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Original Article

Rifampicin resistance patterns and dynamics of tuberculosis and drug-resistant tuberculosis in Enugu, South Eastern Nigeria

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Abstract

Introduction: Tuberculosis (TB) continues to be a public health problem globally. The burden is further exacerbated in developing countries like Nigeria, by poor diagnosis, management and treatment, as well as rapid emergence of drug-resistant TB. This study was conducted to evaluate the prevalence of drug-resistant TB, determine the *rpo*B gene mutation patterns of *Mycobacterium tuberculosis* (MTB) and model the dynamics of multidrug resistant TB (MDR-TB) in Enugu, Nigeria.

Methodology: A total of 868 samples, from patients accessing DOTS services in designated centres within the zone, were screened by sputum-smear microscopy, while 207 samples were screened by Nucleic Acid Amplification (Xpert® MTB/Rif) Test (NAAT). A deterministic model was formulated to study the transmission dynamics of TB and MDR-TB, using live data generated through epidemiological study.

Results: The results showed TB prevalence values of 22.1% and 21.3% by sputum-smear and NAAT assays, respectively. Analysis of the rifampicin resistance patterns showed the highest occurrence of mutations (50%) along codons 523 – 527. Factors such as combination therapy, multiple therapy and compliance to treatment had influence on both prevalence and development of TB drug resistance in the population.

Conclusions: This first documentation of Rifampicin resistance patterns in MTB from Nigeria shows that a majority of *rpoB* gene mutations occurred along codons 523 to 527, contrary to the widely reported codon 531 mutation and that multiple interventions such as combination therapy, with good compliance to treatment are needed to reduce both prevalence and development of TB drug resistance in the population.

Key words: *M.* tuberculosis; Nigeria; *rpo*B; Xpert MTB/RIF; numerical simulation.

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Introduction

The health of approximately 10.4 million individuals worldwide is impacted annually by TB resulting in approximately 1.7 million TB-related deaths, with the majority occurring in resource-limited countries [1]. Available data shows considerable country variation regarding the case fatality ratio, from less than 5% in some developed countries to more than 20% in most countries in Africa [2]. This suggests considerable inequality among countries in terms of TB management and negatively impacts the World Health Organization (WHO) efforts towards universal access to high quality, client-centered TB management. Part of the prerequisite for the implementation of the WHO strategy is rapid detection of *Mycobacterium tuberculosis* (MTB) from sputum samples and drug therapy informed by reliable anti-tuberculosis drug susceptibility profiles [3]. Effective diagnosis and management of TB require rapid detection of both MTB and susceptibility profile of at least one of the two key first-line anti-TB drugs (rifampicin and isoniazid). However, in Nigeria, TB diagnosis still heavily relies on poorly sensitive and poorly discriminatory acid fast (Ziehl Neelsen) staining techniques with only three functional country-wide facilities for TB drug susceptibility testing. The problem is further complicated by the emergence and spread of drug resistant TB and the HIV/AIDS epidemic [1,4]. Currently Nigeria is one of the 14 high burden countries for TB, TB/HIV and multi-drug resistant TB (MDR-TB). The country occupies the sixth position among the 30 high TB burden countries in the world and first in Africa. In the WHO African region especially sub-Saharan countries, the prevalence of MDR-TB is high especially among previously treated TB cases [1,5]. It is reported that approximately 500,000 cases of MDR-TB emerge annually globally with more than 100,000 deaths occurring annually due to MDR-TB [6-8]. In addition, as many as 10% of MDR-TB cases are extensively drug-resistant (XDR) [9,10]. MDR-TB is defined as resistance to both rifampicin and isoniazid while XDR is defined as MDR-TB with additional

