***Original Research Article***

Molecular Detection of *Mycobacterium tuberculosis* and Rifampicin Resistance Isolates in Patients Attending Healthcare Facility in Ogoni, Rivers State, Nigeria

**ABSTRACT**

**Introduction:** In recent times, the emergence of *Mycobacterium tuberculosis* (MTB) resistance to anti-TB drugs has posed a significant public health challenge for effective global TB control. The already precarious situation has been further worsened by the increasing spread of multidrug-resistant TB.

**Aim:** The aim of this research was to identify drug-resistant *Mycobacterium tuberculosis* and its associated genes in tuberculosis-positive individuals visiting health facilities in Ogoni, Rivers State, Nigeria.

**Study design:** The study employed a hospital-based cross-sectional design over the course of 6 months (March 2023 - August 2023).

**Methodology:** A total of 150 sputum samples were collected aseptically from patients presenting clinical symptoms associated with tuberculosis and processed following standard procedures. Phenotypic detection of acid-fast bacilli (AFB) and anti-TB drug testing were carried by Ziehl Neelsen and drug susceptibility testing (DST) methods, respectively. GeneXpert assay was used to detect tuberculosis infection and drug resistance simultaneously while molecular detection of resistant genes (*kat*G and *gyr*A) was carried out using polymerase chain reaction (PCR) method. The results revealed 22.7% and 27.3% prevalence of tuberculosis by Ziehl Neelsen method and GeneXpert assay, respectively.

**Result:** The study revealed a low prevalence of rifampicin (RIF) resistance TB (4.7%) while drug resistance patterns in this study were monoresistance indicating resistance to ethambutol (EMB) (7.32%), isoniazid (IHN) (4.9%) or RIF (2.4%); polyresistance showing EMB-INH (2.4%) or EMB-RIF (2.4%) resistance patterns and multi-drug resistance indicated by EMB-INH-RIF pattern (2.4%). The PCR method detects *kat*G and *gyr*A genes in all the five (5) multidrug-resistant *M. tuberculosis* isolates screened.

**Conclusion:** The study uncovered a significant prevalence of tuberculosis infection, as well as instances of rifampicin-resistant and multidrug-resistant tuberculosis, in Ogoni, Rivers State, with suggestion that the *kat*G and *gyr*A genes are involved in rifampicin and fluoroquinolone resistance among MTB isolates in the region. There is need for effective anti-tuberculosis drug stewardship to mitigate the spread of multi-drug resistance as well as improve MTB treatment outcomes in Ogoni, Rivers State.

**Key word:** Tuberculosis, anti-TB drugs, rifampicin, resistance genes, multidrug-resistant TB

**INTRODUCTION**

Tuberculosis (TB) is one of the most dreaded diseases of mankind with the highest death toll, caused by *Mycobacterium* *tuberculosis* (MTB) (Nwofor *et al.,* 2015). The rise of MTB resistance against anti-TB medications has posed a significant public health challenge in reaching the objective of effective global TB control (WHO End TB, 2022). Drug resistance in M. tuberculosis isolates develops due to spontaneous genetic mutations and can be exacerbated by patients' poor adherence to anti-TB medications (Agarwal, *et al*., 2016). In 2021, the African region was responsible for 23% of newly reported tuberculosis (TB) cases and 31% of deaths connected to TB (WHO, 2023). This amounts to 2.5 million individuals contracting tuberculosis and around 500,000 fatalities. The region continues to experience elevated TB mortality rates, with only a few countries shouldering the majority of disease burden. For example, the estimated number of deaths in 2021 in the Democratic Republic of Congo, Nigeria, and South Africa represented 48% of all TB deaths in the African Region (WHO, 2023).

Worldwide, Nigeria occupies the 6th position among countries with the most significant number of TB cases. According to Azuonwu and colleagues, in 2010, Nigeria was grappling with approximately 2,700 annual cases of multidrug-resistant tuberculosis, and it was estimated that there were around 290,000 cases of MDR-TB in the country (Azuonwu *et al.*, 2017). However, in 2013, the proportion of new tuberculosis cases that were multidrug-resistant was 3.7%. The prevalence of this infection is significantly greater among those who have undergone prior tuberculosis treatment. The World Health Organization estimated that in 2013, there were approximately 0.48 million new cases of MDR-TB worldwide (WHO, 2017). In 2021, the global TB cases rose to an estimated 4.4% (WHO, 2022). From 2000 to 2021, Nigeria saw a rise in tuberculosis cases, escalating from 269,000 in 2000 to 467,000 in 2021, with a prevalence rate of 0.22% (WHO, 2022).

