www.jmscr.igmpublication.org
Impact Factor (SJIF): 6.379
Index Copernicus Value: 79.54
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossrefDOI: https://dx.doi.org/10.18535/jmscr/v6i12.41

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

Genotypic Characterization of Drug Resistant *Mycobacterium tuberculosis* and Non-Tuberculous Mycobacteria in Clinical Isolates from Subjects Resident in Rivers State, Nigeria

Authors
Mac-Fiberesima Gborieneomie[*1], Azuonwu Obioma[2], C. K. Wachukwu[2], S. D. Abbey[2]
[1]South-South Tuberculosis Zonal Reference Laboratory, University of Port Harcourt Teaching Hospital, Nigeria
[2]Department of Medical Laboratory Science, Rivers State University, Port Harcourt, Nigeria
[*]Corresponding Author
Mac-Fiberesima Gborieneomie
Email: *macfibs04@yahoo.com*, Phone: +2348037079310

Abstract
The emergence and increasing prevalence of Mycobacterium tuberculosis strains resistant to first and second line anti-tuberculous medications are exacerbating the global TB epidemic. The aim of this study was to genotypically characterize and identify resistant genes of Mycobacterium tuberculosis and non-mycobacterium tuberculosis isolates recovered from clinical specimens of patients infected with Drug Resistant Tuberculosis strains and test the isolates against first and second line anti tuberculosis drugs. Three hundred and ninety (390) sputum samples were collected from the study participants in University of Port Harcourt Teaching Hospital, Braithwaite Memorial Hospital, Bori, Ahoada, Degema and Chest Clinic Rivers State Port Harcourt. The samples were received and analyzed by decontaminating with NaOH-Citrate NaCl method before conventionally inoculating them unto Lowenstein Jensen slants and incubated at 37°C for 8weeks. GeneXpert was analysed by decontaminating with isopropanol under a biosafety cabinet which were later processed in the GeneXpert machine. DNA was extracted with the chemical method using genolyse following manufacturer's instructions. First line drug susceptibility testing was done genotypically with Genotype MTBDRplus. Second line Drug susceptibility testing was done genotypically with Genotype MTBDRsl kit and sequencing was done with 16S rNA sequence analysis. Total number of participants was higher in females (61.53%) than in males (38.46%) $p<0.05$ and there was no significant statistical difference in prevalence of TB with respect to site. Prevalence of MTB-NTM co-infection was 14.10%. Of the 390 samples tested, 45 (11.53%) were positive for MTB out of which 10(2.56%) were resistant for rifampicin. 52(13.33%) detected MTB out of which 14(3.58%) were resistant for rifampicin respectively. LPA and culture was also compared, of the 390 samples, 45(11.3%) were positive for MTB out of which 10(2.56%) were rifampicin resistant for LPA and 39(10.00%) were culture positive out of which 9(2.30) were rifampicin resistant. Resistance due to isoniazid was 6(1.53%) for LPA and 4(1.02%) for culture. Multiple resistance for rifampicin and isoniazide was 16(4.10) for LPA and 13(3.33) for culture. The same was done for GeneXpert and Culture. Xpert detected 52(13.33%) MTB out of which 14(3.58%) were rifampicin resistant and Culture isolated 39(10.00%) MTB out of which 9(2.30%) were rifampicin resistant. There was no statistical significance observed with respect to type of

assay. 57(14.61%) were tested against second line LPA out of which 4(7.01%) were resistant to the fluoroquinolones and none was resistant to the aminoglycosides which was predominant among age 21-40 years. Seven out of the sixteen isolates were sequenced. Samples 1, 3, 4, 6 and 8 detected amplification for rpoB gene responsible for rifampicin resistance (57.14%) and samples 3, 4, and 8 (42.85%) detected amplification for katG gene. Samples 8, 9, 10, 11, and 12 were NTMs which were detected also for rpoB (100%) and sample 10 (20%) alone detected amplification for katG gene. No resistant gene was detected for inhA. 5(31.25%) NTM from the 16NMTBC isolated from the study were sequenced. Mycobacterium abscessus (40%), Mycobacterium setense (20%), Mycobacterium peregrinum (20%) and Mycobacterium conceptionense (20%). Pragmatic efforts are needed to halt drug resistance from progressing beyond what has been identified. It is recommended that people develop the habit of accessing health care early enough to stop further progress in TB.

Keywords: *Genotype, drug resistance, Mycobacteriun tuberculosis, Port Harcourt, Rivers State.*

Introduction

Mycobacterium tuberculosis (MTB) is a worldwide public health threat responsible for approximately 8.6 million incident cases of tuberculosis (TB) and an estimated 1.3 million deaths annually[1]. The emergence and increasing prevalence of *Mycobacterium tuberculosis* strains resistant to first and second line anti-tuberculosis medications are exacerbating the global TB epidemic[2]. Multidrug resistant (MDR) strains are *Mycobacterium tuberculosis* strains resistant to both rifampicin (RIF) and isoniazid (INH), the most effective first-line drugs. Extensively drug resistant (XDR) *Mycobacterium tuberculosis* strains, are defined as strains with MDR plus resistance to any fluoroquinolone (FQ) and at least one of the second line injectable drugs used commonly for treating TB[3]. As of 2012, the World Health Organization (WHO) estimated the global prevalence of MDR-TB to be 3.6% among new TB cases and 20% among recurrent TB cases[1].