resistance to any fluoroquinolone and at least one of the three second-line injectable drugs such as amikacin, capreomycin and kanamycin [11]. Rifampicin is one of the most effective anti-TB antibiotics and together with isoniazid, pyrazinamide and ethambutol, constitutes the first line treatment regimen against TB. Monoresistance to rifampicin is quite rare and almost all rifampicin-resistant strains are also resistant to other drugs, especially to isoniazid. This makes rifampicin resistance a surrogate marker for MDR-TB [12]. Recently, the Xpert® MTB/Rif assay, which can simultaneously detect the *M. tuberculosis* DNA as well as identify a majority of mutations in the *rpo*B gene that confer rifampicin resistance, has proven an excellent mechanism for the rapid detection of MDR-TB [13-15]. Little information is available regarding drug resistant tuberculosis in Nigeria. In Nigeria, an MDR-TB suspected case is defined as a patient who continues to test positive after the 5th month of standard first line regimen or a symptomatic patient having close contact with MDR-TB confirmed patient. Such imprecise diagnosis could lead to improper treatment and consequently to emergence and transmission of highly resistant MTB. In addition to prompt and accurate diagnosis, prediction and forecasting of future trends are also important in studies of disease epidemiology. In line with this, mathematical models have in recent times become an integral part of epidemiological studies, as tools for forecasting the future trends of a disease process in a given population. This study therefore focused on the rapid identification and elucidation of resistance gene mutation patterns of *Mycobacterium tuberculosis* strains among MDR-TB suspected patients accessing clinical services in selected Directly Observed Treatment Short-course (DOTS) centres in Enugu north geographical zone, using the Xpert® MTB/Rif assay and used a deterministic model to study the transmission dynamics of MDR-TB.

Methodology

Study area

The study was conducted in Enugu north geographical zone, south eastern Nigeria, which currently constitute Enugu north senatorial zone in the state geopolitical arrangement and is comprised of six local government areas (LGAs). The zone has a total ward population of about 1,660,000 [16]. The zone is characterized by low socio-economic indicator, with a majority of the residents engaging in low-income generating activities, such as small trading and peasant farming. The geographical zone has about 35 DOTS centres spread across the six LGAs. Sputum samples were collected from five comprehensive HIV/AIDS treatment sites (2 public health facilities and 3 faith-based organizations) and the other DOTS/ TB Microscopy centres in the zone.

Study population

The study group comprised 868 individuals of different age groups (5 year of age or older) accessing DOTS services in the designated centres across the 6 LGAs in the zone; with productive cough or presumptive diagnosis of tuberculosis, who volunteered to participate in the study. The survey population for NAAT and drug resistant TB comprised 207 stratified randomly selected individuals with productive cough, accessing clinical services in the 35 DOTS centres. The DOTs centres provided the opportunity to collect sputum samples from individuals with high suspicion index for tuberculosis, those newly diagnosed and those on treatment but on follow up visit to the facilities. Individuals 5 years of age or older who clinically/symptomatically screened positive to tuberculosis but did not have productive cough were excluded from the study.

Ethical consideration

The study was carried out in accordance with the tenets of the declaration of Helsinki. The purpose of this study was explained to the subjects in English and Igbo languages. Code numbers rather than names were used to identify them to ensure confidentiality. An Informed Consent Form was developed, explained and administered to clients/client's care givers (for children 5 year of age or older). Only consenting clients were enrolled in the study. Participation in the study was completely voluntary and at no cost to the consenting clients. Clients were also informed that quality of care in the health facility would not in any way, be affected by their decision to participate or not. Approval was obtained from the authorities of district hospitals and the respective DOTS/TB microscopy centres in the zone. Ethical clearance was obtained by the Health Research and Ethics Committee of Enugu State Ministry of Health, Enugu.

Collection and processing of sputum samples

Three sputum specimens (spot, early morning, spot) were collected from each participant into 50 ml screw-capped specimen bottles. The samples were then processed for the standard acid-fast direct smear microscopy using Ziehl-Neelsen staining and one of the spot samples processed for Rifampicin resistance assay

Table 1. Molecular Beacons used for the Nucleic Acid Amplification Test.

