

The Drug resistance patterns

Table 4 showed the Drug resistance patterns of *M. tuberculosis* isolate from HIV patients attending tuberculosis reference centres in northwest, Nigeria showed that the highest resistance patterns were isolates from Kebbi were resistance to INH+ RIF+ FLQ (50.0 %) followed by Kano were resistance to INH+ FLQ (33.3 %) Sokoto were resistance to INH+ RIF+ FLQ (25.0 %) and the lowest was from Kaduna INH+ AMG (20.0 %).

The overall, of the valid results obtained from 22 samples, 17 (77.2 %) were Susceptible to both drugs, 5 (22.7%) were resistant to RIF and/or INH, 2 (9.0 %) were MDR-TB cases and 3 were Monoresistance to INH.

Table 4 Drug resistance patterns of *M. tuberculosis* isolate from HIV patients attending tuberculosis reference centres in northwest, Nigeria

States	INH resistant n(%)	RIF resistant n(%)	AMG resistant n(%)	FLQ resistant n(%)	MDR-TB n(%)
Kebbi (n=2)	1(0.0)	1(0.0)	0(0.0)	1(0.0)	1(50.0)
Kaduna (n=5)	1(20.0)	0(0.0)	1(20.0)	0(0.0)	1(20.0)
Kano (n=3)	1(33.3)	0 (0.0)	0(0.0)	1(33.3)	1(33.3)
Katsina (n=2)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Sokoto (n=8)	2(25.0)	1(12.5)	0(0.0)	1(12.5)	2 (25.0)
Zamfara (n=2)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)

Key; isoniazid (INH), rifampicin (RIF), aminoglycosides (AMG) and fluoroquinolones (FLQ)

Table 5. GenoType MTBDRplus among HIV patients attending tuberculosis reference centres in northwest, Nigeria

Resistant status	**No .% of cases (n=22)**
Susceptible	17 (77.2)
Any resistance	5 (22.7)
RIF	2 (9.0)
INH	5 (22.7)
Monoresistance	
RIF	0 (0.0)

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INH	3 (13.6)
Multidrug resistance	2 (9.0)

Key; isoniazid (INH), rifampicin (RIF)

DISCUSSIONS

The dissemination of antibiotic resistance including the emergence and re-emergence of anti- TB drug resistance strains is linked to pathogen originating from resistance that arises both in newly infected (primary) individuals and in those undergoing retreatment, contributing to the transmission and amplification of resistant *M. tuberculosis* strains [12].

This study investigated the efficacy of anti-tuberculosis drugs studies assessing the effectiveness of the anti-TB drugs in the treatment of MDR -TB isolates from HIV patients under concomitant treatments attending tuberculosis reference centres in northwest, Nigeria**.**

In the present study, the overall prevalence of MDR-TB cases among HIV patients was 4.2%. According to the review paper by Biadg-legne *et al*. [13] the proportion of MDR-TB cases varies from place to place. The finding from this study is comparable to the studies conducted by Getahun *et al*. (2010) that The HIV co-infection was lower than national averages but underreporting is likely. Routine HIV testing was suboptimal, limiting full interpretation of TB/HIV overlap. Findings revealed other African studies where a significant fraction of TB patients have unknown HIV status [14].

The HIV and drug resistance interaction shows that Rifampicin resistance was higher among HIV- individuals. This Suggests MDR-TB is not necessarily concentrated in immunocompromised groups, contrary to some assumptions. The patient Follow-Up tests for treatment monitoring among HIV individuals had majority of the cases that is under diagnosis, with only few follow-up testing. The study analysis show low rate which could possibly reflect loss to follow-up, poor health system support, or financial/transport barriers. A similar distribution was noted in Malaysia[15]. The key implications of the analysis showing high MDR-TB burden in Northwest Nigeria is that there is need for urgent strengthening of surveillance, diagnostics, and treatment programs. The hotspot states (Sokoto, Kaduna, Kano) should be prioritized for interventions. Likewise, the repurposed drugs (levofloxacin, moxifloxacin) may remain highly effective but require monitoring for resistance emergence. The routine HIV testing among TB patients must be improved to guide co-management. In addition, the DST capacity should be expanded, especially for new and repurposed drugs, to ensure early detection and appropriate treatment regimens.

CONCLUSION

The data from this study suggests a notable prevalence of MDR-TB among HIV individuals in northwest Nigeria. The isolates were resistant to Rifampicin, isoniazid. The highest combine resistance recorded in this study was INH+ RIF+ FLQ (50.0 %). However, there is low prevalence of MDR-MTB among HIV-positive patients in the study area.

Compliance With Ethical Standards

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Disclosure Of Conflict of Interest

No conflict of interest among of the authors

Statement of informed consent

Informed consent was obtained from all individual participants included in this study

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