Multidrug-resistant TB is a serious threat and poses significant difficulties for treatment efforts, while extensively drug resistant TB is nearly impossible to treat and must be prevented at all costs. As per the surveillance data on anti-TB drug resistance from the World Health Organization (WHO, 2018), it is estimated that globally, 3.5% of new TB cases and 18% of those previously treated have MDR or rifampicin-resistant (RR) TB.

The use of molecular tools for quickly screening patients at risk of MDR-TB was endorsed by the WHO, based on evidence and expert opinion. Results from the rapid tests can be available within days, which facilitates early and suitable treatment aimed at reducing morbidity and mortality while also halting transmission. The Xpert MTB/RIF, which received WHO approval in December 2010 (WHO, 2010), is a technology that works automatically to quickly and at the same time identify *Mycobacterium tuberculosis* and mutations indicating resistance to Rifampin (RIF) in the *rpo*B gene, all within 2 hours of testing (Zhang *et al.,* 2018). Its limitation, when used in isolation, is that it detects only resistance to Rifampicin. Then, the line probe assay (LPA) was created for the quick and simultaneous identification of MTB and its resistance to RIF and INH (Ling *et al.,* 2008). This assay identifies whether wild type (WT) and/or mutant (MUT) DNA sequences are absent or present within a specific region of three genes: the *rpo*B gene (RIF resistance), the *kat*G gene (high-level INH resistance), and the promoter region of the *inh*A gene (low-level INH resistance) (Brhane *et al.,* 2017). In Akwa Ibom State, research on employing molecular methods to detect and identify resistant strains of *Mycobacterium tuberculosis* in patients with tuberculosis is scarce. Consequently, this research was conducted to identify *kat*G and *gyr*A genes linked to drug-resistant *Mycobacterium tuberculosis* in patients seeking treatment at a health facility in Ogoni, Rivers State.

**MATERIALS AND METHODS**

**Study design and setting:** This study was a hospital-based cross-sectional analysis involving 150 patients who exhibited clinical symptoms related to tuberculosis (TB) at the TB treatment center in Ogoni, Rivers State, during the period from March 2023 to August 2023. Ogoni territory, situated in Rivers State along the Gulf of Guinea coast and east of Port Harcourt city, is part of the Niger Delta region in Southern Nigeria and has a population of approximately 832,000 (according to the 2006 National Census). The area is situated within a 1,050 km² (404 mi²) region and constitutes the world’s third largest mangrove ecosystem.

**Inclusion and exclusion criteria:** To be included in the study, individuals must meet the following criteria: they are male or female patients aged 18 years and older with smear-positive pulmonary TB; they have been diagnosed with drug-sensitive (DS) or multidrug-resistant (MDR) TB, including those resistant to rifampicin and isoniazid.

**Sample size determination:** The sample size was calculated based on Bill Golden's single population prevalence formula. The sample size determined was founded on the prevalence rate of 10.9% from the research conducted in Enugu, Nigeria by Kennethe *et al*. (2021).

**Sample collection:** Each participant was provided with two 50 ml Falcon tubes with lids to expectorate sputum. They received appropriate guidance on how to generate a dep-cough sputum. Each falcon tube was assigned a unique identification number. The tubes were then transported to the South-South Tuberculosis Zonal Reference Laboratory for analysis, maintaining a cold chain and using triple packaging.

**Sputum microscopy using Ziehl‑Neelson (ZN) staining technique:** A clean, grease-free slide was used to create a sputum smear, which was then stained with the ZN method to identify acid-fast bacilli (AFB). This included the application of strong carbol fuchsin as the main stain, 3% acid alcohol for decolorization, and methylene blue as a counterstain.

**Phenotypic drug susceptibility testing (DST):** According to international recommendations, all culture-positive Mycobacterium tuberculosis samples were subjected to DST using the two most potent anti-TB drugs, isoniazid (INH) at 0.2 mg/L and rifampicin (RIF) at 2.0 mg/L. Culture-positive samples were subjected to DST using egg-based Lowenstein Jensen media with added drugs at the South-South Tuberculosis Zonal Reference Laboratory, as outlined by Lawson *et al.* (2011). Results were read after 6weeks of incubation.

