DETECTION OF EXTENSIVELY DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS AMONG PATIENTS ATTENDING NATIONAL TUBERCULOSIS AND LEPROSY TRAINING CENTRE SAYE ZARIA, NIGERIA

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JUNE, 2021

Declaration

I declare that the work in this dissertation entitled "**DETECTION OF EXTENSIVELY DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS AMONG PATIENTS ATTENDING NATIONAL TUBERCULOSIS AND LEPROSY TRAINING CENTRE SAYE ZARIA, NIGERIA**" has been performed by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this project thesis was previously presented for another degree, diploma at this or any other institutions.

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Certification

This dissertation entitled "**DETECTION OF EXTENSIVELY DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS AMONG PATIENTS ATTENDING NATIONAL TUBERCULOSIS AND LEPROSY TRAINING CENTRE SAYE ZARIA, NIGERIA**" by Mikailu SULEMAN meets the regulations governing the award of the degree of Master of Science in Microbiology of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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Dedication

To my beloved parents **Alh. SuleMada** and **HajiyaSalamatu**

Acknowledgement

All praise be to ALLAH our creator for His infinite mercy, Who granted me good health, strength and wisdom to finish this research work. May His peace and blessing be to His prophet Muhammad (S.A.W.), his family and his companion, ameen.

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Mikailu Suleman

ABSTRACT

This study was to detect the Extensively Drug Resistant *Mycobacterium tuberculosis* (XDR-TB) among patients attending National Tuberculosis and Leprosy Training Centre Saye Zaria, Nigeria using both phenotypic and genotypic methods. A total of 6125 patients were involved, out of which 775 (12.6%) were *Mycobacterium tuberculosis* (MTB) positive. Out of the 775, one hundred (100) were resistant to rifampicin by Xpert MTB/RIF with occurrence of 12.9%. Out of 100, 90 (90%) were culture positive while 7 (7%) were culture negative and 3 (3%) were contaminated. All of the ninety (90) samples that were culture positive were confirmed as MTBC using rapid immunochromatoghapic test (TB AgMPT64). Seventy 70 (77.7%) and sixty eight 68 (75.5%) isolates were found to be pan susceptible by Lowenstein Jensen (LJ) and MTBDR*sl* respectively. Overall 20 (22.3%) and 21 (23.3%) of the isolates were resistant to either Fluoroquinolones or Aminoglycosideby LJ and MTBDR*sl* assay respectively. One (1.1%) was detected as XDR-TB by MTBDR*sl* assay while XDR-TB was not detected by LJ proportion method. There was no statically significant in the socio-demographic and risk factors observed among the study population,though the occurrence of pre XDR-TB was among age groups 31-45 and 16-30 years and it was also observed more in male 13 (65%) compared to the female 7 (35%). The occurrence of pre XDR-TB was observed to be12 (60%) among urban resident than those living in rural setting 8 (40%), the occurrence of pre XDR-TB in this study was observed to be among non-reactive HIV patients 12 (60%). The occurrence of pre XDR-TB in relation to alcohol consumption was 18 (90%) among those that have not consume alcohol before and the occurrence of pre XDR-TB was among those that were not previously treated with TB drugs was more than 80%. This study has shown high occurrence of drug resistant

tuberculosis among the study population. Pre-XDR-TB detected by both methods in this study, serve as possible indicator of the future emergence of XDR-TB among patient attending NTBLTC SayeZaria, Nigeria. There is a need for close monitoring of TB patients for proper treatment and compliance to prevent drug resistant tuberculosis. This study has shown an overall high occurrence of drug resistance and it also demonstrate that the occurrence of pre-XDR-TB is high among the study population. Therefore, there is urgent need to increase case detection across Nigeria using rapid molecular test for early and accurate diagnosis of drug resistant tuberculosis.

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List of Abbreviations

16S rRNA	16S ribosomal ribonucleic acid
ACP	Acyl carrier protein
AIDs	Acquired Immuno-Deficiency Syndrome
AFB	Acid Fast Bacilli
ahpC	alkylhydroperoxide reductase
ALA	American Lung Association
A-Lys	Lysis Buffer
AM-A	Amplification Mix A
AM-B	Amplification Mix B
Amk	Amikacin
AMG	Aminoglycosides
A-NB	Neutralizing Buffer
ATP	Adenosine triphosphate
BCG	BacilleCalmette-Guerin
BD	Becton Dickson
BD MGIT TBc complex	Becton Dickson *Mycobacterium* growth indicator tube tuberculosis

BSL	Bio safety level
CAP	Capreomycin
CD4 T	Cluster of Differencial4 T cell
CD8 T	Cluster of Differencial8 T cell
CDC	Centre for Disease Control
CMI	Cell mediated immunity
CRI	Colorimetric Redox Indicator
ddNTPs	Dideoxynucleotides triphosphate
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotides triphosphate
DOTS	Directly Observed Treatment Short Course
DPA	Decaprenylphosphoryl-β-D-arabinose
DRs	Direct Repeats
DST	Drug susceptibility Test
ECDC	European Centre of Disease Prevention and Control
embB	Arabinosyltransferase B gene
embC	Arabinosyltransferase C gene

FLQ	Fluoroquinolones
*gyr*A	Gyrase A
*gyr*B	Gyrase B
H37Rv	Human 37 Rough Virulent Strain
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon gamma
IGRA	IFN-γ-release assays
INH	Isoniazid
inhA	Enoyl [acyl carrier protein] reductase
IV	Intravenous
kasA	β-ketoacylsynthase A gene
katG	Catalase-peroxidaseG gene
KM	Kanamycin
LED	Light Emitting Diodes
LJ	Lowenstein Jensen
LJPM	Lowenstein Jensen Proportion Method
LOD	Limit of Detection

LPA	Line Probe Assay
LTBI	Latent Tuberculosis Infection
MDR-TB	Multi drug resistant Tuberculosis
MIC	Minimum Inhibitory Concentration
MODS	Microscopy Observation Drug-Susceptibility
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MTB/RIF	*Mycobacterium tuberculosis*/Rifampicin
MTBDR*sl*	*Mycobacterium tuberculosis* Drug Resistant second line
NAAT	Nucleic Acid Amplification Test
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NALC-NaOH	N-acetylL-Cystein Sodium hydroxide
NIAID	National Institute of Allergy and Infectious Diseases
NPV	Negative Predictive Value
NRA	Nitrate Reductase Assay
NTBLCP	National Tuberculosis and Leprosy Control Programme

NTBLTC	National Tuberculosis and Leprosy Training Centre
NTM	Non tuberculous Mycobacteria
NTRL	National Tuberculosis Reference Laboratory
oxyR-ahpC	Oxidative stress regulator-alkyl hydroperoxidase C
PCR	Polymerase Chain Reaction
PM	Proportion Method
PP	Purified Protein
PPD	Purified Protein Derivatives
PPV	Positive Predictive Value
Pre XDR-TB	Pre extensively Drug Resistant Tuberculosis
PZA	Pyrazinamides
QRDR	Quinones Resistant Determining Region
RIF	Rifampicin
RR	Rifamficin Resistant
rRNA	Ribosomal Ribonucleic acid
RR-TB	Rifampicin Resistant Tuberculosis
RT-PCR	Real Time Polymerase Chain Reaction

SLID	Second Line Injectable Drugs
SL-LPA	Second Line-Line Probe Assay
TB	Tuberculosis
TlyA	rRNA methyltransferase
TNF	Tumor Necrotic Factor
UV	Ultra Violet
WHO	World Health Organization
XDR-TB	Extensively drug resistant tuberculosis

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Tuberculosis (TB) is one of the world's deadliest infectious disease, it is estimated that one-third of the global population is infected with *Mycobacterium tuberculosis*complex(MTBC), it claims three lives every minute (WHO, 2016). There were an estimated 10 million new TB cases worldwide, 5.7million (57%), 3.2 million (32%), and 1.1 million (11%) were among men, women and children respectively. Overall, 89% and 11% of the cases were among adults and children while male to female ratio was 1.6:1 (WHO, 2019). There were 1.2 million TB deaths among HIV negative people in addition to the 251 000 deaths among HIV positive people while TB remained one of the top 10 causes of death worldwide (WHO, 2019).Nigeria is ranked 4th among 20 high TB burden countries with tuberculosis; the country has an estimated prevalence of pulmonary TB in Adults of 219 and 429 per 100,000 populations between 15 years and above (WHO, 2019).

Multi-drug resistant tuberculosis (MDR-TB) refers to strain of *Mycobacterium tuberculosis* that is resistant to at least two most important anti-TB drugs rifampicin (RIF) and Isoniazid (INH). All cases of rifampicin–resistant TB (RR-TB), including those with multidrug-resistant tuberculosis (MDR-TB), should be treated with a second line MDR-TB regimen (WHO, 2016). The number of reported MDR/RR-TB cases has increased between 2014 and 2015 by more than 20% in four of the 30 high MDR-TB burden countries which are China, Nigeria, Philippines and Ukraine. In Nigeria, there were estimated 4.3% of news cases and 15% among previously treated cases with MDR/RR-TB (WHO, 2019).

