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Phenotypic detection of multi-drug and extensively drug resistant *Mycobacterium tuberculosis* among tuberculosis patients in Kaduna State North West Nigeria

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ABSTRACT

Background: Tuberculosis (TB) is one of the leading infectious diseases that cause death worldwide; the emergence of drug resistant *Mycobacterium tuberculosis* is of great concern for TB control program in Nigeria. **Aim and Objective:** To detect multi-drug and extensively drug resistant *Mycobacterium tuberculosis* among Tuberculosis Patients in Kaduna State North west Nigeria. **Study Design:** This was a cross sectional study among tuberculosis patients attending Directly Observed Treatment Short Course (DOTS) clinics in the three (3) senatorial districts of Kaduna State, Nigeria. **Material and Methods:** A total of 360 presumptive drug resistant tuberculosis patients' samples were collected and decontaminated using modified Petroff method (NaOH-Na-citrate-NALC) from August 2018 to December 2020. Drug susceptibility testing was performed on first- and second-line anti-tuberculosis drugs for the 305 confirmed MTBC isolates using Lowenstein Jensen (LJ) Proportion Method. **Results:** A total of 67(21.8%) isolates were pan-susceptible, 176(57.5%) isolates were MDR-TB, Rifampicin and Isoniazid mono-resistant were 46(15.1%) and 17(5.6%) respectively. For the second line drugs, a total of 271(80.22%) isolates were found to be pan-susceptible and 35(19.88%) isolates were pre-extensively drug resistant TB. **Conclusions:** These findings established higher prevalence of MDR-TB and pre-extensively resistant TB in Kaduna state.

Introduction

Tuberculosis (TB) is one of the leading infectious diseases that cause death worldwide. About one third of the world population is asymptomatically infected with latent *Mycobacterium tuberculosis* (MTB) [1]. Nigeria ranked the 4th among the highest tuberculosis burden countries in the world and 1st in Africa. Multidrug-resistant TB (MDR-TB) is TB that is resistant to at least, rifampicin (RIF) and isoniazid (INH) the two most important first-line drugs with or without other first line drugs. WHO estimates a prevalence of 3.9% of resistance to rifampicin (RR)/ MDR-TB among new cases and 21% among previously treated cases worldwide [2]. In Nigeria, WHO estimated a prevalence of 4.3% among new cases and 25% for retreatment cases [2]. Globally, data show an average cure rate of only 54% for treated MDR-TB patients. In 2016, an estimated 6.2% of people with MDR-TB had extensively drug resistant TB (XDR-TB). XDR-TB patients had a treatment success rate of 30% in 2014 [2]. The emergence and spread of Multi-drug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis

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(XDR-TB) present a major health problem of global significance. MDR-TB and XDR-TB are increasingly posing serious challenges in public health which requires robust, efficient, and quick actions to improve the control and spread of drug-resistant clinical isolates [3]. MDR-TB is prone to sequential accumulation of mutations in the target genes that confer resistance to them [3]. Globally, resistance of the organisms to TB drugs is a major public health problem that threatens the progress made in the area of TB control [1]. Drug resistance arises due to improper use of antibiotics in chemotherapy of drug susceptible organisms [1]. Early detection of the mutant genes associated with TB drug resistance using the advanced molecular techniques will provide a rapid and better understanding of frequency of drug resistance and drug action on the disease. Moreover, it will help for better planning of Nigeria National Tuberculosis Control Programme and treatment of Nigerian TB patients resistant to anti-tuberculosis drugs.

Material and methods

Specimen collection

Sputum sample from deep expectorate was collected from the patients whose sputum samples were xpert mtb/rif resistant positive, one specimen from each patient was collected into well-labeled wide-mouth screw-capped containers and then covered with lids. They were then being transported to the laboratory for processing and were stored at a temperature of 2–8℃ for not more than 4 days before decontamination [4].