Despite the TB burden and high number of drug resistant TB cases in Nigeria, relatively few studies have performed comprehensive analysis of TB using Phenotypic and genotypic assay. Little is known about the MTB complex species associated with TB in Nigeria as a result of limited facilities for TB culture and molecular assay. A better understanding of the circulating MTB complex species and resistance to TB drugs is an essential tool for diagnosis and therapeutic measure which aims at controlling the public health burden of TB in Nigeria. There are new testing molecular techniques which are available for the isolation and characterization of members of the MTB complex including a genotypic MTBC (Hain Assay) that enables quick identification and differentiation of members of MTB complex using growth positive samples or direct clinical specimen with high degree of sensitivity and specificity when compared to conventional method[4-6].

Sequencing DNA for the detection of mutations associated with MTB resistance is another molecular technique not readily available in the commercial setting due to cost effectiveness, technical expertise and time. However it is readily found in some research and public health Laboratories. It remains the gold standard for detecting genetic mutations which is the reason behind drug resistance. The technique results in DNA fragments of various lengths which can be separated by electrophoresis and subsequently DNA is sequenced. The technique is highly accurate and provides the advantage of being able to read larger amounts of DNA[7]. Irrespective of the numerous contributions of the current molecular assays, there is a gap to fill because of the disparities encountered when comparing results of the molecular assays and the conventional technology. The aim of this study is to characterize *Mycobacterium tuberculosis* recovered from clinical specimens of patients infected with drug resistant tuberculosis using phenotypic and genotypic assays.

"MDR-TB is TB that is resistant to at least two of the most widely used anti TB drugs which are rifampicin (RIF) and isoniazide (INH) (WHO, 2013"). "The WHO drug resistant TB surveillance report of 2014 recorded a prevalence rate of 5% new MDR-TB

cases globally and at least half a million cases add up to the existing number yearly". "The primary diagnostic culture method is unable to diagnose TB in a timely manner". "The turnaround time takes a minimum of four weeks to diagnose TB and another minimum of four weeks for the Drug susceptibility testing (DST) to be reported for culture positive cases". "The delay encountered, results to delay in commencing treatment which eventually promotes infectiousness and ultimate mortality. "Therefore, the way forward is to adopt a technology that is timely, robust and efficient".

"A wide range of genotypic assays are in circulation in the developing countries which were introduced by WHO in 2008 and 2010". "The Xpert MTB RIF and Line Probe Assay (LPA) have a shorter turnaround time compared to the conventional culture method". The GeneXpert has the capacity to detect TB and rifampicin resistance simultaneously with a turnaround time of Two hours". "The LPA can simultaneously detect TB and conduct DST for RIF and INH". "It can detect resistance to both RIF and INH because of the presence of the mutation gene on the probes hence requires a highly experienced manpower to be able to interpret the results". The two molecular assays are unable to differentiate MTB complex from NTM". The Xpert is sensitive and requires less technical expertise to manipulate". "However, the two assays are not readily in use as a result of the cost implication hence data on their utilization is scarce in Nigeria especially in South-South". The aim of this study was to genotypically characterize and identify resistant genes of *Mycobacterium tuberculosis* and non-mycobacterium tuberculosis isolates recovered from clinical specimens of patients infected with Drug Resistant Tuberculosis strains and test the isolates against first and second line anti tuberculosis drugs.

Materials and Methods

Study Area

The study area that was selected for this study was Rivers State with the capital city as Port Harcourt. It is a tourist dream center with magnificent architecture that attracts both national and international attention. It is endowed with natural treasure such as crude oil. It is the largest city in Rivers State and the fifth largest in Nigeria with a reported estimated population of 1,865,000. It lies along the Bonny River located in Niger Delta. It was selected as the study area because of the vast classes of people with their different lifestyles based on tradition, culture, beliefs and orientation.

"The study was carried out in three different sites". "Firstly in South-South Tuberculosis Zonal Reference Laboratory University of Port Harcourt Teaching Hospital Choba, Rivers State". Secondly in Medical Laboratory Science Department Niger Delta University Amassoma, Bayelsa State and thirdly in Ingaba Biotechnical Industries Limited Hatfield 0028, Pretoria South Africa between 2016 to 2018".

Study Design

A cross sectional study design was adopted for this study". The study design examines the relationship between diseases and other related health characteristics and variables of interest such as age, sex, marital status, area of residence". It is adequate for quantifying the prevalence of a disease or risk factors and for estimating the accuracy of a diagnostic test". Clinical specimens were randomly collected from six different locations which represent a sub group of South-South in Nigeria". Individuals and sites were randomly selected based on symptoms and prevalence".

Sample Size

The sample size was determined according to[8].