**GeneXpert MTB/RIF technique:** The molecular GeneXpert MTB/RIF method was performed using the automated GeneXpert machine made by Cepheid. In accordance with the Manufacturer’s instructions, samples were examined in a class II biological safety cabinet. Two milliliters of sputum samples was combined with 4 mL of sample reagent (Isopropanol) in a sterile Pasteur pipette and vortex-mixed for 5 minutes. It was permitted to incubate at room temperature for 10 minutes, after which it was agitated once more for an additional 5 minutes. For each sample, a Pasteur pipette was utilized to move the suspension into the GeneXpert cartridge that had a label with the sample's corresponding unique number. The cartridge barcode was photographed with the GeneXpert camera. After entering the participant details into the GeneXpert machine, the cartridges were loaded as per the command. The machine operated for 2 hours, and the results were shown on its monitor.

**Molecular detection of *ka*tG and *gyr*A resistant genes:** Extraction was performed with a ZR fungal/bacterial DNA mini prep extraction kit from Inqaba South Africa, and the extracted DNA was quantified using the Nanodrop 1000 spectrophotometer. Gene amplification by polymerase chain reaction (PCR) was done using specific primers: the *kat*GF: 5'-GGGGCTGATCTACGTGAACC-3' and *kat*GR: 5'-CTCTTCGTCAGCTCCCACTC-3' primers and the *gyr*AF: 5'-CAGCGCAGCTACATCGACTA-3' and *gyr*AR: 5'-CTCAGCATCTCCATCGCCAA-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The amplicons were separated on a 1% agarose gel at 200V for 15 minutes and visualized using a blue light transilluminator.

**RESULTS**

Table 1 displays the phenotypic detection of acid-fast bacilli (AFB) in sputum samples using the Ziehl Neelsen technique. In 34 samples (22.7%), the test identified AFB. According to WHO standards, the infection level was measured as scanty (2%), 1+ (10%), 2+ (8%), and 3+ (2.7%) (Kassa *et al.,* 2021).

Table 1: Phenotypic detection of *Mycobacterium tuberculosis* in clinical samples using Ziehl Neelsen technique

|  |  |  |  |
| --- | --- | --- | --- |
| **No. of AFB** | **Fields** | **Interpretation** | **ZN Test (%)** |
| No AFB seen | Per 100 IF | Negative | 116(77.3) |
| 1 – 9 AFB | Per 100 IF | Positive, scanty | 3(2.0) |
| 10 – 99 AFB | Per 100 IF | Positive, 1+ | 15(10.0) |
| 1 – 10 AFB | Per 50 IF | Positive, 2+ | 12(8.0) |
| > 10 AFB | Per 20 IF | Positive, 3+ | 4(2.7) |
| **Total** |  |  | **150(100)** |

**Key:** AFB=acid fast bacilli; IF=immersion fields; ZN= Ziehl Neelsen

Table 2 shows the prevalence of Mycobacterium tuberculosis and rifampicin-resistant TB in Ogoni, Rivers State, as determined by molecular methods. The GeneXpert analysis of 150 sputum samples revealed that 41 were positive for *Mycobacterium tuberculosis* (MTB), resulting in a prevalence rate of 27.3%. Additionally, 7 samples (4.7%) exhibited rifampicin resistance.

Table 2: Percentage occurrence of *Mycobacterium tuberculosis* and rifampicin resistant TB based on Gene Xpert analysis

|  |  |  |
| --- | --- | --- |
| **Parameters** | **Gene Xpert test result** | **Percentage** |
| MTBD | 41 | 27.3 |
| MTBND | 109 | 72.7 |
| RRD | 7 | 4.7 |
| RRDIV | 7 | 4.7 |
| ERR | 6 | 4.0 |

