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Resistance Profile of Mycobacterial Strains Causing Cluster Pulmonary Infection in Bayelsa 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

Aim: The aim of this study was to profile the anti-microbial resistant of Mycobacterial strains causing cluster pulmonary infections in Bayelsa State, Nigeria.
Study Design: Cross sectional facility based.
Place and Duration: This study was carried out in all the in tuberculosis centers in all the eight local government area in Bayelsa State, Nigeria between January 2019 to February 2021.
Materials and Methods: A total of 102 tuberculosis patients who had been previously diagnosed of Tuberculosis (both new and old cases) participated in the study. Two sputum samples were collected from each subject in a wide mouth screw cape container and falcon tube container respectively. The first was used for GenXpert technique while the second was used for culture on Lowenstein Jensen solid Media. The visible and confirmed Mycobacterium growth was subjected to Line Probe Assay (MTBDRplus assay) and genetic sequencing. The DNA of the isolates where extracted using Genolyse method. The extracted DNA was used to perform a gene mutation profiling using MTBRplus Assay. The 16Sr RNA sequencing was done on the amplified genes using the BigDye terminator kit on a 3510 AB1 sequencer.
Results: Out of the 102 sputum samples analyzed a total of 91(89.2%) were GeneXpert positive.

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Drug resistant profiling by GeneXpert shows 8(7.8) strain mutation at the rpoB gene only while the resistant profiling of the isolated on Lowenstein Jensen solid media had 14(13.7) strain with mutation on genes responsible for first and second line drug resistant. Two non-tuberculosis Mycobacterium species which are *Mycobacteriode abscessus* subsp. abscessus st and *Mycobacterium Kansasii* strain FDAARGOS_1534 where isolated among Tuberculosis patients, both are multidrug-resistant strains. The *Mycobacterium tuberculosis* strains in circulation causing cluster TB infection in Yenagoa especially and other parts of Bayelsa State, Nigeria are MG003 and R2092 strains, but the most predominant strain that is traced to cluster TB infection is MG003. The extraction of MTB gene from a pure culture of a Lowenstein Jensen selected media reduced the possibility of contamination and enhanced the reliability of gene sequencing and Bioinformatics analysis which includes comparing strains of MTB with the once in the National center for Biotechnology information (BLAST) data base.

Conclusion: The resistance profiling of MTB reveals that most strains were resistant to rifampicin. This means that there were more mutation on the rifampicin activation gene (rpoB) than any other gene. Most cases of multi drug resistance is associated with rifampicin resistance while cases of extensive drug resistance is not common.

Keywords: Mycobacterium tuberculosis; abscessus; kansasii; multidrug-resistant; Yenagoa.

1. INTRODUCTION

Tuberculosis is a disease condition caused by Mycobacterium tuberculosis, it is transmitted from one individual to another through aerosolized inhaled droplets. It majorly affects the lungs but can also affect other parts of the body. After the inhalation of TB bacilli it is captured by macrophages, they can evade the immune cells and remain dormant for a long period of time. They could become active when the environment is favorable, because of the peculiarity of MTB virulent nature. After infection the organism is quickly phagocytized by professional alveolar macrophages which in most cases kill the entering bacteria. If the bacilli survives this first line of defense, it starts actively replicating in macrophages and diffuse to nearby cells including epithelial and endothelial cells. it is important to complete the treatment within the expected period which is not less than six months [1]. This is important because MTB has both fast and slow growing strains. However there has being frequent resistance to the first line drugs which is occasioned by relapses and the spread of resistant strains. Therefore, the main aim of this study is to obtain the resistance profile of tuberculosis and non-tuberculosis Mycobacterial strains causing cluster pulmonary infection in Bayelsa State, Nigeria.

Antimicrobial resistance is a major treat in the management of infections caused by bacteria. The danger posed by drug resistant *mycobacterium tuberculosis* is already a global concern. The drug resistant profiling of mycobacterium species requires the isolation of the organism and susceptible testing to both first and second line tuberculosis drugs. The first line drugs are rifampicin (RMP), isoniazid (INH), ethambutol (EM) and pyrazinamide (PZA). The second line drugs are amikacin, kanamycin, capreomycin and fluoroquinoline [2].