$N=z^2pq/d^2$ where:

n=desired sample size, z= standard deviation, P = proportion in target population, q = degree of accuracy, P =0.5, z = 1.96, and d = 0.05. Hence n = $(1.96^2)(0.5)(0.5)/0.05^2$3.8416 × 0.25/0.00250.9604 /0.0025. N= 384 Approximately 390 was finally taken to be the sample size for this study.

Ethical Approval and informed Consent

Having fulfilled the conditions provided by the Ethical Committee of University of Port Harcourt Teaching Hospital and Rivers State Teaching Hospital Port Harcourt, the participants were given questionnaires accompanied with a written informed

consent. All completed questionnaires were recovered from the participants before sputum samples were collected. In order to ensure confidentiality and anonymity, names and address of the participants were not included in the study.

Eligibility Criteria

Patients whose cough has lasted for at least two weeks, and HIV positive patients will be included in the research. Early morning expectorated sputum and on site spot sputum samples without blood will be accepted.

Inclusion and Exclusion Criteria

All participants that consented to the study who are teenagers and adults ranging from age 13 to 70 years were included in the study and those whose cough has lasted for two weeks.

Obvious healthy individuals within the age bracket who consented to the study but did not have signs of cough that has lasted for two weeks were excluded from the study. Also those who consented to the study and are within the age bracket with signs of cough lasting for two weeks but could not produce visible sputum were excluded. Children were also excluded as they are often challenged with difficulty in producing sputum.

Sample Collection

Two hundred and thirty two males and One hundred and fifty eight females who met the criteria for the study made up the study population. Each participant was given two sample collection containers to collect sputum. On return, the sample was given a unique identification number. Two samples were collected to enable the researcher have enough sample to conduct the various analysis.

Sample Collection Procedure

Two 50ml falcon tubes with lid were given to each participant to expectorate sputum. Participants were counseled to wash mouth with clean water in the morning before brushing their mouth or chewing stick. Thereafter, they were asked to breathe inn and out up to four times to expel air into their lungs which will help them to regurgitate to cough. Sputum is then produced into the falcon tubes and then tightly covered with the lid. Identification numbers were given to them and then transported in cold chain with triple packaging to the South-South Tuberculosis Zonal Reference Laboratory.

Laboratory Procedures

GeneXpert

Molecular GeneXpert Mtb/Rif technique was done with the GeneXpert automated machine produced by Cepheid. Following the Manufacturer's instructions [9] samples were analysed in a class 11 biological safety cabinet. Two mililiters of the sputum samples were mixed with 4mls of sample reagent (Isopropanol) with a sterile Pasteur pipette and agitated using a vortex mixer for 5minutes. They were allowed to incubate at room temperature for 10minutes and then agitated again for the second 5minutes. A Pasteur pipette was used per sample to transfer the suspension into the GeneXpert catridge labeled with a corresponding unique number of the sample. The GeneXpert camera was used to snap the catridge barcode. The participant details were entered into the GeneXpert machine and the catridges were loaded into the machine following the command. The machine ran for 2 hours and the results were displayed on the machine monitor

Line Probe Assay (Genotypic Assay)

The Line Probe Assay (LPA) according to Hain Life Science was also used to conduct Drug Susceptibility Testing for First line drugs (rifampicin and isoniazide) and second line drugs (fluoroquinolones and amino glycosides). The assay involves five different steps which includes DNA extraction, Polymerase Chain Reaction (PCR) Amplification, Hybridization and Detection.

Extraction of DNA

"Following the cultivation of *Mycobacterium tuberculosis*, DNA was extracted by the chemical method using the Genolyse Kit". Three hundread micro liter molecular grade water was dispensed with a pipette into sufficient 200µl screw cap tubes for 1 tube per culture". Using sterile disposable inoculation loop of 1µl capacity, bacteria was collected from media with sufficient growth". They were incubated for 20 minutes at 95°C in a water bath"." Thereafter, they were incubated for 15 minutes in ultrasonic bath then centrifuged for 15 minutes at 10,000 x g in a standard table top

centrifuge". "The supernatant was discarded and the pellet resuspended in 100 µl lysis Buffer (A-LYS) by vortexing". The samples were incubated for 5 minutes at 95°C in a water bath and briefly spun down".

100 µl Neutralization Buffer (A-NB) was added to the lysate and the samples were votexed for 5 seconds". They were further spun down for 5 minutes at full speed and 5-10 µl of the supernatant was used for Polymerase Chain Reaction (PCR)".

Master Mix

"The master mix was prepared in a DNA free room by constituting a reagent solution of 45µl which is a solution of 10 µl AMP-A and 35 µl AMP-B". "The DNA was added in a separate room called the sample addition room. 5 µl of DNA was added into the master mix". "This was done for every PCR tube per sample". "The number of samples to run was determined mostly 12 samples in a batch including controls".

Amplification

PCR tubes that have bubbles at the base were removed by swinging arm with tubes in hand in arc. The PCR tubes were transferred to the middle section of the thermo cycler. The program to be used was chosen (clinical). The program parameter was followed by the command given from the menu. The thermo cycler was ran at different temperatures in a given circle.

