ANTI-TUBERCULOSIS DRUG RESISTANCE OF MYCOBACTERIUM TUBERCULOSIS COMPLEX AMONG SMEAR POSITIVE PATIENTS IN PARTS OF ABUJA AND KADUNA STATE, NIGERIA

By

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A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA NIGERIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF A DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

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FEBRUARY, 2021

DECLARATION

I declare that the work reported in this thesis titled **"Anti-tuberculosis Drug Resistance of *Mycobacterium tuberculosis* Complex among Smear Positive Patients in Parts of Abuja and Kaduna State, Nigeria"** has been performed by me in the Department of Microbiology, Ahmadu Bello University.

This thesis is an original work I carried out and no part of it has been presented elsewhere for an award of a degree or diploma. The information derived from literature has been duly acknowledged in the text and a list of references provided.

Mosunmola Oluwaseun Iwakun Signature _____ Date _____

CERTIFICATION

This thesis entitled **"Anti-Tuberculosis Drug Resistance of *Mycobacterium tuberculosis* Complex Among Smear Positive Patients in Parts of Abuja And Kaduna State, Nigeria"** by Mosunmola Oluwaseun IWAKUN meets the regulations governing the award of the degree of Doctor of Philisophy in the Department of Microbiology, Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to God Almighty. The giver of life, the God with whom there is no variableness nor shadow of turning.

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Gratitude being the least of virtues, ingratitude, the worst of vices!

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ABSTRACT

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* has continued to be a major threat worldwide despite the existence of anti-tuberculosis drugs for the past 60 years. Nigeria ranks 7th among the 30 high tuberculosis burden countries and 2nd in Africa with an estimated incidence of 219 per 100,000 population and a case detection rate of 25% implying that transmission continues to occur within populations. This study was conducted to determine the Anti-tuberculosis Drug Resistance of *Mycobacterium tuberculosi*s Complex (MTBC) among Smear Positive Patients in Parts of Abuja and Kaduna State, Nigeria. One hundred (100) Acid Fast Bacilli (AFB) smear positive sputum samples obtained from TB patients attending selected hospitals in Abuja and Kaduna State were processed for culture. The isolates were characterized using the Hain Line Probe Assay MTBC and CM kits. Drug resistance profile of the MTBC was determined using the Hain Line Probe Assay GenoType MTBDR*plus* kit. Questionnaire was used to obtain some demographics and risk factors predisposing to infection. Results obtained and data from the questionnaire were analysed using SPSS version 24 and chi-square analysis was used to determine relationship between TB and the parameters at 95% confidence interval. P-value less or equals to 0.05 was considered statistically significant. Results of the cultures from the 100 AFB smear positive sputum samples showed 86% (86/100) were culture positive, 9% (9/100) were culture negative and 5% (5/100) was contaminated. The prevalence of MTBC and Non tuberculous Mycobacteria (NTM) among culture positive patients was 93% (80/86) and 7% (6/86) respectively. Among the MTBC species, *M. tuberculosis* had the highest prevalence of 91% (73/80) while the lowest (3%: 2/80) was *M. bovis.* Among the Non tuberculous Mycobacteria (NTM) species isolated, *M. fortuitum* had the highest prevalence (50%: 3/6). Other NTMs isolated were *M. mucogenicum, M. gordonae* and *M. intracellulare* with same prevalence of 16.7% (1/6)

each. Pan-susceptible TB occurred with the highest (91.3%: 73/80) frequency among the MTBC isolates. About 8.8% (7/80) of the MTBC isolates were resistant to at least one of the two drugs (Rifampicin and Isoniazid) while only one (1.3%: 1/80) case was Multi- drug Resistant (MDR) TB with resistance to both Rifampicin (RIF) and Isoniazid (INH). The *InhA* gene conferring low level INH resistance occurred more (71.4%: 5/7) compared to the *katG* gene conferring high level INH resistance with a prevalence of (28.6%: 2/7) among the MTBC isolates. The only isolate that was MDR-TB had mutations at *rpoB* WT7 (526-529) and MUT3 (S531L) gene loci. Among the demographic and risk factors examined, none was significantly associated with drug resistant MTBC among the study population. Among the demographic and risk factors examined for NTM infection, there was statistically significant association between livestock farming and NTM infection in this study (P=0.0003). In view of the results obtained in this study, there is need for rapid screening of the drug resistance and speciation of Mycobacteria for prompt initiation of treatment.

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ABBREVIATIONS

AC	Amplification Control
AFB	Acid Fast Bacilli
AIDS	Acquired Immunodeficiency Syndrome
AM	Amikacin
AMAC	Abuja Municipal Area Council
AP	Alkaline Phosphatase
ART	Anti Retroviral Therapy
BCG	Bacillus Calmette-Guérin
BDQ	Bedaquiline
BMI	Body Mass Index
BSC	Biological Safety Cabinet
CC	Conjugate Control
CD4	Cluster of Differentiation 4
CDC	Centre for Disease Control
Cfz	Clofazimine
CM	Common Mycobacterial
CNR	Case Notificaton Rate
CNS	Central Nervous System
DLM	Delamanid
DNA	Deoxyribonucleic Acid
DOTs	Directly Observed Treatment Shortcourse
DST	Drug Susceptibility Test
EMB	Ethambutol
EPTB	Extrapulmonary Tuberculosis
Eto	Ethionamide
FBO	Fixed Base Operators
FCT	Federal Capital Territory
FMOH	Federal Ministry of Health
FQ	Fluoroquinolone

GHA	Garki Hospital Abuja
GTC	Gwagwalada Township Clinic
GyrA	DNA Gyrase (Sub unit A)
GyrB	DNA Gyrase (Sub unit B)
H	Isoniazid drug
HCT	HIV Councelling and Testing
HIV	Human Immunodeficiency Virus
HK	Heat Kill
INH	Isoniazid
InhA	Isonicotinic Acid Hydrazide
IRIS	Immune Reconstitution Inflammatory Syndrome
katG	Catalase Peroxidase
Km	Kanamycin
LAMP	loop-mediated isothermal amplification
LED	Light Emmiting Diodes
LGA	Local Government Areas
LJ	Lowenstein Jensen's
MAC	*Mycobacterium avium* complex
MAF	*Mycobacterium africanum*
MDR	Multi Drug Resistant
MDR-TB	Multi Drug Resistant Tuberculosis
Mfx	Moxifloxacin
MIC	Minimum Inhibitory Concentration
MPT	Mycobacterial antigen
MTBC	*Mycobacterium tuberculosis* complex
MUT	Mutation
NAAT	Nucleic Acid Amplification Tests
NaCl	Sodium Chloride
NADH	Nicotinamide Adenine Dinucleotide
NaOH	Sodium Hydroxide

NIPRD	National Institute for Pharmaceutical Research Development
NO	Number
NRL	National Refernce Laboratory
NTBLCP	National Tuberculosis and Leprosy Control Programme
NTM	Non Tuberculous Mycobacteria
NTP	National Tuberculosis Control Programme
NW	Normal Weight
OB	Obese
OW	Over Weight
PAS	Para Amino Salicyclic
P-DST	Phenotypic Drug Suceptibililty Testing
PCR	Polymerase Chain Reaction
PLWHA	People Living with HIV and AIDs
PMDT	Programmatic Management of Drug Resistant Tuberculosis
POA	Pyrazinoic acid
PTB	Pulmonary Tuberculosis
Pto	Prothionamide
PZA	Pyrazinamide
RH	Rifampicin and Isoniazid
RHZE	Rifampicin, Isoniazid, Pyrazinamide and Ethambutol
RIF	Rifampin
RNA	Ribonucleic Acid
r-RNA	Ribosomal ribonucleic acid
RR-TB	Rifampicin Resistant Tuberculosis
SDA	Strand Displacement Amplification
SL-LPA	Second line - Line Probe Assay
SLI	Second Line Injectibles
SM	Streptomycin
SPSS	Statistical Package for the Social Sciences
TB	Tuberculosis

TB-LAM	Tuberculosis lipoarabinomannan
TB-LAMP	Tuberculosis Loop-Mediated Isothermal Amplification
TCH	Thiophene-2-carboxylic acid Hydrazide
TDR-TB	Totally Drug Resistant Tuberculosis
TUB	Tuberculosis Control
UATH	University of Abuja Teaching Hospital
UC	Universal Control
UW	Under Weight
WHO	World Health Organization
WT	Wild Type
XDR-TB	Extensively Drug Resistant Tuberculosis
ZN	Ziehl-Neelsen

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Tuberculosis (TB) is a common and often deadly infectious disease caused by *Mycobacterium tuberculosis* complex (World Health Organization (WHO) Global TB Report, 2017). Other members of the *Mycobacterium tuberculosis* complex such as *Mycobacterium bovis* also cause tuberculosis, however, they are less common compared to *Mycobacterium tuberculosis* which is predominant. (Kumar *et al.,* 2007; Hassan *et al*., 2017). Almost every organ of the body can be affected with tuberculosis, the lung accounts for over 70%-80% of tuberculosis cases, this is referred to as pulmonary tuberculosis (National Tuberculosis and Leprosy Control Program (NTBLCP) Workers Manual, 2010; WHO Global TB Report, 2017).

Pulmonary tuberculosis is transmitted from person to person through exposure to airborne droplets produced during coughing and sneezing by untreated persons with pulmonary tuberculosis (NTBLCP Workers Manual, 2010; WHO Global TB Report, 2017). These infected droplets remain airborne for a considerable period and may be inhaled by unsuspecting susceptible persons (Glaziou *et al*., 2015). Pulmonary tuberculosis usually occurs in the apex of lungs leading to development of cavities with large populations of tubercle bacilli, this can be detected in a sputum specimen (WHO Global TB Report, 2017). Pulmonary tuberculosis is suggested by symptoms such as: persistent productive cough for three weeks or longer, weight loss, night sweats and chest pains (WHO Global TB Report, 2008; Glaziou *et al*., 2015; WHO Global TB Report, 2017). Extra pulmonary or disseminated tuberculosis is facilitated by the spread of the bacilli to the blood stream or other tissues and organs where secondary tuberculosis lesions can develop (Sharma and

Mohan, 2004). This can be in the peripheral lymph nodes, kidneys, brain and bone (Herrmann and Lagrange, 2005; Adamu *et al*., 2017; WHO Global TB Report, 2017; Jawaid *et al.*, 2018). All parts of the body can be affected by the disease, it rarely affects the heart muscle, skeletal muscles, pancreas and thyroid (Agarwal *et al.*, 2005; Adamu *et al*., 2017; WHO Global TB Report, 2017; Jawaid *et al*., 2018).

Overall, a relatively small proportion (5–15%) of the estimated 2–3 billion people infected with *M. tuberculosis* will develop TB disease during their lifetime and majority of the cases will occur within the first five years after initial infection (Getahun *et al.*, 2015). However, the probability of developing TB is higher in immunosuppressed people e.g. People infected with Human Immunodeficiency Virus (HIV) (WHO Global TB Report, 2015). Other risk factors for developing TB are overcrowding, intensity and duration of exposure to the tubercle bacilli (Glaziou *et al*., 2015; WHO Global TB Report, 2017). Tuberculosis kills over 1.5 million people worldwide each year, more than any other single infectious disease, including HIV/AIDS and malaria (Adamu *et al*., 2017).

Due to the discovery of effective antibiotics, there was a gradual decline in the morbidity and mortality of tuberculosis between 1884 and 1953 and tuberculosis was predicted to be eradicated in 2010 (Cohen, Romero, Farah and Servant-Schreiber,1994). This prediction experienced a downward trend as the emergence of HIV and AIDS led to the rise of TB in the 1980s (Centre for Disease Control and Prevention (CDC), 1990). The rise of resistance to drugs used to treat tuberculosis, has become a significant public health issue in a number of countries and a hindrance to effectively control TB globally (WHO, 2008; Banerjee and Starke, 2016; Zignol *et al*., 2016;

WHO Global TB Report, 2018). The emergence of the multi-drug resistant tuberculosis (MDR-TB) has also threatened global tuberculosis control efforts (Ormerod, 2005).

MDR-TB are strains of *Mycobacterium tuberculosis* which show high level resistance to both isoniazid and rifampicin, with or without resistance to other anti TB drugs (CDC, 2016). The first clinically drug resistant tuberculosis was not described until 1970 (Jean and Richard, 2003). However, the burden of drug resistance has attracted international focus recently (Lange *et al.*, 2018) and Multi-drug resistant tuberculosis (MDR TB) has emerged in epidemic proportions in the wake of widespread of HIV infection in the world's poorest populations, including sub-Saharan Africa (Glaziou *et al*., 2015; Zignol *et al*., 2016). Without treatment, the death rate from TB is high and studies from the pre-chemotherapy era found that about 70% of people with sputum smear positive pulmonary TB died within 10 years; this figure was 20% among culture-positive (but smear-negative) cases of pulmonary TB (WHO Global TB Report, 2015; Zignol *et al*., 2016; WHO Global TB Report, 2018).

Effective drug treatments were first developed in the 1940s and the most effective first-line anti-TB drug, rifampicin, became available in the 1960s (Palomino *et al.*, 2007). The currently recommended treatment for new cases of drug-susceptible TB is a six-month regimen of four first-line drugs: isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) (WHO Global TB Report, 2017). This is divided into 2 months intensive phase and 4 months of continuation phase (NTBLCP Annual TB Report, 2014; Banerjee and Starke, 2016; Zignol *et al*., 2016, WHO Global TB Report, 2018). MDR-TB is characterized with longer duration of treatment (up to 2 years), lower cure rates and also higher default rates unlike 6 months treatment for drug susceptible TB (WHO Global TB Report, 2017).

Rifampicin resistance has been shown to be caused by a change in the β subunit of DNA dependent RNA polymerase encoded by *rpoβ* gene (Traore *et al.,* 2000). More than 95% of rifampicin resistant strains are associated with mutations within an 81-base pair region of the *rpoβ* gene, which is termed rifampicin resistant determinant region (Zaw *et al.,* 2018). Resistance to isoniazid is attributed to mutations at one of the two main sites in the *katG* or *inhA* genes, and these mutations are not directly connected (Piatek *et al.,* 2000). Thus, separate mutations are required for organisms to change from a drug susceptible isolate to MDR-TB (Piatek *et al.*, 2000). Additionally, rifampicin resistance has been considered to be a surrogate marker for checking multidrug resistance in clinical isolates of *M. tuberculosis* because rifampicin resistance is often accompanied by resistance to isoniazid (Traore *et al*., 2000; Sharma and Mohan, 2006).

Understanding the scientific basis of short course 6-month chemotherapy for tuberculosis explains why loss of sensitivity to both isoniazid and rifampicin without resistance to additional drugs, has such major effects on outcome (Han, 2006). Numerous controlled trials have shown that a 6-month regimen of rifampicin and isoniazid, supplemented by pyrazinamide and streptomycin or ethambutol for the first 2 months, will provide a cure in >95% of cases if the medication is correctly taken (British Thoracic Association, 1982). Such a regimen also renders infectious cases non-infectious in 2 weeks (British Thoracic Association, 1982).

Drug resistance in *M. tuberculosis* happens when large bacterial population spontaneously mutate in a random, single step at a low but predictable frequency (Ormerod, 2005). Each drug varies in its ability to kill tubercle bacilli (bactericidal ability), to deal with persistent organisms which are only occasionally metabolically active (sterilizing ability) and to prevent the emergence of drug resistance (Ormerod, 2005). The best bactericidal drug is isoniazid and if mono resistance to this

occurs, treatment with rifampicin and ethambutol has to be extended for 9 – 12 months, in addition to 2 months initial pyrazinamide (Joint Tuberculosis Committee of the British Thoracic Society (JTCBTS), 1998). The best sterilizing drug is rifampicin and mono resistance to this drug requires treatment with isoniazid and ethambutol for 18 months with 2 months initial pyrazinamide (JTCBTS, 1998). Therefore, loss of response to both the main bactericidal drug and the main sterilizing drug means patients remain infectious for much longer time, both in the community and hospital. In addition, the treatment is required for at least 12 and possibly more than 24 months, and a second line drug which is less effective and more toxic have to be used (JTCBTS, 1998).

Although some individuals who have not had TB treatment previously are infected by MDR-TB, this is not usually the case for most patients (Ormerod, 2005). Moreover, most new cases of MDR-TB arise from physician error and poor patient compliance with treatment, this changes organisms which are fully susceptible, or those with less complex resistance patterns into MDR-TB (Ormerod, 2005).

Globally, the treatment success rate for new and relapse cases of TB in 2016 was 82% (WHO Global TB Report, 2018). Among the six WHO regions, the highest treatment success rates were in the Western Pacific Region with 91% and the Eastern Mediterranean Region with 92% (WHO Global TB Report, 2018). The lowest rates of 75% were in the 3 WHO regions of the Americas (due to high level of loss to follow-up and missing data), Europe (due to high rates of treatment failure and death, influenced by the high frequency of MDR/RR-TB) and South-East Asia (due to high proportions of unevaluated cases, especially India) (WHO Global TB Report, 2018). The treatment success rate in the African region was 83% (WHO Global TB Report, 2018). An estimated total of 558,000 cases of multidrug-resistant TB and rifampicin resistant TB (MDR/RR-

TB) occurred in 2017 globally, 82% (457,560) were MDR-TB cases (WHO Global TB Report, 2018). However, only 160,684 cases of MDR/RR-TB were notified in 2017 and 139,114 were enrolled in treatment (WHO Global TB Report, 2018). Forty-seven percent (47%) of the global total of MDR/RR-TB cases were found in India (24%), China (13%) and the Russian Federation (10%) (WHO Global TB Report, 2018). Closing of detection and treatment gaps is still slow with only 25% of the estimated incidence of MDR/RR-TB cases being enrolled in treatment (WHO Global TB Report, 2018). Seventy-five percent (75%) of the gap between enrolment in treatment and the estimated number of MDR/RR-TB incidents were found in ten countries in 2017 with China and India accounting for 40% of the total gap (WHO Global TB Report, 2018).

Nigeria is a Federation of 36 States and a Federal Capital Territory. It is grouped into six geo-political Zones and 774 Local Government Areas (LGAs) (Ikenwa, 2019) with a projected population of about 191 million in 2017. It ranks 7th among the 30 high tuberculosis burden countries and 2nd in Africa (WHO Global TB Report, 2018). Nigeria has an estimated incidence of 219/100,000 population and a case detection rate of 25% (WHO Global TB Report, 2018). The WHO Global TB Report of 2017 showed that a total of 104, 904 of all forms of TB cases were registered and 16% of patients with TB were HIV positive in Nigeria (Kanabus, 2020). Thirty to forty-five percent (30-45%) HIV positive persons in Nigeria present with active TB at one point or the other (Ani *et al.*, 2010). Furthermore, out of the 84,161 (96%) of the TB patients offered HCT in 2014, 16,066 (19%) were HIV infected (NTBLCP Annual TB Report, 2014). Of these patients, 14,569 (91%) and 11,997 (75%) were placed on Co-trimoxazole and Anti-retroviral therapy respectively (NTBLCP Annual TB Report, 2014). Despite the effort put at ensuring that Isoniazid Preventive Therapy (IPT) uptake among children screened for TB is increased, the proportion of under six children placed on IPT among those screened for TB who came in contact

with smear positive TB cases was 3,811 (46%) (NTBLCP Annual TB Report, 2014). Rate of loss to follow-up and death rate among these cases was put at 6% and 5% for the new smear positive TB cases and 6% and 2% for retreatment smear positive cases respectively (NTBLCP Annual TB Report, 2014). However, the failure rates among new smear positive and retreatment TB cases started on treatment in 2013 remained 1% and 7% respectively when compared with the failure rate among those started on treatment in 2012 (NTBLCP annual TB Report, 2014). The distribution of all forms of tuberculosis cases in Nigeria shows that approximately 67% were males mostly within the ages of 35-54 years while about 14% were Children (both boys and girls) aged less than 14 years among all forms of TB cases notified (WHO Global TB Report, 2017).

The number of TB cases notified in the country is highest within the South West and North Central Zones, however, the case notification rates per 100,000 populations also revealed that the highest Case Notification Rates (CNR) in Benue, FCT, Nasarawa, Sokoto and Oyo (NTBLCP Annual TB Report, 2014). All five states recorded Case Notification Rate (CNR) > 100/100,000 population (NTBLCP Annual TB Report, 2014).

1.2 Statement of the Research Problem

Tuberculosis continues to be a major threat worldwide despite the existence of anti-tuberculosis drugs for the last 60 years (Tessema, *et al*., 2013; Zignol *et al*., 2016). Approximately one-third of the world's population is infected with *Mycobacterium tuberculosis*, about 9 million of whom develop "active" tuberculosis each year, which can be spread to others (Glaziou *et al*., 2015). Nigeria ranks 7th among the 30 high tuberculosis burden countries and 2nd in Africa (Kanabus, 2020). Nigeria has an estimated incidence of 219/100,000 population and a case detection rate of 25% (Fadare *et al.*, n.d).

The emergence of the multidrug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) has raised special concern in relation to the international spread of the disease (Zignol *et al*., 2016) and this has become a significant public health problem in a number of countries and an obstacle to effective global TB control (WHO, 2008; Zignol *et al*., 2016; Onyedum *et al.,* 2017). In the same vein, the HIV pandemic has caused dramatic increase in tuberculosis (Zignol *et al*., 2016); and Drug-resistant strains along with HIV/AIDS are the biggest challenge to efficient management and control of TB (Zignol *et al*., 2016; Onyedum *et al.*, 2017). The MDR-TB has reached alarming levels worldwide with the emergence of strains that are virtually untreatable with the existing drugs (Velayati *et al.,* 2018). Multidrug-resistant TB (MDR-TB) is a form of TB that is difficult and expensive to treat because it fails to respond to two important first-line drugs, specifically, rifampicin (RIF) and isoniazid (INH) (Bwanga *et al*., 2010; Glaziou *et al*., 2015; Zignol *et al*., 2016).

Though MDR- TB is often curable, treatment is complex and it requires expert management and frequent monitoring (WHO Global TB Report, 2013). The recommended treatment for MDR- TB lasts 18 to 24 months or longer (WHO Global TB Report, 2013). On the contrary, drug-susceptible TB, takes about 6 to 9 months to treat (WHO Global TB Report, 2013). The global report on anti-tuberculosis drug resistance indicated rates of drug resistant TB in Africa to be among the lowest worldwide (WHO Global TB Report, 2008; Zignol *et al*., 2016; WHO Global TB Report, 2017).

Global efforts for TB control especially in resource limited settings, are challenged by lack of rapid, reliable and inexpensive techniques for the detection of *M. tuberculosis* (Moore *et al.*, 2006; Shiferaw *et al.,* 2007; Brady *et al.,* 2008; Lazarus *et al.,* 2012; Onyedum *et al.,* 2017). The conventional culture detection method currently used consumes time, this hinders early

identification which is key for patient management and controlling transmission of *M. tuberculosis* (Caviedes *et al*., 2000; Shiferaw *et al*., 2007; Mesfin *et al*., 2018). Therefore, faster, inexpensive and reliable tests are needed for the detection of drug resistance tuberculosis (Moore *et al.*, 2006; Leung *et al.*, 2012; Onyedum *et al*., 2017; Mesfin *et al*., 2018).

1.3 Justification of the Study

Multidrug-resistant tuberculosis poses a formidable challenge to TB control due to its complex diagnostic and treatment challenges (Nowshad *et al.*, 2017). Novel technologies for rapid detection of anti-TB drug resistance have therefore become a priority in TB research and development, and molecular line probe assays focused on rapid detection of rifampicin resistance (alone or in combination with isoniazid) are most advanced (Toosky and Javid, 2014; Islam *et al*., 2016; Driesen *et al*., 2017).

Line probe assays allow rapid detection of resistance, and have been recommended by the WHO for rapid detection of drug resistant TB (Hain Lifescience, 2009; Albert *et al*., 2010; Coeck *et al*., 2016; Driesen *et al*., 2017; WHO Global TB Report, 2017**).** The rapid detection of drug-resistant *Mycobacterium tuberculosis* strains facilitates early access to the appropriate therapy, reduce rates of transmission, and improve treatment outcomes (Parsons *et al*., 2004; Zignol *et al*., 2016).

1.4 Aim of the Study

The aim of this study was to investigate the anti-TB drug resistance of *Mycobacterium tuberculosis* complex among smear positive patients in parts of Abuja and Kaduna State, Nigeria.

1.5 Objectives of the Study

The objectives of this study were to:

i. isolate and determine the prevalence of Mycobacteria by culture method from Acid Fast Bacilli (AFB) smear positive sputum samples of TB patients;
ii. characterize the isolated Mycobacteria by Hain Line Probe Assay;
iii. determine the resistance profile of the *Mycobacterium tuberculosis* complex (MTBC) by Hain Line Probe Assay;
iv. determine the frequencies and patterns of mutations at *rpoB, katG* and *inhA* genes of the *Mycobacteria tuberculosis* complex;
v. determine some demographic characteristics and risk factors that predispose to infection with Non-tuberculous Mycobacteria and the drug resistant MTB complex.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Emergence of Tuberculosis Disease

Tuberculosis (TB) was present before the beginning of recorded history and has left its mark on human creativity, music, art, and literature; it has influenced the advance of biomedical sciences and healthcare (Palomino *et al*., 2007). Its causative agent, *Mycobacterium tuberculosis* complex of which *Mycobacterium tuberculosis* predominates, may have killed more persons than any other microbial pathogen (Daniel, 2006). It is reputed that the genus *Mycobacterium* originated more than 150 million years ago (Daniel, 2006).

An early ancestor of *M. tuberculosis* was maybe coexistent and co-evolved with early hominids in East Africa, three million years ago. The modern members of *M. tuberculosis* complex seem to have originated from a common progenitor about 15,000 - 35,000 years ago (Gutierrez *et al*., 2005). TB was documented in Egypt, India, and China as early as 5,000, 3,300, and 2,300 years ago, respectively (Daniel, 2006).

2.2 Classification of *Mycobacterium tuberculosis* Complex

Mycobacterium is a genus of Actinobacteria, and has its family, the Mycobacteriaceae. There are over 190 recognized species in this genus (King *et al.,* 2017). Mycobacteria are classified into major groups based on its diagnosis and mode of treatment (Ryan *et al.,* 2004). The first group is *Mycobacterium tuberculosis* complex (MTBC), it causes tuberculosis and this include: *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti* etc *(*Ryan *et al.,* 2004*)*. Secondly, *Mycobacterium. leprae*, it causes Hansen's disease (Ryan *et al.,* 2004). The third group is the Non

tuberculous Mycobacteria (NTM) also called atypical Mycobacteria. It can cause pulmonary disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease leprosy (Ryan *et al.,* 2004).

2.2.1 *Mycobacterium tuberculosis*

Robert Koch in 1882 first described *M. tuberculosis* as the aetiological agent of human tuberculosis (Koch *et al.,* 1932). The genome of *M. tuberculosis* (4,411,529 bp) was deciphered more than 15 years ago (Cole *et al.,* 1998). *M. tuberculosis* has also been isolated from goats (Hiko & Agga, 2011), cattle (Romero *et al.,* 2011), cats (*Felis silvestris catus*) and dogs (*Canis lupus familiaris*) (Parsons *et al.,* 2008), domestic pigs (*Sus scrofa domestica*) (Jenkins *et al.,* 2011), birds (Schmidt *et al.,* 2008) and also from several wildlife species including zoo animals (Angkawanish *et al.,* 2010).

Phenotyically, it takes four to six weeks to obtain visible small and buff-coloured colonies on Middlebrook, an agar-based culture medium, or Löwenstein-Jensen (LJ), and an egg-based culture medium (Chinedum *et al.,* 2018). In culture, colonies of *M. tuberculosis* are off-white and rough on solid medium (Chinedum *et al.,* 2018). In addition, *M. tuberculosis* can be identified using analysis such production of niacin, nitrate reductase, sensitivity to pyrazinamidase and resistance to thiophene-2-carboxylic acid hydrazide (TCH) (Hoffner *et al.,* 1993; Niemann *et al.,* 2002). Studies showed modern strains of *M. tuberculosis* have an extremely low level of genetic difference, this signifies the entire population of *M. tuberculosis* strains resulted from clonal expansion after an evolutionary bottleneck some 35,000 years ago (Gutierrez *et al.,* 2005; Karboul *et al.,* 2006).