Pre-extensively drug resistant tuberculosis (pre-XDR-TB) is defined as TB with resistance to rifampicin and isoniazide with additional resistance to either a Fluoroquinolone (FLQ) or

Aminoglycosides (AMG) drugs but not against both FLQ and AMG drugs simultaneously (Banerjee *et al*., 2008). Thus pre-XDR-TB cases receive less number of effective drugs under standard MDR-TB regimen. It may amplify further resistance to the effective drugs and progression towards XDR-TB (Extensively drug resistant TB). XDR-TB is usually developed from multidrug-resistant (MDR) TB, which is resistant to rifampin and isoniazid. MDR-TB typically requires two years of treatment with second-line drugs, which is more expensive and more toxic than first-line drugs (Ahmad and Mokaddas, (2014). The low rate of diagnosis and diagnostic delay, the limited access to second-line drugs, and the poor adherence of MDR-TB patients have mainly led to the emergence of XDR-TB (Heysell *et al*., 2012). Most of the XDR-TB and Pre-XDR-TB patients in China were new cases, indicating the transmission of resistant strains (Jain and Dixit, 2008). As a result, MDR-TB and XDR-TB have emerged as significant threats to global TB control (WHO, 2008). The emergence of XDR-TB strains is a reflection of poor tuberculosis management and control, and this situation should be considered as an urgent global health problem, especially in developing countries and those lacking resources (WHO, 2014). The average proportion of MDR-TB cases with XDR-TB was 9.5% (WHO, 2016).

The Xpert MTB/RIF detects DNA sequences specific for *Mycobacterium tuberculosis* and rifampicin resistance by polymerase chain reaction (WHO, 2010). It is based on the Cepheid Gene Xpert system, a platform for rapid and simple-to-use Nucleic Acid Amplification Tests (NAAT). The Xpert® MTB/RIF purifies and concentrates *Mycobacterium tuberculosis* bacilli from sputum samples, isolates genomic material from the captured bacteria by sonication and subsequently amplifies the genomic DNA by real time PCR. Results are obtained in less than 2 hours, with minimal biohazard and very little technical training required to operate (Boehme*et al*., 2010).

Culture of *Mycobacterium* provides the definitive diagnosis of tuberculosis and is considered the gold standard for the bacteriological confirmation of the *Mycobacterium tuberculosis* disease, while also allowing the opportunity to perform drug susceptibility testing. Culture on solid media is the most widely used and Lowenstein Jensen (LJ) is the most commonly used egg-based medium. Like other solid media, LJ allows direct visual recognition of colonial characteristics of *M. tuberculosis* and growth of the contaminants. Cultures on the LJ medium may take as long as eight (8) weeks before they become positive, especially if the inoculated clinical specimen contains few bacilli (Sharma *et al*., 2012).

The conventional phenotypic methods for drug susceptibility testing are based on inoculation of cultured isolates on solid media. The proportion method is inexpensive and highly standardized for testing susceptibility to many drugs, but has a long turnaround time (Sharma *et al*., 2012). Proportion method is the most commonly used method worldwide. The test is based on the exact determination of an inoculum proportion of organisms present in the media that is resistant to a specific concentration of each drug, by comparing quantity of growth in a drug-containing and drug-free control media and when performed in egg-based media, the final reading is done after 42 days of incubation. Furthermore the method is labour intensive and requires a careful quality control of all batches produced with drug susceptible and drug resistant strains for reliable results (Sagonda *et al*., 2014).

In 2008, World Health Organization (WHO) recommended the use of Line Probe Assay (LPA) for rapid screening of MDR-TB in low and middle income settings (WHO, 2008). LPAuse multiplex polymerase chain reaction (PCR) amplification and reverse hybridization to identify *Mycobacterium tuberculosis* complex and mutations to genes associated with rifampicin and isoniazid resistance (WHO, 2008). In 2015, the manufacturer developed and

made commercially available version 2.0 of the MTBDR*sl* assay and was approved by World Health Organization in May 2016. Version 2.0 detects the mutations associated with fluoroquinolone and second line injectable (Kanamycin, Capreomycin and Amikacin) drugs (SLID) resistance detected by version 1.0, as well as additional mutations. Once a diagnosis of rifampicin-resistant TB (RR-TB) or multidrug-resistant TB (MDR-TB) has been established, SL-LPA can be used to detect additional resistance to second-line drugs (Theron, 2016). The MTBDR*sl* assay is coated with 27 probes to detect mutations within genes (*gyr*A and *rrs* for version 1.0 and, in addition, *gyr*B and the *eis* promoter for version 2.0), which are associated with resistance to either fluoroquinolones or SLIDs (Brossier *et al*., 2016).

1.2 Statement of Research Problem

Drug Resistant Tuberculosis (DR-TB) threatens the global burden of tuberculosis care and prevention and it remain a major public health concern (Jassal and Bishai, 2009; WHO, 2019). GeneXpert MTB/Rif assay can only detect *Mycobacterium tuberculosis* and it's resistance to rifampicin (WHO, 2010). Culture on Lowenstein-Jensen solid medium require about 100 viable bacilli/ml of specimen for recovery of Mycobacterium, and is the gold standard for microbiological diagnosis of tuberculosis in developing countries (Morcillo *et al*., 2008). The proportion of MDR/RR-TB with resistance to any fluoroquilones for which testing was done is 20.8% and a total of 13,068 cases of XDR-TB were reported in 2018 (WHO, 2019).

Patientswith XDR-TB have a significantly poorer prognosis than patients with TB caused by drug susceptibility *Mycobacterium tuberculosis*, they have a higher probability of death, treatment failure, longer hospitalization and treatment duration (Johnston *et al*., 2009). Conventional phenotypic method takes weeks to month to fully define the drug resistance profile of *Mycobacterium tuberculosis* isolates due to the slow growth rate of the bacterium (Kent and Kubica, 1985; Kim. 2005). Inappropriate treatment regimen during the period till the Drug Susceptibility Test (DST) result are available may result in increase of resistance and further transmission of these resistant strains (Ajbani *et al*., 2012). Furthermore, due to financial, infrastructural and human resource requirements, widespread implementation of culture-based DST may be challenging in such settings (Albert *et al*., 2010). Specimen transport and specimen contamination issues may also present further challenges (WHO, 2009).

1.3 Justification of the Study

In Nigeria, 31 laboratory confirmed cases of XDR-TB were reported (WHO, 2019). There is increase risk of TB infection and nosocomial transmission of MDR-TB and extensively drug-resistant TB (XDR-TB) in hospitalized patients to health care worker and givers (WHO, 2019).Lowenstein Jensen Proportional method (LJPM) is the gold standard for drug susceptibility testing (DST) of *Mycobacterium tuberculosis* andis widely use (Ajbani *et al*., 2012). Rapid detection of *Mycobacterium tuberculosis* and its drug susceptibility testing (DST) to second line drugs are critical in areas with high rate of RR-TB and MDR-TB and settings with limited conventional DST capacity to the successful control of TB (WHO, 2011; Tagliani, *et al*., 2015).

The molecular based line probe assay (LPA) has a shorter turnaround time (within 48 hours) compared to conventional drug susceptibility testing (Gardee*et al*., 2017). Genotype MBDRsl also enable the detection of mutation involved in resistance to second line injectable (rrs and eis genes) and fluoroquinolones (gyr A and gyr B genes) (Hain Life Science, 2015). Therefore, early detection of drug resistance is crucial to prevent the transmission of drug-resistant TB and averting mortality rate (Barnard *et al*., 2008). Detection and treatment of TB patients with appropriate anti-TB drugs timely and optimally will reduce the infectiousness of the patients in spread of droplet nuclei containing TB bacilli, there by breaking the chain of transmission.

1.4 Aim of the Study

The aim of the study was to detect extensively drug resistant *Mycobacterium tuberculosis* among patients attending National Tuberculosis and Leprosy Training Centre Saye, Zaria.

1.5 Objectives of the Study

The specific objectives of the study were to:

1. Detect rifampicin resistant *Mycobacterium tuberculosis* in sputum from patients attending NTBLTC Saye, Zaria using Xpert MTB/RIF Assay.
2. Isolate and identify *Mycobacterium tuberculosis* using Lowenstein Jensen (LJ) medium and TB Ag MPT64 test.
3. Detect extensively drug resistant *Mycobacterium tuberculosis* (XDR-TB) from patients attending NTBLTC Saye, Zaria using LJ proportion method and Genotype MTBDR*sl* assay.
4. Determine the socio demographic and risk factors associated with XDR-TB patients within the study population.