Probe	Sequence of Molecular Beacon
Probe A	5'-Texas red-TTTTTT-fluorescein-CGAGCTCAGCTGGCTGGTGCGCTCG-dabcyl-3'
Probe B	5'-tetrachlorofluorescein-GCTACGGAGCCAATTCATGGACCAGACGTAGC-dabcyl-3'
Probe C	5'-tetramethylrhodamine- TTTTTT-fluorescein CCGACGCCGACAGCGGGTTGTTCGTCGG -dabcyl-3'
Probe D	5'-rhodamine-TTTTTT-fluorescein-CCACGCTTGTGGGTCAACCCCCGTGG -*dabcyl-3*'
Probe E	*5'-fluorescein-CCTGCCGCCGACTGTCGGCG*CTGGCAGG-dabcyl-3'
16S probe	5'-fluorescein-GCGCCCGCGGCCT ATCAGCTTGTTGGTGGCGC-dabcyl-3'

by Nucleic Acid Amplification Test (NAAT) at Enugu Ezike District Hospital TB laboratory, according to the National guidelines. Stained smears were examined for acid-fast bacilli (AFB) under the microscope using oil immersion (100X) objectives.

Confirmation of MTB and Rifampicin resistance testing

Confirmation of the presence of *M. tuberculosis* and rifampicin resistance were assayed by Nucleic Acid Amplification Test (NAAT), using the GeneXpert/MTB/RIF Kit, in a semi-nested rt-PCR reaction. The tests were run following the manufacturer's instructions. Both conventional and wavelength-shifting molecular beacons were used. The nucleotide sequences of the six molecular beacons are shown in Table 1, while the nucleotide sequences of the primers used to amplify the 189-bp segment of the r*po*B gene and the segment of the mycobacterial 16S rRNA gene that contains a conserved sequence are shown in Table 2.

Mathematical Modeling of the Dynamics of TB and Drug Resistant TB

The model for the dynamics of TB with drug resistance is given below, with the following assumptions that: the population under consideration at time (t) is a heterogeneous population, involving seven compartments that are mutually exclusive; recruitment into the population of the susceptible class is by birth, immigration or by early treatment of latent TB, that is the exposed class; the exposed class may leave the population by natural death, other ailments and not necessarily by the disease (TB); and the population that develops drug resistance may die, move to the non-treated class or recover using another type of drug. The flow diagram of the dynamics of TB infection is shown in Figure 1, where: Λ = rate of recruitment into the susceptible class either by immigration or birth rate; λ = rate of progression from susceptible to exposed class as a result of interaction between susceptible and infectious class; β = rate of progression from exposed to infectious class; α = rate at which exposed individuals return to susceptible class as a result of early detection and treatment; ϕ= Rate at which the infectious class move to be treated; r = treatment control; τ = rate at which treated individuals develop drug resistance; w_1 = natural recovery rate of the non-treated class; w_2 = rate at which treated individuals recover from TB; t = rate at which those with drug resistance move to the untreated class; φ = rate at which the recovered individuals become susceptible again; δ = Death rate of the drug resistance class due to the disease; δ_1= induced death rate of the non-treated individuals as a result of TB; and δ_2 = The rate at which the treated individuals die as a result of the disease.

Figure 1. The flow diagram of the dynamics of TB infection parameters studied.

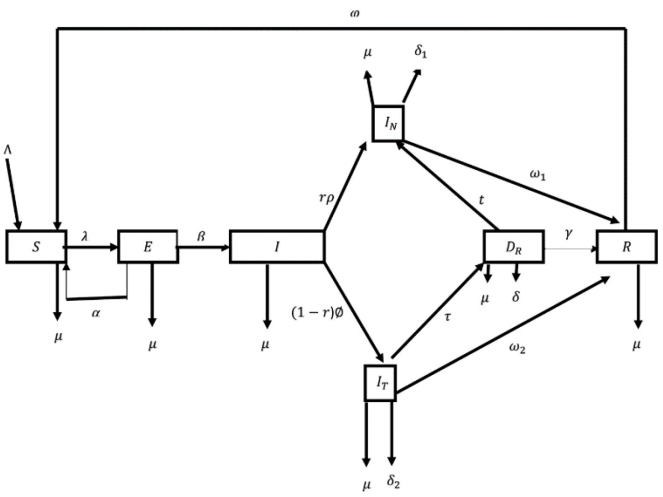


Table 2. Nucleotide sequence of Forward and reverse Primers used for NAAT.