2.2.2 *Mycobacterium bovis*

Mycobacterium bovis causes tuberculosis in warm-blooded animals, such as cattle, dogs, cats, pigs, parrots, badgers, deers, camelids, some birds of prey, and also primates, including humans (Romero *et al.,* 2011). It is the main etiological agent of bovine tuberculosis and has one of the broadest host ranges among the MTBC members (Romero *et al.,* 2011). *Mycobacterium. bovis* colonies on egg-based media are small and rounded, with irregular edges and a granular surface; on agar media, colonies are small and flat (Wayne and Kubica,1986). The genome of *M. bovis* has a size of 4,345,492 bp and a G+C content of 65.6 % (Garnier *et al.,* 2003). The first whole genome sequence of a *M. bovis* strain was completed and found to be much smaller than the *M. tuberculosis* genome (Garnier *et al.*,2003). *M. bovis* evolved from an *M. tuberculosis* like organism (Cole *et al.*,1998). The sequence is >99.9% identical to that of *M. tuberculosis* although, there are some deletions in the genome which resulted in a reduced genome size (Garnier *et al.*, 2003).

2.2.3 *Mycobacterium bovis* BCG

There is also a non-virulent strain of *M. bovis* called Bacillus Calmette Guerin (BCG), which has its origin from a virulent *M. bovis* strain (Calmette, 1928). It came about by 230 in vitro passages of *M. bovis* until the organism lost its virulence by Calmette and Guerin (Bohle and Brandau, 2003). This strain has been used globally as a live attenuated vaccine to immunize people against TB, with highly variable efficacy (Andersen and Doherty, 2005), it might cause disease in humans. Showing protection in children against more serious forms of TB (Colditz *et al.,* 1995; Mittal *et al.,* 1996), although in adults, protection varies from 0 to 80% (Fine, 1995). In addition, in many high TB incident countries, the BCG vaccination is compulsory and free of charge, given on the first three days after birth (WHO, 2014).

2.2.4 *Mycobacterium africanum*

This species was first described as tuberculosis bacilli of the African type in Dakar, Senegal, in 1968 (Castets *et al.*, 1968) and after that it was found almost exclusively in West Africa. *M. africanum,* with a genome size of 4,389,314 bp (G+C content of 65.6%), causes up to half of the cases of human tuberculosis in West Africa (Bentley *et al.*,2012). The strains were described as intermediate between *M. tuberculosis* and *M. bovis*. Nowadays, this pathogen causes half of the human tuberculosis cases in West Africa (de Jong *et al.*, 2009). *Mycobacterium africanum* has been seen in other continents, such as the United States, primarily in people who have lived in Africa (Desmond *et al.*, 2004). *Mycobacterium africanum* is phenotypically diverse, with characteristics common to both *M. tuberculosis* and *M. bovis* (Gomgnimbou *et al.*,2012).

Mycobacterium africanum colonies resembles that of *M. tuberculosis* with physiological and biochemical properties that places the organism between *M. tuberculosis* and *M. bovis* (Smith, 2003). Prior to molecular genetics, the definition of *M. africanum* was difficult and its validity questioned by some authors (Smith, 2003). *Mycobacterium africanum* strains were classified into two major subgroups on the basis of geographical origin and biochemical properties, i.e., *M. africanum* subtype I from West Africa and *M. africanum* subtype II from East Africa (Mostowy *et al.*, 2004). In 2004, *M. africanum* type II was reclassified into *M. tuberculosis sensu stricto* (Mostowy *et al.*, 2004), while *M. africanum* type I was subdivided into West African I, prevalent around the Gulf of Guinea, and West African II prevalent in western West Africa. Subtype II has recently been described as *M. tuberculosis,* based on genotypic analyses thus leaving the West African type as the only *M. africanum* strain with the two subtypes MAF1 and MAF2 (De Jong *et al.*, 2010; Isea-Pena *et al.*, 2012).

2.2.5 *Mycobacterium caprae*

Mycobacterium caprae is also seen in sheep, pigs, wild boars, red deers, and foxes (Romero *et al.*, 2011) and accounted for 31% of human tuberculosis cases, mostly as pulmonary manifestation, in Germany between 1999 and 2001 (Kubica *et al.*, 2003). Easily recognized by its susceptibility to pyrazinamide, its isolation from humans has also been described (Erler *et al*., 2004; Kubica *et al.*, 2003). *Mycobacterium caprae*, with the former names *M. tuberculosis* subsp. caprae (Aranaz *et al.*, 1999) and *M. bovis* subsp. *caprae* (Kubica *et al.*, 2003), was originally described as preferring goats to cattle as hosts (Kubica *et al.*, 2003). The identification results are similar to *M. bovis* and *M. bovis* BCG based on biochemical tests (Kubica *et al.*, 2003). By spoligotyping, *M. caprae* species form a homogeneous cluster easily identifiable by the absence of spacers 1, 3-16, 30-33 and 39-43(Razanamparany *et al.*, 2006). The lack of spacers 39-43 has also been described in *M. bovis* and *M. microti* (Aranaz *et al*., 1999). Genotypically, it was later demonstrated that *M. caprae* is closely correlated to the branches of classical *M. bovis, M. pinnipedii, M. microti*, and *ancestral M. tuberculosis* but separate from modern *M. tuberculosis* (Prodinger *et al*., 2005).

2.2.6 *Mycobacterium canetti*

Mycobacterium canettii, a rare variant of the *M. tuberculosis* complex is the most divergent subspecies within the complex, with a smooth and glossy colony morphology and a rapid growth *in vitro* (Pfyffer *et al.*, 1998). It was first isolated from a Somali-born patient in 1969 by Canetti (Van Soolingen *et al.*, 1997) and preserved and studied extensively at the Pasteur Institute (Daffé *et al.*, 1987; Daffé *et al.*, 1991). The natural reservoir and host range of this pathogen are still unknown but tuberculosis caused by *M. canettii* appears to be an emerging disease in the Horn of Africa (Pfyffer *et al.*, 1998).

However, *M. canettii* is nowadays considered an outgroup of the MTBC due to its divergence from all other members of the complex and the evidence for recombination (Gutiérrez *et al.*, 2005), however the status of *M. canettii* as an outgroup to the MTBC complex has been challenged (Smith *et al.*, 2009a). *Mycobacterium canettii* differs from the other *M. tuberculosis* complex strains by having large amounts of lipooligosaccharides on the cell wall (Daffe *et al.*, 1991). The smooth and glossy colonies produced are highly exceptional for this species (Van Soolingen *et al.*, 1997). This smooth phenotype is however unstable and can switch to a rough colony morphology (Van Soolingen *et al.*, 1997).

2.2.7 *Mycobacterium microti*

Mycobacterium microti, is the causative agent of TB in rodents, such as voles and shrews. *M. microti* also causes naturally acquired tuberculosis in guinea pigs, rabbits, llamas (Oevermann *et al.*, 2004; Alvarez *et al.*, 2011), cats (Rufenacht *et al.*, 2011; Bennett *et al.*, 2011), meerkats and other warm-blooded animals as well as both immunocompetent and immunosuppressed humans (Van-Soolingen *et al.*, 1998; De-Jong *et al.*, 2009b). It was described for the first time by Wells in 1946, in voles (*Microtus agrestis*) from Great Britain (Wells, 1946). It was first reported in 1998 in immunocompromised patients in humans, (Van-Soolingen *et al*., 1998), although human to human transmission of *M. microti* infection seems to be rare (Emmauel *et al.*, 2007). *Mycobacterium microti* fails to grow in culture but has a characteristic "croissant"-like morphology in stained smears (Van-Soolingen *et al.*, 1998). The vole type of *M. microti* can easily be recognized upon spacer oligotyping as it contains an exceptionally short genomic direct repeat region resulting in identical two-spacer sequence reactions (Van-Soolingen *et al.*, 1998). Numerous deletions relative to *M. tuberculosis* have been discovered (Frota *et al.*, 2004).

Based on biochemical properties, this bacterium is difficult to distinguish from *M. tuberculosis*, *M. africanum*, or *M. bovis*, but *M. microti* strains display characteristic Insertion 9 (Zink and Nerlich, 2004).

2.2.8 *Mycobacterium pinnipedii*

Mycobacterium pinnipedii, formerly known as "seal bacillus" due to the host species from which it was first isolated (Forshaw and Phelps, 1991; Cousins *et al.*, 1993), has been classified as a separate species of the MTBC based on its molecular characteristics (Cousins *et al.*, 2003). Its natural host are seal (pinniped) species; *M. pinnipedii* has been reported from captive and wild Australian sea lions (*Neophoca cinerea*), Australian fur seal (*Arctocephalus pusillus doriferus*) and New Zealand fur seals (*Arctocephalus forsteri*) (Forshaw and Phelps, 1991; Cousins *et al.*, 1993; Woods *et al.*, 1995). It has also been isolated from a captive Southern sea lion (*Otaria flavescencs*), wild South American fur seals (*Arctocephalus australis*) and a wild subantarctic fur seal (*Arctocephalus tropicalis*) in Uruguay and Argentina (Bernardelli *et al.*, 1996, Bastida *et al.*, 1999). Furthermore, a Brazilian tapir (*Tapirus terrestris*) and South American fur seals from a zoo in Great Britain were found to be infected (Cousins *et al.*, 2003). Nevertheless, it is capable of infecting other mammalian animal species like dolphins (*Delphinidae*), llama (*Lama glama*), and gorilla (*Gorilla sp.*) (Rodriguez-Campos *et al.*, 2014). Seal trainers who worked with affected seal colonies in Australia and the Netherlands were also found infected with *M. microti* confirming the zoonotic potential of this pathogen (Thompson *et al.*, 1993; Kiers *et al.*, 2008). There were reports that defined *M. pinnipedii spp*. as another member of the *M. tuberculosis* complex (Cousins *et al*., 2003; Mc Nabb *et al*., 2004). The natural host is Pinnipeds but the organism is also pathogenic for guinea pigs, rabbits, and perhaps cattle. It also affects

animals in zoological gardens, e.g., camels (Camelus bactrianus) and tapirs (Tapirus indicus) (Plikaytis *et al*., 1992). Transmission of *M. pinnipedii* to humans has been reported in individuals who are in close contact with marine mammals (Thompson *et al*., 1993; Kiers *et al.,* 2008). The isolates present a distinct spoligotype pattern when likened to other members of the *M. tuberculosis* complex (Cousins *et al.,* 1993).

2.3 Morphology and Metabolism of Mycobacteria

The genus, Mycobacteria are aerobic, bacillary, straight or slightly curved rods between 0.2 and 0.6 µm wide and between 1.0 and 10 µm long (Ryan *et al.,* 2004). Mycobacteria mostly are nonmotile bacteria, except for the species *Mycobacterium marinum*, which has been shown to be motile within macrophages and they are typically acid-fast and cannot be stained by Grams stain procedure (Ryan *et al.,* 2004) with an outer membrane (Niederweis *et al.,* 2010). It has capsules, and typically do not form endospores (Ryan *et al.,* 2004). The species *M. marinum* and maybe *M. bovis* have been shown to sporulate (Ghosh *et al.,* 2009) but was contested by Traag *et al.,* (2010). The mycobacteria cell wall is thicker than in many other bacteria, being hydrophobic, waxy, and rich in mycolic acids/mycolates. The cell wall consists of the hydrophobic mycolate layer and a peptidoglycan layer held together by a polysaccharide, arabinogalactan as shown in Figure 2.1. The cell wall makes a substantial contribution to the hardiness of this genus. The biosynthetic pathways of cell wall components are potential targets for new drugs for tuberculosis (Bhamidi, 2009).

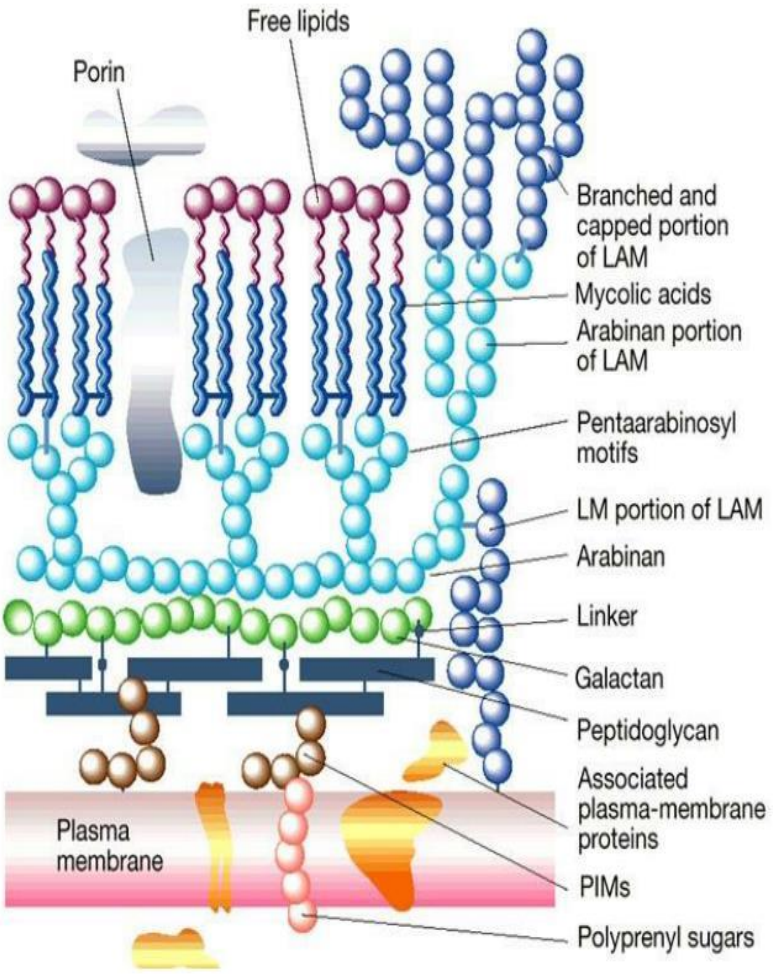

Figure 2.1: Mycobacterial cell wall (Niederweis *et al.*, 2010)

Some Mycobacteria produce carotenoid pigments without light while others require photoactivation for pigment production (Ryan *et al.*,2004). The Photochromogens (Group I) produce nonpigmented colonies when grown in the dark and pigmented colonies only after exposure to light and reincubation examples are *M. kansasii, M. marinum, M. simiae* (Niederweis *et al.,* 2010). The Scotochromogens (Group II) produce deep yellow to orange colonies when grown in the presence of either the light or the dark examples are *M. scrofulaceum, M. gordonae, M. szulgai* (Niederweis *et al.,* 2010). The Non-chromogens (Groups III and IV) form nonpigmentation in the light and dark or have only a pale yellow, buff or tan pigment that does not intensify after light exposure examples are *M. tuberculosis, M. avium-intra-cellulare, M. bovis, M. ulcerans, M. xenopi, M. fortuitum* and *M. chelonae* (Manual of Clinical Microbiology, 2015).

Many *Mycobacterium* species grow on very simple substrates, using glycerol as a carbon source and ammonia or amino acids as nitrogen sources in the presence of mineral salts (Lagier *et al.,* 2015). Optimum growth temperatures vary widely according to the type of species and this temperature range from 25 °C to over 50 °C. Most *Mycobacterium* species, including most clinically relevant species, can be cultured in blood agar (Lagier *et al.,* 2015). A natural division arises between the slowly and rapidly-growing species (Lagier *et al.,* 2015). The species that form visible colonies within 7 days on subculture are termed rapid growers, while those requiring longer periods are termed slow growers (Ryan *et al.,* 2004). The slow growers are as a result of extremely long reproductive cycles; *M. leprae,* may take more than 20 days to proceed through one division cycle (for comparison, some *E. coli* strains take only 20 minutes), making laboratory culture a slow process acid-fast (Ryan *et al.,* 2004).

2.4 Pathogenicity of Mycobacteria

Mycobacteria can colonize their hosts without the hosts showing any adverse signs and this is termed asymptomatic infections of *M. tuberculosis* also called Latent TB (Mandal, 2019). Mycobacterial infections are very difficult to treat because of their cell wall, which is neither truly Gram negative nor positive (Bhamidi, 2009). In addition, they are naturally resistant to a number of antibiotics that disrupt cell-wall biosynthesis, such as penicillin (McCann *et al.,* 2009). They can survive long exposure to acids, alkalis, detergents, oxidative bursts, lysis by complement, and many antibiotics (McCann *et al.,* 2009). *Mycobacterium tuberculosis* produces a number of surface and secreted proteins which include: Free lipids, Porin, Branched and capped portion of LAM, Mycolic acids, Arabinan portion of LAM, Pentaarabinosyl motifs, LM portion of LAM, Arabinan, Linker, Galactan, Peptidoglycan, Associated plasma-membrane proteins, PIMs and Polyprenyl sugars that contribute to its virulence (McCann *et al.,* 2009). However, the mechanism by which these proteins contribute to virulence remains unknown (McCann *et al.,* 2009).

TB Infection occurs when someone inhales droplet nuclei containing tubercle bacilli that reach the alveoli of the lungs (Ahmad *et al.,* 2016). These tubercle bacilli are then ingested by the alveolar macrophages; while the majority of these bacilli are inhibited or destroyed (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). The tubercle bacilli ingested may multiply intracellularly and are released when the macrophages die (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). These bacilli may spread through the lymphatic channels or bloodstream to other tissues and organs (including areas of the body in which TB disease is most likely to develop which are: regional lymph nodes, larynx, apex of the lung, kidneys, brain, and bone) (CDC and Healthcare Infection Control Practices Advisory Committee,

2013). This process of dissemination primes the immune system for a systemic response. The macrophages ingest and surround the tubercle bacilli within 2 to 8 weeks, and these cells form a barrier shell, called a granuloma, that keeps the bacilli contained and under control (LTBI) (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). The bacilli begin to multiply rapidly and develop into TB disease If the immune system cannot keep the tubercle bacilli under control (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). Without treatment, approximately 5% of persons who have been infected with *M. tuberculosis* will develop disease in the first year or 2 after infection, and another 5% will develop disease sometime later in life (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). Also, about 10% of persons with healthy immune systems infected with *M. tuberculosis* will develop TB disease later in life without treatment (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). Infectious individual will show certain features, this includes: presence of cough lasting three weeks or more, involvement of the larynx, failure to cover their mouth and nose while coughing or sneezing and being on inappropriate and inadequate treatment for the infection (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). Individuals showing presence of cavities in the lungs on Xrays, have a positive culture for tuberculosis from their sputum with positive presence (CDC and Healthcare Infection Control Practices Advisory Committee, 2013).

2.5 Transmission of Tuberculosis

Transmission of tuberculosis is from an infected person (with pulmonary or laryngeal tuberculosis) during coughing, talking, shouting, sneezing, laughing etc (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). These activities generate airborne particles called droplet

nuclei, they are tiny water droplets containing infectious bacteria that are 1–5 microns in diameter (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). These tiny droplet nuclei remain suspended in the air for up to several hours and can be inhaled by susceptible persons (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). These droplet nuclei travel via mouth or nasal passages and move into the upper respiratory tract. Thereafter they reach the bronchi and ultimately to the lungs and the alveoli (Mandal, 2019). The factors that determine the risk of transmission of tuberculosis are; If the individual it is transmitted to is prone to the tuberculosis infection, if the individual transmitting the infection is at the infectious stage of the disease, if the environment is suitable for transmission (suitable environment means presence of more droplet nuclei, exposure to the infection in a small, closed and cramped space with poor ventilation and positive air pressure etc (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). Other reasons for transmission are: the length of exposure of the susceptible person to the infected person and Improper handling of laboratory specimen containing the bacteria (Baraboutis *et al.,* 2011). The risk of getting the infection is higher when; the duration of exposure is longer, the frequency of exposure and the proximity of the infected person (Mandal, 2019).

2.6 Epidemiology of Tuberculosis

WHO provides the latest TB situation from the recent global TB report in 2017 based on data collected annually from 216 countries and territories (WHO Global TB Report, 2018). This provides a comprehensive and adequate assessment of the TB prevalence, at various NTP levels across the globe (WHO Global TB Report, 2018). WHO 2018 global report showed 10.0 million new cases of TB (range 9.0-11.1 million) and 1.3 million estimated deaths (range, 1.2–1.4 million) among HIV-negative people, and also an additional 300 000 deaths due to TB (range, 266 000–

335 000) among HIV-positive people (WHO Global TB Report, 2018). The global TB mortality rate (TB deaths among HIV-negative people per 100 000 population per year) is reducing at about 3% per year, and the best estimate for the overall reduction during 2000–2017 is 42% (WHO Global TB Report, 2018). Regionally, the fastest reduction in mortality rates between 2013 and 2017 were in the European Region and South-East Asia Region (11% and 4% per year, respectively) (WHO Global TB Report, 2018). Since 2000, the absolute number of deaths from TB among HIV-negative people have been estimated to have fallen by 29%, from 1.8 million in 2000 to 1.3 million in 2017, and by 5% since 2015 (the baseline year for targets set in the End TB Strategy) (WHO Global TB Report, 2018). In addition, the number of TB deaths among HIV-positive people has fallen by 44% since 2000, from 534 000 in 2000 to 300 000 in 2017, and by 20% since 2015 globally (WHO Global TB Report, 2018). The incidence rate of TB in terms of annual number varied widely among the countries in relation to population sizes (WHO, 2018). However, WHO reported in 2017 that most of the estimated number of cases occurred in South-East Asia Region (44%), African Region (25%) and Western Pacific Region (18%) (WHO Global TB Report, 2018). Fewer cases occurred in the Eastern Mediterranean Region (7.7%), the Americas (2.8%) and the European Region (2.7%). Eight countries: India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%) constituted two thirds of the total estimated TB incident cases worldwide (WHO Global TB Report, 2018). They are part of the 30 high TB burden countries that accounted for 87% of the total estimated incident cases worldwide (Figure 2.2).

The country-specific NTP programs had 6.7 million new TB cases notified to WHO in 2017 (WHO Global TB Report, 2018). At the start of the Directly Observed Short Course Therapy (DOTS) strategy in 1995, there were 3.4 million notified new cases and this showed how the number of

diagnosed TB cases has increased in the past 10-12 years (WHO Global TB Report, 2018). The large disparity between estimated and notified cases for the year 2017 highlights the inability of many NTP programs to correctly diagnose the actual number of TB cases, leaving many undiagnosed and thus untreated (WHO Global TB Report, 2018). This will lead to adverse consequences, particularly in those settings where drug resistance is on the rise (WHO Global TB Report, 2018). In 2017, about 3.6 million of the estimated 10 million people with TB worldwide were either not reported or not diagnosed; many of these people are likely to have been treated by public and private providers who are not linked to the NTP (WHO Global TB Report, 2018). The quality of care provided in these settings is difficult to ascertain and may be substandard (WHO Global TB Report, 2018).

The implementation of combined therapy also known as DOTS was successfully done in many parts of the world, after the declaration of TB a "global emergency" by WHO in 1993 (WHO Global TB Report, 2018). This had an impact on lowering the prevalence of TB. Currently in 2017, the treatment success rate is 82% among new and relapse TB cases globally (WHO Global TB Report, 2018). The remaining 18% of cases is at increased risk of failure, with increasing drug resistance as being one of the underlying reasons (WHO Global TB Report, 2018). With the techniques such as phenotypic drug susceptibility tests (DST) and molecular tools, considerable data on TB drug resistance have become available from many parts of the world (WHO Global TB Report, 2018). Worldwide, 3.5% of new cases and 18% of relapse were estimated to have multi-drug resistance (MDR)-TB as recorded in 2017 (WHO Global TB Report, 2018).

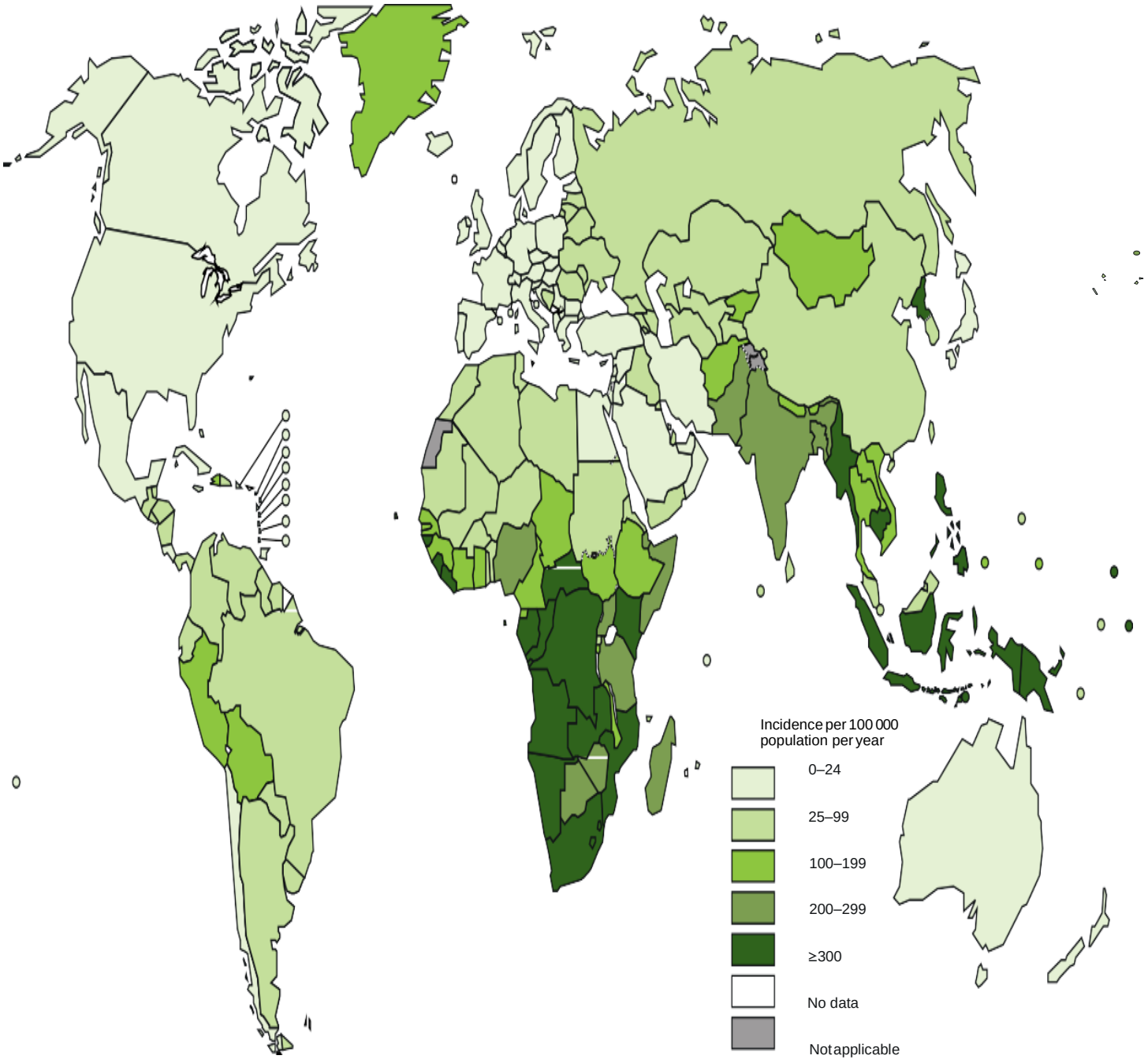

Figure 2.2: Estimated TB incidence rates, 2017 (WHO Global TB Report, 2018)

In May 2018, WHO and FIND released a technical report with updated laboratory parameters for DST of medicines used to treat drug-resistant TB (WHO, 2018). The report summarizes the current evidence used to determine critical concentrations in different culture media for performing DST for specific medicines used in the treatment of drug-resistant TB (WHO Global TB Report, 2018). The Stop TB Partnership has developed a Global Plan to End TB, 2016–2020 which focuses on the actions and funding needed to reach the 2020 milestones of the End TB Strategy (WHO Global TB Report, 2018). The 2020 target of the End TB Strategy are a 35% reduction in TB deaths compared with deaths in 2015, a 20% reduction in the TB incidence rate compared with 2015, and that no TB patients and their households face catastrophic costs as a consequence of TB disease (WHO Global TB Report, 2018). WHO reported in 2017, that there were an estimated 330 000 (range, 310 000–350 000) MDR/RR-TB cases among notified TB patients (WHO Global TB Report, 2018). By the end of 2017, XDR-TB had been reported by 127 WHO Member States (WHO Global TB Report, 2018). Of these, 113 countries and five territories reported representative data from continuous surveillance or surveys regarding the proportion of MDR-TB cases that had XDR-TB (WHO Global TB Report, 2018). Combining their data, the average proportion of MDR-TB cases with XDR-TB was 8.5%, an increase from the 6.2% in 2016 (WHO Global TB Report, 2018). In 2017, 30% (2.0 million) of the 6.7 million new and previously treated

Tuberculosis cases notified globally were reported to have been tested for resistance to rifampicin (WHO Global TB Report, 2018). Coverage was 24% for new TB patients and 70% for previously treated TB patients (Figures 2.3 and 2.4). Globally, 160 684 cases of multidrug-resistant TB and rifampicin-resistant TB (MDR/RR-TB) were notified in 2017 (up from 153 119 in 2016), and 139 114 cases were enrolled in treatment (up from 129 689 in 2016) (WHO Global TB Report, 2018).