Hybridization

The HYB and STR solutions (green and red) were pre warmed at 45°C in a water bath for 15 minutes. Also the Twin Cubator was pre warmed to 45°C. Pipette 20 µl of the DEN solution was (denaturing solution) dispensed with a pipette to each well of tray to be used. Then 20 µl of corresponding amplified DNA sample was added to each well, and mix well by pipetting up and down several times. The tray was incubated for 5 minutes at room temperature. The PCR strips were removed from the tube and placed one after the other in the tray according to the numbers labeled on them. Then 1ml HYB (hybridization solution) was added to each well and gently shaken to homogenize solution. The tray was placed on the Twin Cubator and press "START" to incubate for 30 minutes at 45°C. From this point, the right arrow on Twin Cubator was pressed once to advance steps in protocol. At the end of 1minute, the tray was removed and the HYB solution was gently poured off. The remaining solution was removed by carefully tapping tray against the paper towel on the bench top. 1mi of STR (stringent buffer) was added to each well in the tray and incubated for 15minutes in Twin Cubator at 45°C. While it was incubating, the conjugate and substrate was prepared in a 15ml conical vials by diluting 1:100ml with the corresponding Con-d and Sub-D. The substrate dilution was wrapped in a foil to avoid colour change. The STR was completely removed as it was done for HYB. The rinse solution (1ml RIN) was dispensed into each well and incubated in a Twin Cubator for 1 minute. The rinse was also removed and 1 ml of diluted conjugate dispensed into each well. This was left to incubate for 30 minutes. The solution was removed and the tray washed with 1 ml of RIN per well on the Twin Cubator. The solution was poured out and 1 ml of sterile distilled water used to wash the well for 1minute using the Twin Cubator. The water was removed and 1 ml of diluted substrate added per well. The water was also removed and 1ml of diluted substrate added in each well. This was washed twice with distilled water and water was removed. The PCR strips were removed with colour bands on them for result interpretation. The strips were pasted on a reading map for easy interpretation.

Molecular Identification for Sequencing

Bacterial Genomic DNA Extraction

"Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min". "The cells were re-suspended in 500ul of normal saline and heated at 95^0C for 20 min". "The heated bactererial suspension was cooled on ice and spun for 3 min at 14000rpm". "The supernatant containing the DNA was transferred to a" 1.5ml "micro centrifuge tube and stored at -20^oC for other downstream reactions".

DNA quantification"

"The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer"

"**rRNA Amplification**

"The 16s RRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'- "AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles". The PCR mix included: the X2 Dream taq Master mixsupplied by Inqaba South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of" 0.4M "and the extracted DNA as template". "The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; anealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes". "The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator".

*"**DNA Quantification**"*

"The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer"

Sequencing

"Sequencing was done using the Big Dye Terminator kit on on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa".

"**Phylogenetic Analysis**"

Obtained sequences were edited using the bioinformatics algorithm". Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN". These sequences were aligned using ClustalX". The evolutionary history was inferred using the Neighbor-Joining method in MEGA" 6.0[10]. "The bootstrap consensus tree inferred from 500 replicates[10] is taken to represent the evolutionary history of the taxa analyzed". The evolutionary distances were computed using the Jukes-Cantor method[11].

Results

4.1 Demographic Characteristics of the TB risk factors Stratified by Site

Gender	UPTH Freq (%)	BMSH freq (%)	Bori Freq (%)	Chest Clinic freq (%)	Ahoada freq (%)	Degema freq (%)	Total Freq (%)
TOTAL	**9**	**10**	**12**	**8**	**7**	**6**	**52**
Male	3(4.61)	4 (6.15)	3(4.61)	5(7.69)	2(3.07)	3(4.61)	20(38.46)
Female	6(9.23)	6(9.23)	9(13.84)	3(4.61)	5(7.69)	3(4.61)	32(61.53)
X2 (p-value)							**5.54(0.02)***
AGE							
<20	1(1.53)	2(3.07)	0(0.00)	1(1.53)	0(0.00)	0(0.00)	4(7.69)
20-40	3(4.61)	5(7.69)	8(15.38)	5(7.69)	2(3.07)	3(4.61)	26(50.00)
41-60	4(6.15)	3(4.61)	3(4.61)	2(3.07)	4(6.15)	1(1.53)	17(32.69)
>60	1(1.53)	0(0.00)	1(1.53)	0(0.00)	1(1.53)	2(3.07)	5(9.61)
X2 (p-value)							**33.85(0.001)***
Residence							
Urban	6(9.23)	3(4.61)	8(15.38)	2(3.07)	2(3.07)	3(4.61)	23(44.23)
Rural	3(4.61)	7(10.76)	4(6.15)	6(9.23)	5(7.69)	3(4.61)	29(55.76)
X2 (p-value)							**1.38(0.239)**
Occupation							
Trader	2(3.07)	4(6.15)	6(11.53)	3(4.61)	4(6.15)	1(1.53)	17(32.69)
House wife	3(4.61)	2(3.07)	1(1.53)	1(1.53)	1(1.53)	2(3.07)	10(28.84)
Student	2(3.07)	2(3.07)	4(6.15)	1(1.53)	1(1.53)	3(4.61)	13(23.07)
Health Provider	0(0.00)	0(0.00)	1(1.53)	0(0.00)	0(0.00)	0(0.00)	1(1.92)
Others	2(3.07)	2(3.07)	0(0.00)	3(4.61)	1(1.53)	0(0.00)	8(15.38)
X2 (p value)							**19.10(0.001)***