Target Gene	Primer Sequence
189-bp segment of the r*po*B gene	5'-*GGAG*GCGATCACACCGCAGACGTT-3 (forward) 5-ACCTCCAGCCCGGCACGCTCACGT-3' (reverse)
Mycobacterial 16S rRNA gene	5'-GAGATACTCGAGTGGCGAAC-3' (forward) 5'-GGCCGGCTACCCGTCGTC-3' (Reverse)

Model parameter values taken from the study were: Total participants or susceptible class (S), 868; exposed population (E), 523; infectious population ($I = I_N + I_T$), 490; existing cases (infectious) on treatment, (I_T), 348; new cases or infectious not on treatment (I_N), 142; drug resistant cases (D_R), 6 +1; and recovered class (R), 297.

The equations of the model diagram were:

$$\frac{dS}{dt} = \Lambda + \alpha E + \varphi R - (\lambda + \mu)S$$

$$\frac{dE}{dt} = \lambda S - (\mu + \alpha + \beta)E$$

$$\frac{dI}{dt} = \beta E - [r\rho + (1-r)\phi + \mu]I$$

$$\frac{dI_N}{dt} = r\rho I + tD_R - (\mu + \delta_I + w_I)I_N$$

$$\frac{dI_T}{dt} = (1-r)\phi I - (\mu + \delta_2 + w_2 + \tau)I_T$$

$$\frac{dD_R}{dt} = \tau I_T - (\mu + \delta + \gamma + t)D_R$$

$$\frac{dR}{dt} = w_I I_N + w_2 I_T + \gamma D_R - (\mu + \varphi)R$$

where $\lambda = \theta IS$

The illustration of the numerical solution of the mathematical model for the dynamics of TB with drug resistance was carried out using Matrix Laboratory software (MATLAB).

Results

Prevalence of pulmonary TB among participants by sputum smear and NAAT tests

Out of 868 participants recruited into the study, 192 (22.1%) tested smear-positive for pulmonary TB, while a random testing of 207 samples by NAAT gave a prevalence of 21.3% (44 out of 207 samples).

Prevalence and pattern of Rif Resistance in NAAT-confirmed TB cases

Out of the 44 NAAT-confirmed TB cases, 6 (13.6%) were resistant to rifampicin. Of these, mutations were observed in three out of the five regions of the *rpo*B gene targeted by the different hybridization probes. Three samples (50.0%) showed mutation in the sequence hybridized by probe D, 2 (33.3%) showed mutation in the downstream region (probe E), while only one sample (16.7%) had a mutation in the sequence that hybridized with probe B. Two samples showed partial but stable binding of probes D and E respectively, due to minor change in the nucleotide sequences of the two regions (Table 3). None of the samples showed complete mutation in more than one region.

Performance evaluation of NAAT and Ziehl Neelsen staining techniques

Of the 207 samples subjected to both nucleic acid amplification test and sputum smear microscopy, 11 discordant results were recorded (Table 4). NAAT was able to amplify the genome of *M. tuberculosis* in seven sputum samples that were sputum smear negative. Three of the seven samples were semi-quantified as low, while remaining four samples had semi-quantification of very low *M. tuberculosis*. Two of the three low *M. tuberculosis* samples were from HIV seropositive subjects and one of them was on anti – TB chemotherapy. On the other hand, four smear positive samples (from one HIV seropositive and 3 HIV seronegative subjects) turned out to be negative to NAAT. None of them was on anti TB drugs (Table 4).