1.6 Research Questions

1. Is there XDR-TB among patients attending NTBLTC Saye?
2. If yes, what is the occurrence of XDR-TB among patients attending NTBLTC Saye?
3. What are the socio-demographic and risk factors predispose to XDR-TB among patient attending NTBLTC Saye?

CHAPTER TWO

LITARATURE REVIEW

2.1 Mycobacteria

All species of mycobacteria have rope like structures of peptidoglycan that are arranged in such a way to give them properties of acid fast bacteria (Uhía, 2011). *Mycobacterium tuberculosis* is an acid fast bacterium, which can form acid-stable complexes when certain dyes are added (Uhía, 2011). Mycobacteria are abundant in soil and water, but *Mycobacterium tuberculosis* is mainly identified as a pathogen that lives in the host. Some species of*Mycobacterium tuberculosis* complex have adapted their genetic structure specifically to infect human populations.

2.1.1 Classification of mycobacteria

Kingdom Bacteria

Phylum Actinobacteria

Order Actinomycetales

Family Mycobacteriaceae

Genus *Mycobacterium*

Mycobacteria are classified based on the production of pigments, they can be classified as scotochromogens (produce yellow pigment in the dark) for example *M. scrofulaceum, M. gordonae*; photochromogens (produce an orange pigment in the light) for example *M. kansasii*, *M. marinum* and nonphotochromogens (do not produce any pigment) for example *M. avium, M. intracellularae* and *M. ulcerans*. The cultivable members of the genus can be divided into two main groups on the basis of growth rate; they can be grouped into fast growers, which include *M. fortuitum, M. kansasii, M. smegmatis* and slow growers,

comprising mostly the pathogenic mycobacteria, including *M. tuberculosis, M. bovis and M.kansasii* (Tortoli, 2003; Ryan, 2010).

2.1.2*Mycobacterium tuberculosis*complex

Mycobacterial species that cause TB in humans and other mammalian hosts are collectively known as tubercle bacilli and are grouped within the *Mycobacterium tuberculosis* complex (MTBC). In humans, TB is primarily caused by *M. tuberculosis* and *M. africanum* (sub-Saharan Africa). In addition, several animal-adapted members of MTBC have been identified. These include, *M. bovis* (cattle) and its attenuated derivative *M. bovis* BCG (vaccine strain), *M. microti* (voles and other small rodents), *M. pinnipedii* (seals and sea lions) and *M. caprae* (goats and sheep) (Schaaf and Zumla 2009). The genomic revolution in the last decade has facilitated the application of a variety of powerful tools, in the field of TB research that has led to great advances in the understanding of the biology and pathogenesis of the tubercle bacilli (Schaaf and Zumla 2009).

2.1.3 Genome structure

Mycobacterium tuberculosis has circular chromosomes of about 4,200,000 nucleotides long. The G+C content is about 65% (Ouellet *et al*., 2010). The genome of *M. tuberculosis* was studied generally using the strain *M. tuberculosis* H37Rv. The genome contains about 4000 genes, genes that code for lipid metabolism are a very important part of the bacterial genome and 8% of the genome is involved in this activity (Willia*et al*., 2008). The different species of the *Mycobacterium tuberculosis* complex show a 95-100% DNA relatedness based on studies of DNA homology, and the sequence of the 16S rRNA gene are exactly the same for all the species. Scientists suggest that they should be grouped as a single

species while others argue that they should be grouped as varieties or subspecies of *M. tuberculosis* (Brzostek *et al*., 2009). Plasmids in *M. tuberculosis* are important in transferring virulence because genes on the plasmids are more easily transferred than genes located on the chromosome. One such 18kb plasmid in the *M. tuberculosis* H37Rv strain was proven to conduct gene transfers through conjugation (Brosch *et al.*, 2002).

2.1.4 Cell structure and metabolism

Mycobacterium tuberculosis has a tough cell wall that prevents passage of nutrients into and excreted from the cell, therefore giving it the characteristic of slow growth rate (Vander *et al*., 2007). Mycobacteria are Gram-positive and acid fast bacilli (Brosch *et al.*, 2002). The cell containspeptidoglycan layer, Arabinogalactan and superficial lipids. In addition, there is also a complex structure of fatty acids such as mycolic acids that appear glossy (Vander *et al*., 2007). There are porins in the membrane to facilitate transport. Beneath the cell wall, there are layers of arabinogalactan and peptidoglycan that lie just above the plasma membrane (Thomas *et al*., 2011).

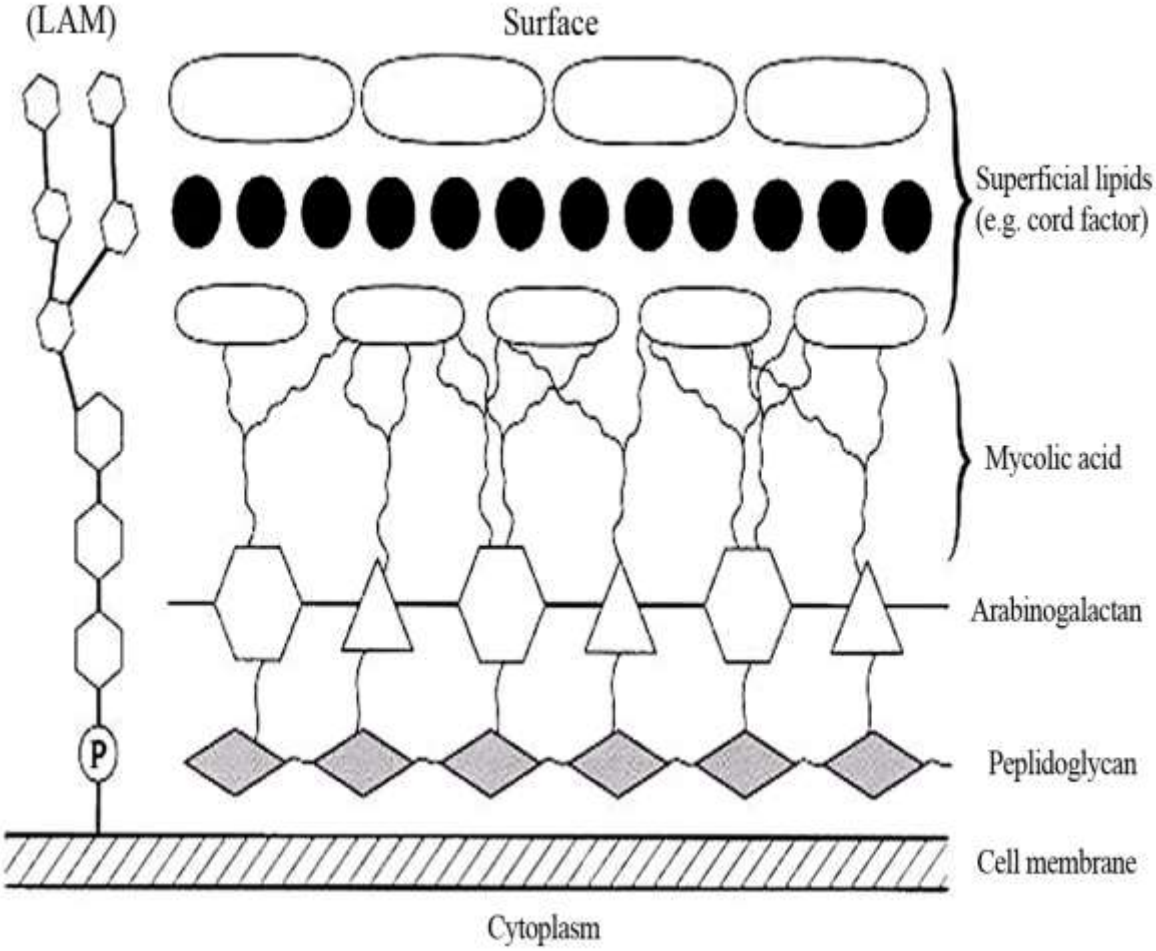


Figure 2.1: Mycobacteria cell wall complex structure; source: *Journal of Tuberculosis Research (*2015) 3:184-205

2.1.5 Growth characteristics

Mycobacterium tuberculosis is obligate aerobes; grows very slow in media, optimum temperature is 37°C and do not grow below 25°C or above 40°C, their reproduction is enhanced by the presence of 5-10% CO_2 in the atmosphere. The generation time of TB is approximately 12-18 hours, so that cultures must be incubated for six to eight weeks at 37°C until proliferation becomes macroscopically visible (Kent and Kubica, 1985). Broth-based culture systems to improve the speed and sensitivity of detection have been developed (Hanna, 1995). In AFB smear-positive specimens, the BACTEC system can detect *M. tuberculosis* in approximately eight days (compared to approximately 14 days for smear-negative specimens (Roberts, 1983).