Table 4.2 Genotypic Drug Resistance to Second Line anti TB Drugs

Age Group (years)	No Tested	Second Line Drugs (Resistance)	
		AMN	FLU
< 20	16(28.07)	0 (0.00)	0 (0.00)
21-40	21(36.84)	0 (0.00)	2 (3.50)
41-60	14 (24.56)	0 (0.00)	2 (3.50)
>60	6(10.52)	0(0.00	0(0.00)
Total	**57 (100.0)**	**0 (0.0)**	**4 (7.01)**

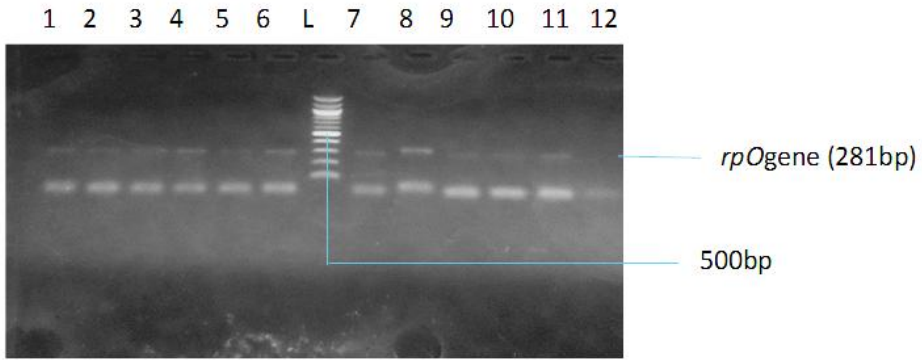


Plate 1: agarose gel electrophoresis of amplified *rpO* gene (281bp). Lane L represents the 100bp molecular ladder. Lanes 1,3,4, 6 and 7 showed amplification for rpoB gene.

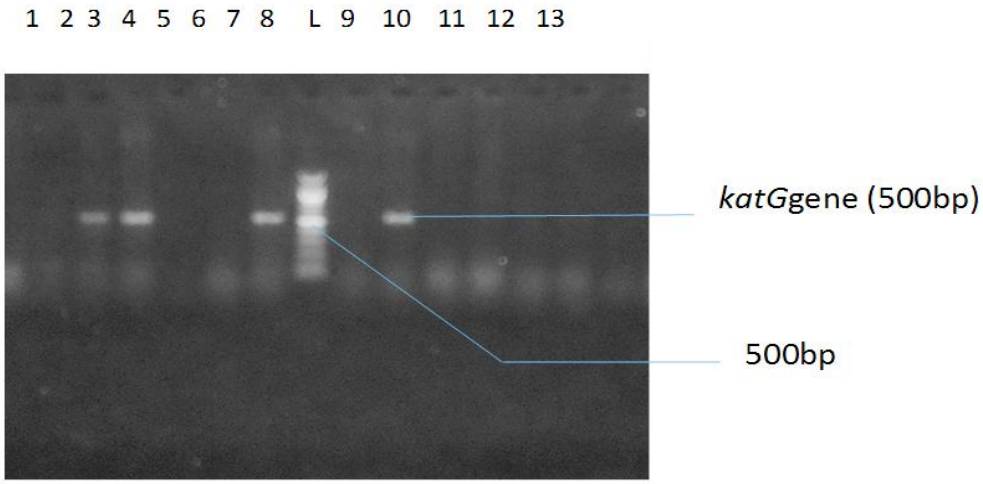


Plate 2: Agarose gel electrophoresis showing the amplified *katG*gene. Lanes 3,4,8 and 10 showed *katG* bands while L represents 100p molecular ladder.