However, due to financial constraints in many countries, only a small proportion of TB cases are currently tested for drug resistance (WHO Global TB Report, 2018)

In addition to being threatened by the emergence of drug resistance, global TB control is further complicated by co-morbidities such as HIV and diabetes (Nieburg *et al.,* 2015). TB disease is relatively higher among HIV infected people (Nieburg *et al.,* 2015). It is also higher in high-risk people such as the undernourished, diabetics, smokers and alcoholics (Nieburg *et al.,* 2015). About 5 to 10% of the estimated 1.7 billion people infected with *M. tuberculosis* will develop active TB in their lifetime (WHO Global TB Report, 2018).

Many new cases of TB are attributable to undernourishment, HIV infection, smoking, diabetes and alcohol (WHO Global TB Report, 2018). This is due to immunosuppression in the host which makes the patients particularly susceptible to TB (WHO Global TB Report, 2018). In the case of HIV-TB co-infection, risk of developing active TB exceeds 10% per year, as opposed to 10% per a life-time in HIV-uninfected individuals (CDC and Healthcare Infection Control Practices Advisory Committee, 2013). HIV/TB co-infection is one of the most common causes of death in least developed countries (Aaron *et al.,* 2004). In 2017, 464 633 cases of TB were recorded among people living with HIV globally, equivalent to 12% of TB patients with an HIV test result (WHO Global TB Report, 2018). The number notified was only 51% of the estimated number of incident cases among people living with HIV but an increase from 49% in 2016 (WHO Global TB Report, 2018).

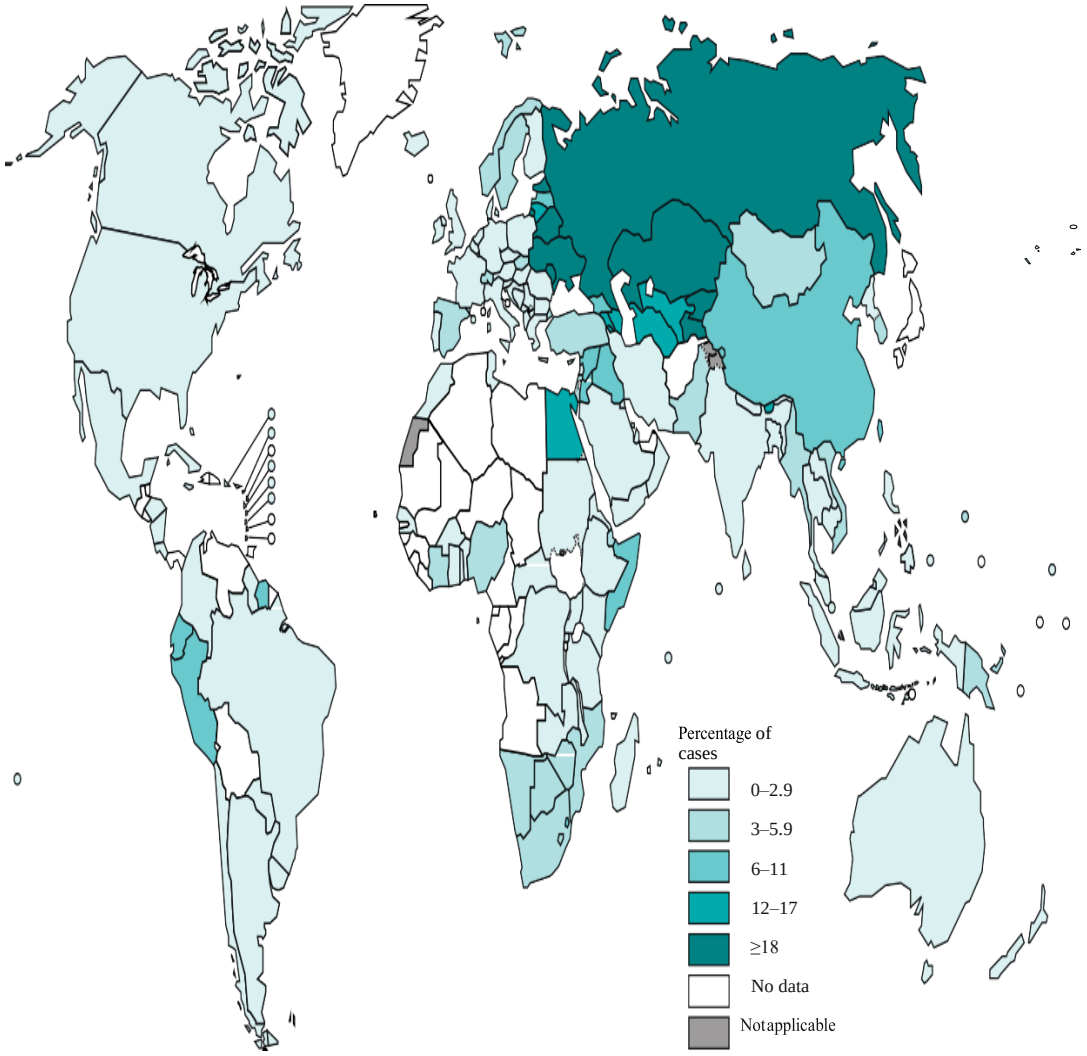

Figure 2. 3: Percentage of new TB cases with MDR/RR-TB[a] (WHO Global TB Report, 2018)

[a] Figures are based on the most recent year for which data have been reported, which varies among countries. Data cover the period 2002–2018.

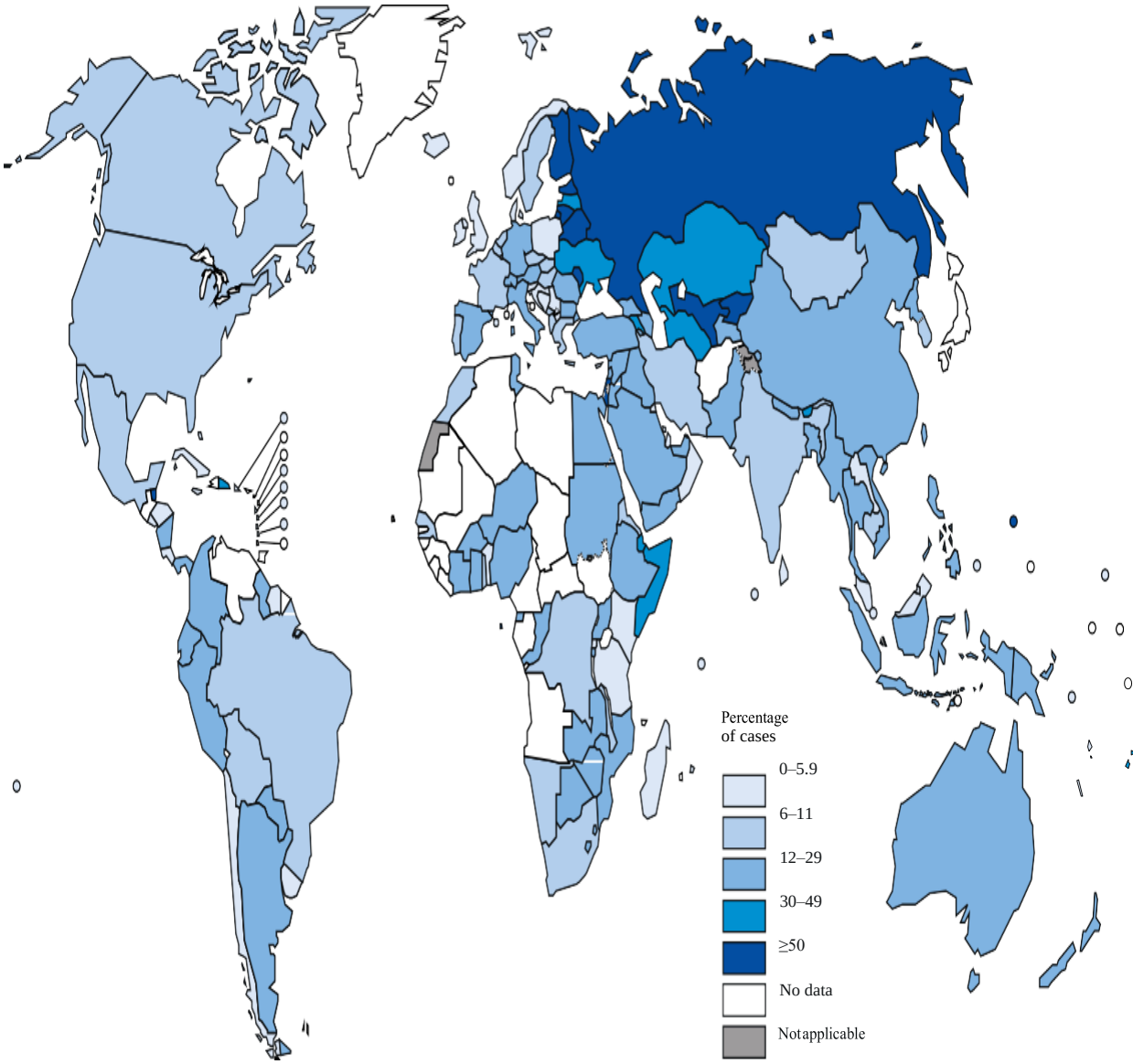

Figure 2.4: Percentage of previously treated TB cases with MDR/RR-TB[a] (WHO Global TB Report, 2018)

[a] Figures are based on the most recent year for which data have been reported, which varies among countries. Data cover the period 2005–2018. The high percentages of previously treated TB cases with RR-TB in Belize, Guam and Sao Tomé and Principe refer to only a small number of notified cases (range: 1–8 notified previously treated TB cases).

Overall, the percentage of TB patients testing HIV-positive has been falling globally since 2008 (WHO Global TB Report, 2018). This decline is evident in all WHO regions with the exception of the WHO European Region, where the proportion of TB patients testing HIV-positive increased from 3% in 2008 to 13% in 2017 (WHO Global TB Report, 2018). The WHO data from the year 2017 estimate shows that the number of TB deaths among HIV-positive people has fallen by 44% since 2000, from 534 000 in 2000 to 300 000 in 2017, and by 20% since 2015 (WHO Global TB Report, 2018). Fifty seven percent (57%) of HIV-positive TB deaths were men and boys and 43% were women and girls (WHO Global TB Report, 2018). Children (aged <15 years) accounted for 10% of total deaths in HIV-positive people. In an effort to better control HIV/TB, screening programmes are being scaled up (WHO Global TB Report, 2018). In 2017, the number of people living with HIV provided with TB preventive treatment was 958 559, based on data from 67 countries (WHO Global TB Report, 2018). These 67 countries accounted for 72% of the estimated number of TB cases among people living with HIV in 2017 (WHO Global TB Report, 2018). WHO in 2006 launched a "Stop TB Strategy" with a vision of "A TB-free world" and plans to "Halt and begin to reverse the incidence of TB by 2015" (WHO Global TB Report, 2017). Also, WHO planned to "reduce prevalence of and deaths due to TB by 50% compared to their levels in 1990 and also to eliminate TB as a public health problem by 2050" (WHO Global TB Report, 2018).

Several publications from 2009 have led to controversies regarding the classification of a new form of TB that is resistant to all anti-TB drugs which has been labelled "totally drug resistant TB (TDR-TB. These reports came from Italy (Migliori *et al.*, 2007), Iran (Velayati *et al.*, 2009), and later also from India (Udwadia *et al.*, 2012). The definition of TDR-TB was greatly argued by WHO and insisted that it could not be used until proper verification and standardization of DST

guidelines have be established that covers all anti-TB drugs which could be applied in all TB diagnostic laboratories (WHO, 2008). In addition, the reproducibility and reliability of second-line DST is limited, and critical concentrations to define resistance have been found to differ in different laboratory settings (WHO, 2008). Finally, new anti-TB drugs are still being evaluated in clinical trials, and the "TDR-TB" has not yet been tested against those drugs (WHO, 2008). The pipeline for new anti-TB drugs in August 2018 has expanded in recent months, and there are now 20 drugs in Phase I, II or III trials, compared with 17 in August 2017 (WHO Global TB Report, 2018). There are 11 new compounds (up by three since August 2017): contezolid, delpazolid, GSK-3036656, macozinone, OPC-167832, pretomanid, Q203, SQ109, sutezolid, TBA-7371 and TBI-166. Two other drugs (bedaquiline and delamanid) have already received accelerated or conditional regulatory approval based on Phase IIb trial results (WHO Global TB Report, 2018). Seven repurposed drugs are undergoing further testing: clofazimine, linezolid, levofloxacin, moxifloxacin, nitazoxanide, rifampicin (high dose) and rifapentine (WHO Global TB Report, 2018, 2018). Most of the new compounds are being developed by not-for-profit organizations, academic institutions, small businesses or government agencies that lack the secure funding and resources available to major pharmaceutical companies (WHO Global TB Report, 2018). This makes the process of progression through trials and then registration more uncertain.

Nigeria is among the high TB, TB/HIV and DR-TB countries globally. The country ranks 7th among the 30 high TB burden countries globally and 2nd in Africa, accounting for 4% of the estimated incidence cases globally (Federal Ministry of Health, 2017; Kwaghe *et al.*, 2020). In 2015, the global estimate for new (incident) TB cases was estimated at 10.4 million. People living with HIV accounted for 1.2 million (11%) of the incident cases (WHO 2017). An estimated

460,000 cases of tuberculosis occur in Nigeria annually (Federal Ministry of Health, 2017). TB prevalence among HIV/AIDS patients rose up to 27% due to increased association of TB with HIV/AIDS (Onyebuchi, 2015). The TB incidence and Mortality rates for the country is 219/100,000 and 39/100,000 population respectively (Federal Ministry of Health, 2017). Nigeria recorded a treatment success of 86% in 2017 (Sariem *et al.,* 2020). Nigeria ranks 7th among the 30 high Multidrug Resistance (MDR) TB burden countries with a drug resistance TB prevalence of 4.3% among new cases and 25% among previously treated cases (Onyedum *et al.,* 2017; Kanabus, 2020).

2.7 Diagnosis

2.7.1 Phenotypic diagnosis and identification of *M. tuberculosis* complex

The diagnosis of TB bacteriologically and the determination of drug susceptibility ensure that a patient is correctly diagnosed and place on effective TB treatment regimen. (WHO Global TB Report, 2014). It is essential to accurately identify members of the *M. tuberculosis* complex particularly in countries with high HIV prevalence, where non-tuberculous Mycobacteria (NTM) have been identified in human, and *M. bovis* remains a problem for cattle (Malama *et al.,*2014). Presence of phenotypic characters still remain the traditional methods of species identification which is based on biochemical testing including growth characteristics on different media and colony morphology (Boero and Bernardi, 2014). The colony morphology varies among the *M. tuberculosis* complex species ranging from flat smooth, domed glossy colonies to dry and rough colonies (Frothingham *et al.,* 1999). Differentiation of *M. tuberculosis* complex species involves the use of biochemical tests such as nitrate reductase, detection of niacin, growth in the presence of Thiophene-2-carboxylic acid Hydrazide (TCH), and catalase activity (Frothingham *et al.,* 1999).

All these tests, although simple and inexpensive to perform, do not clearly differentiate between species and they require experienced personal to interpret the results (Djelouadji *et al.,* 2018).

2.7.1.1 Microscopy

Ziehl-Neelsen (ZN) stain, is a diagnostic technique in which the stains are applied on a sputum smear to detect Acid fast organisms such as *M. tuberculosis* complex (Kradin and Lafrate, 2010). This technique ensures that infectious cases are detected at minimal cost and this is sometimes the only diagnostic tool in resource constraint countries (Nema, 2012). However, this technique has low sensitivity when compared to other diagnostic techniques with limit of detection of bacterial load less than 10,000 organisms/ml sputum sample (Nema, 2012). Smear microscopy, using (ZN) staining technique cannot distinguish between *M. tuberculosis* complex and other acid fast bacilli (Achkar *et al.*, 2011). Fluorescence microscopy, using auramine O staining technique, is 10% more sensitive than ZN staining (WHO Global TB Report, 2011). This method uses a lower power objectives lens (25x, while the ZN uses 100x), that makes the reading faster.

The light-emitting diodes (LED) microscopy now being used for fluorescent microscopy, is less expensive and there is no need of a dark room, which means the same infrastructure as the one for conventional ZN staining, making the implementation much easier (WHO Global TB Report, 2011). Due to 10% increased sensitivity in fluorescent microscopy, WHO recommended the gradual substitution of the ZN microscopy to fluorescent microscopy; this is to improve diagnosis by smear microscopy (WHO Global TB Report, 2011).

2.7.1.2 Culture

The most accurate TB test is culture, which is due to its high sensitivity and specificity (Gelaw *et al.*, 2017). It is labor-intensive and slow and the protocol time for culture in clinical laboratories is between 6 to 8 weeks to achieve maximum sensitivity on solid Lowenstein Jensen (LJ) media

(Moore *et al.,* 2005). Liquid culture (BACTEC MGIT 960) is the most sensitive culture technique for recovery of Mycobacteria from clinical samples (Tortoli *et al.,* 1999). Due to limited resources such as funding**,** reduced number of trained and qualified personnel and proper biosafety management and equipment, the liquid culture it's not currently utilized by all laboratories specifically in low-income countries (Tortoli *et al.,* 1999).

2.7.1.3 Immunochromatographic identification of the M. tuberculosis complex

The immunochromatographic technique have been developed to allow differentiation between the *M. tuberculosis* complex and NTM (Said *et al.,* 2011). This technique is also called lateral flow assays (Said *et al.,* 2011). It uses a monoclonal antibody to detect the MPB64 protein (Rv1980c; also termed as MPT64), which is specifically secreted during growth of *M. tuberculosis* complex bacteria (Harboe *et al.,* 1986). The immunogenic protein MPB64 is highly specific for *M. tuberculosis* complex, except some variants of *M. bovis* BCG (Harboe *et al.,* 1986). Immunochromatographic assays mostly used are commercial kits, including the SD Bioline Ag MPT64 Rapid assay (Standard Diagnostics, Kyonggi-do, Korea), Capilia TB (TAUNS, Numazu, Japan), and the MGIT TBc Identification Test (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD) (Harboe *et al.,* 1986).

2.7.1.4 Lateral flow identification of the M. tuberculosis complex using LipoArabinoMannan Assay

The cell wall lipopolysaccharide lipoarabinomannan (LAM) is Mycobacterial antigen in the urine of patients with pulmonary TB; it is used as a marker for the detection of tuberculosis (Achkar *et al.,* 2011; Minion *et al.,* 2011; Hamasur *et al.,* 2015). The commercial test (Allere-Determine TB LAM in urine) is simple to use, there is no need of instruments, gives rapid result and has low cost (Bjerrum *et al.,* 2019). The LAM test is sensitive in patients with advanced HIV disease but not in

HIV negative adults and HIV positive adults with CD4 counts higher than 100 cells per microliter (Achkar *et al.,* 2011; Lawn *et al.,* 2013; Sarkar *et al.,* 2014; Shah *et al.,* 2014).

2.7.2 Genotypic identification of *M. tuberculosis* complex

Polymerase Chain Reaction have been used successfully to identify *M. tuberculosis* complex with the advantage of being more rapid and accurate than conventional methods (Cormican *et al.,* 1992). Identification of commonly isolated Mycobacteria has been greatly facilitated with the introduction of radioisotope-labelled DNA probes (Fukushima *et al.,* 2003). Similarly, *M. tuberculosis* complex grown in liquid cultures had been successfully identified by commercially available and in-house developed nucleic acid amplification techniques (Yu *et al.,* 2011). Molecular assays based on nucleic acid amplification techniques such as polymerase chain reaction (PCR) have been developed for rapid TB diagnosis and are being implemented in developing countries like Nepal and Namibia (WHO, 2016). Commercially available systems such as the INNO-LiPA (Innogenetics NV, Ghent, Belgium) in which the 16S-23S rRNA spacer region of Mycobacterial species is amplified and the GenoType MTBC targeting the 23 rRNA have been successfully used to directly detect and identify *M. tuberculosis* complex (Hain Lifescience, 2009).

The GenoType MTBC, enables rapid differentiation of *M. tuberculosis* complex bacteria, with higher sensitivity compared to the AccuProbe assay (Richter *et al.,* 2004). Smear microscopy and culture have been the techniques used by many countries until recently that new techniques are changing the landscape of TB diagnostics, presenting a pipeline of various new tools, particularly molecular methods (Pai and Schito, 2015).

2.7.2.1 GeneXpert MTB/RIF Assay

The Cepheid GeneXpert System's MTB/RIF assay is a real-time Polymerase Chain Reaction (PCR) in-vitro diagnostic test that detects *M. tuberculosis* complex DNA and rifampicin resistance (Ioannidis *et al.,* 2011). It involves a single use cartridge-based semi-quantitative assay whose resistance to rifampicin is associated with mutations of the RNA polymerase beta (*rpoB)* gene (Ioannidis *et al.,* 2011). The Xpert MTB/RIF assay has high sensitivity and sensitivity based on a WHO meta-analysis (Denkinger *et al.,* 2014). The test has shown very high sensitivity in sputum samples such as 98% in smear-positive, culture positive and 79% in people living with HIV (Denkinger *et al.,* 2014). In extra-pulmonary samples, the Xpert MTB/RIF test shows high sensitivity when compared to culture in the diagnosis of extra-pulmonary TB from lymph node tissues or aspirates (84.9%), gastric lavage (83.8%), cerebrospinal fluid (79.5%) and other tissue specimens (81.2%), except for pleural fluid samples that have a low sensitivity (43.7%) (Raizada *et al.,* 2018). The specificity is notably high in all groups, more than 92.5% (Raizada *et al.,* 2018)

2.7.2.2 Genotypic Line Probe Assay

The Hain Line Probe Assay is a DNA strip technology that uses the principle of polymerase chain reaction (PCR) for the molecular genetic assay (Hain Lifescience, 2009b). The principle and procedures of the test is the same and the procedure is divided into three namely; DNA extraction, Amplification and Reverse Hybridization (Hain Lifescience, 2009a). The GenoType MTBDR*plus* is a test for the rapid identification of *Mycobacterium tuberculosis* complex and detction of rifampicin resistance (detection mutations of the *rpoB* gene) and isoniazid resistance (testing is enabled by the *katG* gene and the inhA gene) (Hain Lifescience, 2009a). The GenoType MTBC kit allows for the differentiation of the *Mycobacterium tuberculosis* complex from culture material by identifying species belonging to the *Mycobacterium tuberculosis* complex; *M. africanum*, *M.*

bovis BCG, *M. bovis* ssp. *bovis*, *M. bovis* spp. caprae, *M. microti*, and *M. tuberculosis*/*M. canettii* (Hain Lifescience, 2009b). *Mycobacterum tuberculosis* complex which is differentiated by the analysis of any of the most frequently investigated conserved regions (16S ribosomal DNA, ITS, 23S ribosomal DNA) (Hain Lifescience, 2009b). The GenoType CM kit (Hain Lifescience GmbH, Nehren, Germany) allows for the differentiation of common Mycobacteria and they are *M. avium, M. chelonae, M abscessus, M forturtum, M gordonae, M intracelluare, M scrofulaceum, M interjectum, M kansasil, M malmonense. M. marinum, M ulcerans, M. tuberculosi complex, M peregrinum* and *M xenopi* (Hain Lifescience, 2009b).

2.7.2.3 Loop-mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is a unique temperature-independent way of DNA amplification (Wong *et al.,* 2018). It is facilitated by a visual optic readout in an instrument that is robust and can be used at the peripheral health center level where microscopy is performed (Wong *et al.,* 2018). Commercial nucleic acid amplification tests (NAAT) for the detection of TB and other Mycobacterial diseases have come into routine use in industrialized countries because of their great advantage of speed compared to culture (CDC, 2014). The complexity of existing commercial NAAT formats, the necessity of a high precision instrument, a high degree of technical support and well-trained staff made it unsuitable for developing country settings (CDC, 2014). Recently, novel isothermal amplification methods, such as loop-mediated isothermal amplification (LAMP) and strand displacement amplification (SDA), have been developed (Zanoli and Spoto, 2013). The speed of the reaction (less than 1 hour), the absence of need for a thermocycler, and the easy visual readout make these methods excellent platforms for the development of a simple and sensitive tool for molecular detection of TB in developing country settings (WHO, 2016). TB-LAMP (Eiken Inc.) is a manual assay that requires less than one hour to perform and can be read

with the naked eye under ultraviolet light (WHO, 2016). This first LAMP research prototype for the detection of TB was evaluated in Peru, Bangladesh and Tanzania between January and May 2006 (Boehme *et al.,* 2007). In the relatively small number of samples, LAMP was highly specific and significantly more sensitive than microscopy, detecting M. tuberculosis (MTB) DNA in almost all smear-positive sputum specimens and half of smear-negative, culture-positive specimens (Boehme *et al.,* 2007).

2.8 Tuberculosis Control Programme in Nigeria

The National TB and Leprosy Control Programme (NTBLCP) was launched in 1991 under the Federal Ministry of Health (FMOH) in response to the public health concern caused by tuberculosis which is a high priority disease (NTBLCP, 2014). The DOTS Strategy was officially adopted in 1993, but nationwide rollout began only in 2003 (NTBLCP, 2014). In Nigeria, 100% of the LGAs have at least two DOTS centres and there are DOTs centres in all 36 states and FCT of the country (NTBLCP, 2014). By the end of 2018 there were 9,625 DOTs centers, 2,875 facilities contained laboratories with microscopes and had the capacity to run AFB diagnosis and 395 GeneXpert machines. There is capacity for diagnosis and treatment of multi drug resistant tuberculosis, with 27 DR-TB Treatment centers, 2 National, 6 Zonal, 1 State and 1 Private Reference Laboratories (NTBLCP, 2014).

The NTBLCP is built on strategies which ensures services are provided in an encompassing way that guarantees that TB, Leprosy & Buruli ulcer services are integrated into the existing health system infrastructure and general health care services in all the 774 Local government areas in Nigeria (NTBLCP Management and control Guideline, 2015). In addition, there is a road map which is centered on demand creation, provision of access and ensuring social re-integration of

those affected by TB into the community (NTBLCP Management and control Guideline, 2015). The services provided by the NTBLCP are implemented at the various levels of health care with the involvement of the public, private and the community health providers and these are in conjuction with the HIV/AIDS Control Program (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013). At the moment, mixed model of management of drug resistant TB activities is being implemented with both hospital and community-based care for the patients (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013).

In Nigeria, private sector involvement in TB program remains suboptimal. Private healthcare providers account for more than 60% of care-seeking, yet these providers contributed only 4,698 cases or 4.5% of the reported1 and 1.2% of estimated TB cases (NTBLCP Management and Control Guideline, 2015). As at December 2017, there was a total of 70,598 private sector health providers (547 FBOs, 13,448 Private for profit, 2,103 private labs, and 54,500 community pharmacist and patient medicine vendors) (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013). Out of this only 1,762 (277 FBOs, 646 Private for profit, 96 private laboratories, and 743 community pharmacist/patient medicine vendors) representing 2.5% were engaged to provide TB services in 2017 (NTBLCP PMDT Guideline, 2017). Efforts to engage private providers over the last 20 years have been sporadic, small and short-lived. The TB National Strategic Plan (2015 – 2020) had included a target of 30% of total notifications to be contributed by private providers, but these aspirations were not backed-up by commensurate resource allocation (NTBLCP Management and Control Guideline, 2015).