Mycobacterium tuberculosis (TB) resistant, which ranges from mono drug resistance TB (DR-TB), multiple drug resistance (MDR-TB) to extensive drug resistance (XDR-TB) is a leading challenge in the management of tuberculosis in Bayelsa State, Nigeria. Genexpert method of diagnosis can only identify MTB strains that are resistant to rifampicin and in some cases produce indeterminate resistant results. it is therefore important to carry out a more advanced and systematic resistant profiling that has the ability to detect anti-microbial resistance of tuberculosis [3].

2. MATERIALS AND METHODS

A cross sectional facility bases study was carried out in some approved tuberculosis treatment center in Bayelsa State. These facilities were General Hospital Sagbama, Cottage Hospital Okologba Kolokuma/Opocuma, Tuberculosis and leprosy control center Igbogene Yenagoa, Federal medical center (FMC) Yenegoa, Comprenhensive Health Centre Ekeremor, FMC Otuoke Ogbia, General Hospital Amasoma Southern Ijaw, Genaeral Hospital Nembe and General Hospital Brass. The State has an estimated population of 1.7 million people. It is located between 4 '15' north and 5 '23' south latitude and 5 '22' west longitude 6 '45' east. It

has boundary with Delta State in the north, Rivers State in the east, and the Atlantic Ocean in the west and south part.

Ethical clearance was gotten from Bayelsa State Ministry of Health before the commencement of this research. Informed and writing Consent were obtained from each subject before collecting demographic information, data and sputum samples.This study were carried out in accordance with the Declaration of Helsinki (ethical considerations).A questionnaire were issued to each of the participants in other to obtain information about their age, gender and address.

A total number of 102 sputum Samples were collected from TB patients who had previously been diagnosed with tuberculosis. Both untreated (new) and treated (old) cases were considered. A 50ml capacities falcon tubes were used to collect a minimum of 2ml sputum samples for TB culture, while a wide mouth sputum cup was used for GeneXpert. Each participant produce sputum into a falcon tube and a wide mouth sputum cup.

The GeneXpert MTB/Rif machine was used for the identification and probing of resistance to Rifampicin. Sputum samples were processed according to the operating procedures for GeneXpert MTB/RIF assay as described in a publication by Audu et al., 2017 [4]. Results were automatically generated indicating if *M. tuberculosis* was detected or not and if the detected *M. tuberculosis* was rifampicin resistant. The GeneXpert System is built with GX 2.1 software / computer, printer and barcode wand-reader and the GeneXpert real time Polymerase Chain Machine. The machine is always available in a one, two, four or 16-module build up. The one used in this research has four module configuration, with serial number 805757.

Line Probe Assay (MTBDRplus assay) method were used for resistant profiling of isolates gotten from MTB positive Lowenstein Jensen solid culture media. Genomic of Mycobacterial culture was extracted by incubating the colonies dissolved in 300 μL of molecular biology grade water for 20 minutes at 95◦C in water bath followed by 15 minutes in ultrasonic bath and centrifugation for 5 minutes at 12000 rpm. Polymerase chain reaction (PCR) and hybridization were performed following manufacturer's recommendations (Hain Lifescience, Nehren, Germany). Colorimetric method (using streptavidin conjugated with alkaline phosphatase and substrate buffer) was used to detect hybridized amplicons. The strip containing hybridized amplicons were interpreted following manufacturer's instructions [5].

Drug resistance (R) and drug Susceptibility (S) were determined by the presence of mutation or no mutation. The resistance profiling of all the isolated was carried out with MTBDRplus assay. This molecular technique targets the genes that were responsible for drug resistance of both the first and second line drugs. If there are indication of mutation on either rpoB, KatG, inhA, gyr, rrs & eis gene it were considered as drug resistant, reported as R. If there was no indication of mutation it were represented as S meaning no mutation.