**Key:** MTBD=Mycobacterium tuberculosis detected

MTBND=Mycobacterium tuberculosis not detected

RRD=Rifampicin resistance detected

RRDIV=Rifampicin resistance detected invalid

ERR=Error

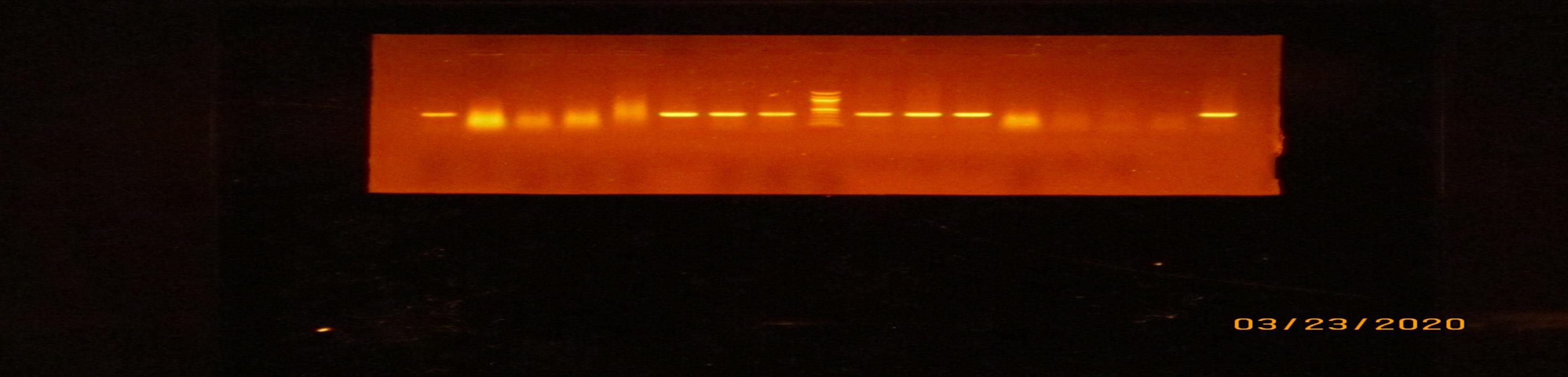
The *in vitro* drug resistance pattern of *Mycobacterium tuberculosis* isolates is shown in Table 3. The results showed three levels of isolates’ resistance to the first line drug used for the treatment of tuberculosis. Of the 41 isolates, 6 (14.6%) were monoresistant, 2 (4.9%) were polyresistant while 1(2.0%) was multi-resistant. The rates of monoresistant isolates to ethambutol (EMB), isoniazid (INH) and rifampicin (RIF) were 3 (7.31%), 2 (4.9%) and 1(2.4%), respectively. Resistance to drug combinations: EMB-INH and EMB-RIF as well as EMB-INH-RIF were found among polyresistant and multi-drug resistant isolates, respectively.

Table 3: Drug resistance patterns of *Mycobacterium tuberculosis* isolates

|  |  |  |
| --- | --- | --- |
| **Resistance profile** | **Number of isolates (N=41)** | |
| **n** | **%** |
| **Monoresistant** |  |  |
| EMB | 3 | 7.31 |
| INH | 2 | 4.9 |
| RIF | 1 | 2.4 |
| **Polyresistant** |  |  |
| EMB-INH | 1 | 2.4 |
| EMB-RIF | 1 | 2.4 |
| **Multidrug resistant** |  |  |
| EMB-INH-RIF | 1 | 2.4 |

The agarose gel micrographs of *gyr*A and *kat*G genes in multi-drug resistant *Mycobacterium tuberculosis* (MTB) isolates are shown in Figure 1 and 2. Figure 1 shows the agarose gel micrograph of *kat*G genes in multi-drug resistant MTB isolates. Lanes 1 – 5 represents the 350 base pairs of amplified *kat*G genes. Lane M contains the 100 bp molecular ladder. The *gyr*A gene in multi-drug resistant MTB isolates is shown in Figure 2, which is an agarose gel micrograph. Lanes 1–5 show the 300 base pairs of amplified *gyr*A genes. Lane Z shows the 100 bp molecular ladder.