Lanes 3, 4, 8, and 10 detected amplification for katG gene

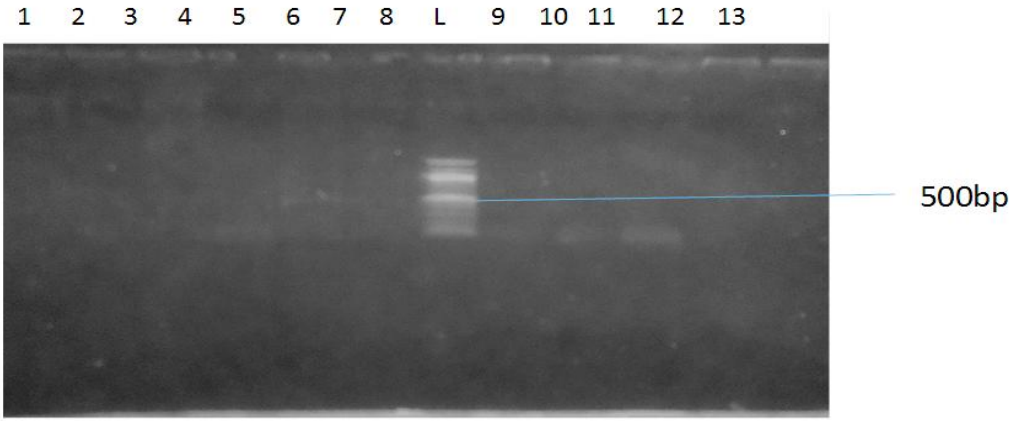
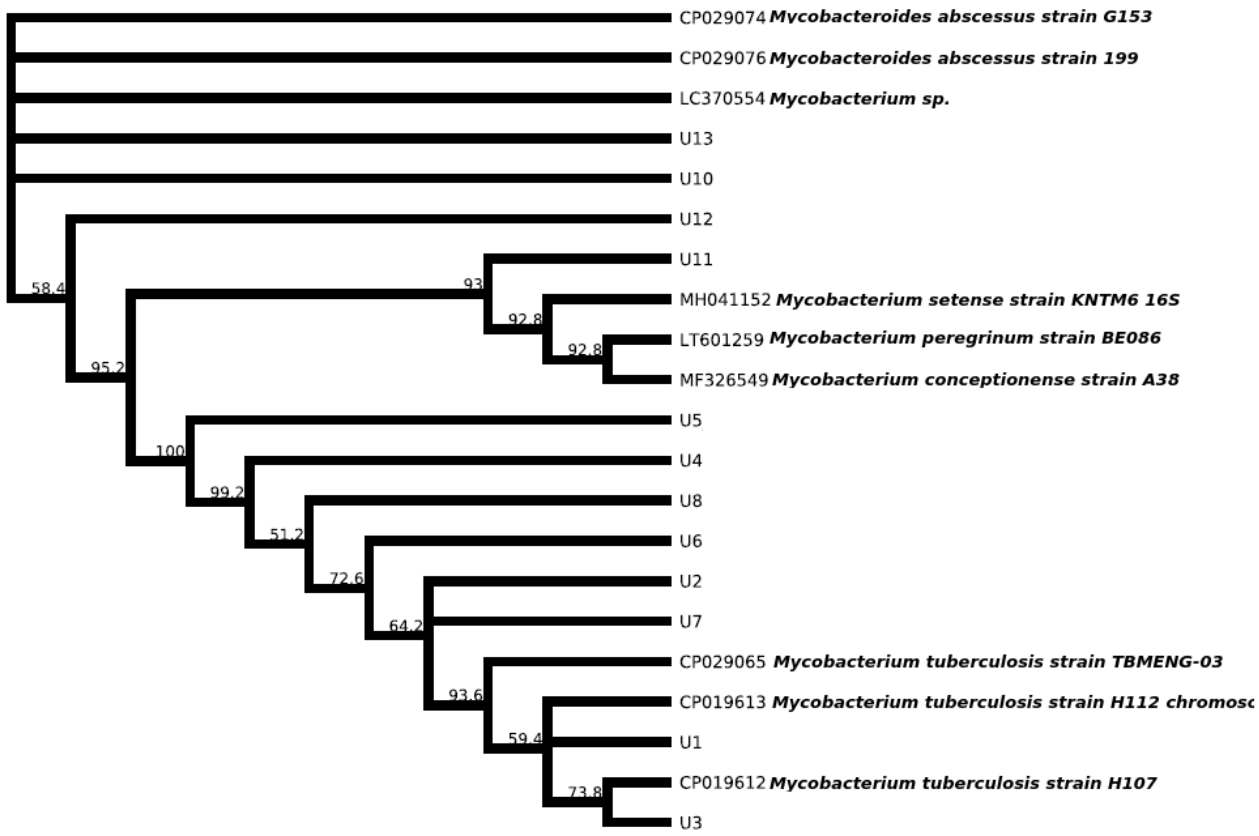


Plate 3: Agarose gel electrophoresis of the amplified *inhA* gene showing no amplification. Lane L represents the 100bp molecular ladder.

Table 4.3 Distribution of Mutation with Line Probe Assay

Locus	Mutant	Codon	No. of Strains
rpoB –WT1 –WT8	Mut 1	D516V	2(15.38%)
	Mut 2A	H526Y	2 (15.38%)
	Mut 2B	H526D	1(7.69%)
	Mut 3	S531L	2(15.38%)
	Unknown		1(7.69%)
	NMTBC		5(38.46%)
		Total	13
katG – WT	Mut 1	S315T1	4(30.76%)
	Mut 2	S315T2	2(15.38)
	Unknown		2(15.38%)
		Total	8
inhA – WT1 – WT2	Mut 1	C15T	1(15.38%)
	Mut 2	A16G	
		Total	2

Distribution of Mutations with Line Probe Assay



Phylogenetic tree showing the evolutionary relationship among the *Mycobacterium sp* obtained from the patients.

Figure 4.1: Phylogenic Tree showing Evolutionary relationship among the *Mycobacterium sp.* obtained from patients

Discussion

The aim of this study was to characterize Drug Resistant Mycobacterium tuberculosis isolated from the study participants using genotypic assay. Different approaches were used to achieve this aim. Obvious, significant results were achieved in most of the demonstrations however, some variables were less significant.