16S rRNA Sequencing of isolated Mycobacterium species was done using the BigDye Terminator kit on on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min and phylogenic analysis was carried.

Statistical Package for Social Science (SPSS) version 21 was the statistical tool used to analyse data after collation with Microsoft excel spread sheet. Frequency, percentage and prevalence were calculated. Chi square was used to measure association between variables. Mann Whitney test and kruskal Wallis were used to compare difference between two and more than two variables respectively. All tests of significance was at 0.05 alpha level. Results were presented on tables.

3. RESULTS

Subjects who are positive to rifampicin resistance tuberculosis while using the GeneXpert method across the Local Government Area are shown in Table 1. The results on Table 1 shows Rifampicin resistant TB among males and female in each of the Local Government Area using GeneXpert technique. Yenagoa L.G.A had the highest rifampicin resistance case but not statistically significant. Females had more cases of rifampicin resistance than males.

In this research GeneXpert confirm 8 MTB strains that has mutation on the rpoB gene (Table 1). Kolokuma\Opukuma (KOLGA) local government has the lowest TB prevalence cases of 1 (1.0%) Table 1. The highest TB prevalence was in Yenagoa, with a prevalence value of 48(47.1%). Samples were gotten from symptomatic TB cases across the state, therefore increasing the likelihood of having more positive cases of TB.

Rifampicin Resistant TB across the various age group Using GeneXpert technique is shown on Table 2, the statistical correlation of the various number of participants across the various age group and the number of rifampicin resistance cases are mostly significant statistically. Subjects who are positive to rifampicin resistance tuberculosis while using the GeneXpert method across the various age groups are shown in Table 2. The age interval of 31- 40 years had the highest MTB positive cases of 30(29.4%) using the GeneXpert technque. The GeneXpert technique shows that individuals within the age intervals of 31-40 years has the highest prevalence of TB than other age group.

Rifampicin resistance as a result of mutation on *rpoB* gene detection using Line Probe Assay (MTBDRplus assay) technique is shown on Table 3. Rifampicin resistance profiling of all the culture isolates using the line probe assay technique are shown in Table 3. The Mycobacterial species and strains are identified by gene sequencing and phylogenetic analysis. The strains identified in this research were lineage 4(Euro- American) strains.

Table 1. GeneXpert rifampicin resistant TB by Local Government area

L.G.A	No. examined	Male	Female	Total	P-value
BRASS	4	0(0.0)	1(1.0)	1(1.0)	0.67
EKEREMOR	8	0(0.0)	0(0.0)	0(0.0)	0.92
KOLGA	1	0(0.0)	1(0.0)	1(1.0)	0.67
NEMBE	6	0(0.0)	0(0.0)	0(0.0)	0.92
OGBIA	9	0(0.0)	0(0.0)	0(0.0)	0.92
S/IJAW	16	1(1.0)	0(0.0)	1(1.0)	0.67
SAGBAMA	4	0(0.0)	0(0.0)	0(0.0)	0.92
YENAGOA	54	2(2.0)	3(2.9)	5(4.9)	0.67
Total	102	3(2.9)	5(4.9)	8(7.8)	0.35

Number in parenthesis = percentages. P-value = 0.32 (not significant)

Table 2. Rifampicin Resistant TB by Age group Using GeneXpert technique

Age (Years)	No. Examined	Rifampicin resistant	P-value
≤20	15	1(1.0)	**0.00**
21 – 30	29	1(1.0)	**0.00**
31 – 40	31	2(2.0)	**0.00**
41 – 50	12	3(2.9)	**0.00**
51 – 60	8	0(0.0)	**0.00**
61 – 70	5	1(1.0)	0.05
>70	2	0(0.0)	0.38
Total	102	8(7.8)	**0.00**

Table 3. Changes in Amino Acid and rpoB Gene Mutation Pattern

Isolates	No. of Isolate	MTBDRplus assay mutation (rpoBgene)	Result
M. abscessus	1(1.0)	WT8	RIF^R
M. kansasii 1534	1(1.0)	WT3,4 MUT1	RIF^R
MTB R2092	4(3.9)	WT2,3 MUT8	RIF^R
MTB MG003	8(7.8)	WT8,MUT3	RIF^R
MTB MG003	1(1.0)	No Mutation	RIF^S