1 2 3 M 4 5

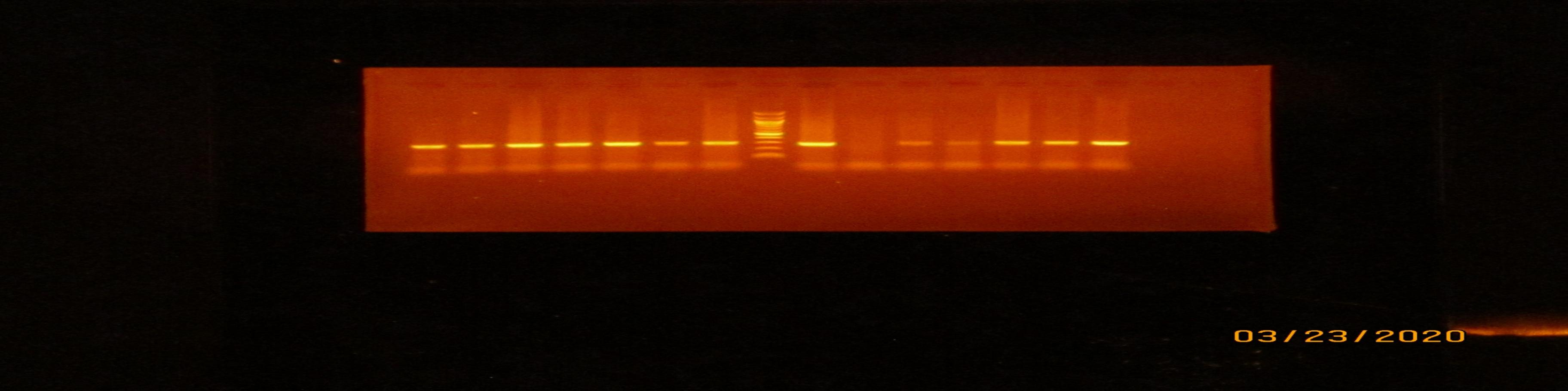


1500bp

350bp

**Figure 1:** Agarose gel electrophoresis of the *kat*G gene of selected *Mycobacterium tuberculosis* isolates

1 2 3 4 Z 5



300bp

1500bp

**Figure 2:** Agarose gel electrophoresis of the *gyr*A gene of selected *Mycobacterium tuberculosis* isolates

**DISCUSSION**

The appearance of rifampicin-resistant (RR) and multi-drug-resistant (MDR) strains of *Mycobacterium tuberculosis* poses a significant challenge to worldwide tuberculosis control efforts (Malenfant and Brewer, 2021). The issue of tuberculosis treatment arises from TB isolates acquiring resistance genes against both first and second-line anti-TB medications. To mitigate this threat, it is essential to use the GeneXpert MTB/RIF assay alongside the phenotypic AFB ZN method for early detection of drug-resistant MTB strains. This is crucial for precise diagnosis and epidemiological aims. The phenotypic detection of AFB in sputum samples yielded a result of 22.7% in this study. This figure is less than the 32.8% prevalence of AFB recently reported in Kaduna, Nigeria by Olatunde *et al.* (2023). The variation in AFB detection rates could be attributed to the detection methods employed and the geographical areas, which are known to influence TB prevalence.

The study found that the overall prevalence of tuberculosis (TB) infection and rifampicin resistance was 27.3% and 4.7%, respectively, using the GeneXpert method. The results of this finding are comparable to the recently reported TB infection and rifampicin-resistant prevalence rates of 40.4% and 1.25%, respectively, by Olatunde *et al.* (2023) within the group of presumptive TB patients visiting hospitals in Kaduna, northern Nigeria. It aligns with the TB infection rates of 35.6%, 31.4%, and 37% found in studies conducted in Akure, Nigeria (Bello *et al.,* 2014) and Pakistan (Butt *et al.,* 2004), respectively. However, a much higher TB prevalence has been reported from previous studies by Oyefabi *et al.* (2017) in Zaria, North Western Nigeria (88.6%) and by Rikoto (2015) among patients attending National Tuberculosis/Leprosy Center and Teaching Hospital, Saye Zaria (40.5%). In contrast, studies conducted in Borno (Denue *et al.,* 2018), Makurdi (Vange *et al.,* 2019), Ogun (Babajide *et al.,* 2014), Calabar (Kooffreh *et al.,* 2016) and Ethiopia (Mulu *et al.,* 2017) have reported lower prevalence rates of 19.1%, 25.5%, 16.7%, 24.8% and 23.2%, respectively. The observed differences in prevalence rates may be attributed to variations in the MTB detection method employed, the nature and type of subjects screened, the type of hospital used for the study, the level of tuberculosis infection endemicity in the study area, and the geographical area from which the study population was drawn (Olatunde *et al.,* 2023).