Our study performed identification of resistant genes among the isolated Mycobacterium Multi-resistant isolates using the 16S rNA sequencing. Seven out of the sixteen isolates were sequenced. Samples 1, 3, 4, 6 and 8 detected amplification for rpoB gene responsible for rifampicin resistance (57.14%) and samples 3, 4, and 8 (42.85%) detected amplification for katG gene. Samples 8, 9, 10, 11, and 12 were NTMs which were detected also for rpoB (100%) and sample 10 (20%) alone detected amplification for katG gene. No resistant gene was detected for inhA. Xiaoliang *et al*, 2012 reported detection of rpoB gene mutation from MDR-TB isolates at a proportion of 44.8% and 55.8% for katG which is closely comparable to the findings of our study.[12] 14% of MTBC identified were rifampicin resistant out of which 3 were NTM with Genotype MTBDRplus. Three NTMs that were resistant to isoniazid and rifampicin by phenotypic and genotypic methods did not match with

sequencing results. It was disclosed in another study that the samples from JUTH with a mutation in codon 526 of the *rpoB* gene showed a different point substitution that is responsible for resistance. The most common mutation spanning the *rpoB*^{wt8} codon in NIMR samples was S531L. Only 2 out of the 16 resistant samples had discordant results with the GenoType MTBDR plus test and sequencing. There was concordance with sample LG7 which was still resistant to RIF, and one of the three mutations indicated by GenoType MTBDRplus (*rpoB*^{wt4} D516A).

Also,[13] in their research in Ibadan, Nnewi and Abuja, Nigeria reported the distribution of MDR-TB isolates from phenotypic Drug Susceptibility Testing (DST) in BACTEC-MGIT® (Becton Dickinson, NJ, USA). They isolated 29 (7%) from 407 isolates as MDR among which 23 belonged to the CAM family and 17 were SIT 61. Resistant level of drugs among the CAM and T clades were reported to be 53% and 54% respectively. Only one out of the 53 *M. africanum* isolates was MDR and 17 (32%) were resistant to one drug. Highest proportion of MDR (28%) was seen among the H family. In this study, there was mutation disparity between the two molecular methods. Table 4.3 showing mutation with LPA detected inhA resistance for isoniazid (Mut 1 and Mut 2) which was not detected by sequencing. Also resistance to NTM by Genotype MTBDRplus gave a proportion of 5(35.46%) against Sequencing (100%) for rpoB and (20%) for katG. These disparities can be attributed to differences in site and expertise of personnel in reading and interpreting the results. The primers used in the assay could be a source why there is discordance in results.

Speciation of NTN with molecular methods particularly sequencing will give a more reliable prospect for appropriate patient management. We sequenced 5(31.25%) NMTBC isolates from the 16 NMTBC isolated from the study. The identified NTMs were *Mycobacterium abscessus* (40%), *Mycobacterium setense* (20%), *Mycobacterium peregrinum* (20%) and *Mycobacterium conceptionense* (20%). Haican *et al*, 2016 in China identified *M. intracellulare*, followed by *M. avium* and *M. abscessus* from a collection of 27 NTM that was sequenced.[14] also identified 1.4% of *Mycobacterium peregrinum* out of 69(15%) of NTM isolated.[15] also identified 1249 (39%) of *M. abscessus* which were likely isolated from older age with P <0.0001. A phylogenic analysis identified *M. setense* and *M. conceptionense* and sample was collected from a bone graft of a 66years old man where sequencing result showed above 97% for rpoB gene. It is evident that NTM infection in humans is no longer new and its implication in disease control has become more paramount than previously taught.

The report of this study has shown that NTM infection is not limited to the advanced countries where modern diagnostic tools are often provided. It is not surprising to isolate NTM from this part of the country reason being that it is an endemic area for TB disease and just a few facilities are provided with appropriate skilled personnel capable of identifying NTM from sputa by conventional or molecular methods. It is timely however to note that some of the diseases of unknown origin may perhaps be implicated with NTM infection owing to the basic fact that NTM is predominant in the environment, water and soil. The study area may have also encouraged the prevalence of NTM disease as the area is predominantly surrounded with water and occupation of the indigenes are mainly farming and fishing. It has been reported that NTM cause mycobacteriosis in fish. The predominant NTM isolated from fish include but not limited to *M, abscessus* and *M. peregrinum* as reported by[16-18]. NTM can be isolated from immune-competent and immune-suppressed individuals. Bearing this in mind, it is possible that NTM have been transmitted into portable water which can be aerosolized and contracted by other susceptible individuals.[19] It has been reported that *M. avium, M. abscessus, M. lentiflavum,* and *M. kansasii* have been isolated from household water of people infected with NTM. Further studies are required to highlight more facts about NTM disease.

Conclusion

The findings of this study highlights the importance of using conventional and genotypic methods to diagnose tuberculosis. It was discovered in the current study that prevalence of tuberculosis is increasing. Morbidity and mortality resulting from the disease is also increasing as the organism is constantly mutating to resist the potency of drugs. Different forms of the disease has emerged. Drug resistant TB, Multidrug resistant TB, pre-Extensively drug resistant TB and Extensively drug resistant TB which may show up in the near future within the study area with further studies are all raging war against the human race. Infection with Non-Tuberculous Mycobacteria (NTM) was discovered from the findings of this study. Pragmatic efforts are also needed to halt drug resistance from progressing beyond what has been identified. It is recommended that people develop the habit of accessing health care early enough to stop further progress of TB.