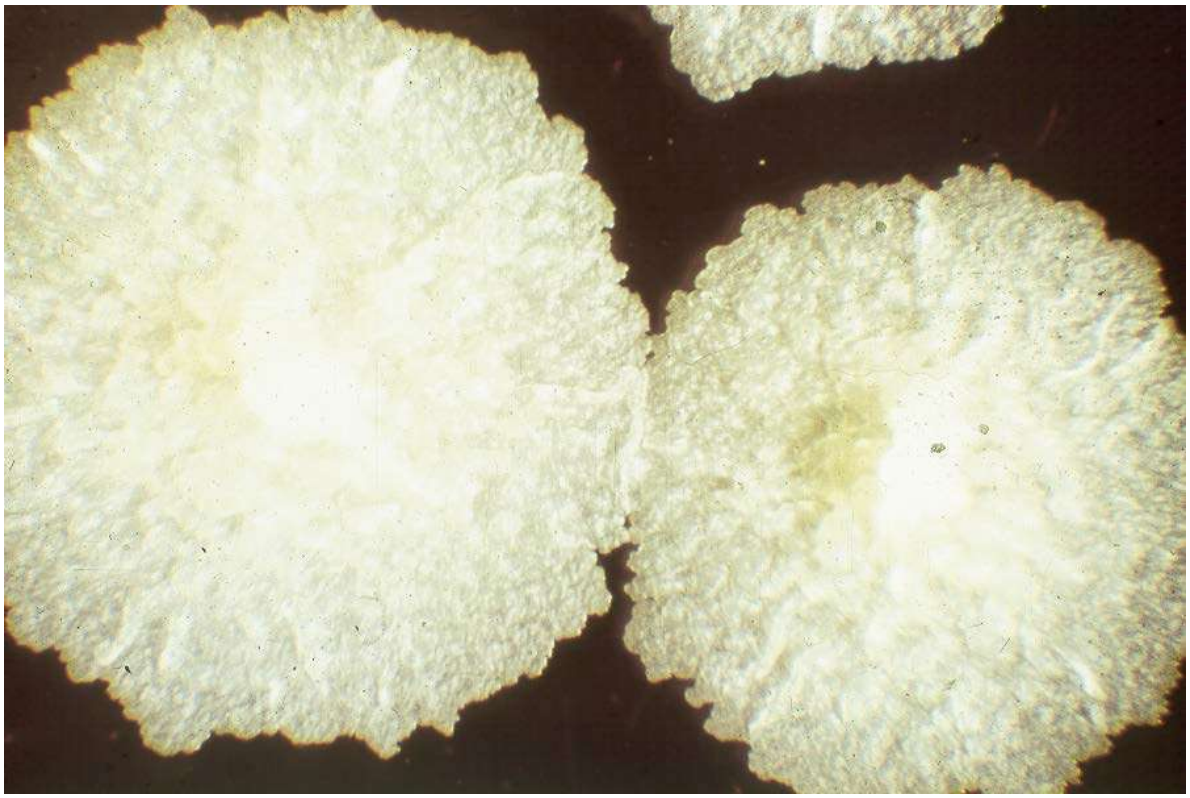


Figure 2.2 Colony morphology of MTB on LJ media; **Source:** ***Journal of tuberculosis research*****, 2015, 3:**184-205

2.1.6 Tuberculosis

Tuberculosis (TB) remain a major public health problem andone of the top 10 causes of death worldwide, ranking above HIV/AIDS as one of the leading causes of death from an infectious disease (WHO, 2015). Tuberculosis is an infectious disease caused by the bacillus *Mycobacterium;* it usually affects lungs known as pulmonary TB but can also affect other part of the body known as extrapulmonary TB. The disease is spread when people who are infected with active pulmonary TB expel bacteria into the air; through coughing, sneezing, talking e.t.c. Small proportion (5–15%) of the estimated one third of the world population infected with *M. tuberculosis* will develop TB disease during their lifetime. However, the risk of developing TB disease is much higher among people infected with HIV (WHO, 2016).

2.1.7 History of Tuberculosis

Archaeological evidence from Egyptian mummies indicated manifestation of TB as early as 5000 BC (Schaaf and Zumla 2009). TB was designated "The Captain of all the Men of Death" as it continued to claim lives through the ages (Daniel 2006). In the 18th and 19th centuries, TB reached epidemic proportions in Europe and North America and was referred to as "The Great White Plague" (Anna, 2013). In 1882, the German scientist Robert Koch identified *Mycobacterium tuberculosis* as the causative agent of TB which changed the course of TB. The tubercle bacilli discovery paved the way for great advances in the areas of tuberculosis prevention, diagnosis and control. As at 1907, tuberculin skin test was developed by Clemens von Pirquet for diagnosing TB and used it to demonstrate latent TB infection in asymptomatic children (Daniel, 2006).

In 1908, Albert Calmette and Camille Guérin attenuate *Mycobacterium bovis* which finally led to the development of the BCG (Bacille Calmette- Guérin) vaccine in 1921 (Calmette 1931; Behr, 2001). BCG remains the most widely used vaccine in the world. In the decades that followed, a therapeutic revolution took place with the discoveries of para-amino salicylic acid (PAS) in 1943 and streptomycin in 1944 (Daniel 2006; Zumla *et al.* 2009). Isoniazid became the first oral anti-TB drug in 1952, followed by rifampicin in 1963 (Daniel 2006). In the early 1970s, short-course chemotherapy regimens was developed and proved to be highly efficient in the treatment of TB (Schaaf and Zumla, 2009; Zumla *et al.* 2009). Even before the introduction of BCG and chemotherapy, improvements in the socio economic conditions and living standards had led to a decrease in the death toll from TB in the Western world. As tuberculosis was no longer considered a threat to developed countries funding for tuberculosis research declined. However, at the same time, TB assumed greater prevalence in many poor and developing countries (Anna, 2013). There was a global resurgence of TB by the late 1980s primarily due to the arrival and spread of HIV infection and the emergence of drug resistant strains of MTB. This combined with the immigration from high-incidence countries, the deterioration in the quality of TB control, and intermittent adherence to treatment fuelled the TB epidemic further (Murray 2004; Zumla *et al.* 2009).

In the 1990s, it was estimated that one-third of the world's population had been infected with MTB(LTBI) putting these individuals at risk of developing active TB at a later stage (Dye *et al.* 1999). HIV infection was identified as the most powerful risk factor for progression of TB from latency to active disease, along with diabetes, malnutrition, old age and other factors leading to immunosuppression (Aaron *et al.* 2004; Corbett *et al.* 2006; Lawn and Zumla, 2011). The World Health Organization (WHO) declared TB a global

emergency in1993, and re-established its TB control program by promoting the Directly Observed Therapy Short-Course (DOTS) strategy in 1994. The DOTS strategy relies on efficient case detection, standardized chemotherapy, uninterrupted drug supply, standardized recording and reporting systems and government commitment in order to cope with the high burden of TB (Espinal *et al.* 1999; Schaaf and Zumla, 2009). In 2001, the Stop TB Partnership was established with the goal of eliminating TB as a public health problem, the partnership outlined a global plan to reduce TB prevalence and mortality by 2015 and to eliminate TB as a public health problem by 2050 (Stop TB Partnership, 2006). Despite the growing number of local, national and international research and TB control initiatives in the past decade TB is still among the top causes of death and morbidity worldwide, especially in the low and mid-income countries (WHO, 2019).

2.1.8 Evolution

Tuberculosis is caused by members of the specie *Mycobacterium tuberculosis* complex (MTBC), which includes: *Mycobacterium tuberculosis* (*MTB*), the etiologic agent of TB in humans; *M. africanum*, that causes TB in humans only in certain regions of Africa; *M. bovis*, *M. caprae* and *M. pinnipedii*, *M. canettii* causing TB in wild and domesticated mammals; *M. microti*, that causes TB only in voles. With the identification of new properties and allowed the reconstruction of the history of *MTB* as a global human infectious agent (Brosch*et al*.,2002). *Mycobacterium tuberculosis* emerged as a human pathogen in Africa around 70.000 years ago and then spread out of the continent following human migrations (Brosch*et al*., 2002; Gutierrez *et al.*, 2005). It is now widely accepted that the ancients *M. tuberculosis* strains originated from environmental mycobacteria, that can still be isolated from immunocompromised patients in certain parts of east Africa, are

unable to cause chronic persistent infection in the immuno-competent host and are not transmitted among humans (Supply *et al*., 2013). These ancient *M. tuberculosis*strains evolved, through a genetic bottleneck, so to persist in low density populations, causing disease reactivation following long period of latent infection (Blaser *et al*.,2007). Following domestication, humans were able to transmit the disease to animals and *M. bovis* emerged as a pathogen of domesticated and wild animals (Brosch *et al*.,2002). The introduction of agriculture, civilization and the increase in human population density in urban areas led to the selection of *MTB* strains with enhanced virulence and transmissibility that are named modern *MTB* strains (Wirth *et al*., 2008; Comas *et al*., 2013). The modern *MTB* strains spread throughout the world causing the TB epidemics that ravaged mankind for centuries and these strains are responsible for most of the TB cases nowadays (Gagneux, 2011).