2.9 Tuberculosis Treatment

2.9.1 Standard treatment protocols for treating drug-susceptible tuberculosis in Nigeria

Two standardized treatment regimens have been adopted for the treatment of all susceptible TB cases in Nigeria. Each regimen is divided into two phases (intensive and continuation) and the drugs used come in fixed dose combinations as shown in Table 2.1 and the doses are as shown in Table 2.2.

2.9.1.1 Regimen 1 (Six Months Regimen)

This is for all forms of TB (PTB and EPTB cases both new and previously treated) with the exception of TB meningitis and Osteo-articular TB cases (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013). All Adults and children weighing more than 18kg diagnosed with Rifampicin susceptible TB (with the exception of TB meningitis and Osteo-articular TB) are started on the Regimen 1 treatment during the intensive phase, 4 drugs (isoniazid, rifampicin, pyrazinamide, and ethambutol) are used to rapidly kill the tubercle bacilli and infectious patients become less infectious within approximately 10-14 days of starting treatment (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013). Two drugs (isoniazid and rifampicin) are used in the continuation phase, over a period of 4 months. The sterilizing effect of these drugs eliminates the remaining bacilli and prevents subsequent relapse (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013). DOT is observed throughout the period of treatment either through direct supervision by the health worker or through the engagement of a treatment supporter (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013).

2.9.1.2 Regimen 2 (Twelve Months Regimen)

The twelve months treatment regimen is in use to treat severe or complicated diseases like TB meningitis and TB of the bones or joints (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013). The intensive phase remains two months and the continuation phase is prolonged to ten months (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013). Therefore, all adults and children weighing more than 18kg diagnosed with Rifampicin susceptible TB of the meninges, bones and joints (TB meningitis and Osteo-articular TB) should be started on the Regimen 2 for treating TB meningitis and Osteo-articular TB (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013). DOT is observed throughout the period of treatment either through direct supervision by the health worker or through the engagement of a treatment supporter (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013). Streptomycin is no longer used in the Regimen 1 and 2 of the NTBLCP but may be used in special situations such as in liver disease when Rifampicin and Isoniazid cannot be used (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013).

2.9.2 Standard treatment protocols for treating drug-resistant Tuberculosis

The eligibility criteria for starting DR-TB treatment regimens include the following as contained in the NTBLCP PMDT Guideline (2017).

Patients (adults and children) with confirmed rifampicin resistance: All patients enrolled in care in the implementation sites who have been diagnosed with RR-TB (RR/MDR/Pre-XDR/XDR TB) are eligible for the DR-TB treatment options (NTBLCP PMDT Guideline, 2017).

Table 2.1: NTBLCP recommended regimen for drug susceptible TB

Type of TB Disease	Intensive Phase	Continuation Phase	Length of treatment
PTB and all forms of EPTB except TB meningitis & Osteo-articular TB (spine, joints) in adults	2 RHZE	4 RH	6 months
TB meningitis and Osteo-articular TB (spine, joints) in adults	2 RHZE	10 RH	12 months
PTB and all forms of EPTB except TB meningitis & Osteo-articular TB (spine, joints) in children	2 RHZ+E	4 RH	6 months
TB meningitis and Osteo-articular TB (spine, joints) in children	2 RHZ+E	10 RH	12 months

(National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013)

Keys: R=rifampicin. H=isoniazid. Z=pyrazinamide. E=ethambutol. Numeral refers to number of months of the regimen example- 2 RHZE refers to two months of daily rifampicin, isoniazid, pyrazinamide and ethambutol (National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013).

Table 2.2: Regimen and doses of anti-tuberculosis drugs for new Pulmonary TB patients (adult)

Regimen	Pre- treatment weight			
	30-37kg	38-54kg	55-70kg	>70kg
Intensive phase (2 months) Combined tablet RHZE (150mg+75mg+400mg+275mg)	2	3	4	5
Continuation phase (4 months) Combined tablet RH (150mg + 75mg)	2	3	4	5

(National Guidelines for Tuberculosis, Leprosy and Buruli Ulcer management and Control, 2013).

Keys: R=Rifampicin, H-Isoniazid, Z=Pyrazinamide, E-Ethambuthol

Other patients at high risk for rifampicin resistant TB to be considered for enrollment: In the absence of bacteriological confirmation, the following are eligible for second-line treatment after considering all circumstances around the patient (NTBLCP PMDT Guideline, 2017):

i. Young children who are diagnosed with active TB, with a close contact, (especially a parent or caregiver) who has bacteriologically confirmed DR-TB
ii. PLHIV with active TB who are close contacts of known DR-TB patients
iii. EPTB who are close contact of a known DR-TB patient
iv. Failure of first-line TB treatment

There are two options for treating DR-TB in Nigeria; the shorter DR-TB regimen or an individualized DR-TB regimen (NTBLCP PMDT Guideline, 2017). A systematic approach is be followed to determine if the patient should be treated with either options. Triage is done on the patient and this includes clinical evaluation to determine the patient's risk of resistance or intolerance to Fluroquinolone (FQ) and/or Second Line Injectible (SLI), and bacteriological testing of pre-treatment specimen to determine the strain's resistance to Fluroquinolone (FQ) and Second Line Injectible (SLI) drugs (NTBLCP PMDT Guideline, 2017). In addition, two sputum samples must be sent for second-line Line Probe Assay (SL-LPA) and for culture, as well as first line and second line phenotypic drug susceptibility tests (FL/SL PDST) before the start of treatment (NTBLCP PMDT Guideline, 2017). Once the results are available, the initial regimen can then be adjusted if necessary, to the appropriate regimen (NTBLCP PMDT Guideline, 2017). If the patient has mono- or poly-drug resistant TB with resistance to rifampicin (susceptible to isoniazid), will receive the same regimens as RR/MDR-TB (shorter or individualized according to eligibility criteria), with isoniazid high dose included (NTBLCP PMDT Guideline, 2017). If the patient has

mono- or poly-drug resistant TB with resistance to isoniazid (susceptible to rifampicin), care should be taken to evaluate the medical history for possible amplification of resistance which may have developed, but may not be apparent from the laboratory results (NTBLCP PMDT Guideline, 2017). As such, treatment for mono- and poly-drug resistant TB should never rely solely on DST results (NTBLCP PMDT Guideline, 2017). It is important to assess the history of previous TB treatment, contact history, risk of amplification of resistance, extension of the disease, and patient's condition (NTBLCP PMDT Guideline, 2017). There is no updated international recommendation on the treatment of isoniazid mono- and poly-drug resistance at the time of developing these guidelines (NTBLCP PMDT Guideline, 2017). For isoniazid poly-drug resistance, MDR-TB regimen can be considered (NTBLCP PMDT Guideline, 2017). For isoniazid mono-resistance, the patient summary can be referred to the national DR-TB committee for guidance (NTBLCP PMDT Guideline, 2017).

2.9.2.1 Shorter treatment regimen

This is given as a standardized regimen with little or no room for customization with substitutions or switches during treatment (NTBLCP PMDT Guideline, 2017).

i. The intensive phase consists of Kanamycin (Km), high dose Moxifloxacin (Mfx), Clofaximine (Cfz), Ethionamide (Eto), Pyrazinamide (Z), Ethambuthol (E) and Isonaizid (H) daily for four months.
ii. The criteria to shift to continuation phase is based on smear conversion and clinical response. The patient should have smear negative results and be clinically improving. All attempts should be made to send monthly samples for culture and trace results. It is important to have Second Line (SL) Line Probe assay (LPA), culture and SL phenotypic (Drug Suceptibility Testing) DST results available to exclude resistance to

Fluoroquinolone (FQ) and Second Line Injectible (SLI) (though SL LPA test when performed in smear negative samples can have uninterpretable results).

iii. If the smear conversion is not achieved at month four, the intensive phase shall be extended to a maximum total duration of six months (until smear conversion and one negative culture result). Then Kanamycin (Km) (Amikacin (Am) or Capreomycin (Cm)) may be given thrice-weekly (on alternate days), from the fourth month onwards (fifth and sixth months).

iv. If the patient remains smear and/or culture positive at six months, will be declared as a treatment failure and switched to an individualized regimen.

v. Failure declaration and a switch to an individualized treatment will be considered earlier than six months in patients with clear lack of response (clinically, smear grading, culture). The decision to switch earlier must be made by the expert committee of state consilium (and if required national consilium).

vi. The continuation phase consists of high dose Mfx, Cfz, E and Z for a fixed duration of five months. Shorter Regimen: Short course regimen drug dosages for adults and adolescents > 30 kg

vii. Treatments for 4-6 months Km-Mfx-Cfz-Pto-Z-E-Hh / 5 Mfx-Cfz-E-Z. The dosage is as shown in Table 2.3

viii. Add vitamin B6 100 mg

Table 2.3: Dosage of medicines to use in the WHO recommended shorter DR-TB regimen for adults

Drug	Weight Group		
	Less than 30 kg	**30 –50 kg**	**More than 50 kg**
Moxifloxacin	400 mg	600 mg	800 mg
Clofazimine	50 mg	100 mg	100 mg
Prothionamide	250 mg	500 mg	750 mg
Pyrazinamide	1000 mg	1500 mg	2000 mg
Ethambutol	800 mg	800 mg	1200 mg
Isoniazid	300 mg	400 mg	600 mg
Kanamycin*	15 mg / kg body weight (maximum 1 gm)		

(NTBLCP PMDT Guideline, 2017)

***For adults over 59 years of age, the dose will be reduced to 10 mg/kg (maximum dose 750 mg)

2.9.2.2 Individualized regimen

Patients with DR-TB who are not eligible for treatment with the shorter regimen will be treated with the individualized regimen (NTBLCP PMDT Guideline, 2017). The total duration is 20 months or longer. Regimen design for individualized treatment as described in Table 2.4 and doses in Table 2.5.

i. The regimen will be designed based on the patient's most recent DST results and history of previous drug use and/or exposure.

ii. Standard duration of the intensive phase is for at least 6 months and duration of the continuation phase will be at least 14 months. The duration of the intensive phase (injectable agent) depends on the number of effective drugs in the regimen, patient response to treatment and tolerability. At least 5 effective drugs during the intensive phase (one chosen from Group A, one from Group B and at least two from Group C). If the minimum number of TB medicines cannot be composed as above, an agent from D2 and other agents from Group D3 should be added to bring the total to five. At least 3 effective drugs are strongly advised to be used after culture conversion (until the end of treatment).

iii. Bendaquilin (Bdq) or Delaminid (Dlm) is used for 6 months and 12 months as and when required. The use of Bdq or Dlm can be extended by DR-TB expert committee in cases where the remaining regimen is insufficient (less than 3 effective drugs) and treatment tolerability is good. The following are also noted in the management of the DR-TB patients:

a. Patients who are enrolled for treatment with regimens containing new drugs (Bdq or Dlm) must have informed consent should be obtained as per WHO guidelines.

b. In HIV-infected patients, ART will be prescribed within the first eight weeks of DR-TB treatment initiation. For patients on ART, Dlm (if available) is preferable because of the drug-drug interaction between Bdq and Efavirenz (and Lopinvir/Ritonavir).

c. Criteria to include new drugs Bdq or/and Dlm in the Individualised regimen are: DR-TB patient with additional resistance or intolerance to Fluoroquinolone and/or SLI (Pre-XDR, XDR), and those with extensive lesions and advanced disease and others with likelihood or poorer outcome. If indicated, Dlm or Bdq can be used also for children with proper safety measures. However, for children more than 6 years old (>20 Kg) and adolescents, Dlm should be the drug of choice.

d. The patient will be provided with materials in local languages that explain DR-TB treatment procedures.

Table 2.4: Individualized regimens for DR-TB in Nigeria

Types of DR-TB	Phases of treatment		Total duration
	Intensive	**Continuation**	
Pre-XDR TB (resistant to second line injectable)	6 Mfx-Bdq-Lzd-Cfz-PAS-Hh-Z	14 Bdq(6)-Lzd-Cfz-PAS-Z	20 months
Pre-XDR TB (resistant to fluoroquinolones)	6 Cm/Km-Bdq-Lzd-Cfz-PAS-Hh-Z	14 Bdq(6)-Lzd-Cfz-PAS-Z	20 months
XDR-TB	6 Cm/Dlm-Bdq-Lzd-Cfz-PAS-Hh-Z	14 Bdq(6)-Lzd-Cfz-PAS-Z	20 months

(NTBLCP PMDT Guideline, 2017)

Keys: Bdq=Bendaquilin, Dlm=Delaminid, Km=Kanamycin, Mfx=Moxifloxacin, Cfz=Clofaximine, Eto= Ethionamide, Z=Pyrazinamide, E-Ethambuthol, H=Isonaizid (H), PAS= Para Amino Salicyclic Acid,

Table 2.5: Dosage of medicines to use in the WHO recommended individualized DR-TB regimen for adults ≥30

Drugs	Daily dose	30-35kg	36-55kg	46-55kg	56-70kg	>70kg
Isoniazid	4-6 mg/kg once daily	150mg	200mg	300mg	300mg	300mg
Rifampicin	8-12 mg/kg once daily	300mg	450mg	450mg	600mg	600mg
Pyrazinamide	20-30 mg/kg once daily	800 mg	1000 mg	1200 mg	1600 mg	2000 mg
Ethambutol	15-25 mg/kg once daily	600 mg	800 mg	1000 mg	1200 mg	1200 mg
Rifabutin	5–10 mg/kg once daily	300 mg	300 mg	300 mg	300 mg	300 mg
Levofloxacin	750–1000 mg once daily	750 mg	750 mg	1000 mg	1000 mg	1000 mg
Moxifloxacin	400 mg once daily	400 mg	400 mg	400 mg	400 mg	400 mg
Ethionamide	500–750 mg/day in 2 divided doses	500 mg	500 mg	750 mg	750 mg	1000 mg
Prothionamide	500–750 mg/day in 2 divided doses	500 mg	500 mg	750 mg	750 mg	1000 mg
Cycloserine	500–750 mg/day in 2 divided doses	500 mg	500 mg	500 mg	750 mg	750 mg
p-aminosalicylic acid	8 g/day in 2 divided doses	8 g	8 g	8 g	8 g	8–12 g
Bedaquiline	400 mg once daily for 2 weeks then 200 mg 3 times per week					
Delamanid	100 mg twice daily (total daily dose = 200 mg)					

Clofazimine	200–300 mg daily (2 first months) then reduce to 100 mg daily (alternative dosing 100 mg daily)					
Linezolid	600 mg once daily	600 mg	600 mg	600 mg	600 mg	600 mg
Amoxicillin/Clavulanic acid 7/1	80 mg/kg/day in 2 divided doses	2600 mg	2600 mg	2600 mg	2600 mg	2600 mg
Amoxicillin/Clavulanic acid 8/1	80 mg/kg/day in 2 divided doses	3000 mg	3000 mg	3000 mg	3000 mg	3000 mg
High-dose isoniazid	16–20 mg/kg once daily	600–1000 mg	1000–1500 mg	1500 m	1500 m	1500 m
Imipenem / Cilastatin	1000 imipenem/1000 mg cilastatin twice daily					
Meropenem	1000 mg three times daily (alternative dosing is 2000 mg twice daily)					

(NTBLCP PMDT Guideline, 2017)

2.9.2.3 Adjuvant therapy

This is as described in the NTBLCP PMDT Guideline, 2017.

i. Vitamin B6 (pyridoxine) preventive therapy at a dose of 100mg/day up to 150 mg/day should be given to all patients receiving isoniazid, cycloserine or linezolid to minimize peripheral neuropathy, neurological adverse event and myelosuppression.

ii. Corticosteroids (Prednisolone 1 mg/kg and gradually decreasing by 10 mg per week when a long course is indicated) may be used in the following conditions: TB Meningitis (and other compromise of central nervous system (CNS), TB Pericarditis, Immune Reconstitution Inflammatory Syndrome (IRIS).

2.9.2.4 Surgery

This is as described in the NTBLCP PMDT Guideline, 2017.

Patients with DR-TB with the following conditions should be considered for surgical intervention:

i. Patients who remain smear positive, while on fully monitored treatment for more than six months and

ii. Have resistance to more than two of medicines and

iii. Have localized pulmonary disease.

The most common operative procedure in patients with pulmonary DR-TB is resection surgery. Generally, at least two months of therapy should be given prior to resection surgery to decrease the bacterial load in the surrounding lung tissue. Even with successful resection, an additional 12-24 months of chemotherapy should still be given.

2.10 Vaccination

To date, the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine remains the only licensed vaccine for the prevention of TB (Smith *et al.*, 2012). It is traditionally given to newborns, and protects newborns (Smith *et al.,* 2012). Nevertheless, this is not sustained, and generally, the vaccine provides little protection in adults (Smith *et al.*, 2012).

It was first administered in 1921 to an infant as oral vaccine (Murphy *et al.*, 2008). BCG is a live attenuated strain of *Mycobacterium bovis*, obtained by Albert Calmette and Camille Guérin through 230 *in vitro* passages over a 13 year-period (Liu *et al.*, 2009). Thereafter, daughter strains of BCG have been distributed around the world to produce vaccine (Liu *et al.*, 2009). Genetic and antigenic differences have emerged between vaccine strains (Behr, 2002). The BCG is widely used in TB endemic countries, newborns are immunized immediately after birth with a single intradermal dose (Hesseling *et al.,* 2008). Recent estimates shows that BCG has been administered over 4 billion times and 120 million children receive BCG every year globally (Dalmia and Ramsay, 2012).

Reports from meta-analyses of published studies has confirmed BCG prevents against meningeal TB and disseminated forms in children (Colditz *et al*., 1994). However, randomized clinical trials estimates protection against pulmonary TB that vary from nil to 80% (Colditz *et al*., 1994; Behr, 2002). Therefore, BCG has little impact in limiting TB spread as pulmonary TB is the most prevalent form of disease in adolescents and adults and the most impactful source of TB transmission (WHO, 2021). Several causes have been considered to elucidate the variable efficacy of BCG, they are: Differences in BCG strains, host genetic and nutritional factors, variable virulence among *Mycobacterium tuberculosis*, interference of environmental Mycobacteria,

switch to a type 2 immunological response in presence of helminthic infection and variation among trial methods (Fine, 1995; Liu *et al*., 2009; Ottenhoff and Kaufmann, 2012; Dalmia and Ramsay, 2012; WHO, 2017). Several studies revealed a booster dose of BCG did not improve protection against TB (Rodrigues *et al.*, 2005).

The BCG is a safe vaccine in healthy infants though there may be loco-regional adverse reaction, such as regional adenitis and are usually self-limiting (WHO Global TB Report, 2017). However, BCG-osis, a severe disseminated disease in patients affected by some primary immunodeficiency, such as severe combined immunodeficiency and chronic granulomatous disease (Norouzi *et al.*, 2012). There have been reports of increased risk of disseminated BCG disease in HIV-infected children, even if asymptomatic at time of vaccination and this is revealed by different studies (Fallo *et al.*, 2005; Hesseling *et al.*, 2007). This necessitated WHO Global Advisory Committee on Vaccine Safety to recommend that BGC should not be administered in HIV-infected patients which is a huge limitation as TB endemic countries have high HIV prevalence (Global Advisory Committee on Vaccine Safety, 2007). Thus, a safer and more effective vaccine is urgently required.

2.11 Mechanism of drug resistance in *Mycobacterium tuberculosis*

Resistance is a phenotype, the ability of a bacterial cell to survive the presence of a drug at a concentration that normally kills or inhibits growth. Resistance is caused by mutation and can be differentiated from tolerance, which is a conditional phenotype mediated by the physiological state of the bacilli (Brauner *et al.*, 2016). Drug susceptibility of *M. tuberculosis* is measured by testing Mycobacteria cells in the exponential phase of growth (Coban *et al.*, 2004). The MIC is thus defined as the minimum concentration of the drug which kills 99% of cells (Goins, 2017).

Drug resistance in *M. tuberculosis* can be either intrinsic (or natural) or acquired (Nieto *et al.,* 2014). Intrinsic resistance refers to resistance due to unique characteristics of *M. tuberculosis* such as its natural resistance to penicillin or clarithromycin (Nieto *et al.,* 2014). Acquired resistance refers to susceptible *M. tuberculosis* becoming resistant to drugs as a result of mutations (Nieto *et al.,* 2014). Bacteria can become resistant to antibiotics or antibacterial agents by a number of common strategies, including cellular target modification, target overexpression, barrier mechanisms, drug-inactivating enzymes, inactivation of drug-activating enzymes, and drug extrusion mechanisms (Table 2.6). Since drugs must enter bacterial cells to be active, bacteria can become resistant by preventing entry of the drug (Stewart *et al.,* 2004).

Table 2.6: Mechanisms of drug action and resistance in Mycobacteria

Drug *a*	MIC (ug/ml)	Gene(s) involved in resistance	Gene function	Role	Mechanism of action	Mutation frequency (%)	Allele type
Isoniazid	0.02-0.2	*kat*G	Catalase-peroxidase	Prodrug conversion	Inhibition of mycolic acid biosynthesis and other multiple effects on	20-80	Recessive
		*inh*A	Enoyl ACP reductase	Drug target	DNA,	15-43	Dominant
		ndh	NADH dehydrogenase II	Modulator of INH activity	lipids, carbohydrates and NAD	10	Recessive
		*ahp*C	Alkyl hydroperoxidase	Marker of resistance	metabolism	10-15	
Rifampin	0.5-2	*rpo*B	RNA polymerase	Drug target	Inhibition of transcription	96	Dominant
Pyrazinamide	16-50 (pH5.5)	*pnc*A	Nicotinamidase/pyrazinamidase	Prodrug conversion	Acidification of cytoplasm and de-energized membrane	72-97	Recessive
5-Chloro-pyrazinamide	8-32	*fasI*	FASI	Drug target	Inhibition of FASI	Na *b*	Dominant
Ethambutol	1-5	*emb*CAB		Drug target	Inhibition of arabinogalactan synthesis	47-65	Dominant
Stretomycin	2-8	*rps*L	S12 ribosomal protein	Drug target	Inhibition of protein synthesis	52-59	Recessive
		rrs	16S rRNA	Drug target		8-21	Dominant
Amikacin/kanamycin	2-4	*rrs*	16S rRNA	Drug target	Inhibition of protein synthesis	76	Dominant
Quinolones	0.5-2.5	*gyr*A	DNA gyrase subunit A	Drug target	Inhibition of DNA gyrase	75-94	Dominant
		gyrB	DNA gyrase subunit B	Participates in drug binding?		In vitro	
Ethionamide	2.5-10	*eta*A/*eth*A	Flavin monooxygenase	Prodrug conversion	Inhibition of mycolic acid biosynthesis	37	Recessive
		inhA		Drug target		56	

a: Rifampin, aminoglycosides and fluoroquinolones (underlined) are broad-spectrum antibiotics, whose mechanism of resistance in M. *tuberculosis* is the same as in other bacteria; b: NA, not available (Stewart *et al.*, 2004).

2.11.1 Isoniazid

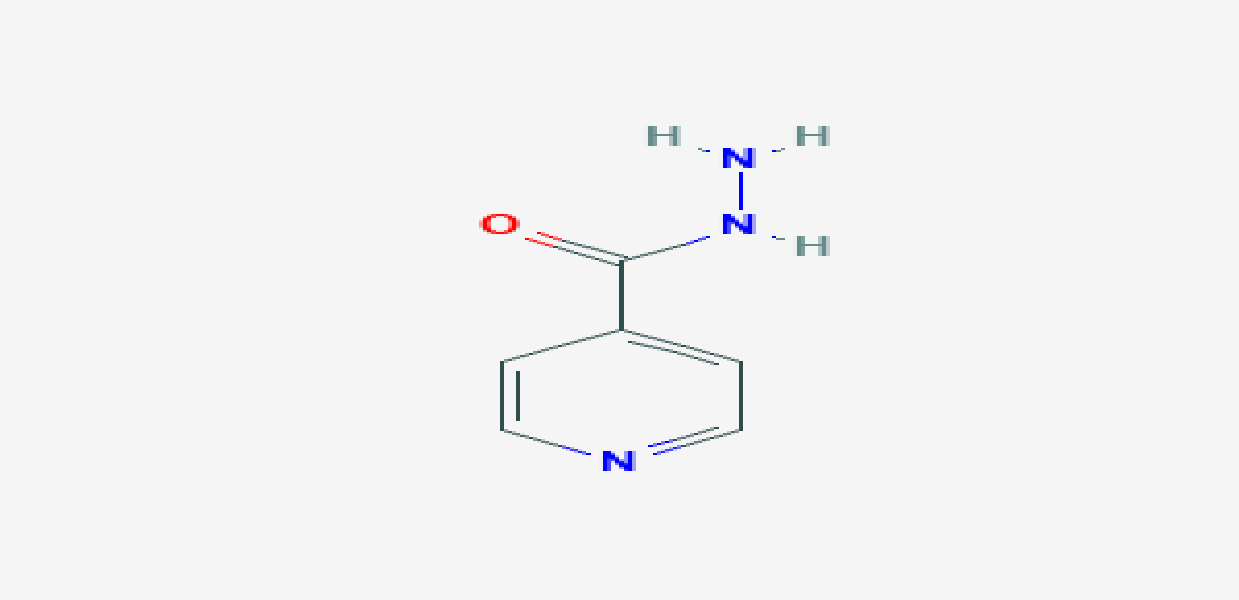

Figure 2.5: Structure of Isonaizid (National Center for Biotechnology Information, 2021a).

Isonaizid (INH) is active against growing tubercle bacilli but not resting bacilli (Schaefer, 1954; Mitchison and Selkon, 1956). INH requires activation by the *katG*-encoded catalase-peroxidase (*katG*) because it is a prodrug (Zhang *et al.*, 1992). Isonicotinic acyl radical is the active species and it attacks the nicotinamide group of NAD^+ to form a covalent INH-NAD adduct (Rozwarski *et al.*, 1998), which inhibits *InhA*, resulting in a blockage of FASII activity which leads to accumulation of FASI end products. This will make the Mycobacteria unable to obtain mycolic acids required for the synthesis of the cell wall, the Mycobacterial cells lyse (Stewart *et al.*, 2004). Mutations conferring resistance to INH are located in several genes and loci (Banerjee *et al.*, 1994). INH resistance has been associated mainly with mutations in *kat*G, *inhA, ahpC, and kasA* (Banerjee *et al.*, 1994).

Isonaizid (INH) resistance includes the following mechanism; Loss of *katG* function due to mutations in *katG* which reduces the ability of *KatG* to activate the prodrug INH, thus leading to INH resistance, and this represents a novel mechanism of drug resistance. Overexpression or alterations in the INH target, *InhA* (Banerjee *et al.,* 1994). In an overexpressed *inhA* from a multicopy plasmid, the MIC of INH increased by 20- to 80-fold above the MICs of three independent *M. tuberculosis* strains (Larsen *et al.,* 2002). Mutations in *M. tuberculosis of* INH-resistant clinical isolates, have been mapped to the promoter region and the *inhA* structural gene (Larsen *et al.,* 2002). These mutations usually occur in 15-43% of *M. tuberculosis* strains that are INH-resistant and are usually associated with a low level of INH resistance (MIC, ≤1μg/ml) (Banerjee *et al.,* 1994; Heym *et al.,* 1994; Morris *et al.,* 1995; Rouse *et al.,* 1995; Musser *et al.,* 1996; Basso *et al.,* 1998; Kiepiela *et al.,*2000; Piatek *et al.,* 2000; Kim *et al.,* 2003; Ramaswamy *et al.,* 2003). Loss of NADH dehydrogenase II activity: a modulator of INH-NAD and ETH-NAD formation or binding. The NADH dehydrogenase type II gene, *ndh*, speeds up the rate of INH resistance by complementation of an INH-resistant, temperature-sensitive *M. smegmatics* mutant with a genomic library of *M. tuberculosis* (Miesel *et al.,* 1998). Alterations of overexpression of *KasA*. Slayden and Barry have reported that overexpression of *kasA* conferred low-level resistance to INH in *M. tuberculosis* (Sladen & Barry, 2002).