This study recorded an overall rifampicin resistant TB (RRTB) of 4.7%. A similar study in Kaduna reported a very low prevalence of 1.25%. The finding was similar to the 4.2% prevalence of rifampicin resistant TB among patients that have been previously treated for pulmonary TB in Northwestern region of Nigeria as reported previously (Fadeyi *et al.,* 2017). This outcome aligns with the Global TB reports published by WHO in 2016, which indicated that MDR/RRTB levels were low (< 3%) among new TB patients across different regions worldwide (WHO, 2016). outcomes reported in prior research conducted both within Nigeria and abroad have differed. As an example, Borno State in Nigeria reported 6.1% of MDR/RRTB cases (Denue *et al.,* 2018) and 7.3% in Delta State (Ukwamedua *et al.,* 2018). In addition, studies carried out in India have reported a comparatively higher prevalence of 49.1%, 18.8%, 13.6%, 14.7%, and 29.4% (Bello *et al.,* 2014), Yenagoa (Rikoto *et al.,* 2015), Saye Zaria (Vange *et al.,* 2019), Akure (Menon *et al.,* 2012) and Makurdi (Ikuabe *et al.,* 2018), respectively. Variations in prevalence rates can be attributed to factors such as the extent and burden of MDRTB/RRTB in the geographical area, as well as the testing methods and sample sizes employed. Variations in rifampicin resistance rates observed in studies may also be influenced by exposure to anti-TB drugs and treatment practices (Caminero, 2010). The drug resistance patterns observed in this study included monoresistance to EMB (7.32%), IHN (4.9%), or RIF (2.4%); polyresistance with EMB-INH (2.4%) or EMB-RIF (2.4%) combinations; and multi-drug resistance represented by the EMB-INH-RIF pattern (2.4%).

Prior research has indicated that resistance of *M. tuberculosis* to anti-TB medications is typically linked to mutations in particular genes. The drug-resistant loci examined in this study were *kat*G (catalase-peroxidase) and *gyr*A (DNA gyrase A). All five (5) MDR MTB isolates screened showed the presence of both genes as detected by the PCR analysis. This finding is consistent with reports from earlier studies (Salvato *et al.,* 2019). The identification of *kat*G genes suggests that the isolates exhibit resistance to isoniazid, a primary anti-TB drug. Reports have indicated that the mechanism of mutation conferring resistance involves enoyl-acyl carrier protein reductase, leading to resistance through a drug titration mechanism. Mutations in the ahpC-oxyR and *kas*A gene loci can also lead to resistance to isoniazid (Siddiqi *et al.,* 2002). The possession of *gyr*A genes in MDR MTB isolates is responsible for resistance to fluoroquinolones. It is widely recognized that fluoroquinolone resistance mechanisms are driven by mutations in the quinolone resistance-determining region (QRDR) of the genes for subunit A or B of DNA gyrase (*gyr*A or *gyr*B). Nonetheless, this study analyzed and detected fragments comprising gyrA. In tuberculosis treatment, the secondary drug regimen consists of fluoroquinolones. When the *gyr*A gene is detected, it suggests that the isolates may be resistant to ofloxacin. This could be partly attributed to the misdiagnosis of tuberculosis as a bacterial infection and the prevalent overuse of fluoroquinolones in the population. The majority of codons in *gyr*A exhibit polymorphism and can provide resistance to several fluoroquinolone antibiotics apart from ofloxacin (Salvato *et al.,* 2019).

**CONCLUSION**

The study uncovered a significant prevalence of tuberculosis infection, as well as instances of rifampicin-resistant and multidrug-resistant tuberculosis, in Ogoni, Rivers State. The study has suggested that the *kat*G and *gyr*A genes are involved in rifampicin and fluoroquinolone resistance among MTB isolates in the region. The noted resistance to first- and second-line TB medications is concerning, suggesting that there are geographic differences in the molecular genetic mechanisms of drug resistance in M. tuberculosis within the Ogoni region of River State.

**Ethical Approval and consent:** Ethical approval was obtained from Ethical Review Committee of the Rivers State Ministry of Health with assigned No: RSHMB/RSHREC/2023/028. Written informed consent was also obtained from all subjects before their inclusion in the study.

**CONFLICT OF INTEREST**

The authors declared that there was no conflict of interest to this manuscript.

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Details of the AI usage are given below:

1.

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3.

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