2.1.9 Epidemiology of Tuberculosis

Tuberculosis (TB) has existed for decades and its remains a major global health problem. It causes disease in millions of people each year and tuberculosis is one of the 10 high ranking causes of death globally in 2015, ranking above HIV/AIDS as one of the leading causes of death from an infectious disease (WHO, 2016). This is despite the fact that with a timely diagnosis and correct treatment, most people who develop TB disease can be cured. It has been estimated that one-third of the world's population is infected with *M. tuberculosis* and roughly 10% of these individuals will develop active tuberculosis within their lifetime. Based on the world health organization report, there were an estimated 10.4 million new TB cases worldwide, 5.9million (56%), 3.5 million (34%), and 1.0 million (10%) were among men, women and children respectively. Overall, 90% and 10% of the cases were among adults and children while the ratio of male to female was 1.6:1.

People living with HIV accounted for 1.2 million (11%) of all new TB cases, the proportion was highest in countries in the WHO Africa region, and exceeded 50% in parts of Southern Africa. In addition to the 1.4 million TB deaths among HIV negative people, there were 0.4million deaths from TB among HIV positive people in 2015 (WHO, 2016). With the rise in HIV infections, tuberculosis has been on the rise and death due to tuberculosis in HIV-infected people is two-fold higher in individuals with only HIV infection.

Nigeria is ranked 4th among 20 high TB burden countries with tuberculosis; the country has an estimated prevalence of pulmonary TB in Adults of 318 and 524 per 100,000 populations between 15 years and above (WHO, 2016). There were estimated total TB incidence of 586 and 100 among HIV-negative and HIV-positive TB per 100,000 respectively, with an estimated mortality rate of 180 and 57 per 100,000 among HIV-negative and HIV-positive respectively (WHO, 2016).Anyone can get TB; however, some groups are at higher risk to active TB disease. People such as those with HIV infection, close contact with those known to be infectious with TB, with medical conditions such as diabetes, or people undergoing treatment with drugs that can suppress the immune system (such as long-term use of corticosteroids) from countries with high TB rates who work in or are residents of long-term care facilities (nursing homes, prisons, some hospitals) who are malnourished and who are alcoholics or intra venous (IV) drug users (ALA, 2009).

2.1.10 Tuberculosis (TB) Pathogenesis

Mycobacterium tubeculosis infection occurs when few tubercle bacilli dispersed in the air from a patient with active pulmonary TB reach the alveoli of the host. However, not everyone infected with TB bacteria becomes sick, people who are infected but not sick have latent TB infection and those who have a latent infection are asymptomatic and are not

contagious while only 5-10% of people infected with *M. tuberculosis* actually develop TB. At this stage, *MTB* is quickly phagocytized by professional alveolar macrophages that most often can kill the entering bacteria due to the innate immune response (Urdahl *et al*., 2011). If the bacilli can survive this first line of defense, it starts actively replicating in macrophages; diffuse to nearby cells including epithelial and endothelial cells, reaching in few weeks of exponential growth a high bacterial burden (Wolf *et al.,* 2008). During these early steps of infection, *MTB* can diffuse to other organs through the lymphatics and by haematogenous dissemination where it can infect other cells (Giovanni *et al*., 2013). Thereafter, once the adaptive immune response kicks in, migration to the site of primary infection of neutrophils, lymphocytes and other immune cells form a cellular infiltrate that later assume the typical structure of a granuloma (Ottenhoff and Kaufmann, 2012).

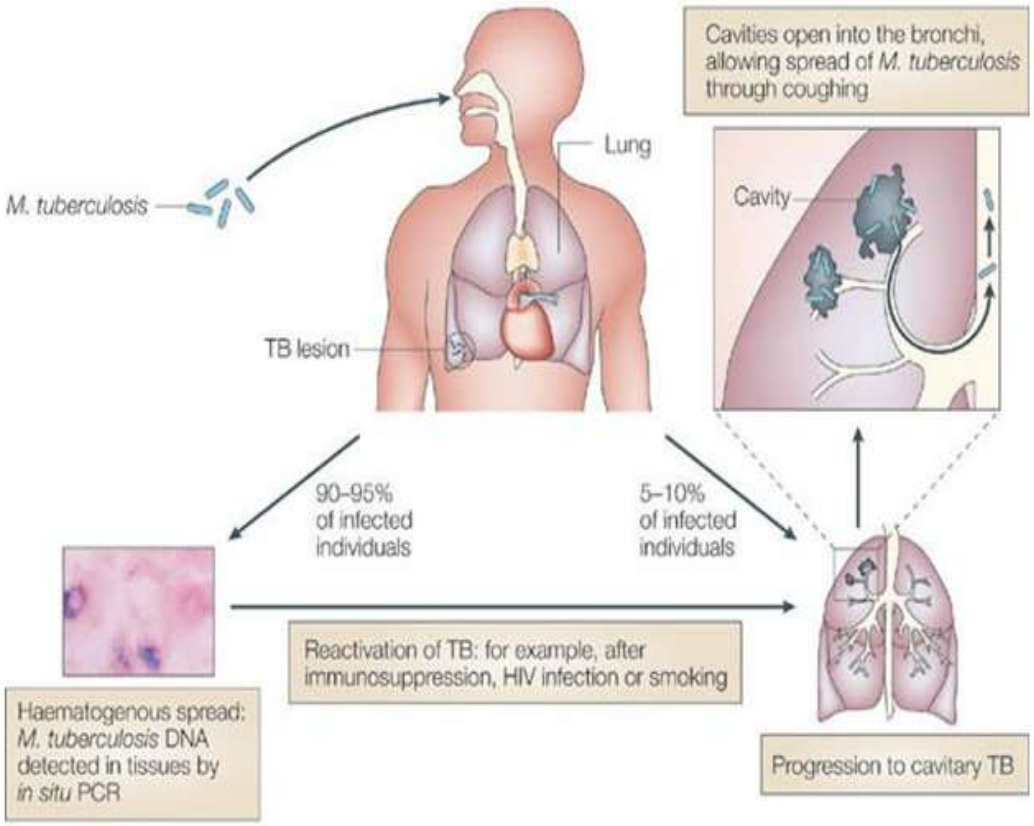


Figure 2.3: *Mycobacterium tuberculosis* infection and disease progression, Nature review |Immunology (2005).

Fibrotic components cover the granuloma that becomes calcified such that bacilli remain encapsulated inside and protected by the host immune response. This primary lesion, classically termed the Ghon complex was thought to be the "sanctuary" of *MTB* during latent infection, with bacilli persisting in a dormant, non-metabolically active state, for years, decades, or most often for lifetime. In this scenario, during latent infection, for unknown reasons, bacilli would start replicating inside this primary lesion, active disease would ensue (Bishai, 2000). A major corollary of this hypothesis, with relevant patho-physiological and clinical implications, was that reactivation of TB originated from this very primary site of infection. This hypothesis was challenged since the early 20th century, when it was shown that viable and infective bacilli were found in unaffected portion of lung tissues of infected guinea pigs or human necropsy rather than from the central core of the tuberculous lesions (Bishai, 2000). Despite these early findings, only in 2000 Hernandez-Pando using normal lung tissues isolated at necropsy from patients who had died for causes other than TB in a TB endemic country, were able to detect by *in situ* PCR *MTB* DNA in non-phagocytic cells, fibroblasts and endothelial cells, clearly suggesting that in latent TB subjects *MTB* bacilli can persists in tissues and cells not associated with the granuloma or the Ghon complex (Hernandez-Pando *et al*., 2000). Using similar experimental settings, *MTB* was detected in the fat tissue surrounding several organs, residing intracellularly in adipocytes, where it can survive protected from the host immune response (Neyrolles *et al*., 2006). All these evidences suggest that during latent tuberculosis infection (LTBI) *Mycobacterium tuberculosis* can reside in different organs, tissues and cell types, not associated with the site of primary infection and lacking any sign of the typical granulomatous lesions (Neyrolles *et al*., 2006).