2.11.2 Rifampin

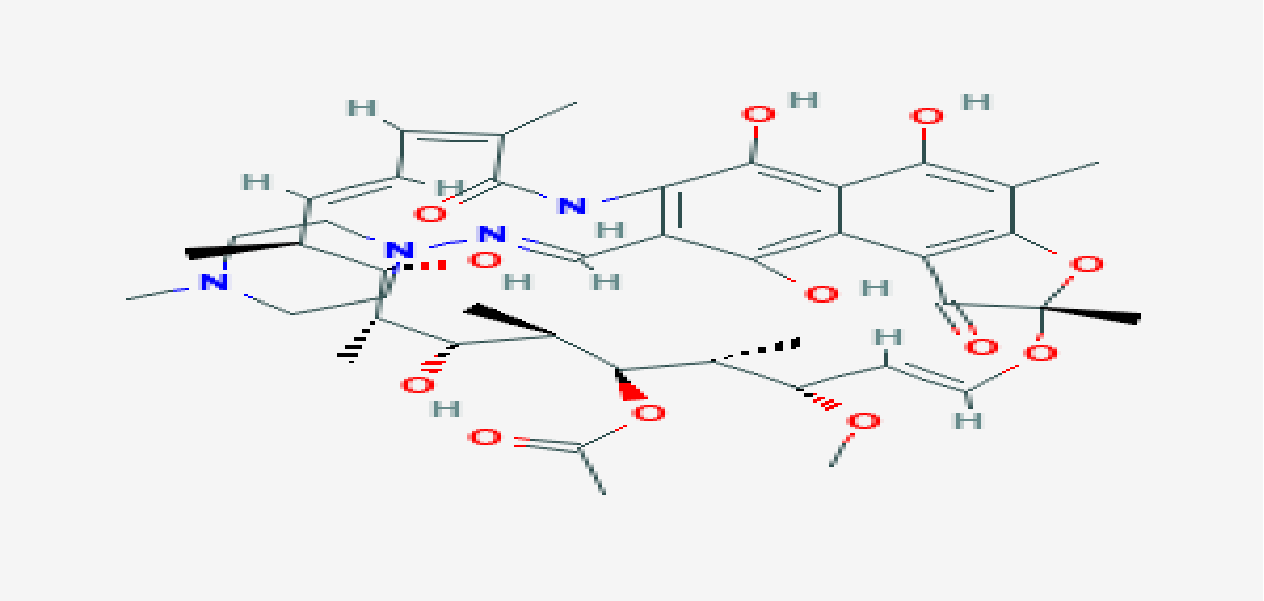

Figure 2.6: Structure of Rifampicin (National Center for Biotechnology Information 2021b).

Rifampicin (RIF) is a broad-spectrum rifamycin derivative that inhibits RNA synthesis by binding to bacterial RNA polymerase, an oligomer consisting of a core enzyme, formed by four chains ($\alpha_2\beta\beta'$) that associates with the σ subunit to specifically initiate transcription from promoters (Shan,2010). The position of the RIF binding site is shortly upstream of the catalytic center (Mustaev *et al.*, 1994), keeping with the model that RIF plugs the product exit channel (McClure and Cech, 1978). Analysis of RIF-resistant mutants has elucidated the definition of a priming nucleotide site overlapping with a larger site holding the RNA product in the active center during elongation (McClure and Cech, 1978). RIF has demonstrated activeness against growing tubercle bacilli and also stationary-phase bacilli with reduced metabolism (McClure and Cech, 1978). The activity of RIF against the stationary-phase bacilli is thought to be important in shortening the duration of treatment (Mitchison, 1985; Stewart et *al.*, 2004).

Resistance to RIF occurs at a frequency of 1 in 10^7 to 10^8 bacilli in *M. tuberculosis*. Mutations in a defined region of *rpoB* have been found >95% of RIF-resistant clinical isolates of *M. tuberculosis* like in other bacteria (Telenti *et al.,* 1993). *Mycobacterium. leprae* has the same mechanism of resistance to RIF (Honore and Cole, 1993). Although *rpoB* mutations have been found in RIF-resistant *M. avium* (Williams *et al.,* 1994), many isolates from the *M. avium-intracellulare* complex present a significant level of natural resistance to RIF, probably due to decreased permeability (Guerrero *et al.,* 1994; Hui *et al.,* 1977). The involvement of *rpoB* mutations in causing resistance has been confirmed by genetic transformation experiments (Miller and Shinnick, 1994; Williams *et al.,* 1998). Mutation in *rpoB* generally results in high-level resistance (MIC >32µg/ml) and cross-resistance to all rifamycins. However, specific mutations in codons 511, 516, 518 and 522 are associated with lower-level resistance to RIF and rifapentine but retained susceptibility to rifabutin and new rifamycin KRM1648 (Bodmer *et al.,* 1995; Moghazeh *et al.,* 1996; Williams *et al.,* 1998). Low-level resistance (MIC, 4µg/ml) has been associated with an L176F mutation in a separate region of *rpoB* corresponding to *E.coli* L146F, a codon mutated in 2% of RIF-resistant *E.coli* strains (Severinov *et al.,* 1994). Ribosylation, which is associated with a degradative mechanism of resistance to RIF, has been found in the rapidly growing Mycobacteria such as *M. smegmatis, M. chelonae, M. flavescens,* and *M. vaccae* (Dabbs *et al.,* 1995; Quan *et al.,* 1997), but not in *M. tuberculosis* (Stewart *et al.,* 2004).

2.11.3 Pyrazinamide

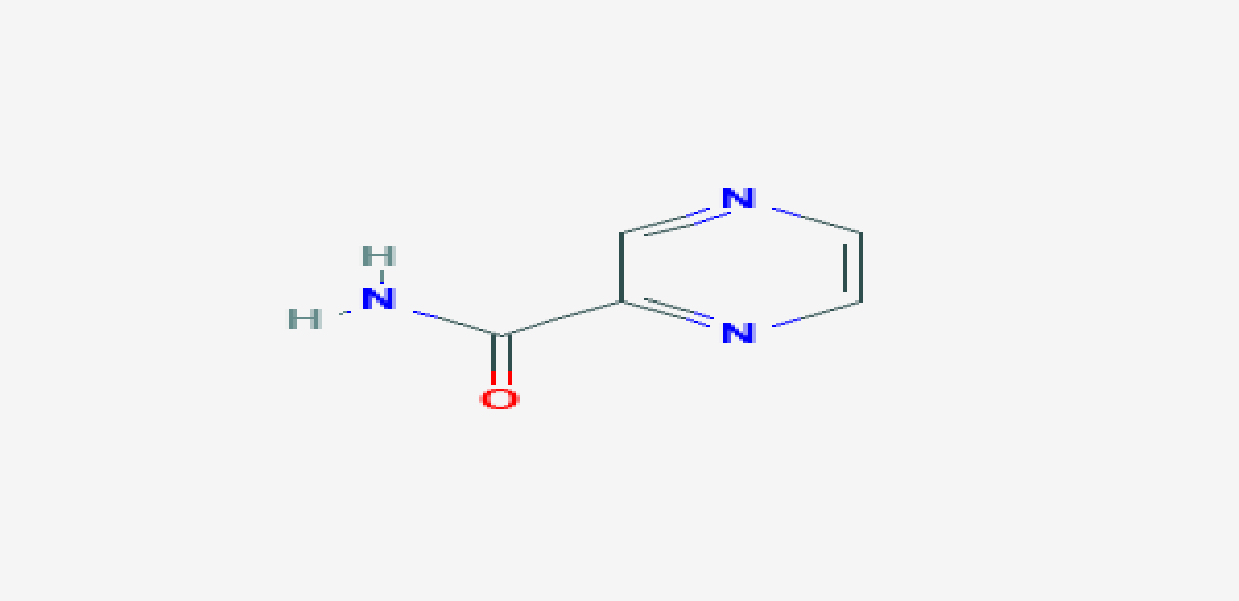

Figure 2.7: Structure of Pyrazinamide (National Center for Biotechnology Information 2021c).

Pyrazinamide (PZA) is an unconventional and paradoxical TB drug that has high in vivo activity but poor in vitro activity (Zhang and Mitchison, 2003). Pyrazinamide is a prodrug that is converted to the active form, pyrazinoic acid (POA), by bacterial pyrazinamidase (Pzase)/nicotinamidase (Konno *et al.*, 1967; Scorpio and Zhang, 1996). Studies have proposed the following model for the mode of action of PZA. The PZA drug enters the bacilli through passive diffusion and is converted into POA (a strong weak acid with pK_a of 2.9) by the cytoplasmic PZase (Scorpio and Zhang, 1996; Zhang *et al.*, 1999; Zhang *et al.*, 2003). The uptake and conversion of PZA to POA in *M. tuberculosis* are much slower than in the normally resistant non tuberculosis Mycobacteria or *E. coli*, presumably due to higher PZase activity in other bacteria (Zhang *et al.*, 1999). The excretion of POA from the cell is by passive diffusion and a weak efflux mechanism in *M. tuberculosis* (Zhang *et al.*, 1999). Acidic pH does not facilitate the uptake of There is accumulation of POA in *M. tuberculosis* cells because of the acidic PH (Zhang *et al.*, 1999). The HPOA brings

protons into the cell, and this could eventually cause cytoplasmic acidification, such that vital enzymes are inhibited, as a potential mechanism of POA action (Zhang *et al.*, 1999). In addition, HPOA could potentially deenergize the membrane by collapsing the proton motive force and affect membrane transport as a possible mechanism of POA action (Zhang and Telenti, 2000).

Microbacterium. tuberculosis strains are exclusively susceptible to PZA at MIC of about 50µm/ml at pH 5.5, whereas nontuberculous Mycobacteria and other bacteria are innately resistant to PZA; e.g the MIC for *M. smegmatics* and *E. coli* is >2,000µg/ml (Boshoff and Mizrahi, 1998; Zhang and Telenti, 2000). In *M. tuberculosis*, PZA susceptibility relates with the presence of PZase activity (Zhang and Telenti, 2000). Mutations in *pncA* as a result of defective PZase activity is the major mechanism for PZA resistance in *M. tuberculosis* (180), as confirmed by numerous other studies (Hirano *et al.*, 1997; Sreevatsan *et al.*, 1997; Sachais *et al.*, 1998; Hoashi *et al.*, 1999; Hou *et al.*, 2000; Hui *et al.*, 1977; Lemaitre *et al.*, 1999; Martilla *et al.*, 1999, Mestdagh, *et al.*, 2000; Morlock *et al.*, 2000; Park *et al.*, 2001). The *pncA* mutations recognized are from amino acid substitutions due to missense mutations and in some cases nucleotide insertions or deletions, non-sense mutations in the *pnc*A structural gene, or non-sense mutations in the putative promoter region with frequently occurring mutation at -11 (putative promoter region) was found in several studies (Scorpio *et al.*, 1997; Sreevatsan *et al.*, 1997; Cheng *et al.*, 2000; Mestdagh *et al.*, 2000; Lee *et al.*, 2001; Park *et al.*, 2001). The *pncA* mutations are highly diverse and are scattered along the gene with some degree of clustering that affects three regions (regions 3 to 17, 61 to 85 and 132 to 142) of the PncA protein (Lemaitre *et al.*, 1999; Scorpio *et al.*, 1997) that are likely to contain catalytic sites. The crystal structure of the *Pyrococcus horikoshii* PncA (37% identity to *M. tuberculosis PncA*) has provided some structural basis for understanding the *pncA* mutations in *M. tuberculosis* that cause PZA resistance (Du *et al.*, 2001), since the three regions where they

cluster correspond to three of the four loops that contribute to the scanfold of the active site. Although most PZA-resistant *M. tuberculosis* strains have mutations in *pncA* (Scorpio *et al.*, 1997; Cheng *et al.*, 2000), there are some resistant strains that do not, and these include PZase-negative strains (Lemaitre *et al.*, 1999; Martilla *et al.*, 1999; Cheng *et al.*, 2000) with a high level of resistance. This indicates that mutations in an undefined *pncA* regulatory gene may be involved in PZA resistance. Another type of such strain has low-level resistance and positive PZase activity, presumably due to an alternative mechanism of resistance.

2.11.4 Ethambutol

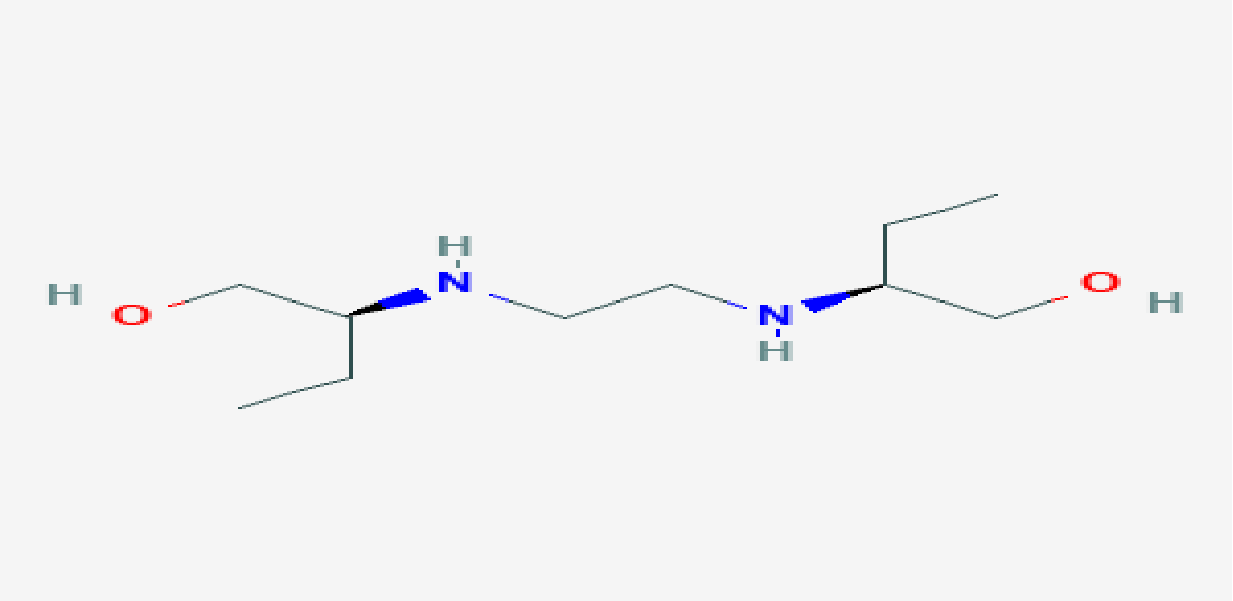

Figure 2.8: Structure of Ethambutol (National Center for Biotechnology Information 2021d).

Ethambutol (E) ((S,S′)-2,2′-(ethylenediimino)-di-1-butanol) inhibits the biosynthesis of arabinogalactan, the major polysaccharide of the Mycobacterial cell wall (Takayama and Kilburn, 1989). Ethambutol interferes with the polymerization of cell wall arabinan of arabinogalactan and of lipoarabinomannan (Mikusova *et al.*, 1995) and induces the accumulation of β-D- arabinan biosynthesis (Wolucka *et al.*, 1994; Lee *et al.*, 1995). The target of ethambutol is Arabinosyl-

transferase, an enzyme involved in the synthesis of arabinogalactan, (Belanger *et al.,* 1996). This enzyme is encoded by *embB,* which is part of an operon comprising the *embA* and *embB* genes in *M. avium* (Belanger *et al*., 1996) and the *embC, embA* and *embB* genes in other Mycobacteria such as *M. smegmatis* (Alcaide *et al.,* 1997; Telenti *et al.,* 1997).

Ethambutol proteins are about 65% identical to each other and are predicted to be integral membrane proteins with 12 transmembrane-spanning domains (Telenti *et al.,* 1997). Though, the precise mechanism of how EMB inhibits *embB* is not known. Mutations in the *embCAB* operon were identified in up to 65% of ethambutol-resistant clinical isolates of *M. tuberculosis* (Telenti *et al.,* 1997). Mutations at codon 306 of *embB* occur most frequently (Streevatsan *et al.,* 1997; Telenti *et al.,* 1997; Ramaswamy *et al.,* 2000), but mutations at amino acid residues Asp328, Gly406, and Glu497 are also found (Ramaswamy *et al.,* 2000; Streevatsan *et al.,* 1997). The codon 306 region is highly conserved among the various Emb proteins and among different Mycobacteria (Alcaide *et al.,* 1997; Lety *et al.,* 1997; Telenti *et al.,* 1997), although the E-resistant *M. leprae, M. chelonae,* and *M. abscessus* display variant amino acids at this position. Genetic transfer experiments involving these *emb* alleles supported the role of this region in determining natural resistance to E (Alcaide *et al.,* 1997). Studies have shown that the significance of *embB* mutations in the *emb-embA* intergenic region have inter related in strains that also had resistance-associated amino acid substitutions in *EmbA* or *EmbB* (Ramaswamy *et al.,* 2000), and these may play a secondary or compensatory role in resistance.

Ramaswamy *et al.* (2000) reported that *embR* homologue is located 2 Mb apart from the *embCAB* locus in *M. tuberculosis* and mutations have been associated with E resistance as a result of a Q379R replacement and an A insertion at position 137 upstream of the *embR* start codon. A mutation 24 bp upstream of the start codon of the *Rv*0340 gene, which precedes the INH and EMB-

inducible *iniBAC* operon (Alland *et al.*, 1998), was associated with EMB resistance (Ramaswamy *et al.*, 2000). Mutations in *rmlD* (S257P and T284L, or a G- to -T nucleotide change at position 71) and *rmlA2* (D152N), both of which are involved in the modification of rhamnose, were found to be associated with E resistance (Ramaswamy *et al.*, 2000). Despite these occurrences, more newgenes being identified as involved in EMB resistance, about 24% of E-resistant *M. tuberculosis* strains do not have mutations in any of the genes described above (Ramaswamy *et al.*, 2000). There is need for further genetic and biochemical studies to confirm the role of the above genes in E resistance.

2.11.5 Streptomycin

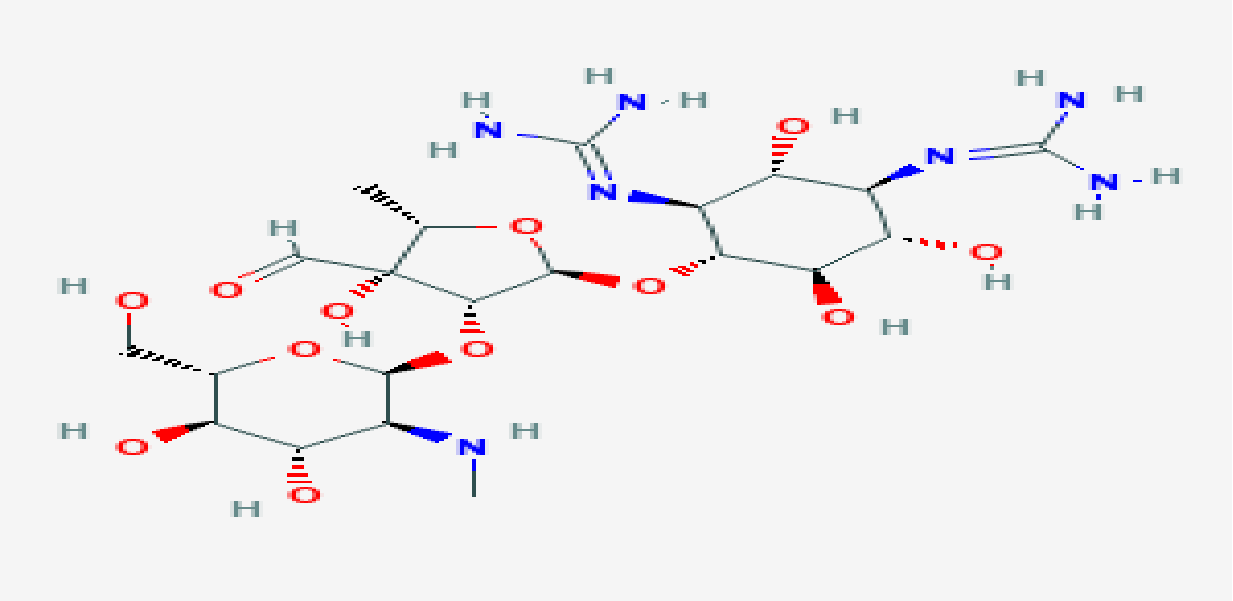

Figure 2.9: Structure of Streptomycin (National Center for Biotechnology Information 2021e).

Streptomycin (SM) is an aminoglycoside antibiotic that primarily interferes with protein synthesis but has some other effects such as inhibition of respiration, damage to the cell membrane and stimulation of RNA synthesis (Gale *et al.*, 1981). In addition, SM can cause misreading or miscoding of the genetic code (Davies *et al.*, 1964). The 30S subunit of the ribosome is the site of

action of SM, specifically at ribosomal protein S12 and the 16S rRNA (Garvin *et al.,* 1974). The mode of action of SM in *M. tuberculosis* is recognized to be the same as in *E. coli,* by the presence of mutations in the same target, i.e., ribosomal S12 protein (encoded by *rpsL)* and 16S rRNA (encoded by *rrs*) by interfering with protein translation process (Garvin *et al.,* 1974).

Mutation of the SM target in the ribosome makes the *M. tuberculosis* to become resistant. The principal site of mutation to SM is the *rpsL* gene, encoding ribosomal protein S12 (Finken *et al.,* 1993; Honore and Cole, 1994, Kenney and Churchward, 1994; Nair *et al.,* 1993; Sreevatsan *et al.,* 1996). Residues 42 and 88 are the most important in the development of SM resistance just like we have in *E.coli*. Another mechanism of resistance in *M. tuberculosis* is in *rrs,* the gene encoding 16S rRNA (Honore and Cole, 1994). While most bacteria have multiple copies of *rrs*, *M. tuberculosis* and other slow-growing Mycobacteria have a single copy while rapidly growing Mycobacteria have two copies of *rrs*s (Honore and Cole, 1994). An SM-dependent mutant of *M. tuberculosis* contained an insertion of cytosine in the 530 loop as a likely cause of its SM dependence (Honore *et al.,* 1995). A previously described nucleotide change at position 491 of the *rrs* gene, in two clinical isolates resistant to SM (Meier *et al.,* 1994; Sreevatsan *et al.,* 1996), is a polymorphism that is not associated with SM resistance. However, mutation in the loops of 16S rRNA, the highly conserved 530 loop and on the adjacent 915 region, that interact with the S12 protein constitute an easily selected resistance site (Finken *et al.,* 1993). Mutations in *rpsL* and *rrs* structure are identified in 50% and 20% of SM-resistant clinical isolates, respectively, and result in high-level (MICs >1,000µg/ml) or intermediate (MICs, 64 to 512µg/ml) resistance (Cooksey *et al.,* 1996). In addition, another mechanism accounting for low-level resistance (MICs, 4 to 32µg/ml) remains unidentified, but it may involve changes in drug uptake (Cooksey *et al.,* 1996).

2.11.6 Other drugs

Other studies on bacteria have reportedthe presence of quinolone resistance mutations in (i) the DNA gyrase (composed of subunit g*yrA* and g*yrB*) (ii) topoisomerase IV, and (iii) the cell membrane proteins that regulate the intracellular concentration of the drug by meditating drug permeability and efflux (Yoshida *et al.,* 1990; Yoshida *et al.,* 1991; Ferrero *et al.,* 1995; Gensberg *et al.,* 1995; Avalos *et al.,* 2015). High level resistance can arise from the stepwise accumulation of mutations in several of these genes. In *M. tuberculosis*, *gyrA* mutations cause cross-resistance to other fluoroquinolones (e.g ofloxacin) (Cambau *et al.,* 1994) and resistance to ciprofloxacin (Takiff *et al.,* 1994). Fluoroquinolones resistance has been associated mainly with mutations in the *gyrA* and *gyrB* genes respectively (Cambau *et al.,* 1994). It accounts for 80-90% resistance in fluoroquinolones and the level of susceptibility to quinolones arises from amino acid sequences in *GyrA* (Guillemin *et al.,* 1998).

The main targets of the quinolones are the desoxyribonucleic acid (DNA) gyrase, a type-II DNA topoisomerase composed of two A and two B subunits encoded by genes *gyrA* and *gyrB*, respectively **(**Cambau *et al.,* 1994**)**. The DNA gyrase gene inhibit bacterial replication by blocking their DNA replication pathway. This unwinding of DNA in the bacteria is done by enzymes in the bacteria called DNA gyrase (Guillemin *et al.,* 1998). DNA gyrase is an enzyme that unwinds the DNA by introducing negative supercoils and can also help relax positive supercoils (Guillemin *et al.,* 1998). Fluoroquinolones inhibit this enzyme by binding to it's A-subunit, making the bacteria unable to replicate or even synthesize proteins (Cambau *et al.,* 1994). They relieve the coils that form in DNA when the helix is being opened in preparation for replication/ transcription/repair. Aminoglycosides resistance has been associated with mutations on the rrs gene (Honore *et al*., 1995). Aminoglycosides are associated with mutations in the *rrs* gene encoding 16S rRNA

(Honore *et al*., 1995). Capreomycin, like streptomycin and kanamycin, inhibits protein synthesis through modification of ribosomal structures at the 16S rRNA which affects polypeptide synthesis, ultimately resulting in inhibition of translation (Honore *et al*., 1995). Resistance to streptomycin and the other aminoglycosides in *M. tuberculosis* usually develops by mutation of the ribosome target binding sites **(**Honore *et al*., 1995**)**.

The aminoglycosides; kanamycin and capreomycin, inhibit protein synthesis through the modification of 16S rRNA ribosomal structures and this is also the same mechanism of resistance to streptomycin **(**Honore *et al*., 1995**)**. High-level resistance to kanamycin and amikacin (Taniguchi *et al.,* 1997; Alangaden *et al.,* 1998; Suzuki *et al.,* 1998) are observed at mutations at *rrs* position 1400 are associated. Cross-resistance between kanamycin and capreomycin may also be observed (Taniguchi *et al.,* 1997; Alangaden *et al.,* 1998; Suzuki *et al.,* 1998). The mechanism of resistance to Para Amino Salicyclic Acid is unknown. Two possible mechanisms have been proposed for the mechanism of PAS and they are interference with folic acid biosynthesis and inhibition of iron uptake. Ethionamide (Eto) is also a prodrug and inhibits mycolic acid biosynthesis (Tomlinson, 2011). And mutations in *inhA* have been associated with cross-resistance to INH and Eto (Barnejee *et al.,* 1994; Morlock *et al.,* 2003). Cycloserine obstructs the synthesis of peptidoglycan by blocking the action of D-alanine racemase and D-alanine: alanine synthase (Chetty *et al*., 2017). The D-alanine racemase enzyme, encoded by *alrA*, has been cloned from *M. smegmatis* or *M. bovis* BCG results in resistance to D-cycloserine (Caceres *et al.,* 1997). Inactivation of *alrA* in *M. smegmatis* caused increased sensitivity to D-cycloserine (Chacon *et al.,* 2002).

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Study Area

The study was conducted in Abuja and Kaduna State. Abuja the capital of Nigeria, located in the North Central Geo-Politocal Zone of the country. It is a planned city that was developed primarily during the 1980s. However, Abuja is not just the fastest-growing city in Africa but is also one of the fastest-growing in the world. The last census taken in 2006 put the population at 776,298. However, between 2000 and 2010, the population grew by almost 140%, with more recent 2021 estimates showing that the population is 3,464,123 with an annual growth of 35% making it one of the fastest-growing cities in the world (World Population Review, 2021a). The latitude of Abuja, Nigeria is 9.072264, and the longitude is 7.491302. The gps coordinates are 9° 4' 20.1504" N and 7° 29' 28.6872" E (Latlong.net, n.d). Abuja has six Local Government Areas (LGAs), namely: Abaji, Abuja Municipal (AMAC), Gwagwalada, Kuje, Bwari and Kwali. The sites where the samples were collected were located in the AMAC and the Gwagwalada LGAs of the city (Mfonobong, 2020).

Kaduna State is located in the North West Geo-Political Zone of Nigeria and its 2021 population is now estimated at 1,133,430 (World Population Review, 2021b). Kaduna has grown by 20,220 since 2015, which represents a 1.82% annual change. The latitude of Kaduna, Nigeria is 10.609319, and the longitude is 7.429504. The gps coordinates are 10° 36' 33.5484" N and 7° 25' 46.2144" E (Latlong.net, n.d). Kaduna has 23 local Government areas and three Senatorial districts in North, Central and South. The NTBLTC Zaria Hospital is a referral hospital for TB

serving all the Northern States (North Central, North East and North West) and is located with the Northern Senatorial district of the State (Media Nigeria,2018).