Results of Numerical Simulation

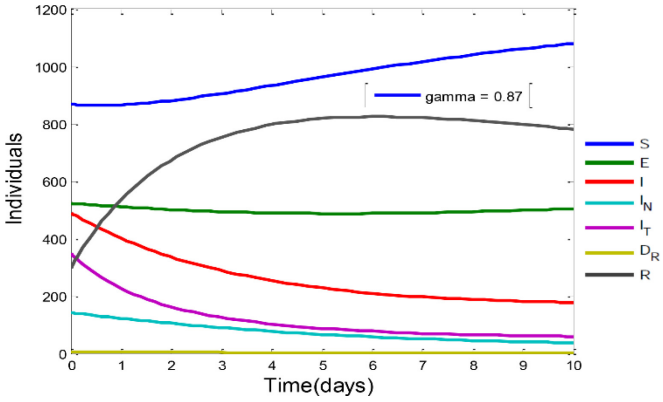
Simulation studies using parameter values fitted from study generated data showed that during the initial condition of 2 months, both the susceptible and recovered classes increased within the first 20 days before gradual and steady decline. Once treatment commenced, many people presented as though they had recovered, but as the duration of treatment progressed, the number dropped and almost stabilized at the end of two months' intensive period. However, the infectivity of tuberculosis steadily increased even with sign of early recovery. Development of rifampicin resistance remained zero at the initial condition of treatment. The simulation results also showed that placing a client on multiple chemotherapy reduced the rate of infectivity while the recovery rate increased noticeably and with increased recovery, the number of susceptible class maintained a positive slope. However, the number of individuals in the exposed or susceptible class reduced.

Table 3. Frequency of Mutant Genotypes in *rpo*B Detected by Different Probes.

Probe	Sequence	Codons Spanned	No of samples with mutations (%)	Number with partial binding (%)
A	5′ – GCACCAGCCAGCTGA - 3′	507 – 510	0	0
B	5′ – GAGCCAATTCATGGACCAGA - 3′	511 – 517	1 (16.7)	0
C	5′ – AACAACCCGCTGTCGG - 3′	518 – 522	0	0
D	5′ – GGGGTTGACCCACAAG - 3′	523 – 527	3 (50.0)	1 (16.7)
E	5′ – CGCCGACTGTCGGCGCTG -3′	528 – 533	2 (33.3)	1 (16.7)

If the intervention (multiple drug therapy) was sustained, the rate at which the population developed drug resistant tuberculosis remained zero. Increased intervention produced identical result among the individuals in the various epidemiological classes or compartments as the case with multiple chemotherapy (Figure 2).

Figure 2. Dynamics of TB in the studied population when the infectious is on multiple therapy. Note that the susceptible class (S) remains on a steady rise. However, the recovered class (R) increases, while the infectious population (I) and those with drug resistance (D_R) decrease.



Discussion

In this study, 868 participants were enrolled, comprising: suspected, newly diagnosed and existing cases of tuberculosis. The study was a hospital-based analysis and the findings may not be generalized in terms of prevalence of TB, considering the fact that most of the subjects were accessing chest clinics. However, the trends are important and will be compared with similar or related studies. The overall prevalence of pulmonary TB among participants was between 21 and 22% (22.1% by sputum smear and 21.3% by NAAT). Although this was a cross sectional (sentinel) study of clients accessing chest clinics, the overall prevalence was found to be higher than WHO projected national prevalence, but lower than a previous report from northern Nigeria, which recorded 26.3% prevalence [1,17].

In this study, also, the prevalence of rifampicin resistant MTB was 13.6% among subjects currently on anti-TB chemotherapy. This finding is lower than the report of a study in southern Nigeria, with prevalence 23.4%, but relatively high in comparison with many other reports from Nigeria and other African counties such as Ethiopia and Burundi [17-22]. The variation in reported prevalence even within the same country may reflect the variations in sample size, access to health care facilities, and effectiveness of TB control programs. According to a WHO report, across the globe, 3.5% of new TB cases and 20.5% of previously treated cases were estimated to have MDR-TB. This result suggests that current projections of MDR-TB in Nigeria may be lower than the actual prevalence [23]. Analysis of rifampicin resistance patterns showed the highest (50 %) resistance occurring along codons 523 – 527, representing probe D. This was followed by codons 528 – 533 (33.3%), representing probe E and the least resistance (16.7%) occurred along codons 511 – 517, corresponding to probe B. Of the rifampicin resistant isolates, 1 (16.7%) was recovered from a subject co-infected with HIV, the rest were isolated from HIV seronegative subjects. No mutations were recorded in sequences complementary to probes A and C. Two samples had isolates that showed partial but stable binding of probes D and E respectively, due to minor change in the nucleotide sequences of the two regions. None of the isolates showed complete mutation in more than one region. These results are in contrast to many reports across the globe, including some reports from East Africa, that had reported the most frequent mutations in codons 531 [24,25]. Although it had been

Table 4. Performance evaluation of NAAT and Ziehl Neelsen staining Technique.