Studies carried out in the non-human primate model of TB further corroborated these findings indicating that during latent infection *MTB* is metabolically active and replicates in host tissues despite the lack of any clinical sign or symptom of disease (Ford*et al*., 2011; Gideon and Flynn, 2011). Interestingly, in a single monkey with active TB it was possible to observe many different type of lesions, ranging from liquefied cavities with massive loads of bacilli, to necrotic or caseous hypoxic lesions with variable number of bacteria, to sterile lesions (Barry *et al*., 2009). A similar scenario was observed in patients with pulmonary TB, where diverse lesions were observed simultaneously and with lesions responding differently to chemotherapy (Barry *et al*., 2009), suggesting that they represent distinct *MTB* subsets in different micro-environments.

Based on the new understanding of the biology of *MTB*, its different metabolic states, the dynamic host immune responses occurring during infection and on the spectrum of conditions that are observed during infection, it has been proposed that during latent infection most bacilli persist in a dormant state with fewer *MTB* found in an active replicating state (Anna, 2013). These replicating bacilli, named "scouts" are processed and killed by the host immune defenses and as a result they are responsible for the induction of the large number of memory T cells directed against *MTB* antigens that are found in the peripheral blood (Chao and Rubin, 2010) Hence, during latent TB dormant bacteria constantly replenish the bulk of actively replicating bacilli readily killed by the host. When, for any reason, host immune responses fail to control these scouts, uncontrolled bacterial replication promotes diseases manifestations and active disease ensues (Gengenbacher and Kaufmann, 2012).

Classical examples are highlighted by HIV infection that affects CD4 T cells that play a pivotal role in controlling *MTB* multiplication; (Beham *et al*., 2011).Treatment with biological therapies with anti-TNF that are known to increase the risk of developing TB disease up to 25 times in latent TB subjects as a result of the disruption of granuloma organization and depletion of certain populations of CD8 T cells known to play a role in controlling *MTB* (Bruns *et al*., 2009; Beham *et al*., 2011), treatment with corticosteroids, vitamin D deficiency and any other condition affecting T cell function are also known to increase the risk of active TB in latent TB subjects. Cancer patients, including those with haematological diseases, are also at increased risk of developing TB and in these patients clinical outcomes are usually very aggressive, may present as systemic infections with a high fatality rate and diagnosis is usually delayed (Cordonnier *et al*., 2004; Maartens and Wilkinson, 2007).

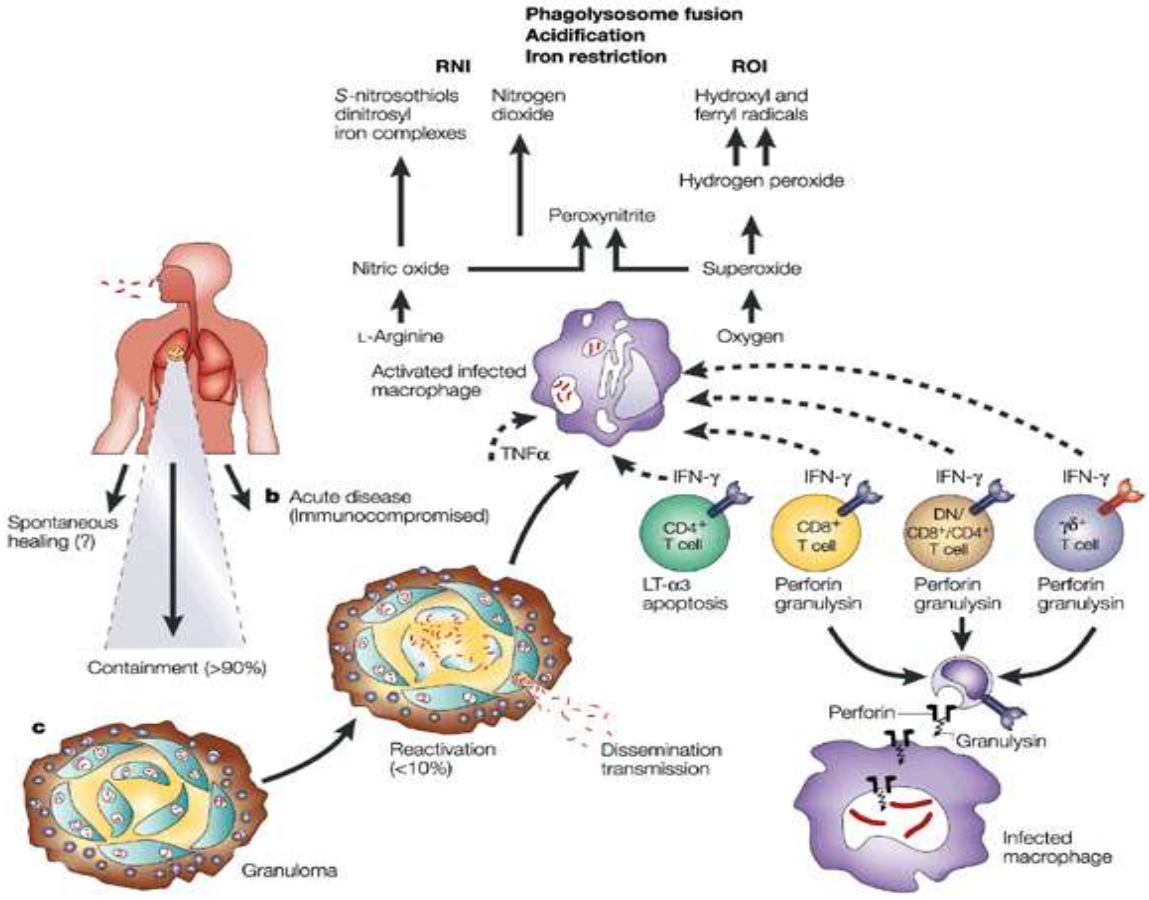


Figure 2.4: Ways in which immunology contribute to the control of tuberculosis. S. Kaufmann, (2001). Macmillan Publishers Ltd: Nature Reviews Immunology, Oct; 1 [17]

2.1.11 Primary Infection

The tubercle bacilli establish infection and develop rapidly in the lungs after they are carried as droplets (5 to 10 microns) to reach the alveolar spaces. When the defense system of the host fails to eliminate the infection, the bacilli proliferate inside alveolar macrophages and eventually kill the cells. The infected macrophages produce cytokines and chemokines that attract other phagocytic cells, including monocytes, other alveolar macrophages and neutrophils, which eventually form a nodular granulomatous structure called the tubercle (dormant bacterium). If the bacterial multiplication is not controlled, the tubercle enlarges and the bacilli enter local draining lymph nodes. This leads to lymphadenopathy, a characteristic clinical manifestation of primary tuberculosis (TB). The lesion produced by the expansion of the tubercle into the lung parenchyma and lymph node involvement is called the Ghon complex. Bacteremia may accompany initial infection (Robert, 2013). The bacilli continue to proliferate until an effective cell-mediated immune (CMI) response develops, usually two to six weeks after infection. Failure by the host to mount an effective CMI response and tissue repair leads to progressive destruction of the lung (Robert, 2013).

2.1.12 Reactivation of tuberculosis disease

Reactivation of TB results from proliferation of a previously dormant bacterium seeded at the time of the primary infection. Among individuals with latent infection and no underlying medical problems, reactivation disease occurs in 5 to 10 per cent (Giovanni *et al.,* 2013). Immunosuppression is associated with reactivation of TB, although it is not clear what specific host factors maintain the infection in a latent state and what triggers the latent

infection to become overt for immunosuppressive conditions associated with reactivation of TB (Sarafino-wani, 2012). The disease process in reactivation TB tends to be localized (in contrast to primary disease) there is little regional lymph node involvement and less caseation. The lesion typically occurs at the lung apices, and disseminated disease is unusual unless the host is severely immunosuppressed. It is generally believed that successfully contained latent TB confers protection against subsequent TB exposure (Robert, 2013).

2.1.13 Symptoms

Early symptoms of active pulmonary TB include cough for more than two (2) weeks, fever, weight loss, night sweats, chest pain, tiredness and loss of appetite (CDC, 2009). The infection can either be latent or become more serious with the onset of chest pain and coughing (NIAID, 2009).

2.2 Diagnosis

2.2.1 Mantoux (Intradermal Tuberculin Test)

First introduced in 1890, the TST is an intradermal injection of purified protein derivative (PPD). The PPD is a crude antigenic mixture, shared among *M. tuberculosis*, *M. bovis*, and other non-tuberculous mycobacteria (NTM) (Lee and Holzman, 2002). The test measures *in vivo* delayed-type hypersensitivity reaction based on immunological recognition of mycobacterial antigens in exposed individuals. Mycobacterial antigens are injected below the epidermal layer, causing infiltration of antigen-specific lymphocytes and the elaboration of inflammatory cytokines. This inflammatory reaction results in the characteristic

indurated area at the site of injection, tuberculin reaction is classified as positive based on the diameter of the induration in conjuction with patient-specific risk factors (ECDC, 2016). A positive reaction for TB infection only reports that a person has been infected with TB bacteria. It does not tell whether or not the person has active disease, a sample of sputum are needed to determine whether the person has active disease (Nahid *et al*., 2006).