Number in parenthesis = percentage; M = Mycobacterium; MTB = Mycobacterium tuberculosis; RIF = Rifampicin

Table 4. *Mycobacterium tuberculosis* resistant and gene mutation

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
rpoB	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
KatG	S	S	S	R	S	S	S	R	S	R	S	S	S	S	S
inhA	S	S	R	S	S	S	S	R	S	S	R	S	S	R	S
gyr	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
rrs	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
eis	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S

S1- S15= isolates; R=resistance; S=susceptible

The isolates confirmed and harvested from the Lowenstein Jensen culture media are represented as S1- S15 in Table 4. The results gotten from the profiling of isolates gene whose mutation decides drug resistance is shown on Table 4.

4. DISCUSSION

GeneXpert molecular technique was used to detect MTB resistance to rifampicin, Majority of the isolated strains are resistant to rifampicin. The Line Probe Assay technique also reveals that most of isolates from Lowenstein Jensen culture media had mutation on the rpoB gene. The second most common form of mutation occurred at the katG gene, further confirming that most cases of MDR-TB cases starts from rifampicin resistance. GeneXpert molecular technique was only able to detected mutation at the rpoB gene, it cannot offer an insight into the genetic characterization of MTB. It also lack the ability to identify non tuberculosis mycobacterium specie but has being proven to be more sensitive and specific than ZN staining technique. When MTB is resistant to rifampicin, the chance of being resistant to the first line TB drug becomes high. Line Probe Assay provides the possibility of detecting resistance to all the first and second line tuberculosis drugs. GeneXpert technique had being considered as a gold standard for TB management in some hospitals across Bayelsa State because of unavailability of an accredited facility for TB culture. This situation possesses a greater risk because the physiologic and genetic characterization of Mycobacterium is an unavoidable tool in the management of multiple drug resistant tuberculosis which is currently the highest treat in TB program across Bayelsa State. The possibility for the identification and prediction of extensive drug resistant MTB depend on the genetic fingerprinting of all mycobacterium isolates in Bayelsa State, Nigeria.

The ability of the Genxpert molecular system to identify mutation on the rpoB gene which is the receptor for rifampicin was a major mile stone in the fight against drug resistant tuberculosis. Yenagoa has been identified as the highest local government with the highest TB prevalence rate in Bayelsa state, this also agrees with study carried out by Obioma and kpomasirichi, [3].

Prolong use of drug is one of the major reasons while MTB is resistant to rifampicin. GeneXpert being a molecular technique is said to be more sensitive and specific than ZN AFB staining technique. Therefore its diagnostic value is very important, several journals such as (4) Calabar, [4] Nasarawa and [6] Adamawa all in Nigeria have shown that individuals within 31-40 years age interval tend to have more positive TB cases than persons within other age intervals.

The isolation of *Mycobacterium Kansasii* (MBK) and *Mycobacteriode abscessus* (Table 3) among subjects in Yenagoa could be as a result of the concentration of frequent migrants. These organism are predominately reported in the United States as some of the Mycobacterium species responsible for non-tuberculosis pulmonary mycobacterium infection [7]. This research further reveal the reason for emphasis on rifampicin resistance by World Health Organization in 2019 [8] as shown in Table 3. Gene mutation on the rifampicin resistance determinant site is more common than any other form of mutation that is likely to occur with MTB genome. A total of 13.7% of isolates where resistant to rifampicin, using the MTBDRplus assay. Therefore resistance to rifampicin is the first point of concern in the genetic profiling of resistant genes of MTB Table 3. According to a similar research in Ibadan, Nnewi and Abuja, Nigeria [9]. Eight percent of all MTB cultured samples isolate are resistant to rifampicin, while in our study in Bayelsa State 13.7% of all cultured mycobacterium isolates are resistant to rifampicin. [10] also reported that lineage 4(Euro-American) identified among pastoralists in Nigeria are also responsible for most cases of MTB drug resistance in Nigeria. MTB drug

resistance is majorly caused by treatment interruption, lack of information on MTB characterization, lack of adherence to W.H.O recommended principles and guideline in the management of TB patients and prolong drug intake [10].