HIV Status	Follow up	AFB	MTB Load	Cycle Threshold (CT) Value					SPC
1	1	0	L	28.7	28.6	28.4	29.2	30	25.5
0	0	0	L	30.6	29.3	29.7	31.3	32.1	24.7
1	0	0	L	30.1	29.1	28.8	29.6	31	30.1
0	0	1	0	0	0	0	0	0	28.1
1	0	1	0	0	0	0	0	0	25.7
0	0	1	0	0	0	0	0	0	25.7
0	0	1	0	0	0	0	0	0	25.3
0	0	0	VL	28.3	28.5	28.4	29.1	30	25.8
0	1	0	VL	34.7	33.3	33.3	0	38.8	27.4
0	0	0	VL	28.7	28.7	28.6	29.6	30.5	26
0	1	0	VL	26.7	27.7	27.3	27.5	28.2	28.4

0: Absence/No; 1: Presence/Yes; L: Low; VL: Very Low; SPC: Sample processing Control; AFB: Acid Fast *Bacillus*; MTB: *Mycobacterium tuberculosis*.

suggested that variations may exist in different ethnic groups and geographic locations [24], most reports have shown mutations in codon 531 to be the most frequent, leading to the conclusion that codon 531 is the most prevalent region associated with rifampicin resistance [24-30]. Given the observation in this study, however, there may be need to carry out further investigation into the geographical variations. The non-existence of mutations along the nucleic acid sequence hybridized by probe C has been reported by other authors [27]. It is believed that this particular region is less prone to mutations conferring rifampicin resistance.

Performance evaluation of nucleic acid amplification test and sputum smear microscopy, revealed discordant results between the two test techniques. Seven samples tested positive to NAAT but could not be picked by smear microscopy. On the other hand, four samples which stained positive to smear microscopy tested negative by NAAT. Similar reports of discordant results have been reported in different studies [31,32]. Smear microscopy is most likely to miss MTB in specimens with low bacilli/ml, as microscopy requires at least 5000 bacilli/ml of sputum to produce a positive result [31]. This implies that smear microscopy is less sensitive that NAAT. Moreover, these discordant results were quantified as low or very low by NAAT. Two of the three low *Mycobacterium tuberculosis* samples were from HIV seropositive subjects and one of them was on anti-TB chemotherapy. On the other hand, because of the specificity of the primers and probes used, the inability of the NAAT to identify MTB in four samples that stained positive to ZN could be interpreted as false positive results, because microscopy will identify both tuberculosis and non-tuberculosis *Mycobacterium*. It could also mean that the mutations in these isolates were outside the hotspot region targeted by the Xpert MTB/Rif assay, as some studies have indeed documented such uncommon mutations, which could not be detected by the Xpert assay [29].

In this study, a deterministic model for the transmission dynamics of TB was formulated and analyzed using live data generated through epidemiological study. The numerical simulation showed that the rate of recovery of those infected with drug resistance was low, but with multiple drug therapy, recovery rate could increase. This means that multiple interventions such as combination therapy, with good compliance to treatment are needed to reduce both prevalence and the development of TB drug resistance in the population. The results of mathematical modeling in this study are comparable to reports in other studies [33-35]. The model predicted that TB drug resistance can emerge by infection (primary resistance) or as a consequence of poor adherence to treatment.

This study represents the first documentation of Rifampicin resistance patterns in MTB from Nigeria and shows that a majority of the mutations occurred along codons 523 to 527 of the *rpo*B gene, contrary to the widely reported codon 531 mutation. Furthermore, numerical simulation, showed that multiple interventions such as combination therapy, with good compliance to treatment are needed to reduce both prevalence and development of TB drug resistance in the population.

Conclusion

Enugu State, south eastern, Nigeria, has a high prevalence of pulmonary tuberculosis, including resistant tuberculosis, which may be controlled through multiple interventions, such as combination therapy and adherence to treatment regimen. In addition, the TB resistance genotypes in the zone differ from the most commonly reported genotypes and warrants further study.

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