2.2.2 Chest X-ray

In pulmonary TB, lesions on x-rays are often seen in the apical segments of the upper lobe or in the upper segments of the lower lobe. However, lesions may appear anywhere in the lungs, especially in HIV-positive and other immunesuppressed person (CDC, 2010). Chest x-rays may be used to rule out the possibility of pulmonary TB in a person who has a positive reaction to the tuberculin skin test and no symptoms of disease (Nahid *et al*., 2006).

2.2.3 Sero-diagnosis of tuberculosis

Recent meta-analyses and systematic reviews concluded that currently available commercial serological tests such as (Anda-TB IgG)provided inconsistent results (Steingart *et al*., 2011) due to cross reactivity and poor sensitivity. The WHO currently recommends against their use for the diagnosis of pulmonary and extrapulmonary TB (WHO, 2011). Further research is needed to develop immune response-based or serodiagnostic tests with appropriate performance.

2.2.4 Interferon gamma (IFN-γ)

Gamma Interferon (IFN-γ) plays a critical role in regulating cell-mediated immune responses to *M. tuberculosis* infection led to the development of alternative tests for the

diagnosis of TB infection; the interferon gamma release assays (IGRAs) are in vitro blood tests of cell-mediated immune response; they measure T cell release of interferon gamma following stimulation of a blood sample from a patient by TB-specific antigens, ESAT-6 and CFP-10, unique to *M. tuberculosis* complex. There are two commercially available IGRAs; Quantiferon TB Gold test and T-SPOT.TB test. Several published studies have demonstrated a better performance of these tests over the TST in the diagnosis of a TB infection (CDC, 2010). Despite these studies, the lack of a reference standard test for latent tuberculosis infection (LTBI) makes it difficult to access the true accuracy of these assays. IGRAs cannot distinguish between latent infection and active tuberculosis (TB) disease and should not be used for diagnosis of active TB.

2.2.5 Smear Microscopy

Microscopic examination of sputum specimens has been the basis of TB case detection for over 100 years and still, in resource limited settings, the diagnosis of TB relies on Ziehl-Neelsen stain using light microscope. Smear microscopy with the bright field microscope is a relatively less sensitive for the diagnosis of TB and only detects about 60-70% of the TB cases. An alternative for the bright field microscope is the fluorescence microscope, reported to be 10% more sensitive, the fluorescent bacilli of *M. tuberculosis* can be seen at lower magnification and the smears can be examined in only 25% of the time taken to read with the bright field microscope. However, due to the higher cost associated with purchase of the microscope with a mercury vapor lamp, the need for frequent replacement of the UV lamp, which lasts only 200–300 h, and the need for a dark room for reading the slides (Marais, 2008). A light-emitting diode (LED) with a long life-span, resulted in LED

fluorescence microscopy which is reletively inexpensive and more sensitive (about 10%) than bright field microscopy is used (Marais, 2008).

2.2.6 Xpert MTB/RIF Assay

The Xpert MTB/RIF assay (ceipheid) is areal time polymerase chain reaction (RT-PCR) technology, which is self enclosed, rapid molecular test for the simultaneous detection of *Mycobacterium tuberculosis* (MTB) and its resistance to rifampicin (RIF).Xpert MTB/RIF integrates sputum processing; the DNA extraction and the amplification in a one-step sample preparation. This automated cartridge-based assay detects, directly from sputum in two hours. The technology is based on the Gene Xpert platform, the platform enables the detection of rifampicin resistance via the detection of mutations in the rpoB gene. The closed system ensures that there is no risk of contamination and no requirement for bio safety facilities. It has high TB detection among culture-confirmed cases by 23% in comparison to smear microscopy. For rifampicin resistance detection, the assay pooled sensitivity was 95% and pooled specificity was 98% (Steingart *et al.,* 2011).

2.3Mechanism of Resistance to First and Second Line Drugs

2.3.1 Phenotypic characteristics

Resistance describes the ability of a bacterial cell to survive the presence of a drug at a concentration that normally kills or inhibits growth. Drug resistance is caused by genetic changes in the bacterium which are passed to subsequent generations (Anna, 2013). This can be differentiated from tolerance, which is a conditional phenotype typically mediated by the physiological state of the bacteria (Wiuff *et al*., 2005). Most antibiotics act against

actively growing bacteria, and the lack of susceptibility of non growing bacteria to antibiotics is due to changes in bacterial metabolism or physiological state. Another type of phenotypic resistance relates to the phenomenon of persisters and dormant bacteria. Persisters are a small number of bacteria, from actively growing cultures, that are not killed after exposure to antibiotics (Wakamoto *et al*., 2013).

The concept of persistence it not equivalent to dormancy, even though the terms in some cases can be `used interchangeably. Dormant bacteria are in a state with no or low metabolic activity and do not form colonies directly on solid medium, however, can be resuscitated to form colonies on plate under appropriate conditions. On the other hand, persisters may or may not form colonies on plates, an outcome which depends on the environment (Zhang, 2004). It is due to the survival of a small number of bacteria during antibiotic exposure despite lacking genetic resistance mechanisms that the minimum inhibitory concentration (MIC) is commonly defined as the lowest concentration of antibiotic that kills or inhibits growth of 99% of a bacterial population (Anna, 2013). The presence of persistent or dormant TB bacteria is believed to be one of the reasons for the lengthy duration of TB chemotherapy, since current drugs are not effective in eliminating persistent or dormant bacilli (Zhang *et al*., 2012).

2.3.2 Genotypic characteristics

Drug resistance can either be intrinsic or acquired. Intrinsic resistance refers to non susceptibility of a bacterium due to its unique characteristics. *M. tuberculosis* is naturally resistant to penicillin due to its production of beta-lactamases which deactivate the drug (Flores *et al*., 2005). Acquired resistance refers to susceptible bacteria becoming resistant to drugs as a result of genetic alterations. Resistance is classified as acquired when drug-

resistant mutants are selected as a result of suboptimal treatment, and as primary when an individual is infected with an already drug-resistant strain. Drug resistance in *M. tuberculosis* is conferred through spontaneous mutations in the chromosomal DNA not like other bacterial species that are mediated by mobile genetic elements, such as plasmids, or transposons (Heym *et al*., 1994; Anna, 2013). Base substitutions most commonly confer drug resistance in *M. tuberculosis*; however, insertions and deletions of single bases, or longer regions, are also observed (Sandgren *et al*., 2009).

Mycobacterium tuberculosis, like other bacteria, becomes resistant by a number of strategies, including target modification, target over expression, drug-inactivating enzymes, inactivation of drug-activating enzymes, and possibly efflux pump mechanisms (Zaunbrecher *et al*., 2009; da Silva *et al*., 2011). Pleiotropism refers to the phenomenon when a single gene affects several phenotypes (King *et al*., 2006). In *M. tuberculosis*, no pleiotropic mutation that mediates multidrug resistance has been reported; instead resistance to several drugs is caused by sequential accumulations of mutations in the chromosome. Cross-resistance arises when a strain acquires resistance to one drug through direct exposure, and at the same time also becomes resistant to one or more other drugs to which it has not been exposed to but the mechanism of resistance drugs is the same (Anna, 2013).

2.3.3 Rifampicin

Rifampicin is a rifamycin derivative introduced in 1972 as an antituberculosis agent. It is one of the most effective anti-TB antibiotics and together with isoniazid constitutes the basis of the multidrug treatment regimen for TB. Rifampicin is active against growing and non-growing (slow metabolizing) bacilli (Mitchison, 1979). The mode of action of rifampicin in *M. tuberculosis* is by binding to the β-subunit of the RNA polymerase,

inhibiting the elongation of messenger RNA (Blanchard, 1996). The majority of rifampicin-resistant clinical isolates of *M. tuberculosis* harbor mutations in the *rpoB* gene that codes for the β-subunit of the RNA polymerase. As a result of this, conformational changes occur that decrease the affinity for the drug and results in the development of resistance (Telenti *et al.,* 1993). In about 96% of *M. tuberculosis* isolates resistant to rifampicin, there are mutations in the so-called hot-spot region of 81-bp spanning codons 507–533 of the *rpoB* gene. This region is also known as the rifampicin resistance-determining region (Ramaswamy and Musser, 1998). Mutations in codons 516, 526 and 531 are the most commonly associated mutations with rifampicin resistance in the majority of studies (Somoskovi *et al.,* 2001; Caws *et al.,* 2006).

