Resistance pattern of *Mycobacterium tuberculosis* Complex Isolated from Re-treatment Patients to First Line Anti-Tuberculosis Drugs in Zaria Nigeria

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Abstract: Tuberculosis (TB) is one of the leading infections that cause death worldwide. The emergence of drug-resistant *Mycobacterium tuberculosis* is of great concern for National TB control programmes wich hinders the control of tuberculosis; therefore it has become important to screen infected patients for resistant TB. A total of 437 sputum samples were collected from re-treatment TB patients. The samples were screened for Acid Fast Bacilli (AFB) and positive samples were cultured on Lowenstein Jensen medium. The isolates were confirmed as *Mycobacterium tuberculosis* Complex (MTBC) using immune-chromatographic test and their resistant pattern was detected using proportion method. The results revealed that 72 (16.47%) of the samples were AFB Smear positive, out of which, 62 (86%) were culture positive, 6 (8%) were culture negative and 4(6%) were contaminated. Also 57 (91.9%) of the 62 cultures positive samples, were *Mycobacterium tuberculosis* Complex (MTBC). Among the57 isolates, 33(58%) isolates were found to be resistant to streptomycin, 47 (82.5%) and 44(77%) were found be resistant to isoniazid and rifampicin respectively. While 35 (51%) isolates were found to be resistant to ethambutol. Additionally, 41(71.93%) of the isolates were identified as multi-drug resistant tuberculosis (MDR-TB). The study detects high resistance of *M. tuberculosis* to the two of the most important first anti-TB drugs as well as exhibited high multidrug resistance. The study identifies the need for fast and accurate diagnosis of resistant TB especially among re-treatment cases to interrupt transmission chain which will go a long way in providing a successful TB control.

Key word: Tuberculosis, First Line anti-TB Drugs, Resistance Pattern, Re-treatment TB Patients.

INTRODUCTION

Tuberculosis remains one of the leading causes of morbidity and mortality throughout the world. One-third of the world's population is estimated to be infected by members of the MTB complex which are collectively responsible for about three million deaths each year and over 95% of which occur in developing countries (Raviglione *et al*., 1995: Kooffreh *et al*., 2016). The World Health Organization (WHO) estimated that in 2018, there were an estimated 10.0 million incident TB cases worldwide along with 1.7 million people death, People living with Human immunodeficiency virus (HIV) accounted for 10% of the total infection (WHO, 2018).

Multi- Drug-Resistant Tuberculosis (MDR-TB) is a Tuberculosis (TB) caused by MTB that are Resistance to isoniazid and rifampicin whether there is resistance to other drugs or not. In Nigeria, the national MDR-TB survey showed a prevalence of 4.3% among new cases and 21% for retreatment cases (WHO, 2018).

Drug Susceptibility Testing (DST) to First line anti-tuberculosis drug is a major challenge in a resource limited settings where Culture laboratories are limited (WHO, 2013). Factors contributing to the development of drug resistance include improper Directly observed treatment short course (DOTs) inadequate dosage, inadequate treatment regimens, and poor-quality drugs, sale of medications over the counter, poor infection control practices, poor patient treatment adherence counseling and malabsorption of high bacillary load (Balaji *et al*., 2010; Zhao *et al.,* 2012).

A patient who develops active disease with a drug-resistant TB strain can transmit this

form of TB to other individuals. Drug-resistant bacteria especially MDR and extensively drug resistant Tuberculosis (XDR TB), persists as a global public health problem (Vander *et al*., 2012). The use of effective diagnosis tools and anti-tuberculosis drugs have greatly assisted in the analysis of disease transmission and correct diagnosis of drug-resistant MTB strains in turn may result in decreased transmission of sensitive and drug-resistant MTB strains in a population. The study aims at detecting the resistance pattern of *Mycobacterium tuberculosis* complex isolated from re-treatment TB patients to first line anti-TB drugs in Zaria, Nigeria.

MATERIALS AND METHODS

Study area: The study was conducted at the National Tuberculosis and Leprosy Training Center (NTBLTC), Zaria, which is the largest TB referral center in northern Nigeria and serves as the National Training Center for community health workers and laboratory health personnel's involved in diagnosis of tuberculosis and treatment at the peripheral, state and zonal levels. This center is one of the two National TB reference laboratory that is equipped with TB biosafety level 3 (BSL-3) and TB molecular diagnostic laboratories.

Study population: The study population comprised patients with presumptive previously treated TB who presented at NTBLTC for evaluation. A total of 437 sputum samples were collected and screened from these re-treatment TB patients. Inclusion was based on smear positivity for Acid Fast Bacilli (AFB), ensuring that only patients with microbiologically confirmed TB were included for culture and drug susceptibility testing

Sample size: A total of 437 sputum sample was screened for Acid fast bacilli (AFB)

Ethical approval: All participants who provided sputum specimens were given a written or witness verbal inform consent. Ethical consideration was obtained from the Research Ethics Committees of the National Tuberculosis and Leprosy Training Centre (NTBLTC) Zaria.