Sample processing

Patients whose sputum samples were xpert mtb/rif resistant positive, were decontaminated using to N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method. Equal volume of NALC-NaOH-Na-Citrate solution and sputum samples was added into the 50 mL falcon tube and the tube was vortexed at 5 minutes interval for 20 minutes. Sterile phosphate buffer solution of pH 6.8 was added to make up to 45 mL. Then the 50 mL falcon centrifuge tube was centrifuged at 3000 ×g for 15 minutes. Supernatant was discarded into container containing disinfectant (5% lysol) and pellet would resuspend with 1-2 mL phosphate buffer solution. The resuspended pellet was used for smear preparation, inoculation into solid culture media and molecular analysis [5]

Isolation of Mycobacteria

An aliquot of 0.1mL of the digested sputum sample was transferred on to tube containing egg-based Lowenstein-Jensen medium. The inoculated tubes were incubated at 37°C in slanted position. The caps of the tubes were kept loose for the first 48 hours of incubation to let the inoculums dry. After 48hours, the caps of the tubes were tightened to avoid dehydration of the medium. Within 5 to 7 days, the tubes were examined to permit the early detection of rapidly growing mycobacteria and enable prompt removal of contaminated cultures. All cultures were incubated for 8 weeks with weekly examination of growth. Presence or absences of *M. tuberculosis* in slants were preliminarily determined by observing colony morphology on LJ slant. Presence or absence of contamination on solid culture media were carefully observed [6]

Ziehl-Neelsen staining for confirmation of positive cultures

Ziehl-Neelsen staining was performed to confirm growth of acid-fast bacilli on LJ medium. Smear was prepared for microscopic examination to detect the acid-fast bacilli [4].

Rapid identification method

Colonies from the positive culture slants were harvested and transferred in a 2 ml cryovial containing 1ml of buffer and vortexed for 30 second one hundred microliter (100 ul) of suspension was added into the sample well of the Rapid immunochromatographic test kits (SD bioline Kit). The result was read within reaction and was observed for 15 minutes. Presence of both test and control indicate positive, while absent of test band indicate negative. Invalid, only one reddish band appears in the test window [7].

Preparation of Lowenstein- Jensen medium with drugs

Isoniazid (INH), Rifampicin (RIF), Ethambutol (EMB), Amikacin (AMK), Kanamycin (KAN), Capreomycin (CAP) and Ofloxacin (OFL) was obtained as powder from Sigma Aldrich (Bornem, Belgium). Each drug was prepared at a concentration of 10 mg/ml in sterile distilled water with the exception of RIF, which was dissolved in dimethylsulphuroxide (DMSO). Stock solutions were filtered through a mesh of (0.45µm) sterilized and stored at -70°C and used within six months. Different concentration 0.2µg/ml INH, 2µg/ml EMB, 40µg/ml RIF, 30µg/ml AMK, 30µg/ml KAN, 30µg/ml CAP and 2µg/ml OFL, were incorporated

into LJ medium and then inspissated at 85°C for 45 minutes. After preparation, the media were incubated for 48 hours at room temperature for sterility check before use [8].

Drug susceptibility testing using Lowenstein Jensen Proportion Method

Drug susceptibility of the isolates to isoniazid (INH), rifampicin (RIF), ethambutol (EMB), amikacin (AMK), kanamycin (KAN), capreomycin (CAP) and ofloxacin (OFL) was performed by standard proportion method (Strong and Kubica, 1981). Briefly, LJ media with drug incorporated in various concentrations (0.2ug/mL isoniazid, 40 ug/mL rifampicin, 2ug/mL ethambutol, 30ug/mL amikacin, 30ug/mL kanamycin, 30ug/mL capreomycin and 2ug/mL ofloxacin) and plain LJ medium for control were used. The growth from a 3-4-week-old culture was scraped with a loop and bacterial suspension was made in sterile distilled water and matched with McFarland turbidity tube No.1. Dilutions of 10^{-2}, 10^{-3} and 10^{-4} were made and inoculated on control C1, C2 and C3 respectively and 10^{-2} were inoculated on drug containing media and incubated at 37°C. The first reading was taken on 28th day of incubation and the second on 42th day. The percentage resistance (R) was calculated as the ratio of the number of colonies on the drug containing media to those on the control medium [8].

Drug susceptibility testing to the second line drugs was performed for the 176 isolates using Lowenstein Jensen Proportion Method. A total of 141(80%) isolates were pan-susceptible while 35(20%) isolates were pre-extensively drug resistant TB as presented in **Table(4, 5)**.