Various genetic profile studies in different part of the world had reveal great diversity in the genetic evolution of mycobacterium. A study in Ethiopia had revealed the existence of extensive mycobacterium drug resistance. This was achieved as a result of the phylogenic and spoligotyping profile of various isolates of *Mycobacterium tuberculosis* [5]. Drug resistant pattern of occurrence has also been linked to clusters of *Mycobacterium tuberculosis* strain that is predominant in some certain areas [11]. We were able to observe in this study that the cluster of *Mycobacterium tuberculosis* strain MGOO3 is the most common strain associated with MTB drug resistant in Bayelsa State. Progress has been made in understanding of the molecular mechanisms that determine the epidemiological success of certain *M. tuberculosis* lineages. However, the molecular basis for the high epidemiological suitability of the M strain remains unclear [5].

The genetic resistant profiling of the isolated strains includes the observation of nucleotide or amino acid changes within the gate keeper genes. Mutation profiling using the MTBDRplus assay was able to show gene mutation that has the ability to confer resistance to first and second line TB drugs. *KatG* gene mutation is the determinant factor for the effectiveness of isoniazid, *KatG* gene mutation is not as common as *rpoB* gene mutation. Total of 2.9% of isolates had mutation on the KatG gene, while 2.0% had mutation on the *inhA* gene. MTB resistance to isoniazid depends on the mutation of both *KatG* and *inhA* gene therefore those isolates that had mutations on *KatG* and *inhA* gene are resistant to isoniazid Table 4. It is important to note that certain drugs had been recommended for treatment by the National Tuberculosis and leprosy control program. Therefore, for a more precise contribution to knowledge this research is focus on the gene mutation which will lead to resistant of the approved first and second line drugs. Studies had also revealed that when there is mutation on rpoB gene, there is always a high possibility of having mutation on *KatG* or *inhA* gene [12].

Flouroquinolones are major second line drugs, activation of flouroquinolones is determined by gyr gene. In this research the genetic profiling of gyr gene reveals no mutation or amino acid changes. Therefore flouroquinolones are highly recommended for management of MDR-TB cases in Bayelsa State. This further confirms the need for the use of flouroquinolones as a major second line anti tuberculosis agent. There is also no mutation on the rrs gene of all the isolates in this research. This shows that all the isolates are sensitive to other second line drugs like Amikasin, Kanamycin and caporemycin. 1.0% of the isolate had mutation on the eis gene which is responsible for the activation of kanamycin and Amikasin. Therefore 1.0% of the isolate will not respond to kanamycin and Amikasin Table 4.

5. CONCLUSION

Genexpert technique is the most common technique uses for multi-drug resistance tuberculosis diagnosis across Bayelsa State because of unavailability of an accredited facility for TB culture, this situation poses a greater risk because the phylogenic and genetic characterization of mycobacterium is an unavoidable tool in the management of multiple drug resistant tuberculosis which is currently the highest treat in TB program across Bayelsa State. The possibility to identify and predict extensive drug resistant MTB depend on the genetic fingerprinting of all mycobacterium isolate in Bayelsa State. The most predominate multidrug resistant strain of mycobacterium circulating in Bayelsa State is *mycobacterium tuberculosis* MG003. In this research this strain had been identified as the cause of a cluster infection in Amarata community of yenagoa local government area in Bayelsa State. The second most predominate MTB strain is *Mycobacterium tuberculosis* R2092 while the non-tuberculosis species causing pulmonary multi drug resistance are *Mycobacteriode abscessus* and *Mycobacterium kansasii.*

CONSENT

Written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

Ethical approval was gotten from Bayelsa State Ministry of Health Yenagoa.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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