Inclusion criteria: All patients who consented to participate in the study and whose sputum samples were smear positive for acid fast bacilli.

Exclusion criteria: All patients who did not consent to participate in the study or those whose sputum samples were acid fast bacilli negative were excluded.

Sample collection: Sputum samples were collected from the patient attending NTBLTC: two specimens from each patient were collected into well-labeled wide-mouth screw cap containers and then covered with lids. They were then transported to the laboratory for processing. Salivary specimen instead of sputum was also processed following World Health Organization (WHO) tuberculosis guidelines (WHO, 2010).

Microscopy: Each specimen was smeared, air dried, heat fixed and stained with Ziehl-Neelsen (Z-N) reagents using a known acid-fast bacilli (AFB)-stain slide as positive control and a stain slide made of egg albumin as negative control. Results were recorded according to the grading system of the IUATLD as Negative (0), scanty (1-9AFB/100 field), +1(10-99AFB/100 field), 2+ (1-9AFB/field in 50 field) or 3+ (10 or more AFB/in at least 20 field) (WHO, 2010).

Media preparation: The culture media was prepared by weighing 37.4g of commercial Lowenstein Jensen medium powder and dissolved n 600ml distilled water then 12ml glycerol was added and mixed thoroughly, the solution was heated with frequent agitation just until the media boils, the solution was autoclaved at 121^{o}C for 30 minutes and cooled, 1000 mls of homogenized eggs was then added and mixed. The media was then dispensed into 15ml falcon tubes and were inspissated at 85^{o}C or 45 minutes; the media were incubated at 37^{o}Cfor 48 hours for sterility checks (Concencious *et al.,* 2001).

Specimen digestion and decontamination: All specimens that were AFB smear positive

were processed immediately at the NTBLTC. Each specimen was processed with N-acetyl-L-cysteine–NaOH–sodium citrate solution. The specimen was then centrifuged at a speed of 3000 g for 15-20 minutes. After centrifugation, tubes were then allowed to sit for 5 minutes to allow aerosols to settle. Then carefully, the supernatant was decanted into a suitable container containing a mycobactericidal disinfectant. A small quantity of (1-2 ml) phosphate buffer (pH 6.8) was added and the sediment re-suspended using a pipette and vortex mixer. The sediment in each tube was reconstituted with phosphate buffered saline (PBS) to 2 ml, mixed well and then pooled into one tube that served as the common inoculum source for all subsequent tests (Kubica,1963).

Isolation: An inoculum of 0.1ml of the sediments was inoculated into tubes of Lowenstein Jensen (LJ) media and incubated at 37^0C in slanting position. The tubes were observed after three (3) days to check for fast growers and contaminant and weekly thereafter till eight weeks. Cultures showing evidence of growth at any time during this period were then checked for morphology by making smear and staining by the Zielh-Neelsen procedure (Concepcion *et al*., 2001; Martin *et al*., 2005; Srivastava*et al*., 2008).

Identification of Mycobacterial Isolates: Rapid identification of MTBC from AFB-positive LJ media colonies was made using the Rapid kit (SD Bioline). Two to three colonies from LJ slopes were emulsified in 200 µl of antigen diluent and 100 µl of the emulsion was used as inoculum. The results of identification culture test (ICT) were read within 15 minutes. Positive test had two red to purple bands, one for internal control and the second line for the test. Negative had only one band in internal control slot. Strong or light bands with any intensity were considered to be positive (Swapna *et al.,* 2012).

Preparation of Lowenstein- Jensen Medium with anti- TB Drugs: Isoniazid (INH), Streptomycin (STR), Rifampicin (RIF) and Ethambutol (EMB) were 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 (0.45µm) sterilized and stored at -70^{o}C and used within six months. Different concentration of 0.2µg INH, 2µg EMB, 40µg RIF and 4µg STR, were incorporated into LJ medium and then inspissated at 85^0C for 45 minutes. After preparation, the media were incubated for 48 hours at room temperature for sterility check before use (Canetti *et al.,* 1963).

Drug Susceptibility Testing by Proportion Method: The indirect PM (IPM) was performed on LJ medium with the same recommended critical concentrations of antibiotics as mentioned previously. The culture of M. *tuberculosis* strain was harvested and suspended in tube containing sterile distilled water with 5-7 sterile glass beads then vortexed for about 30 seconds to homogenize the bacterial suspension and then allowed to stand for 15-30minutes for large aggrevates of bacteria to settle and turbidity of bacterial suspension was adjusted to match the McFarland turbidity No. 1. The original bacterial suspension was further diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. The tubes were arranged in order of C_1, C_2, C_3, S (streptomycin), I (isoniazid), R (rifampicin) and E (ethambutol) respectively.