Table 1. Occurrence of MTB and NTM among TB patients in Kaduna State

Mycobacteria	No. Positive	Percentage (%)
MTB	306	98.4
NTM	5	1.60
Total	311	100

Key: MTB = *Mycobacterium tuberculosis*, NTM = Non-tuberculous mycobacteria

Table 2. Resistance of *Mycobacterium tuberculosis* to second line anti -tuberculosis drugs by Lowenstein Jensen Proportion Method among TB patients in Kaduna State

Drug resistance pattern	No positive	Percentage (%)
Pre-XDR-TB	35	20
Pan-susceptible	141	80
Total	176	100

Figure 1. Percentage distribution of *Mycobacterium tuberculosis* culture among TB patients in Kaduna State

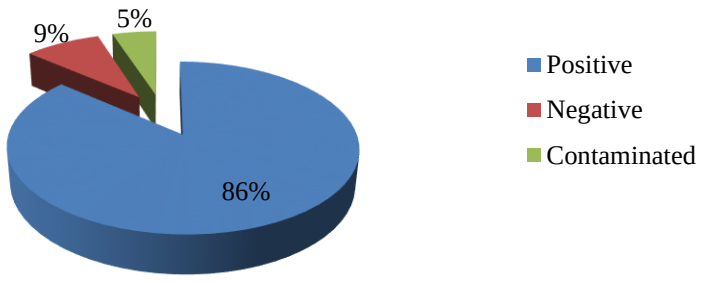
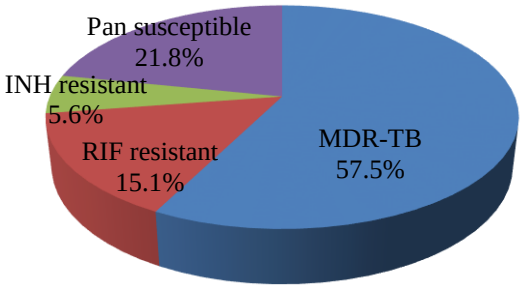


Figure 2. Resistance pattern of *Mycobacterium tuberculosis* to first line anti -tuberculosis drugs by Lowenstein Jensen Proportion Method among TB patients in Kaduna State



Discussions

Contamination rate was observed to be 18(5%) which could be due to incomplete digestion, aged samples and some contaminants are resistant to 4% NaOH, improper or under decontamination of specimen; very mucoid specimens that are hard to liquefy may result in high contamination; long storage and transportation time of the specimen after collection, especially in hot weather when bacteria tend to overgrow and become hard to kill by routine decontamination procedure. This contamination rate is lower than 14.7% reported by **Gambo et al**. [9] in Northern Nigeria; 11.9% in North Central by **Molina-moya et al.** [10] and **Chihote et al.** [11] who reported 8.3% in South Africa and are higher than 3.0% reported by **Ahmadu et al.** [12].

Xpert MTB/Rif positive culture negative samples were found to be 31(8.6%) and this could be attributed to the presence of dead Acid-Fast Bacilli, low number of viable bacilli that LJ media cannot detect or could be due to longer exposure of TB bacilli to 4% NaoH or the number of viable AFB inoculated on Lowenstein Jensen. It is pertinent to note that, the treatment status of patients also plays an important role. Specimens from chronically treated patients with drug resistant TB take a longer time to grow as drugs may cause injury or death to *mycobacteria* during TB treatment and the processing of specimen influences the positivity as well. A high pH or very low pH may cause injury or death to *mycobacteria* during processing of the specimen. Thus, it takes longer time for the revival and growth of viable *mycobacteria* in some instances and, as many as 33% of the *mycobacteria* are killed during processing. This above result agrees with the observation by **Aliyu et al.**[13] who reported 7% and **Ahmadu et al.**[12] who reported 6% but, higher than 4.9% reported by **Chihote et al.**[11] in South Africa.

The high culture positive rate (86.4%) in xpert MTB/RIF positive sputum obtained in this study may be due to the fact that all samples were transported in cold chain, processed within the acceptable time limit and samples processed in a high-quality laboratory. The culture positivity rate is in agreement with the 85% rate reported by **Selvakumaret al.**[14] in India and 91% reported by **Ahmadu et al.**[12] in Kaduna. Also, **Molina-moya et al.**[10] reported 79.5% and close to the 100% rate reported by **Simeon et al.** [15] and **Suleman et al.** [16] who obtained 90% culture positive among Xpert MTB/RIF positive.