Abuja and Kaduna were selected due to the cosmopolitan nature and the centres were selected due to the workload. This study was carried out at five Directly Observed Treatment Short course (DOTs) centers in Kaduna and Abuja. These were University of Abuja Teaching Hospital (UATH) Gwagwalada, Gwagwalada Township Clinic (GTC), National Institute for Pharmaceutical Research Development (NIPRD) Idu and Garki Hospital (GH) within Abuja and the National Tuberculosis and Leprosy Training Centre (NTBLTC), Saye, Zaria in Kaduna.

3.2 Study Design

This was a cross sectional hospital-based study and all consenting TB patients were enrolled in the study. TB positive sputum samples that were previously tested by AFB microscopy were used for the analysis. The study was carried out between March - Septmeber 2016.

3.3 Study Population

The study population consisted of One hundred TB patients enrolled in the five DOTs Clinics of the selected hospitals. The TB patients enrolled per sites were as follows: UATH (9 patients), GTC (11 patients), GHA (15 patients), NIPRD (14 patients) and NTBLTC (51 patients). They consisted of males and females that were either employed or unemployed.

3.4 Ethical Approval and Consent

Ethical approvals (Appendices I and II) were obtained from the Federal Ministry of Health and FCT Health Research Ethics Committee (NREC). Signed consents (Appendix III) were collected from all patients where the samplings were done.

3.5 Inclusion and Exclusion Criteria

The inclusion criteria were confirmed smear positive patients from the laboratory and those that were willing to give consent. Exclusion criteria were patients that were smear negative and unwilling to consent.

3.6 Sample Size Determination

The sample size used for the study was determined using a formula recommended by WHO (1990) shown below and a previous prevalence of 4.8% MDR TB determined in Nigeria. (Nigeria Drug Resistant TB Prevalence Survey Report of 2012)

$$n = \frac{z^2 p.q}{d^2}$$

Where n= sample size

z= standard normal distribution at 95% confidence interval (1.96)

d= allowable error taken as 0.05

p= Known prevalence rate of the infection

q= 1-p

p= Known prevalence rate of MDR TB infection (4.8%)

$$n = \frac{(1.96^2) \times 0.048 \times 0.98}{(0.05)^2} = 70$$

An acceptable error margin of 5% and 95% confidence interval was used. The calculated sample size was seventy (70). However, to adjust for expected loses through contaminated cultures, non-growth and other factors, the calculated sample size was increased by 20% (WHO, 2003) giving a total sample size of eighty-four (84) but because of the different locations, one hundred (100) samples were collected.

3.7 Questionnaire Administration

One hundred structured questionnaires (Appendix IV) were used to collect data on socio demographics, risk factors and clinical history from the patients.

3.8 Collection of Sputum Samples

A total of 100 positive sputum samples that were tested within 24 hours by AFB microscopy were used for the analysis. The sputum samples were collected in standard screw-capped leak-proof sputum containers labelled with specific clinic identification number and study number. The samples were kept in a refrigerator at 2-8 ^{o}C and then moved within 72 hours of collection to TB Reference laboratory for testing.

3.9 Sputum transportation

The fresh AFB positive sputum samples were transported in cold boxes within 3 days at 2-8^{0} Celsius to the NTBLTC, National TB Reference Laboratory (NRL) for investigations by a designated carrier. The cold boxes were studded with ice packs and temperature monitored with a thermometer.

3.10 Analysis of Samples

The samples that were smear positive after microscopy were subjected to decontamination, culture and Hain Line Probe Assay. The flow chart for the methodology is shown in Appendix V.

3.10.1 Determination of Acid-Fast Bacilli (AFB) using the Ziehl Neelsen (ZN) staining Procedure

Smears were made from processed sputum samples (to retest) and culture isolates. The smears were flooded with 1% carbol fuchsin and heated intermittently with spirit lamp until the steam rose. The stain was allowed to act on the smear for 5 minutes and then rinsed with water followed by decolourization of smears with 3% acid alcohol for 3 minutes. The slides were rinsed with water thoroughly and counterstained with 1% methylene blue for I minute. The slides were rinsed with water and allowed to drain dry before examination under the microscope using x100 oil immersion (Salaman and Sabine, 2006). AFB appeared as pink rod shapped bacilli against a blue background. The Acid fast (AFB) load was graded according to the International Union against Tuberculosis and Lung Disease (2000) grading system (Appendix VI).

3.10.2 Processing of sputum samples and isolation of Mycobacteria

All clinical specimens were processed in a Biological Safety Cabinet (BSC) using the NALC-NaOH method as previously described in (Steingart *et al*., 2006). Equal volume (5ml) of NALC-NaOH and sputum was mixed. This was vortexed and incubated for 15 minutes, 35ml Phosphate buffer was added to the NALC-NaOH-Sputum mixture and the tubes centrifuged at 3000xg for 15 minutes in a refrigerated centrifuge (ROTINA 420R) at 4^{o}C. The supernatant was carefully discarded and the sediment was resuspended in 2ml buffer. The sediment was used to make smears and also inoculated Lowenstein Jensen's (LJ).

3.10.3 Identification of Mycobacteria using the rapid immunochromatographic method

Mycobacteria were identified using the simple, rapid immunochromatographic method (SD-Bioline Ag MPT64 Rapid assay) that detects MPB 64 predominant protein Ag, secreted by MTB complex strains (Standard Diagnostics, Kyonggi-do, Korea). The isolates were harvested and suspended in a 2ml cryovial containing 1ml of molecular grade water. Thereafter 100 ul of the isolate suspension was added to the sample well of the SD Bioline kit and reaction was left for 10 minutes at room temperature before viewing. A positive result is indicated by a clear distinguishable reddish-purple band appearing in the control and test windows. A Negative result is indicated by only one reddish-purple band appearing in the control window while the presence of only one reddish band in the test window show an invalid result (Patrick Orikiriza *et al.,* 2017) as shown in Appendix VII.

3.10.4 Drug Resistance determination of the *Mycobacterium tuberculosis* complex species and Characterization of Mycobacteria using the Genotypic Hain Line Probe Assay

Drug resistance determination of MTBC and the characterization of the Mycobacteria species were done using The Hain Line Probe Assay. The procedure is divided into three namely; DNA extraction, Amplification and Reverse Hybridization as described by the manufacturer (Hain Lifescience GmbH, Nehren, Germany, 2009).

3.10.4.1 DNA Extraction of the Mycobacteria isolates

The Mycobacteria isolates were harvested into into a 2ml cryovial and 300µl of molecular grade water was added. The Mycobacteria suspension was mixed by vortexing gently for 30 seconds. The tubes containing the Mycobacteria suspension were incubated in a water bath at 95^{o}C for 20 minutes to heat kill (HK) all viable Mycobacteria. The tubes containing the Mycobacteria suspension were removed and incubated for 15 minutes in an ultrasonic water bath to break cell

walls to release DNA material. This was followed by centrifugation (Eppendorf A12233) at 10,000rpm for 5 minutes in a microcentrifuge (to remove cell debris) and the supernatant containing the extracted DNA transferred to clean labelled microcentrifuge tubes using sterile disposable transfer pipettes (Hain Lifescience GmbH, Nehren, Germany, 2009).

3.10.4.2 Amplification of the Mycobacteria DNA

The DNA harvested from the procedure above was amplified by multiplex PCR amplification with biotinylated primers. The amplification reagents were prepared by the addition of 35 µl primers nucleotide mix, 5 µl PCR buffer, 2 µl Magnesium Chloride, 2.8 µl molecular grade water, 0.2 µl Hot start *Taq.* polymerase enzyme and 5µl DNA all mixed in a PCR tube. The amplification reagents and DNA mix were amplified in a thermocycler according to the heating cycles detailed as follows: 15 minutes at 95^{o}C for 1 cycle, 30 seconds at 95^{o}C and 2 minutes at 65^{o}C for 10 cycles, 25 seconds at 95^{o}C, 40 seconds at 50^{o}C, 40 seconds at 70^{o}C for (30 cycles- clinical, 20 cycles-culture); 8 minutes at 70^{o}C for 1 cycle (Hain Lifescience GmbH, Nehren, Germany, 2009).

3.10.4.3 Reverse Hybridization of the Mycobacteria DNA

The amplified products (amplicon) were detected using the reverse hybridization. The amplification products from the procedure above were denatured by the addition of 20µl denaturation solution to 20µl of the amplicon. This was followed by the addition of 1ml of hybridization buffer to the single stranded biotin labelled amplicon to facilitate the binding of the membrane bound probes to the amplicon. The membrane was washed by the addition of 1ml of stringent wash solution. One (1) ml streptavidin/alkaline phosphatase (AP) conjugate was added to mediate an alkaline phosphatase (AP) staining reaction by the addition of 1ml of substrate solution to the membrane bound probes (Hain Lifescience GmbH, Nehren, Germany, 2009).

3.10.4.4 Results Interpretation

The membrane strips were dried and evaluated using the interpretation charts according to the manufacturer's instruction (Hain Lifescience GmbH, Nehren, Germany, 2009). The strips were pasted in the designated fields by aligning the bands Conjugate Control (CC), Universal Control (AC), Amplification Control (AC) and Tuberculosis Control (TUB), with respective lines on the evaluation sheet (Hain Lifescience GmbH, Nehren, Germany, 2009) as shown in Appendix VIII. Interpretation of Genotype MTBDR*plus* Result: There are three gene loci on the GenoType MTBDR*plus* strips and they are *rpoB* gene locus with 8 wild type probes and 4 mutation probes, *katG* gene locus has 1 wild type probe and 2 mutation probes, and *inhA* gene locus has 2 wild type probes and 4 mutation probes. In addition to the three control loci; Amplification Control (AC), Conjugate Control (CC) and Tuberculosis Control (TUB). The strips are evaluated based on manufacturers instructions (Hain Lifescience GmbH, Nehren, Germany, 2009). The presence of a brown-coloured line on the Conjugate Control (CC) Zone indicates correct binding of the conjugate on the strip and a correct chromogenic reaction. The test is invalid if CC is missing. The presence of a brown-coloured line on the Amplification Control (AC) Zone indicates that the amplification procedure was successful. A missing AC band in case of a negative result indicates errors during amplification set up, or carry-over of amplification inhibitors. The presence of a brown-coloured line on the TUB zone shows that the organism belongs to the *Mycobacterium tuberculosis* complex. If this zone is missing, the organism does not belong to the *Mycobacterium tuberculosis* complex and cannot be evaluated by this test system. There are locus control zones for *rpoB, katG, inhA* genes. Respective locus is present and must always be positive when the TUB has documented the presence of a *M. tuberculosis* strain. If neither the Locus Control probe nor the Wild type or Mutation probes of one of the three genes examined are developed, the test cannot be evaluated.

Interpretation of GenoType MTBC Result: The GenoType MTBC strip has 13 reaction zones and 3 Controls; Conjugate Control (CC), Universal Control (UC) and MTBC. The presence of a brown-coloured line on the CC indicates conjugate binding and substrate reaction and test is invalid if CC is missing. The presence of a brown-coloured line on the UC detects all Mycobacterial and members of the group of gram-positive bacteria with a high Guanine and Cytosine content. For MTBC, this zone hybridizes, as known with amplicons generated from all members of the *Mycobacterium tuberculosis* complex. MTBC negative does not belong to member of the MTB Complex and cannot be evaluated by the MTBC kit.

Interpretation of GenoType GenoType CM Result: The GenoType CM strip has 17 reaction zones and 3 Controls; CC, UC and GC. The presence of a brown-coloured line on the CC indicates conjugate binding and substrate reaction. The test is invalid if CC is missing. The presence of a brown-coloured line on the UC, it detects all Mycobacterial and members of the group of gram-positive bacteria with a high Guanine and Cytosine content. The presence of a brown-coloured line on the GC indicates that the organism belongs to the Mycobacteria group. However, the GC band may drop out in spite of the presence of Mycobacterial DNA, as long as a species-specific banding pattern is present, the amplification reaction was performed properly, the test result is valid.

3.11 Data Analysis

The data obtained from the study were reduced to percentages and presented in tables, charts and figures. The data were analysed with the aid of SPSS software, version 24 using chi-square at p-value of 0.05 and 95% confidence interval to test for association between demographics characteristics, risk factors, TB drug resistance and NTM infection.

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence and distribution of Mycobacteria, MTBC and NTM among the Population Screened

The study population consisted of 100 TB patients that were AFB positive. The results of the cultures from the 100 samples showed 86% were culture positive, 9% were culture negative and 5% were contaminated (Figure 4.1).

4.2: Distribution of MTBC and NTM among the Culture Positive Isolates

The results of the 86 culture positive isolates revealed that 93% were MTBC and 7% were NTM (Figure 4.2). Out of the 80 MTBC isolates characterized by GenoType MTBC kit, majority were *M. tuberculosis* (91%) and the least was *M. bovis* (3%) as shown in Table 4.1. Out of the 6 NTM isolates characterized by GenoType CM kit, majority (50%) of the isolates were *M. fortuitium* while there were equal distributions (16.7%) for *M. mucogenicum*, *M. gordonae*, and *M. intracellulare* as shown in Table 4.2.

4.3: Resistance Profile of *Mycobacterium tuberculosis* Complex

The resistance profile of the 80 MTBC isolates showed that 91.3% of the MTBC isolates showed no resistance (pan susceptible) to any of the two drugs (rifampicin and isoniazid) tested, 7.5% of the isolates were resistant to only isoniazid, 1.3% was resistant to both drugs (multi drug resistance) and 8.8% of the MTBC isolated had resistance to at least one of the two drugs (any resistance) as presented in Table 4.3.

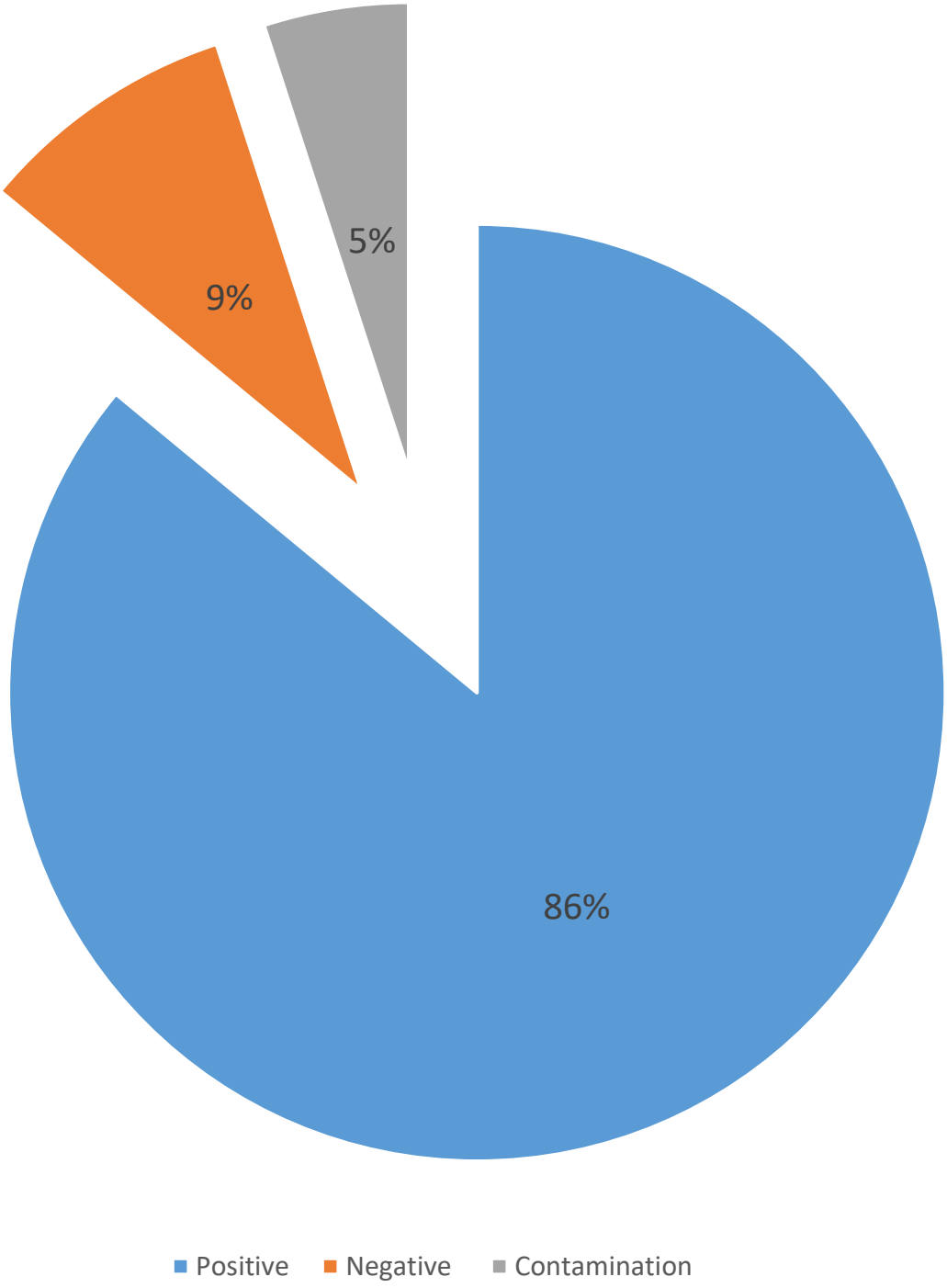

Figure 4.1: Prevalence of Mycobacteria by Culture technique among smear positive patients in the study population

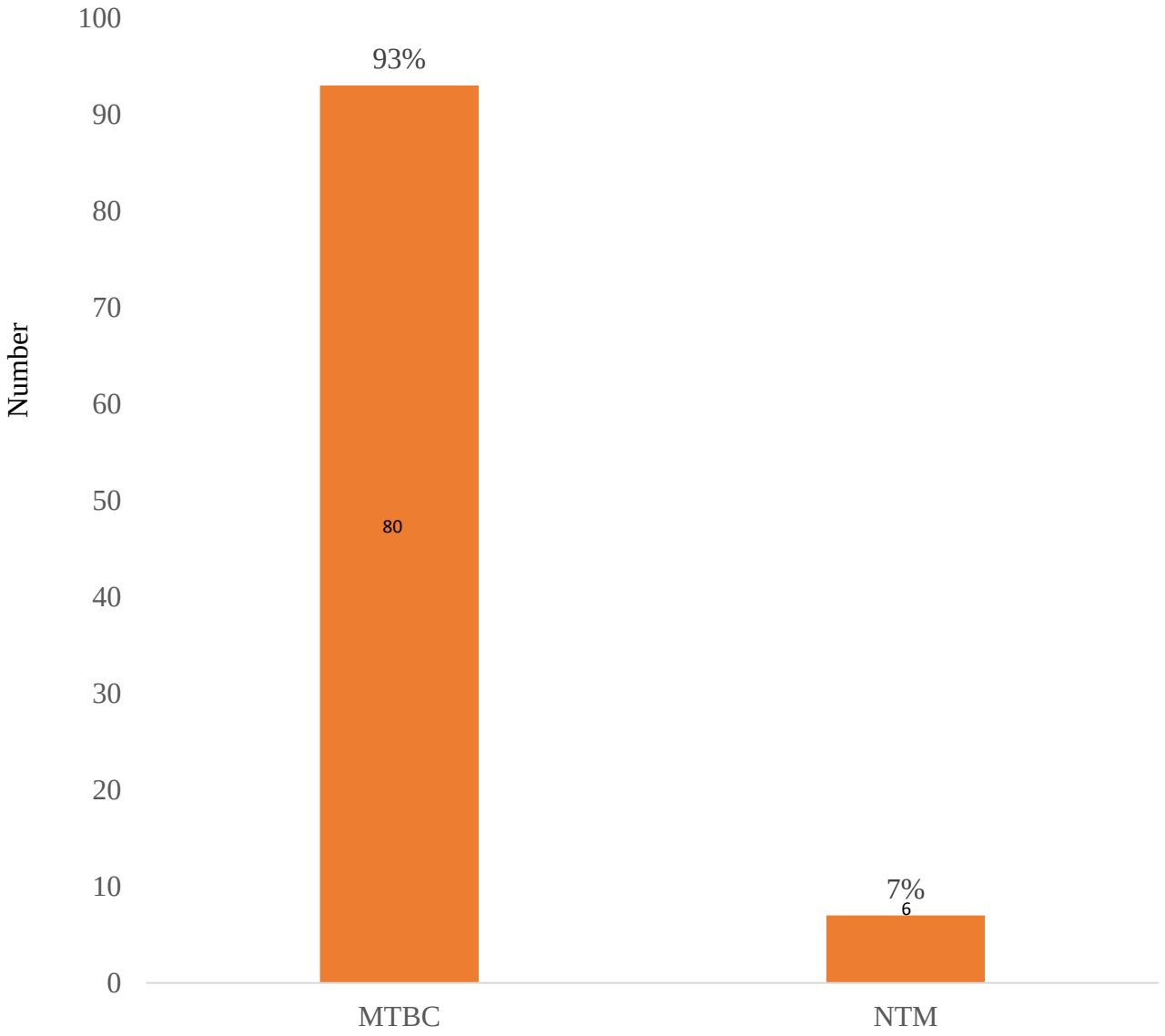

Figure 4.2: Distribution of MTBC and NTM among the culture positive isolates of smear positive patients in the study population

Keys: MTBC: *Mycobacterium tuberculosis* complex, NTM: Non tuberculous Mycobacteria

Table 4.1: Distribution of *Mycobacterium tuberculosis* complex among the culture positive isolates of smear positive patients in the study population

Species of MTBC	FCT	Kaduna	Total Frequency	Percentage (%)
M. tuberculosis	35	38	73	91.3
M. africanum	4	1	5	6.3
M. bovis	1	1	2	2.4
Total	**40**	**40**	**80**	**100**

Keys: MTBC: *Mycobacterium tuberculosis* complex

Table 4.2: Distribution of Non tuberculous Mycobacteria among culture positive isolates of smear positive patients in the study population

Species of NTM	Frequency	Percentage (%)
Mycobacterium mucogenicum	1	16.7
Mycobacterium gordonae	1	16.7
Mycobacteriumfortuitum	3	50
Mycobacteriumintracellulare	1	16.7
Total	**6**	**100**

Keys: NTM: Non tuberculous Mycobacteria

Table 4.3: Resistance Profile of the *Mycobacterium tuberculosis* complex among isolates of smear positive patients in the study population

Resistance Profile of MTBC	Frequency	%
No resistance (Pan susceptible)	73	91.3
INH Mono Resistant	6	7.5
MDR (resistant to RIF and INH)	1	1.3
Total MTBC	**80**	**100**

Keys: MTBC- *Mycobacterium tuberculosis* complex, RIF- Rifampicin, INH- Isoniazid

MDR- Multi drug resistant

4.4: Frequencies and Patterns of Mutations at *rpoB, katG* and *inhA* Genes of the *Mycobacterium tuberculosis* Complex

The results of the analysis based on the pattern and prevalence of gene mutations among the drug resistant MTBC is shown in Table 4.4. The result revealed that the mutation in the *rpoB* gene (analyzed for rifampicin resistance) is as a result of mutations in codons 526-529 (100%). Similarly, mutations to isoniazid were found in the *katG* and *inhA* genes analyzed. Out of the 7 INH-resistant strains found in this study, majority (71.4%) had mutation in the *inhA* gene conferring low level INH resistance. Analysis of the *katG* gene revealed that 28.6% of the INH resistant strains had deletion of the WT probe at codon 315 and 28.6% of the INH resistant strains had the presence of *katG* MUT1 mutation band at codon 315.

Further analysis of the *inhA* gene revealed that 71.4% of the INH resistant strains had deletion of the WT1 probe at nucleic acid position -15/-16 and 57.1% had the presence of *inhA* MUT1 mutation band at nucleic acid position -15. The only MDR- TB isolate simultaneously harbored the mutations at the *rpoB* gene (deletion of WT7 at codons 526-529 and presence of *rpoB* MUT3 mutation band at codon 531) in addition to mutation at the *katG* gene (deletion of WT probe at codon 315 and presence of *kat* MUT1 mutation band at codon 315).

Table 4.4: Frequencies and patterns of mutations at *rpoB, katG* and *inhA* genes of the *Mycobacterium tuberculosis* complex of smear positive patients in the study population

Gene	Band	Mutation at codon analyzed	Frequency of mutation (%)
rpoB	WT1	506-509	0 (0)
	WT2	510-513	0 (0)
	WT3	513-517	0 (0)
	WT4	516-519	0 (0)
	WT5	518-522	0 (0)
	WT7	526-529	1 (100.00)
	WT8	530-533	0 (0)
	MUT1	D516V	0 (0)
	MUT2A	H526Y	0 (0)
	MUT2B	H526D	0 (0)
	MUT3	S531L	0 (0)
KatG	WT	315	2 (28.57)
	MUT1	S315T1	2 (28.57)
	MUT2	S315T2	0 (0)
InhA			
	WT1	-15/-16	5 (71.43)
	WT2	-8	0 (0)
	MUT1	C-15T	4 (57.14)
	MUT2	A–16G	0 (0)
	MUT3A	T–8C	0 (0)
	MUT3B	T–8A	0 (0)

Keys: MUT- Mutation, WT- Wild Type

4.5: Demographic Characteristics that Predispose to Iinfection with Drug Resistant *Mycobacterium tuberculosis* Complex

The data was analyzed based on the demographic characteristics and the result is shown in Table 4.5. The age distribution of the patients with no drug resistance revealed the highest prevalence of 31.5% within the age group of 21-30 years and the lowest prevalence of 6.8% was recorded among the older generation of the age group above 51 years. Majority (71.4%) of patients with drug resistant TB were within the age range of 21–30 years while the least (28.6%) was within the age group of 31-40 years. While the distribution of age among the isoniazid mono resistant patients revealed the highest prevalence of 83.3% in patients within the age group of 21-30 years and the least (16.67%) within the age group of 31-40 years. The only patient with MDR-TB was 35 years within the age group of 31-40 years. There was no significant association between age and drug resistance (χ^2=12.23, df=4, P=0.662).

Pan suceptibility and Isoniazid resistance were found in males 72.6% and 66.7% respectively. The only patient with MDR-TB was male. There was no significant association between sex and drug resistance (χ^2=0.49, df=1, P=0.922).

Analysis of the results based on Body Mass index (BMI) showed that 52.1% of the pan susceptible patients were within the normal weight with BMI of 18.5-24.9kg/m^2 while least prevalence of 4.1% (3/73) were within obese patients with BMI of ≥30 kg/m^2. Equal prevalence of 42.9 % were recorded for underweight (BMI of 0 -18.49kg/m^2) and normal weight (BMI of 18.5-24.9kg/m^2) in patients with drug resistance. Fifty percent (50%) of the patients with isoniazid mono resistance were underweight, BMI 0-18.49kg/m^2 and least prevalence of 16.7% being overweight with BMI of 25-29.9kg/m^2. The only patient with MDR-TB had BMI 22.32kg/m^2 was within the normal

weight range. There was no significant association between BMI and drug resistance (χ^2=4.86, df=3, P=0.847).

4.6: Risk Factors that Predispose to Infection with Drug Resistant *Mycobacterium tuberculosis* Complex

The result of analysis of the data based on risk factors is shown in Table 4.6. A total of 66 out of 73 (90.4%) patients that were pan susceptible (no resistance) were HIV negative. All the patients with isoniazid resistance were HIV negative (100%), the only patient with MDR-TB was HIV positive. There was no significant association between HIV infection and drug resistance (χ^2=9.521, df=1, P=0.230). Majority (97.3%) of the pan susceptible patients do not have history of diabetes. Among the patients that are resistant to isoniazid and MDR-TB, none of them had diabetes history. There was no significant association between diabetes history and drug resistance (χ^2=0.39, df=1, P=0.942).

The data was also analyzed according to the TB treatment history of the patients. In this study, 79.5% (58) of the pan susceptible patients were new TB cases while 20.6% were re-treatment cases. All the patients with isoniazid mono-resistance and MDR-TB were new TB cases. There was no significant association between previous treatment and drug resistance (χ^2=3.48, df=1, P=0.324).