One in hundred dilutions (10^{-2}) was inoculated into control (C_1) and four (4) drugs (S I R E), 10^{-3} dilution was inoculated into C_2 and 10^{-4} was inoculated into C_3 and then all tubes were incubated at 37°C for six weeks.

Growth was recorded at 28 days and at 42 days as follows: 3+ for confluent growth, 2+ for more than 100 colonies, and 1-100 actual numbers of colonies. Susceptibility or resistance was recorded when the proportion of bacteria in drug-containing medium to that of drug free medium is < 1 or ≥ 1 respectively. *M. tuberculosis* reference strains H37Rv (ATCC 27294) sensitive to first-line anti-tuberculosis drugs, RIF-

resistant (ATCC 35838), INH-resistant (ATCC 35822), EMB-resistant (ATCC 35837) and STR-resistant (ATCC 35820) were used as susceptible and resistant controls. All strains were sub cultured in Lowenstein–Jensen (LJ) medium for 4 weeks before being studied (Canetti *et al.,* 1963).

RESULTS

Out of the 437 re-treatment tuberculosis suspected samples collected from patients attending National Tuberculosis and Leprosy Training Center, Zaria, 72 were AFB smears positive giving a prevalence of 16.47% (Figure 1). Out of the 72 AFB smear positive samples, 62(86%) were culture positive, while 6 (8%) were culture negative and 4 (6%) were contaminated as shown in Figure 2. Out of the 62 culture positive 57 (91.9%) were confirmed as MTBC using the immunochromatographic test (ICT) while only 5(8.1%) were negative which were considered to be non-tuberculosis Mycobacteria (NTM) as shown in Table 1. Among fifty-seven (57) isolates, 33(58%) isolates were found to be resistant to streptomycin. Forty-seven (82.5%) and 44 (77%) were found be resistant to isoniazid and rifampicin respectively. While 35 (51%) isolates were found to be resistant to ethambutol. Isoniazid recorded the lowest of 17.5%, followed by rifampicin 22.8% and streptomycin 42%. Ethambutol (39%) had the highest susceptibility (Table 2). Forty-one MTBC isolates were found to be MDR-TB by LJ PM given a prevalence of 72.0% (41/57). The prevalence of poly-resistance to anti-TB drugs using was 14% whereas pan susceptible isolates had a prevalence of 14% (Table 3).

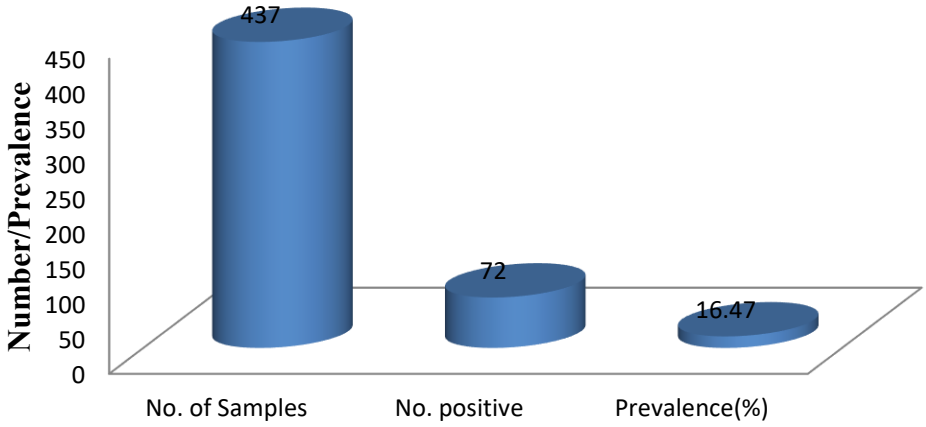

Figure 1: Prevalence of TB by microscopy

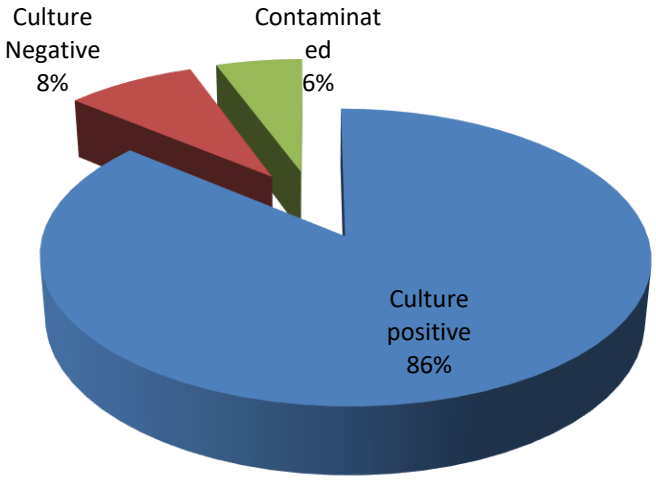

Figure 2: Percentage distribution of culture

Table 1: Prevalence of MTBC by rapid immunochromatographic test among culture positive