The *Mycobacterium tuberculosis* prevalence of 98.4% in this study is higher than 80.5% reported by **Ahmadu et al.** [12] in Kaduna but, similar to the study conducted by **Suleman et al.** [16] with prevalence rate of 100%. The studies by **Aliyu et al.** [13] and **Cadmus et al.** [17] in Nigeria and USA reported lower rates of 77% and 48% *Mycobacterium tuberculosis* prevalence respectively. The higher prevalence of MTB obtained from the study could be due to the fact that all sputum samples were screened using Xpert MTB/RIF assay for the detection of MTB and RIF resistant as Xpert MTB/RIF detect only MTB and reason for lower prevalence by other studies could be due to the fact that they used microscopy as screening tool for MTB as microscopic examinations cannot differentiate between MTB and NTM viable and non-viable organisms.

The identification of Non-tuberculous *mycobacteria* from culture as much as 1.6% is probably due to many factors, including the secretion of proteins that are not enough to be

detected by MPT64 rapid test kits or MPT64 gene mutations, the possibility of a mixed infection between NTM and *M. tuberculosis* or only a single infection NTM. Non-tuberculous *mycobacteria* grow faster and suppress the growth of *M. tuberculosis* growth; therefore, NTM colonies grow more than *M. tuberculosis* colonies [18,19]. The results of NTM observed in this study is lower than the study by **Aliyu et al.** [13] and **Ahmadu et al.** [12] who reported 10% and15% of NTM respectively. Furthermore, the result of this study is higher than study conducted by **Suleman et al.**[16] who reported 0%. Thus, the higher prevalence of NTM reported by **Ahmadu and Aliyu** [12, 13]could be due to the fact that, they all used AFB microscopy as a screening tool for TB and the zero-prevalence obtained by **Suleman et al.** [16] is because all samples were screened by Xpert MTB/RIF Assay which only detect MTB not NTM.The prevalence of MDR- TB in Kaduna State was found to be 57.04%. This may be related to the fact that majority of the study population were patients who had history of previous anti-tuberculosis treatment and are therefore a high-risk group for the development of MDR-TB. However, it is commonly used globally that rifampicin resistant tuberculosis is the surrogate maker for multi-drug resistant tuberculosis [20]. This is higher than the result obtained by **Aliyuet al.** [13] who reported 5% of MDR-TB patients among patients in Kaduna state and prevalence reported by **Surya Kant et al.** [21] who reported 20.7% prevalence MDR-TB in India. The global TB reports 2010 estimated that 78% of MDR-TB was detected from Rifampicin resistant tuberculosis cases [4] and lower than the results obtained by **Daniel and Osama** [22] who reported 76.40% in south west Nigeria. The overall prevalence of pre-XDR-TB among fluoroquinolones and aminoglycosides in this study was 35/176(19.88%). The emergence of pre-XDR-TB is a major concern to the TB control program in Nigeria, as these highlights possible effects of the use of fluoroquinolones and aminoglycosides in the treatment of non-tubercular infections. This can be explained by the fact that fluoroquinolones are also the most widely prescribed class of antibiotics in the world today. This is lower than the result reported by **Sulemanet al.**[16] who reported 22.2% of pre-XDR-TB among fluoroquinolones and aminoglycosides resistant among patients attending National Tuberculosis and Leprosy Training Center Zaria. Globally, the number of pre-XDR-TB strains being identified has been in increasing. A study in South West Nigeria showed a pre-XDR prevalence of 17% by **Daniel et al.** [23]. The rates of pre-XDR in majority of studies outsides Nigeria reported higher rate of pre-XDR TB in India (56%), China (34%), Pakistan (24%), Bangladesh (16%) and South Africa (17%) [24,25,26,27].

Conclusions and recommendations

These findings established higher prevalence of MDR-TB and pre-extensively resistant TB in Kaduna state. This study recommend that all drug resistance cases should be detected and treated with appropriate drugs, combinations or dosages and they should always adhere to their treatments without interruption or irregular treatments and incomplete treatments duration. There is also need for further detection of drug resistant TB in Nigeria.

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