Table 4.5: Demographic Characteristics that Predispose to Infection with Drug Resistant *Mycobacterium tuberculosis* complex among smear positive patients in the study population

Demographic Factor	Drug Susceptibility Status								
Factor	**Pan susceptible Number= 73**		**Isoniazid Mono resistance Number= 6**		**MDR Number= 1**		**All resistant Number= 7**		**P-values**
	F	**(%)**	**F**	**(%)**	**F**	**(%)**	**F**	**(%)**	
Age group (years)									**0.662**
11-20	8	(10.96)	0	(0)	0	(0)	0	(0)	
21-30	23	(31.50)	5	(83.33)	0	(0)	5	(71.4)	
31-40	21	(28.77)	1	(16.67)	1	(100)	2	(28.6)	
41-50	16	(21.91)	0	(0)	0	(0)	0	(0)	
51 and above	5	(6.84)	0	(0)	0	(0)	0	(0)	
Sex									**0.922**
Male	53	(72.6)	4	(66.67)	1	(100)	5	(71.4)	
Female	20	(27.4)	2	(33.33)	0	(0)	2	(28.6)	
BMI (kgm^{-2})									**0.847**
0-18.49 (UW)	16	(21.92)	3	(50)	0	(0)	3	(42.9)	
18.5-24.9 (NW)	38	(52.05)	2	(33.33)	1	(100)	3	(42.9)	
25-29.9(OW)	16	(21.92)	1	(16.67)	0	(0)	1	(14.3)	
≥30 (OB)	3	(4.11)	0	(0)	0	(0)	0	(0)	
Location									**0.416**
Abuja	40	(50)	4	(66.67)	1	(100)	5	(71.43)	
Kaduna	40	(50)	2	(33.33)	0	(0)	2	(28.57)	

Keys: F- Frequency, MDR-Multi drug resistant, BMI- Body Mass Index, UW-Under Weight, NW- Normal Weight, OW-Over Weight, OB- Obese

Table 4.6: Risk Factors for Infection with Drug Resistant *Mycobacterium tuberculosis* complex among smear positive patients in the study population

Risk Factor	Drug Susceptibility Status								
Factor	**Pan susceptible (N= 73) F (%)**		**Isoniazid mono resistance (N= 6) F (%)**		**MDR (N= 1) F (%)**		**All resistant (N= 7) F (%)**		**P- values**
HIV Status									**0.230**
Reactive	7	(9.59)	0	(0)	1	(100)	1	(14.29)	
Non-Reactive	66	(90.41)	6	(100)	0	(0)	6	(85.71)	
Diabetes History									**0.942**
Yes	2	(2.74)	0	(0)	0	(0)	0	(0)	
No	71	(97.26)	6	(100)	1	(100)	7	(100)	
Previous TB treatment									**0.324**
New case	58	(79.45)	6	(100)	1	(100)	7	(100)	
Re-treatment	15	(20.55)	0	(0)	0	(0)	0	(0)	

Keys: MDR- Multi drug resistant, N- Number, F- Frequency, HIV-Human Immunodeficiency Virus

4.7: Demographic Characteristics that predispose to infection with Non-tuberculous Mycobacteria

The data was analyzed based on the socio-demographics factors and the result is shown in Table 4.7. The majority (73.8%) of the patients infected with MTBC were male. The analysis also showed equal distribution (50%) of gender among patients infected with NTM. There was no statistically significant association between NTM infection and sex (χ^2=2.80, df=1, P=0.247). The distribution of age among patients with NTM revealed equal age distribution (50%) for patients within age groups 21-30 and 31-40. There was no statistically significant association between NTM infection and age (χ^2=10.37, df=4, P=0.409). Analysis of the results based on Body Mass index (BMI) showed 51.3% of the patients infected with MTBC were within the normal weight with BMI of 18.5-24.9kg/m^2 while 3.8 % of the patients were Obese with BMI of >30 kg/m^2, 66.67% of patients were underweight (BMI of 0 -18.49kg/m^2) and 33.3% had Normal Weight with BMI 18.5-24.9 kg/m2. There was no statistically significant association between NTM infection and BMI (χ^2=6.535, df=3, P=0.366).

4.8: Risk Factors for Infection with Non-tuberculous Mycobacteria

The data was analyzed based on the risk factors and the result is shown in Table 4.8. A total of 72 out of 80 patients with MTBC infection were HIV negative while 10% were HIV positive. Among the patients with NTM infection, (66.7%) were HIV negative while 33.3% were HIV positive. There was no significant association between NTM infection and HIV (χ^2=2.96, df=1, P=0.228). Among patients infected with MTBC showed that 97.5% had no history of diabetes and 2.5% had history of diabetes. While all the 6 (100%) patients infected with NTM had no history of diabetes. There was no statistically significant association between NTM infection and diabetes (χ^2=0.51,

df=2, P=0.775). The analysis of data based on alcohol consumption revealed that 60% (48/80) of the patients with MTBC infection did not consume alcohol and 40% consumed alcohol. While 66.7% of patients with NTM infection did not consume alcohol and 33.33% consumed alcohol. There was no statistically significant association between NTM infection and alcohol consumption (χ^2=1.80, df=2, P=0.407). Analysis of results based on consumption of milk among patients with MTBC infection showed a higher prevalence of 61.3% among those that did not consume milk compared to those that did consume milk with a prevalence of 38.8%.

The analysis of results based on consumption of milk among patients with NTM infection showed a higher prevalence of 66.7% among those that did not consume milk compared to those that did consume milk with a prevalence of 33.3%. There was no statistically significant association between NTM infection and local milk consumption (χ^2=0.11, df=1, P=0.949). Majority (85%:) of the patients infected with MTBC isolated were not involved in livestock farming. In addition, majority (83.3%) of the patients with NTM infection were involved in livestock farming. There was statistically significant association between NTM infection and livestock farming (χ^2=48.42, df=1, P=0.003).

Table 4.7: Demographic Characteristics that Predispose to Infection with Non-tuberculous Mycobacteria among smear positive patients in the study population

Factor	MTBC Isolated N= 80		No Mycobacteria isolated N= 14		NTM isolated N= 6		P- values
	F	(%)	F	(%)	F	(%)	
Age group (years)							**0.409**
11-20	8	(10)	2	(14.29)	0	(0)	
21-30	28	(35)	6	(42.86)	3	(50)	
31-40	22	(27.50)	4	(28.57)	3	(50)	
41-50	17	(21.25)	0	(0)	0	(0)	
51 and above	5	(6.25)	2	(14.29)	0	(0)	
Sex							**0.247**
Male	59	(73.75)	12	(85.71)	3	(50)	
Female	21	(26.25)	2	(14.29)	3	(50)	
BMI (Kgm^{-2})							**0.366**
0-18.49 (UW)	19	(23.75)	5	(35.71)	4	(66.67)	
18.5-24.9 (NW)	41	(51.25)	6	(42.86)	2	(33.33)	
25-29.9 (OW)	17	(21.25)	3	(21.43)	0	(0)	
≥30 (OB)	3	(3.75)	0	(0)	0	(0)	
Location							**0.233**
Abuja	40	(50)	8	(57.14)	1	(16.67)	
Kaduna	40	(50)	6	(42.86)	5	(83.33)	

Keys: MTBC- *Mycobacterium tuberculosis* complex, NTM- Non tuberculous Mycobacteria, N- Number, F- Frequency, BMI- Body Mass Index, UW-Under Weight, NW- Normal Weight, OW- Over Weight, OB- Obese

Table 4.8: Risk factors for infection with species of *Mycobacterium tuberculosis complex* and Non- tuberculous Mycobacteria among smear positive patients in the study population

Factors	MTBC Isolated N= 80		No mycobacteria isolated N= 14		NTM isolated N= 6		P- values
	F	(%)	F	(%)	F	(%)	
HIV Status							**0.228**
Reactive	8	10	2	14.29	2	33.33	
Non-Reactive	72	90	12	85.71	4	66.66	
Diabetes History							**0.775**
Yes	2	2.50	0	0	0	0	
No	78	97.50	14	100	6	100	
Drinking Alcohol							**0.407**
Yes	32	40	3	21.43	2	33.33	
No	48	60	11	78.57	4	66.67	
Local milk Consumption							**0.949**
Yes	31	38.75	5	35.71	2	33.33	
No	49	61.25	9	64.29	4	66.67	
Livestock Farming							***0.003**
Yes	12	15	4	28.57	5	83.33	
No	68	85	10	71.43	1	16.67	

Keys: MTBC- *Mycobacterium tuberculosis* complex, NTM- Non tuberculous Mycobacteria, N- Number, F- Frequency, %- Percentage, *- Significant association

CHAPTER FIVE

5.0 DISCUSSION

The prevalence of Mycobacteria isolated from acid fast bacilli smear positive samples was found to be 86%. The 86% smear positive culture positive (Mycobacteria) obtained in this study might be due to validated microscopy results, and showed that the Mycobacteria is present in the samples. This could also be due to the fact that the smear positive status of the patients is caused mainly by Mycobacteria. The result obtained in this study is similar to the result obtained from Selvakumar *et al*. (2012) who reported 85% of smear positive culture positive in India. Studies from Ibadan and Calabar revealed lower prevalence of Mycobacteria to be 75% and 70.8% (Olusoji *et. al.*, 2011; Benjamin *et. al.*, 2012). In addition, Sobh *et al.* (2016) and Addo *et al.* (2007) reported prevalence of 82.5% and 91% in Egypt and Ghana respectively. The reason for this variation may be attributed to inclusion criteria (smear positive TB Patients) used in our study. The variation in the prevalence of Mycobacteria may be also as a result of initial host range susceptibility (De Groote and Huitt, 2006; Griffth *et al.*, 2007).

Contamination rate observed in this study was 5%. This could be due to inappropriate sample collection, sub-optimal patient instructions, sub-optimal specimen storage, etc. The result of contamination rate is in agreement with the established standard of 3–5% for solid LJ culture method (Cornfield *et al*., 1997). It is also similar to findings from Addo *et al.* (2007) who reported 4.3% contamination rate. However, it contrasts the findings from Uganda by Kennedy *et al*. (2014) who reported the contamination rates for solid LJ to be 31%, which is greater than the recommended threshold of 5% for laboratories that receive freshly collected sputum sample. The

difference might be due to batching of samples, storage temperature (> 2-8^{o}C) and transit time of samples (> 72 hours) and methodologies used.

The 9% AFB smear positive but culture negative obtained from this study might be associated with the number of viable AFB inoculated into LJ tube, and may also depend on quality of the specimen. It could also depend on the presence of other AFB positive organisms like *Nocardia, Actinomyces, Rhodococcus, Legionella micdadei* or cysts of *Cryptosporidium* species (Clavel *et al.,* 1996). This result is similar to the result by Mamuda *et al.* (2017) in Nigeria where he reported 8% smear positive culture negative and results obtained by Addo *et al*. (2018) in Ghana with report of 9.8% smear positive culture negative samples.

The 93% prevalence of MTBC recorded in this study is higher than 7% for NTM. This could be due to the transmission mechanism of MTBC which is from person to person or animal to man unlike NTMs which are mostly environmental Mycobacteria. This result is in accordance with report of Premraj *et al.* (2014) who reported that the prevalence of MTBC was higher than the prevalence of NTM. A number of cases (7%) who sought clinical treatment for tuberculosis in this study were caused by NTM. This could be due to the medical importance of environmental Mycobacteria. The prevalence of NTM obtained in this study was 7% which is lower than the outcomes of previous studies conducted within Nigeria; between 23.1%-26.6% in Northern Nigeria (Mawak *et al.,* 2006), 16.5% reported in the south-south region (Pokam *et al.,* 2012) and 15% prevalence was reported in Kaduna (Aliyu *et al.,* 2013b). The reason for the low prevalence of NTM recorded in this study could be attributed to the fact that the study was conducted outside of the harmattan period. Harmattan is a West African trade wind that occurs during the winter and is characterized by heavy amount of dust in the air, low humidity and reduced visibility (Weinstein *et al.* 2010). There are higher risks for environmentally acquired pulmonary Mycobacterial

infections for individuals with exposures to dust. Since this study was conducted outside the harmattan period, exposure to dust was limited hence the lower prevalence of NTM in the study.

In this study the MTBC isolated were predominantly (91.3%) *M. tuberculosis* and least (2.4%) was *M. bovis*. This could be due to the transmission mechanism of *M. tuberculosis* transmission within the population which is being transmitted from person to person unlike *M. bovis* which is commonly transmitted from livestock and products to man. The higher distribution of *M. tuberculosis* obtained in this study is simillar to studies conducted in different parts of Nigeria; 61.5% in Jos (Mawak *et. al*., 2006), 90.9% in Ibadan (Olusoji *et al.,* 2011), 83.5% in Cross River (Benjamin *et al.,* 2012) and 94.4% in Kaduna (Aliyu *et al*., 2013). However, bovine TB has been reported to be more prevalent in the Northern part when compared to the Southern part of Nigeria (Mawak *et al.,* 2006). Studies from other West African nations have shown the prevalence of *M. africanum* to be between 9% - 28%. These studies also reported a very low prevalence of *M. bovis*; 3% in Ghana (Niobe-Eyangoh *et al*., 2003), 0.8% in Mali (Addo *et al*., 2007), 0.2% in Cameroun (Traore *et al*., 2012) and 0% in Burkina Faso (Gomgnimbou *et al*., 2012). *Mycobacterium bovis* infection generally results from spread from livestock or their products and may be transmitted through routes other than the respiratory system. The low proportion of TB cases attributed to *M. africanum* in this study also suggests decreased transmissibility. Reasons for decreased transmission of *M. africanum* are unknown but could include decreased infectiousness or decreased progression to disease compared with *M. tuberculosis*, as was previously reported (de Jong *et al.,* 2010*).*

In this study, the predominant (50%) species of NTM isolated was *M. fortuitum* while others *M. mucogenicum, M. gordonae and M. intracellulare* had prevalence of 16.7%. The distribution of NTM species isolated from pulmonary samples differs by geographic regions. In a study

involving 30 different countries from six different continents, a higher prevalence of *M. avium* complex was documented from North America, South America, Australia, Asia and Europe (Hoefsloot *et al.,* 2013). However, most of these data are from the developed world and sub-Saharan Africa is under represented (Hoefsloot *et al.,* 2013). Although there are now emerging NTM disease data from Asia and parts of Africa, significant knowledge gaps still exist especially in sub-Saharan Africa with nine of the world's 22 high burden tuberculosis countries. (Buijtels *et. al.,* 2005; Hoefsloot *et al*. 2008; da Costa *et al*. 2013). Pulmonary non-tuberculous Mycobacterial (NTM) disease epidemiology in sub-Saharan Africa is not as well described (Okoi *et al.,* 2017). Earlier reviews of global NTM epidemiology only included subject-level data from one sub-Saharan Africa country. The prevalence of pulmonary NTM colonization in 37 articles in sub-Saharan Africa was 7.5% and *M. avium* complex predominated (27.7%) as shown by (Okoi *et al.,* 2017).

The observed high (91.3%) pan susceptibility in this study indicates that, well supervised short course chemotherapy regimens quite often result in a lower proportion of treatment failure and shows success of DOTs programme in Nigeria. In addition, the finding that RIF had the lowest resistance in the MDR-TB (1.3%) among the anti-TB drugs used (RIF and INH) had an important implication for national and global MDR-TB diagnosis because rifampicin resistance has been used as a surrogate marker for MDR-TB. The 7.5% INH mono-resistance in this study is in agreement with the findings from Aliyu *et al.* (2013b) who reported that isoniazid resistance is higher than rifampicin resistance and invariably low rate of MDR-TB. He also reported 23 cases (6.1%) had resistance to at least one of the two drugs- INH and RIF (any resistance); of those, 13 (3.5%) cases had resistance only to isoniazid; 5 (1.3%) had resistance only to rifampicin while the remaining 5(1.3%) cases had resistance to both drugs (MDR-TB). In the study, the 7.5%

prevalence of INH mono-resistance is higher than the 4.8% INH mono-resistance of the 2012 Nigeria MDR TB Survey as well as the 1.3% MDR-TB being lower than the 4.8% of the 2012 Nigeria MDR TB Survey. The 8.8% rate of drug resistant TB is lower than the global percentage of 20% for any form of anti-TB drug resistance (WHO, 2010). These variations could be due to the small sample size used in the study which is lower than samples used for the 2012 MDR TB Survey.

In this study, two genes associated with INH resistance were examined which are *inhA* (codes for low level resistance) and *katG* (codes for high level resistance). This study revealed higher (71.4%) INH resistance due to mutations at the *inhA* gene (codes for low level resistance). This could mean that there is ongoing transmission of the strains harboring this low-level resistance which are currently in circulation and are being transmitted from one person to another. There is scarcity of reports from Nigeria to establish the proportion of high-level resistance (at the *katG* gene) to that of low-level resistance (at the *inhA* gene). Studies from other countries have confirmed the variability in the contribution of different mutations to INH resistance (Mokrousov *et al*., 2002; Baker *et al*., 2005). The reasons for the variations could be because TB is endemic in Nigeria (Nigeria has the fourth largest TB epidemic in the world) and with HIV prevalence (Second largest HIV epidemic in the world). In order to prevent TB co infection among People living with HIV and AIDs (PLWHA), IPT is routinely given. The frequent use of INH at 300mg/daily for 6 months in the population predisposes to INH resistance.

The analysis of the amino acids at the *inhA* gene region showed that WT1 (-15/-16) accounted for majority (71.4%) of mutation while MUT1 (C-15T) had the least prevalence of 57.1%. The higher prevalence (71.43%) of mutations in the amino acid positions -8, -15 and -16 in the promoter region of the *inhA* gene of the INH resistant strains found in this study contrasts reports from other

known studies that show that 8% to 20% of INH resistance are defined as the low-level drug resistance (0.1-lg/ml) mainly caused by the mutations in the promoter region of *inhA* gene, involving -15, -16 and -8 loci (Zhang *et al*., 2011). The frequency of INH-resistant strains harbouring a single C15T (MUT 1) mutation in the *inhA* regulatory gene in this study is similar to reports from South-Africa (Barnard *et al*., 2008) and China (Huang *et al*., 2009). This study shows that 28.6% each INH resistance was mediated by *katG* codon 315 (WT) and S315T1 mutations (MUT1) which conferred high-level resistance to INH; this is lower than the 84.6% *katG* S315T1 mutation found in the study carried out in Southern China (Zhang *et al*., 2010). In this study, MDR strain simultaneously harbored the mutations in the *rpoB* WT7 (526-529), *katG* WT (315) and *katG* MUT1 (S315T1) and this contrasts findings from South-Africa which revealed that *rpoB* S531L mutation (MUT3 band) occurred most commonly with 70.5% of all RIF-resistant strains (Barnard *et al*., 2008). However, the occurrence of only one MDR-TB isolates could not justify the true picture of this in the study area.

This study examined the relationship between demographics characteristics and drug resistant MTBC. Among the demographic factors examined, none was statistically associated with drug resistant tuberculosis. Vast majority of drug resistant cases was within the age group of 21-30 years. The magnitude of drug resistance among younger age (21-40 years) is more likely to be indicative of recent transmission than among older age groups, who are more likely to be harboring older infections (WHO, 2009). Uzoewulu *et al*. (2014) reported that majority of patients with drug resistant TB were within the age range of 21–40 years, but she reported in her study that the age distribution did not differ statistically significantly between the resistant and susceptible groups. There was no statistical association between sex and drug resistant tuberculosis in this study.

The result showed that, TB drug resistant cases were more in males than females which may be due to differences in health seeking habits of people arising from the stigma associated with tuberculosis. This study highlights the social role of males and cultural habits that influence risk of exposure have also been implicated as possible reasons for higher prevalence of drug resistance in male. Uzoewulu *et al*. (2014) reported that, drug resistance cases were found to be more likely in male 27.7% than female 18.3% while a study in Georgia revealed that women were at higher risk of MDR TB compared with men (Lomtadze *et al.,* 2009). There was no statistical association between sex and drug resistant tuberculosis in this study.

This study revealed equal (42.9%) distribution of drug resistance patients that are under-weight and are of normal weight. This could be due to the small sample size used in this study. Cholesterol –rich diet has also been reported to accelerate bacteriologic sterilization in pulmonary tuberculosis (Masijedi *et al*., 2008). This finding is contradictory to other findings where low BMI has been reported to be associated with poor TB treatment outcome and overweight (BMI > 25kg/m^2) to be significantly associated with lower risk of active TB than normal weight (BMI, 18.5 -25 kg/m^2) individuals (James *et al*., 2009). There was no statistical association between BMI and drug resistant tuberculosis in this study.

The study examined the relationship between risk factors and drug resistant MTBC. Among the risk factors examined, none was statistically associated with drug resistant tuberculosis. In this study, majority (85.71%) of the patients with TB drug resistance were HIV negative. Gomes *et al*., (2014) did not find a significant association between HIV co-infection and drug-resistance, although this association has been described by other studies (Casal *et al*., 2005; Faustini *et al*., 2006; Kliiman and Altraja, 2009). Lack of such an association could be caused by inadequate testing (WHO, 2010). Nevertheless, the epidemiological impact of HIV on the epidemic of drug

resistant TB is not well established and may depend on several factors such as testing amongst others. There was no statistical association between HIV and drug resistant tuberculosis in this study.

This study revealed that the absence of diabetes among TB drug resistant patients implied that there may be other associated factors for development of drug resistance in the study population. The mechanism by which diabetes can cause development of drug-resistant TB is unclear as reported by Gomes *et al.*, (2014). Possible malabsorption of anti-TB drugs among diabetic patients, thus reducing the treatment effect, has been suggested (Dooley and Chaisson, 2009; Mi *et al*., 2013). There was no significant association between diabetes and drug resistance in this study.

All the drug resistant TB cases were found among new TB patients in this study and this indicates a high level of emerging *M. tuberculosis* being resistant among new TB cases showing that the drug resistant TB was by primary transmission of drug resistant strains. Although drug-resistant TB can be transmitted between individuals (primary resistance), most cases arise after inadequate treatment allowing a drug-resistant strain to become dominant (Suarez-Garcia *et al*., 2009).

This study showed that there was very high level of TB drug resistant among new TB cases which is similar to findings from Uzoewulu *et al.* (2014) that revealed 31.1% of TB drug resistance cases from new TB cases. Range *et al*. (2012) also reported 84.6% TB drug resistance among new cases. In contrast, some studies have reported previous treatment to be one of the risk factors for drug resistance or poor treatment outcome (James *et al*., 2009). There was no significant association between case type and drug resistance in this study.

This study examined the relationship between demographics characteristics and NTM infection. Among the demographic factors examined, none was statistically associated with NTM infection.

The observed distribution of age among patients with NTM revealed equal age distribution for patients within age groups 21-30 and 31-40. Pulmonary NTM infections usually infect people with middle-aged or older adults and this could be due to local pulmonary changes or immunological factors, but younger individuals seem to be largely protected from NTM infection. However, host defense and immunological aging have been suggested as a predisposing reason for NTM infection (Beerman *et al*., 2010). However, this is not in line with the report from Taiwan in which most cases of NTM colonisation was between 65-84 years of age (Jung-Yien *et al.,* 2014). There was no significant association between age and NTM infection in this study.

In this study there was equal distribution (50%) of NTM infection among males and females. This could be due to the sources of exposure to the infectious agents. This finding is in disagreement to that of Cassidy *et al.,* (2009) in Oregon, US which showed a strong association of NTM frequent infection in women than men. Our findings are not in line with published reports from institutional cases series and experts who believe the epidemiology of this disease has changed during the last several decades to affect women more frequently than men (Griffith *et al*., 2007). Although small sample size used for this study makes it difficult to make definitive conclusions. There was no significant association between sex and NTM infection in this study.

The study examined the relationship between risk factors and NTM infection. Among the risk factors examined, none was statistically associated with NTM infection There was higher NTM prevalence among HIV negative individuals than among HIV positive individuals. The higher burden of NTM among the HIV negative cannot be ignored. Factors such as the duration of exposure to infectious agents not minding the HIV status of the patient and dose could be reasons for this finding. This observation is contrary to the report of a recent study where all the patients from whom NTM were isolated were all HIV-negative (Borroni *et al.,* 2015). However, a report

showed that these strains of Mycobacteria are the commonest opportunistic infection of the immunosuppressed individuals (Wen Lee *et al*., 2011). There was no significant association between HIV and NTM infection in this study.

There was higher percentage of NTM among patients that do not consume local milk than the patients that consumed local milk. This could be due to extreme exposure to other environmental factors that predispose to NTM infection like the presence of NTM in the water supply systems, where Mycobacteria can easily form biofilms, which are almost impossible to remove and in other sources like the intense air pollution. This contrasts studies where the presence of NTMs were found to be predominantly detected in raw and pasteurized milk (Leite *et al.*, 2003; Konuk *et al.*, 2007). There was no significant association between local milk consumption and NTM infection in this study.

There was higher NTM prevalence among patients that do not consume alcohol compared to patients that do consume alcohol. This could be due to other predisposing factors and sources of infection. A study carried out by Knut *et al*. (2008) stated that there was no association between alcohol use and mycobacteriosis. In contrast, another study carried out by Ahmadu *et al*. (2019) stated that majority of their patients with NTM infection were alcohol drinkers. There was no significant association between alcohol consumption and NTM infection in this study.

Diabetes was another risk factor that was investigated in this study with regards to NTM infection. None of the patients with NTM infection from this study had diabetes. This could be due to other predisposing factors and sources of infection. This is in contrast to other reports were NTM infected patients with diabetes have been found among their studies. Some of the studies include Yu *et al*. (2014) which showed that 8.9% of their patients had diabetes. Andréjak *et al*. (2010)

stated that 3.9% of their patients with NTM infections had diabetes and Umrao *et al*. (2016) stated that 13.3% of their NTM infected patients had diabetes (Umrao *et al*., 2016). Diabetes is a strong risk factor for the development of other infectious diseases such as melioidosis, pneumococcal disease and tuberculosis. In addition, there were other studies that showed diabetes to be a significant risk factor for disease due to NTM. There was no significant association between diabetes and NTM infection in this study.

This study revealed that 83.3% of patients with NTM infection were involved in livestock farming. Studies have shown that the most common NTM isolated from humans are listed are also environmental opportunistic Mycobacteria (Prince *et al.*, 1989; Lilo *et al*., 1990; Alcaide *et al.*, 1997; Costrini *et al.*, 1981, Aubry *et* al., 2002; Zaugg *et al.*, 1993; Conger *et al.*, 2004). In addition to their isolation from patients suffering from mycobacterial disease, most have been recovered from environmental habitats. Humans and their agronomic animals are literally surrounded by NTM, thus close physical contact between humans, animal and their environment with inadequate disease control measures in animals and humans can facilitate the transmission of NTMs and this could also be a reason for this occurrence (Cosivi *et al.*, 1998). In addition, due to the presence of NTM in the environment, human activities have had direct impacts on their ecology and hence their epidemiology (Kankya *et al*., 2011) and as humans have very substantially altered the living environment this may provide new niches that some Mycobacteria can exploit and thereby increase our risk of exposure (Falkinham, 2010). An example of this is water supply systems, where Mycobacteria can easily form biofilms, which are almost impossible to remove (Falkinham, 2011). In all the habitats, where NTM have been recovered (Michel *et al*., 2007), the Mycobacteria are part of the normal flora, existing as stable, resident and growing populations. The variation in the rates of NTM disease and colonization among different populations may also reflect differences

in the risk for exposure to environmental Mycobacteria. There was significant association between livestock farming and NTM infection in this study (P=0.0003).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This study established that out of 100 AFB positive study participants whose samples were cultured using Lowenstein Jensen's media, 86% (86/100) were culture positive, 9% (9/100) were culture negative and 5% (5/100) were contaminated.

The distribution of Mycobacteria within the study population revealed that MTBC was the dominant species with prevalence of 93% (80/86) while prevalence of NTM was 7% (6/86).

Among the MTBC species, *M. tuberculosis* had the highest prevalence of 91% (73/80), while majority (50%: 3/6) of the NTM species isolated were *M. fortuitum*.

The study also demonstrated higher prevalence of pan-susceptible TB among study population with 91.3% (73/80) and1.3% (1/80) prevalence of MDR.

The INH resistance in the *inhA* gene that codes for low level resistance was more with prevalence of 71.43% (5/7).