Mycobacteria	No positive	Percentage (%)
MTBC	57	91.9
Non- MTBC	5	8.10
Total	62	100

Keys: MTBC = Mycobacterium Tuberculosis Complex; Non-MTBC= Non-Mycobacterium Tuberculosis Complex

Table 2: Drug susceptibility pattern of *Mycobacterium tuberculosis* complex using proportion method obtained from sputum samples of re-treatment TB patients

Drugs	Streptomycin No (%)	Isoniazid No (%)	Rifampicin No (%)	Ethambutol No (%)
Resistance	33(58)	47(82.5)	44(77.2)	35(51)
Susceptible	24(42)	10(17.5)	13(22.8)	22(49)

Table 3: Resistance pattern of *Mycobacterium tuberculosis* complex by LJ using proportion method obtained from sputum samples of re-treatment TB patients

Drug resistance pattern	No of patients	Percentage (%)
MDR-TB	41	72
Poly-resistance	8	14
Pan susceptible	8	14
Total	57	100

Keys: DR-TB = Multidrug Resistant Tuberculosis LJPM = Lowenstein Jensen proportion Method

DISCUSSION

In this study the overall prevalence of TB by AFB smear microscopy among re-treatments patients attending NTBRL, Zaria was found to be 16.47%. This prevalence rate may result to the different patient's category used for the study subjects. The 16.7% rate is lower than the 18.6% reported by Aliyu *et al*. (2020) while similar to the report by Ahmadu *et al*., (2019) who reported 13.6% prevalence by AFB smear Microscopy.

This study showed 86% culture positive rate of mycobacteria among AFB smear positive samples which is lower than the reports of Ahamadu *et al*. (2019) and Selvakumar *et al*. (2012) who obtained prevalence of 91% and 85% culture positive rate in Kaduna and india respectively. This may be due to the fact that in all samples were transported, processed within the acceptable time period and moderately decontaminated. Contamination rate was observed to be 6% which could be due to incomplete digestion, use of contaminated reagent, media or equipment. This percentage (6%) is lower than 14.7% contamination reported by Gambo *et al.* (2013) in northern Nigeria. Smear positive culture negative sample were found to be 8%, this is could be attributed to the dead Acid-Fast Bacilli, a smaller number of viable bacilli that LJ media cannot detect or could be due to action of NaOH used for decontamination.

Five (7%) of the isolates were non-tuberculosis Mycobacteria. This percentage of NTM found may be due to environmental Mycobacteria that are abundant in soil and water or exposure to Harmattan dust. This is similar to the result obtained in northern Nigeria and America by Gambo *et al.* (2013) and Sharan *et al.* (2013) where they reported 5.7% and 4.3% of NTM respectively.

This study showed a prevalence rate of 72%. The higher prevalence of MDR-TB cases is mostly reported among Re-treatment patients who have previously exposed to TB treatment (WHO, 2008; Sharma *et al*., 2011). Our findings showed that the percentage of MDR-TB is high among patients with re- treatment cases. This may

reflect referral or selection bias of hospital-based studies similar to results from other countries in the region. More than half of MDR-TB isolates were resistant to all four first-line anti tuberculosis drugs tested. Re-treatment cases were significantly more likely to have MDR-TB than non-MDR-TB. Previous studies have shown that resistance to rifampicin is a significant predictor of resistance to isoniazid and streptomycin; isolates resistant to rifampicin were also resistant to isoniazid and streptomycin (Mdivanin *et al.,* 2008). The results of drug susceptibility testing by LJPM indicate that the resistance rate was higher against isoniazid and rifampicin, which are the most important first line drugs used for the treatment of tuberculosis. This could be as a result of overuse of these drugs for the treatment of TB naïve patients. This is in agreement with the work of Mahadev *et al.* (2004) and Iqbal *et al.* (2000) who reported higher resistance to rifampicin and isoniazid.

CONCLUSION

The study detects high resistance of *M. tuberculosis* to the two of the most important first anti-TB drugs as well as exhibited high multidrug resistance. The study identifies the need for fast and accurate diagnosis of resistant TB especially among re-treatment cases to interrupt transmission chain which will go a long way in providing a successful TB control.

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