References

1. WHO. Global Tuberculosis Report 2013. Geneva, Switzerland: World Health Organization.
2. Al-Mutairi, N.M., Ahmad, S., Mokaddas, E. (2011). First report of molecular detection of fluoroquinolone resistance associated gyrA mutations in multidrug-resistant clinical Mycobacterium tuberculosis isolates in Kuwait. *Biomed Central Research Notes*, 4,123-124
3. Ando, H., Mitarai, S., Kondo, Y., Suetake, T., Kato, S., Mori, T. (2011). Evaluation of a line probe assay for the rapid detection of gyrA mutations associated with fluoroquinolone resistance in multidrug-resistant Mycobacterium tuberculosis. *Journal of Medical Microbiology*, 60(2), 184–188.
4. Somoskovi, A, Jillian D, Jeremy R, Maria P, Melinda M, &Max S (2008).Direct Comparison of the GenoType MTBC and Genomic Deletion Assays in Terms of Ability to Distinguish between Members of the *Mycobacterium tuberculosis* Complex in Clinical Isolates and in Clinical Specimens, *Journal of Clinical Microbiology,* 10 (1128), 105-107
5. Siddiqi, Kamran (2003). Clinical diagnosis of smear-negative pulmonary tuberculosis in low-income countries: the current evidence, *The Lancet Infectious Diseases*, 3,(88): 1-3.
6. Kirwan, Daniela E (2012). Same-day diagnosis and treatment of tuberculosis, *The Lancet Infectious Diseases*, 3:1-3.
7. Ameeta, S.K. & Majid, S.,Y. (2014) in Nigeria *International Journal of Mycobacteriology*
8. Araoye, M.O. (2003). Sample Size determination. In: Research methodology with Statistics for Health and Social Sciences. Ilorin: Nathadex.
9. Cepheid GeneXpert MTB/RIF-10.Package insert. September, 2010
10. Saitou, N. & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406-425.
11. Jukes, T.H. & Cantor, C.R. (1969). Evolution of protein molecules. In Munro HN, editor, Mammalian Protein Metabolism, pp. 21-132, Academic Press, New York.
12. Nwofor, A.C., Nyamngee, A., Nwabuisi, C., Iwakun, M., Gidado, M., Mensah, C., Dakum, P., Agbede, O.O., Ndembi, N., Blattner, W.A. & Abimiku, A.G. (2015). Performance of Genotype MTBDRplus in the Detection of Resistance to Rifampicin and Isoniazid among Clinical Mycobacteria Isolates in Ilorin, Nigeria.Curriculum for HIV Resistance,13(4), 308-314.
13. Lawson, L., Zhang, J., Gomgnimbou, M.K., Abdurrahman, S.T., Le Moullec, S. & Mohamed, F. (2012). A Molecular Epidemiological and Genetic Diversity Study of Tuberculosis in Ibadan, Nnewi and Abuja, Nigeria. *PLoS ONE*, 7(6), 1-2.

14. Gambo, A., Samer, S., El-Kamary, A., Abimiku, N., Ezati, I., Mosunmola, L., Hungerford, C., Brown, K. J., Tracy, J. O. & William, B. (2013) Mycobacterial Etiology of Pulmonary Tuberculosis and Association with HIV Infection and Multidrug Resistance in Northern Nigeria. *Tuberculosis Research and Treatment*, 8(5), 3-5.
15. Adjemian, J., Olivier, KN, & Prevots, D.R. (2018).Epidemiology of Pulmonary Nontuberculous Mycobacterial Sputum Positivity in Patients with Cystic Fibrosis in the United States, 2010-2014. *Annals of American Thoracic Society*, 15(7), 817-826.
16. Chang, T. C., Hsieh, C. Y., Chang, C. D., Shen, Y. L., Huang, K. C., Tu, C., … Tsai, S. S. (2006). Pathological and molecular studies on mycobacteriosis of milkfish *Chanos chanos* in Taiwan. *Diseases of Aquatic Organisms*, 72, 147–151.
17. Guz, L., Grądzki, Z., Krajewska, M., Lipiec, M., Zabost, A., Augustynowicz-Kopeć, E. & Szulowski, K. (2013). Occurrence and antimicrobial susceptibility of *Mycobacterium peregrinum* in ornamental fish. *Bulletin of the Veterinary Institute in Pulawy*, 57, 489–492.
18. Beran, V., Matlova, L., Dvorska, L., Svastova, P., & Pavlik, I. (2006). Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. *Journal of Fish Diseases*, 29, 383–393.
19. Rachel, T., Carla, T., Robyn, C., Chris, C., Flavia, H. & Megan, H. (2013). Isolation of Nontuberculous Mycobacteria (NTM) from Household Water and Shower Aerosols in Patients with Pulmonary Disease Caused by NTM. Journal of Clinical Microbiology, 51(9), 3006–3011.