RESISTANT PROFILING OF MYCOBACTERIUM STRAINS CAUSING CLUSTER PULMONARY INFECTION IN BAYELSA STATE, NIGERIA.

Comment [MEM1]: Resistance profile of Mycobacterial strains causing cluster pulmonary infection in Nigeria.

ABSTRACT

Aim: The aim of this study is to profile the anti-microbial resistant of tuberculosis and non-tuberculosis Mycobacterium 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: 102 tuberculosis suspects 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. Drug resistant profiling by GeneXpert shows 8(7.8) strain mutation at the rpoB gene only while the resistant profiling of the isolated on LJ 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 TB patients, both are multi drug 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.

Comment [MEM2]: Did these patients have TB or not? See the material and methods section...

Comment [MEM3]: What does it mean?

Comment [MEM4]: The first time an abbreviation appears, the full name must be entered.

Comment [MEM5]: You mean: multidrug-resistant strains?

Keywords: [mycobacterium tuberculosis, abscessus, kansasii, drug, resistance, ~~profiling~~)

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1. INTRODUCTION

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.

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 ~~tuberculosis~~ and non-tuberculosis mycobacterium species requires the identification of mycobacterium species that is susceptible to the 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 [1].

Tuberculosis which 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 acrophages, 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, it is important to complete the treatment within the expected period which is not less than six months. 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. The aim of this research therefore is resistant profiling of tuberculosis and non-tuberculosis Mycobacterium strains causing cluster pulmonary infection in Bayelsa State, Nigeria.

2. MATERIAL AND METHODS

Ethical clearance was gotten from Bayelsa State Ministry of Health. Informed Consent was obtained from each subject before collecting demographic information, data and sputum samples. This study was conducted in all the eight local government areas of Bayelsa State. 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. 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 [2]. 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.

Comment [MEM7]: (Mtb)...

Comment [MEM8]: ...tuberculosis (TB)...

Comment [MEM9]: Please write the abbreviations in capital letters.

Comment [MEM10]: ..TB...

Comment [MEM11]: ...macrophages, ...

Comment [MEM12]: Therefore, the main aim of this study is to obtain the resistance profile of tuberculous and nontuberculous Mycobacterial strains causing cluster pulmonary infection in Bayelsa State, Nigeria.

Comment [MEM13]: First, the authors should start by describing why TB is a critical illness, then what problems occur with its treatment (multi-resistance, etc.), and in the end, why this study is essential to improve knowledge of this disease. The first paragraph must be the last, and the third paragraph must be the first.

Comment [MEM14]: The authors must declare that this study was carried out in accordance with the Declaration of Helsinki (ethical considerations).

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~~The Gene Xpert cartridge is a single-use Xpert MTB/RIF cartridges, where the genetic extraction, amplification and detection of MTB takes place. It also has a sample reagent of a maximum volume of 8ml which can be used for diluting just one sample. A permanent marker pen is also required for proper sample labeling. The GeneXpert System also comes with a sterile disposable pipette which is already marked for minimum value of diluted sample to be transferred to the cartridge, each sterile pipette is used for one sample. A sterile screw cap wide mouth specimen container is also one of the materials required in the GeneXpert system.~~

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 [3].

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 analysis were carried out on the data's obtained.

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Comment [MEM19]: The description of the study design should be more detailed, it would be a good idea to use subsections, such as Population study, Ethical considerations, Sampling, Resistance study, Culture study, Statistical analysis, etc.

3. RESULTS AND DISCUSSION

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. ~~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.~~ In this research GeneXpert confirm 8 MTB strains that has mutation on the rpoB gene (Table 1).

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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. 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, [6].~~

Comment [MEM21]: Discussion section.

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)

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.

~~Prolong use of drug is one of the major reasons while MTB is resistant to rifampicin.~~ The age interval of 31- 40 years had the highest MTB positive cases of 30(29.4%) using the GeneXpert technque. ~~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, [2] Nasarawa and [5] 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 GeneXpert technique shows

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that individuals within the age intervals of 31-40 years has the highest prevalence of TB than other age group.

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**

Rifampicin resistance as a result of mutation on *rpoB* gene detection using Line Probe Assay (MTBDRplus assay) technique is shown on table 3. ~~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 [9].~~

~~This research further reveal the reason for emphasis on rifampicin resistance by World Health Organization in 2019 [7] 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 [8]. 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.

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The strains identified in this research were lineage 4(Euro- American) strains, ~~[11] 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 [11].

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* [3]. Drug resistant pattern of occurrence has also been linked to clusters of *Mycobacterium tuberculosis* strain that is predominant in some certain areas [10]. 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 [3].

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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.

The isolates confirmed and harvested from the Lowenstein Jensen culture media are represented as S1- S15 in table 4. 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 are 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 is considered as drug resistant, reported as R (Table 4). If there is no indication of mutation it is represented as S meaning no mutation as indicated in Table 4.

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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.

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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.

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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

4. CONCLUSION

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.

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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 DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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