Among the demographics and risk factors examined, only livestock farming was significantly associated with NTM infection among the respondent (P= 0.003)

6.2 RECOMMENDATION

Based on the findings of the study, appropriate patient education and sample collection for TB patients for appropriate samples collection is recommended.

There should be public awareness on the risk and mode of transmission of TB and NTM considering the occurrence of MTBC and NTM in the study population.

There should be rapid speciation and identification method that distinguishes MTBC from NTM. This should be an important prerequisite for the proper management of patients with mycobacterial infections, and should be introduced as a required standard into routine laboratory diagnostics.

There should be rapid screening TB for drug resistance for prompt initiation of treatment.

Infection control mechanism should be in place at all levels.

This study revealed significant association between livestock farming and NTM infection (P=0.0003).

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Appendices

Appendix I: Approval A

FEDERAL CAPITAL TERRITORY
Health Research Ethics Committee
Research Unit, Room 10, Block A Annex, HHSS, FCTA Secretariat,
No. 1 Kapital Street Area 11, Garki, Abuja - Nigeria

Name of Principal Investigator	Iwakun Mosunmola
Address of Principal Investigator:	Dept. of Microbiology, Ahmadu Bello University, Zaria, Kaduna State.
Date of receipt of valid application:	02/03/2016

Notice of Research Approval
Protocol Approval Number: FHREC/2016/01/19/14-03-16

Study Title: Genetic Diversity of Mycobacterium Tuberculosis Complex among Tuberculosis Patients in Some Parts of Federal Capital Territory (FCT) and Kaduna State, Nigeria.

This is to certify that the FCT Health Research Ethics Committee (FCT HREC) has approved the research described in the above stated protocol.

Effective Date: - **14/03/2016**
Expiration Date: - **13/03/2017**

Note that no activity related to this research may be conducted outside of these dates. Only the FCT HREC approved informed consent forms may be used when written informed consent is required. They must carry FCT HREC assigned protocol approval number and duration of approval of the study.

The National Code of Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations, and with the tenets of the code. The FCT HREC reserves the right to conduct compliance visit to your research site without prior notification.

Modifications: Subsequent changes are not permitted in this research without prior approval by the FCT HREC.

Problems: All adverse events or unexpected side effects arising from this project must be reported promptly to FCT HREC.

Renewal: This approval is valid until the expiration date. If you are continuing your project beyond the expiration date, endeavor to submit your annual report to FCT HREC early, and request for renewal of your approval to avoid disruption of your project.

Closure of Study: At the end of the project, a copy of the final report of the research should be forwarded to FCT HREC for record purposes, and to enable us close the project.

Desmond Emerebnyeokwe
For: Secretary, FCT HREC
March 14, 2016

Appendix II: Approval B

FEDERAL MINISTRY OF HEALTH

DEPARTMENT OF PUBLIC HEALTH
NATIONAL TUBERCULOSIS AND LEPROSY TRAINING CENTRE

Saye Village Old Zaria - Kaduna Road,
P.M.B. 1089 Zaria, Kaduna State, Nigeria.
Tel: 069-332726, Telefax: 069-333205
E-mail: ntbltc@hotmail.com

Ref No: NTBL/TRG/ZA/182/Vol.IV
Date: 4/03/2016

Head of Department,
Microbiology,
Faculty of Science,
Ahmadu Bello University,
Zaria.

LETTER OF APPROVAL

ATTENTION: **IWAKUN MOSUNMOLA O (Ph.D/SCIEN/12268/2011-12)**

Your research proposal titled: **"GENETIC DIVERSITY OF MYCOBACTERIUM TUBERCULOSIS COMPLEX AMONG TB PATIENTS IN SOME PARTS OF FEDERAL CAPITAL TERRITORY AND KADUNA STATE, NIGERIA"** refers.

This is to convey approvals for ethical clearance for the commencement of the research and to use the National TB Reference Laboratory to analyze your samples. You are required to keep this office informed on the progress being made on the research and to abide by all the rules and ethics of the research.

Yours Faithfully,

Dr Kenneth O. Adagba
Chair, Ethical Committee

Appendix III: Informed Consent Form

Title of Research Study: The aim of this study is to investigate the anti-TB drug resistance of *Mycobacterium tuberculosis* complex among smear positive patients in parts of Abuja and Kaduna State, Nigeria.

What is the Purpose of this Study?

TB causes both death and sickness that prevents people from working and taking care of their families. The Government of Nigeria is concerned that the germs that cause TB (the disease you are being treated for in this clinic) are sometimes not killed by drugs used to treat them. When this happens it is called drug resistance. This study wants to know investigate the anti-TB drug resistance of the *Mycobacterium tuberculosis* complex in the sputum sample.

Who will take part in the study?

Persons found to be coughing and have reported in TB treatment centers and are being considered for treatment or repeat treatment if they have been treated before.

What will be done to me?

As part of your routine TB care at your clinic site, you will cough and spit into a small cup to provide the clinic with a sputum sample that will be looked at under a microscope to see if the TB germs are present. As is the normal procedure to diagnose TB, you will submit 2 sputum sample. The sample will be checked for TB germs using a microscope. Both your diagnosis and treatment will continue whether you decide to participate in this research study or not as it is the normal step in diagnosing TB. If you agree, the TB germs in your sputum/cough, if any, will be grown and tested against the drugs used for treating TB and information will be collected from your medical records about your TB disease. Information about your HIV status will be obtained from your

clinic records. Other information will include your age, TB disease and treatment information. The study staff may also ask you questions related to your TB disease.

Confidentiality

The information collected from you and your medical record will be kept secret.

Potential Risks and Discomforts

The potential risks of this study include answering questions about your practice that may make you shy or uncomfortable. Risks also include other people finding out what you say. The interview will be conducted in a private area with only you and the study staff. The study staff will not put your name on any of the material, only your card number will be taken in order to identify the samples you provided to the hospital. All study materials will be kept under lock and key in a secured office. Like other results to be used in this study, your HIV result will not carry your name only the unique number given to you. Some participants may experience additional personal problems because of their participation. Spouses, other family members, or sexual partners may react differently.

Potential Benefits

All study participants will be educated on the importance of adhering to their TB treatment regimens. Results from this study may also benefit the people of Nigeria.

Subject's Right

Your participation is voluntary without penalty or loss of clinic benefits to which you are otherwise entitled. If you decide to stop participating, if you have any questions, concerns, complaints or if you need to report a medical injury related to the research, please contact Iwakun Mosunmola 08032113266, Prof Steve Olonitola Department of Microbiology, Ahmadu Bello University, Zaria 08035890195 at any time to discuss your feelings.

Alternatives:

You do not have to participate in this study. If you decide to participate, you are free to withdraw at any time however the samples you have submitted cannot be withdrawn. Refusal to participate will not involve any penalty or loss to your clinic benefits.

Subject Costs and Payments

There are no costs to you as a consequence of your participation in this research study. You will not be paid to participate in this research study.

Signature of Subject or Legally Authorized Representative:

I have read (or someone has read to me) the above information. I agree to participate in this study. I have been given a copy of this form.

_____ _____ _____

Signature/Thumbprint Date Printed Name

Questionnaire Identification Number:

Signature of study staff obtaining Consent Date (must be same as subject's):

Appendix IV: Questionnaire

No	Questions and filters	Coding categories
95	Unique ID No	
96	Study ID No	
97	Sex	M F
98	Weight (Kilograms) …….	
99	Height (Meters)……	
100	BMI (Kg/m^2)	0-18.49 (UW) 18.5-24.9 (NW) 25-29.9 (OW) ≥30 (OB)
101	HIV Results R/NR……	R NR
102	What is the highest level of formal school education you completed	NONE…….. 0 PRIMARY……..…2 JUNIOR SECONDARY……..….3 SENIOR SECONDARY……..4 CERTIFICATE/DIPLOMA…...5 UNIVERSITY……6
103	To which ethnic group (tribe) do you belong?	HAUSA……..2 FULANI……..3 YORUBA……4 IGBO…… 5 OTHER…… 6 (if OTHER please write……)
104	Country of Origin	NIGERIAN

		OTHERS (SPECIFY)
105	State of Origin	…….
106	Town of Residence	……
107	State of Residence	…… FCT NIGER KADUNA LAGOS
108	Religion	CHRISTAIN…….0 MUSLIM……...1 TRADITIONAL ……..2 OTHERS (SPECIFY)……3
109	Are you currently married, single, divorced, widowed or separated?	MARRIED……2 WIDOWED…….3 DIVORCED……..4 SEPARATED……5 SINGLE/NEVER MARRIED…6
110	Could you tell me the number of people in your household?	1- 5……..…2 6- 10…….….3 >10……..4
111	The number of persons per room?	1-2……...2 3-5…….…..3 >5…….….… 4
112	What is your occupation:	FULL TIME FARMING…….2 PART TIME FARMING……3 ANIMAL HEALTH WORKE….4 HUMAN HEALTH WORKE….5 WORK AT ABBATTIOR…….6 MEAT VENDOR……..…7 BUSINESS/TRADING……8

		CIVIL SERVANT ……9 STUDENT……..10 ARTISAN……..11 DRIVER……..12 HOUSEWIFE…….13. MILITARY/SECURITY……..14 TEACHER……..15 COMMERCIAL SEX WORKER.. 16 UNEMPLOYED……..17 OTHER(specify)……18
113	For how long have you been sick?	……..Weeks ……Months
114	Please describe the signs and symptoms you noticed when you first became sick? FATIGUE, CHEST PAIN, COUGH WITH SPUTUM, NIGHT SWEATS, COUGH WITHOUT SPUTUM, BLOOD IN SPUTUM, WEIGHT LOSS, FEVER, CHILLS	NO……0 YES……1
115	Are you currently receiveing ART?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED…….99
116	Have you had X-ray examination for TB before?	NO……0 YES……1 DON'T KNOW NOT SURE……97

		REFUSED……..…… 99
117	Did you have X-ray examination during this current illness?	NO YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..…… 99
118	X-ray findings (cavitary disease e.t.c)	1…… …… 2…… ……. 3…… ……. 4…… …….
119	Do you have TB in other organs other than the lungs?	YES…… ……. NO …… ……
120	Do you know if any member of your family is having similar symptoms?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..…… 99
121	Have you had sputum examination for TB before?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..…… 99
122	Are you currently receiving treatment for TB?	NO…….. ….0

		YES……..… ……1 DON'T KNOW NOT SURE…..…97 REFUSED……..… 99
123	Was your treatment delayed before you commenced and if yes for how long	SPECIFY THE NO OF WEEKS…… UNKNOWN……
124	Have you ever received treatment of TB before?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..… 9
125	TB treatment was for how long	SPECIFY THE DAYS…… SPECIFY THE MONTHS……
127	Previous treatments	NEW CASE ……. RETREATMENT …….. UNKNOWN ……
128	No of previous treatments	0 1……. > 1 ……..
129	Outcome of the last treatment	NOT APPLICABLE UNKNOWN ……. CURED …….. TREATMENT COMPLETED …… FAILURE …… TREATMENT UNCOMPLETED …… TRANSFERRED OUT DATA MISSING
130	Does any member of your family or a close contact currently has a cough or receive treatment for TB?	NO…… …0

		YES……1 DON'T KNOW NOT SURE……97 REFUSED……99
131	Does any member of your family or a close contact have TB in the past?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……99
132	Does any member of your family has a swelling around the neck, receive treatment for neck swelling or have a swollen neck in the past?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……99
133	If any of the questions about your family receiving TB treatment/ received TB treatment in the past or having swelling in the past is true, does this person tend to cattle or other livestock?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……99
134	A BCG immunization is usually given only once in a person's lifetime and is given in the right arm. Have you ever had a BCG immunization or a sigh in your right arm suggestive of BCG immunization?	NO……0 YES……1 DON'T KNOW NOT SURE……97

		REFUSED……..…… 99
135	Have you ever smoked cigarettes in your life?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..…… 99
136	Have you smoked at least 100 cigarettes in your entire life? NOTE: 5 PACKS= 100 CIGARETTES	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..… 99
137	If you smoked up to 100 cigarettes in your life, how many cigarettes do you now smoke in a day?	NOT ANY MORE……0 Less than 1……2 1-2…….3 3-5…….4 More than 5……5 DON'T KNOW/NOT SURE…..97 REFUSED……. 99
138	Have you ever drunk alcohol?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……. 99

139	In the past 30 days, have you had at least one drink of any alcoholic beverage?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..…… 99
140	Are you an intravenous drug user	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..…… 99
141	Have you ever been tested for HIV?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..…… 99
142	Have you ever been told by a doctor, nurse or other health professional that you have diabetes mellitus?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..… 99
143	Are you currently being treated for diabetes mellitus?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..… 99
144	If yes, for how long?	…… Weeks ……Months

		…….Years
145	Have you ever been told by a doctor, nurse or other health professional that you have cancer?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……..99
146	Are you currently being treated for Cancer?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……..99
147	If yes, for how long?	…… Weeks ……Months …….Years
148	Are you having any other complicating health conditions? PNEUMOCOCONIOSIS……, MENTAL ILLNESS ……, LUNG INFECTION …….., OTHERS (SPECIFY)……..	NO YES
149	Have you been treated for any of the following in the past one year? READ OUT Gonorrhea, Syphilis, HPV(for female less than 50 years)	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……..99
150	Do you have unprotected sex with multiple partners within the last one year?	NO……0

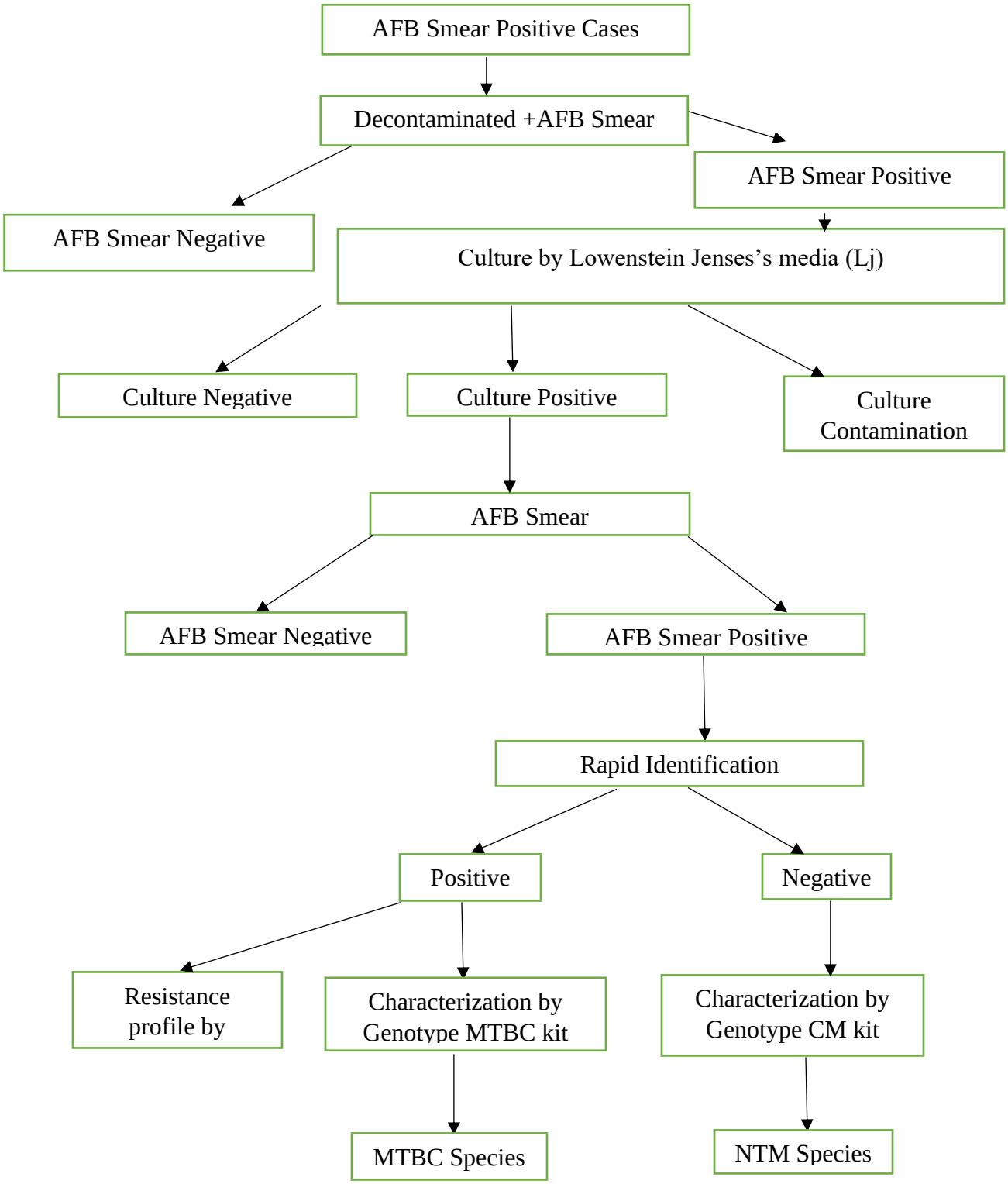
	Unprotected sex: having sex without using a new latex or polyurethane condom every time	YES……1
	Multiple partners: extra marital partners	DON'T KNOW NOT SURE……97 REFUSED……99
151	Do you have unprotected sex with someone who is HIV –positive?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……99
152	Have you ever receive a blood transfusion or blood products?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……99
153	Are you currently engaged in cattle rearing or other livestock farming?	NO……0 YES……1 DON'T KNOW NOT SURE…97 REFUSED…….99
154	How much time of the day do you spend tending to cattle or other livestock?	NO TIME SPENT……..…0 THE WHOLE DAY…….2 LESS THAN 1 HOUR……...3 1-3 HOURS……..…….4 4-6 HOURS……..…….5 >6HOURS < A DAY……..…….6
155	Which of the following livestock do you spend the time with?	CATTLE…….….. .2 SHEEP……..… …3 GOAT……..… …4

		MIXED (specify type)…….……5 OTHER(specify type)…….…….6 NOT APPLICABLE
156	Livestock (herd) size you spend time tending to?	0…… ….0 <10……..…… …2 10- 20……..……..3 21- 30……..……..4 >30……..…….. 5
157	Were there any sick cattle in your herd or in the neighborhood within the last 3 months?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……..… 99
158	Were there any cattle in 3 the past 3 months from your herd or in the neighborhood that had coughed or exhibited substantial loss of weight?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED…… 99
159	Were there any cattle in 3 the past 3 months from your herd or in the neighborhood that WAS found to have an animal TB?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED…… 99

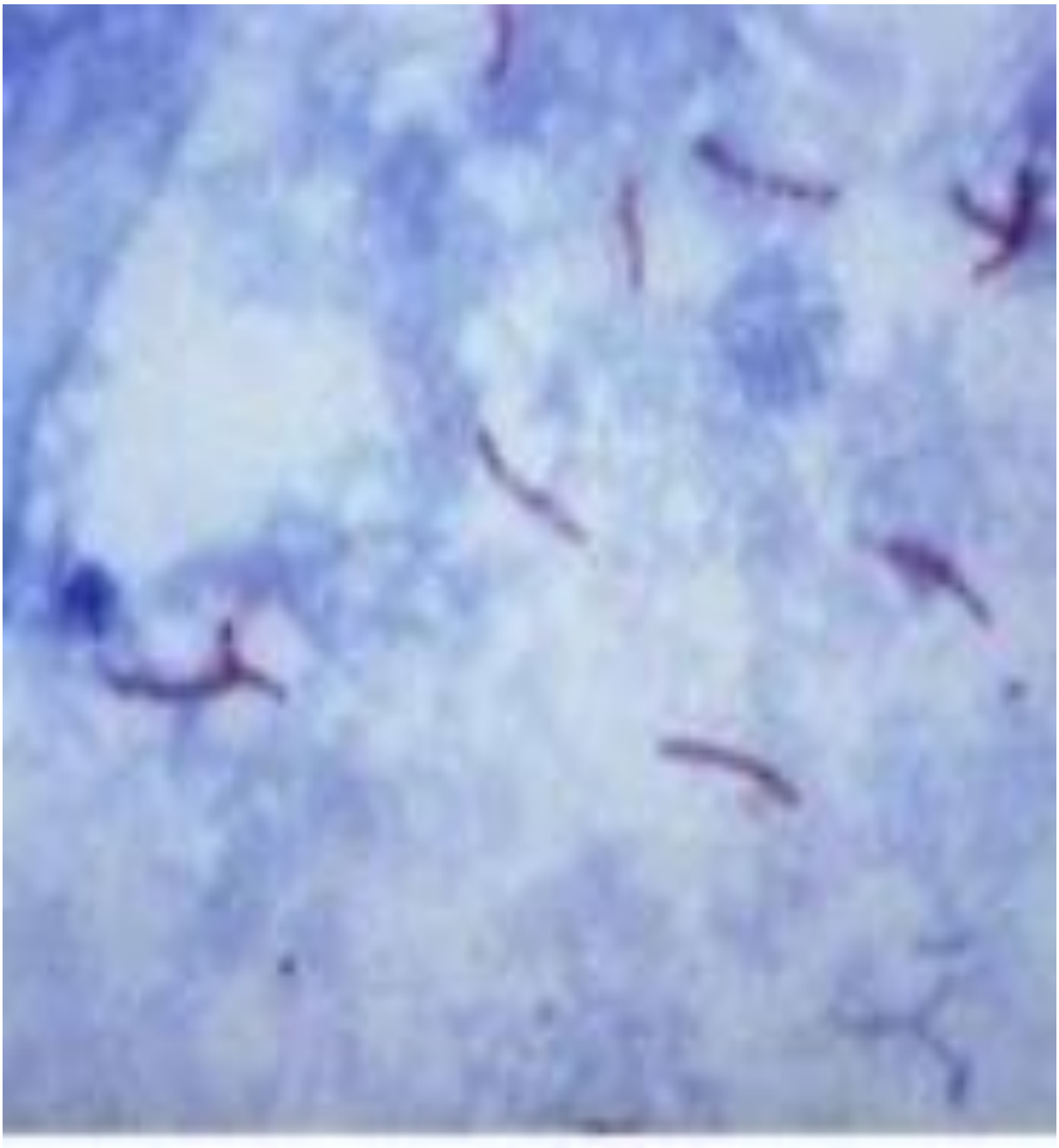
160	Do you observe any cattle from your herd with a swelling around the herd, neck or other parts of the body?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……..99
161	Any new cattle introduced into the herd within the last 3 months?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……..99
162	Do you milk breast feeding livestock? Cows? Goats? Other?	NO……0 YES……1 DON'T KNOW NOT SURE……97 REFUSED……..99
163	Do you consume the locally expressed milk from livestock or meals consisting of it?	NO……0 YES……1 DON'T KNOW NOT SURE...…97 REFUSED……..99
164	How often do you take the milk or meal consisting of it?	EVERY DAY……2 SOME DAYS……..……3 NOT AT ALL……..……4 DON'T KNOW/NOT SURE…97 REFUSED……99
165	In which form or forms do you consume the milk?	UNBOILED……..…..2

		BOILED……..….. 3 BOTH BOLIED AND UNBOILED.4 DON'T KNOW NOT SURE…..97 REFUSED……..… 99 NOT APPLICABLE OTHERS
166	Do you consume raw meat or blood?	NO…… …0 YES…… ……1 DON'T KNOW NOT SURE……97 REFUSED……. 99
167	If you cook meat before consumption for how long do you cook it?	5-10 minutes…….…..2 11-20 minutes……...3 21-30 minutes……4 Over 30 minutes……..…..5
168	Type of sample:	SPUTUM…… ….6 OTHERS (SPECIFY)……7 ……
169	Date of Sample collection:	……

Appendix V: Flow Chart of the Methodology for the anti-tuberculosis drug resistance of *Mycobacterium tuberculosis* complex among smear positive patients in the study population



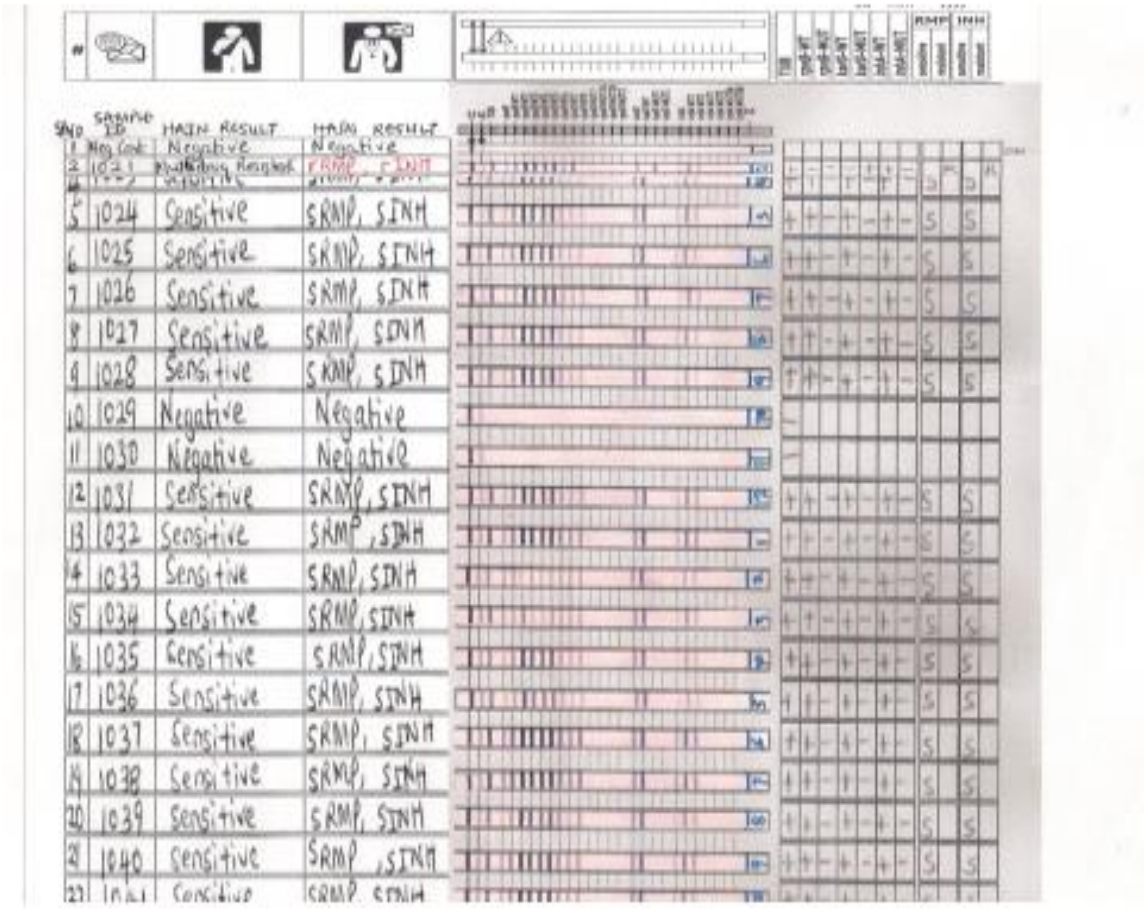
Appendix VI: Acid Fast Bacilli on stained slide



Appendix VII: Rapid Identification (SD Bioline) cartridge

a) Negative **b) Positive**

Appendix VIII: Hain Line Probe Assay Strips for GenoType MTBDRplus



SNo	Sample ID	HAIN RESULT	HAIN RESULT
1	Neg Cont	Negative	Negative
2	1023	Multidrug Resistant	RMP, INH
5	1024	Sensitive	SRMP, SINH
6	1025	Sensitive	SRMP, SINH
7	1026	Sensitive	SRMP, SINH
8	1027	Sensitive	SRMP, SINH
9	1028	Sensitive	SRMP, SINH
10	1029	Negative	Negative
11	1030	Negative	Negative
12	1031	Sensitive	SRMP, SINH
13	1032	Sensitive	SRMP, SINH
14	1033	Sensitive	SRMP, SINH
15	1034	Sensitive	SRMP, SINH
16	1035	Sensitive	SRMP, SINH
17	1036	Sensitive	SRMP, SINH
18	1037	Sensitive	SRMP, SINH
19	1038	Sensitive	SRMP, SINH
20	1039	Sensitive	SRMP, SINH
21	1040	Sensitive	SRMP, SINH
22	1041	Sensitive	SRMP, SINH