Although less frequent, some reports have also noted the occurrence of mutations outside of the hot-spot region of *rpoB* (Heep *et al.,* 2000 and Siu *et al.,* 2011). Cross-resistance with other rifamycins can occur. Mutations in some codons (e.g., 518 or 529) have been associated with low-level resistance to rifampicin but still susceptible to other rifamycins, such as rifabutin or rifalazil (Yang *et al*., 1998, Cavusoglu *et al*., 2004). This is important for TB patients that need to receive antiretroviral therapy since rifabutin is a less effective inducer of the cytochrome P450 CYP3A oxidative enzyme (Burman and Jones 2001). On the other hand, monoresistance to rifampicin is quite rare and almost all rifampicin-resistant strains are also resistant to other drugs, especially to isoniazid. This is the reason why rifampicin resistance is considered as a surrogate marker for MDR-TB (Traore *et al*., 2000).

Recent genome sequencing studies have uncovered the acquisition of compensatory mutations in *rpoA* and *rpoC*, encoding α and β subunits of RNA polymerase, in rifampicin-resistant strains with mutations in *rpoB* (Comas *et al*., 2011). These compensatory mutations would be responsible for restoring the fitness of these strains *in vivo* and have

also been associated with a higher transmissibility in some settings (Brandis and Hughes, 2013; De Vos *et al.*, 2013).

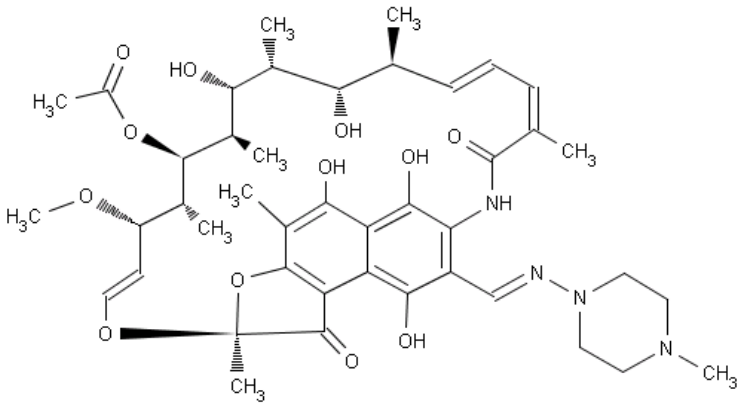


Figure 2.5: Chemical structure of Rifampicin, Source:**drugbank.ca/drug/DB01045**

2.3.4 Isoniazid

Isoniazid was introduced in 1952 as an anti-TB agent and it remains, together with rifampicin, as the basis for the treatment of the disease. Unlike rifampicin, isoniazid is only active against metabolically-active multiplying bacilli. Also known as isonicotinic acid hydrazide, isoniazid is a pro-drug that requires activation by the catalase/peroxidase enzyme KatG, encoded by the *katG* gene, to exert its effect (Juan and Anandi, 2014). Isoniazid acts by inhibiting the synthesis of mycolic acids through the NADH-dependent enoyl-acyl carrier protein (ACP)-reductase, encoded by *inhA* (Rawat *et al.*, 2003). Although simple in its structure, resistance to this drug has been associated with mutations in several genes, such as *katG*, *inhA*, *ahpC*, *kasA* and NDH. The two main molecular mechanisms of isoniazid resistance are associated with gene mutations in *katG* and *inhA* or its promoter region. Indeed, numerous studies have found mutations in these two genes as the most commonly associated with isoniazid resistance (Ramaswamy *et al.*, 2003, Hazbon *et*

al.,2006). Among these, the most prevalent gene mutation has been identified as S315T in *katG* resulting in an isoniazid product deficient in forming the isoniazid-NAD adduct needed to exert its antimicrobial activity (Vilchèze and Jacob, 2007).

This mutation has been consistently associated with high-level resistance (MIC > 1 μg/mL) to isoniazid (Fenner *et al.,* 2012) and occurs more frequently in MDR strains (Hazbon *et al.,* 2006). The most prevalent mutation found is at position −15C/T and is more commonly associated with low level resistance to isoniazid (MIC < 1 μg/mL). Mutations in *inhA* not only cause resistance to isoniazid but also to the structurally related drug ethionamide, which shares the same target (Banerjee *et al.,* 1994, Larsen *et al.,* 2002). A recent study found that a mutation in the *inhA* regulatory region together with a mutation in the *inhA* coding region produced high-level isoniazid resistance and also cross-resistance to ethionamide (Machado *et al*., 2013).

In *M. tuberculosis*, *ahpC* encodes an alkyl hydroperoxidase reductase that is implicated in resistance to reactive oxygen intermediates and it was initially proposed that mutations in the promoter of *ahpC* could be used as proxy markers for isoniazid resistance (Juan and Anandi, 2014). It is now better understood that mutations in the promoter of *ahpC* are compensatory mutations for the loss of catalase/peroxidase activity rather than the cause for isoniazid resistance (Juan and Anandi, 2014). Moreover, overexpression of AhpC does not confer resistance to isoniazid (Juan and Anandi, 2014). Several studies have found single nucleotide polymorphisms in other genes in isoniazid resistant clinical isolates of *M. tuberculosis*, including *kasA* and the *oxyR-ahpC* and *furA-katG* intergenic regions (Cardoso *et al.,* 2007; Ando *et al.*, 2011). However, their direct role as a cause of isoniazid resistance has not been fully demonstrated. On the other hand, co-resistance to isoniazid and ethionamide has been clearly demonstrated to be caused by mutations in ndh in *M.*

smegmatis and M. bovis BCG, by altering the NADH/NAD ratios inside the cell, leading to a competitive inhibition of the INH-NAD adduct (Vilcheze *et al.,* 2005). A recent study has also found that a silent mutation in mabA conferred isoniazid resistance through upregulation of *inhA* in *M. tuberculosis* (Ando *et al.,* 2014).

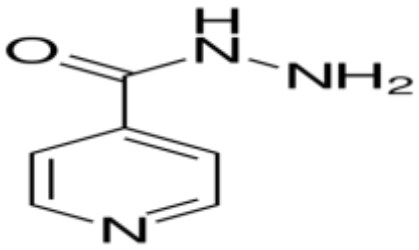


Figure 2.6: Chemical structure of Isoniazid, Source:**drugbank.ca/drug/DB00951**

2.3.5 Ethambutol

Ethambutol was first introduced in the treatment of TB in 1966 and is part of the current first-line regimen to treat the disease. Ethambutol is bacteriostatic against multiplying bacilli interfering with the biosynthesis of arabinogalactan in the cell wall (Juan and Anandi, 2014). In *M. tuberculosis*, the genes *embCAB*, organized as an operon, code for arabinosyl transferase, which is involved in the synthesis of arabinogalactan, producing the accumulation of the intermediate D-arabinofuranosyl-P-decaprenol (Juan and Anandi, 2014). The recognized mechanism of resistance to ethambutol has been linked to mutations in the gene *embB* with mutations at position *embB*306 as the most prevalent in most of the studies performed (Juan and Anandi, 2014). Some studies, however, have also found mutations in *embB*306 in ethambutol susceptible isolates (Ahmad *et al*., 2007).

Moreover, a study with a large number of *M. tuberculosis* isolates found that mutations in *embB*306 were not necessarily associated with resistance to ethambutol but with a predisposition to develop resistance to increasing number of drugs and to be transmitted

(Hazbón *et al*., 2005). In fact, allelic exchange studies have shown that individual mutations causing certain amino acid substitutions produced ethambutol resistance, while other amino acid substitutions had little or no effect on ethambutol resistance (Safi, 2008). The same authors have more recently reported that mutations in the decaprenylphosphoryl-B-D-arabinose (DPA) biosynthetic and utilization pathway genes, Rv3806c and Rv3792, together with mutations in *embB* and *embC* accumulate, giving rise to a range of MICs of ethambutol depending on mutation type and number (Safi *et al*., 2013). These findings could have influence on the correct detection of ethambutol resistance by current molecular methods. Mutations in *embB*306 then, cause variable degrees of ethambutol resistance and are required but are not enough to cause high-level resistance to ethambutol. There remain about 30% ethambutol resistant strains that do not present any mutation in embB stressing the need to identify other possible mechanisms of drug resistance to this drug

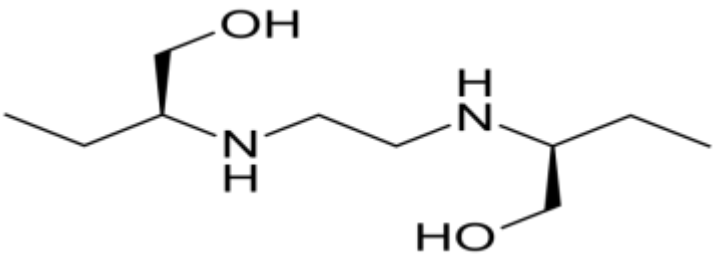


Figure 2.7: Chemical structure of Ethambutol, Source:**drugbank.ca/drug/DB00330**

2.3.6 Second-Line Anti-TB Drugs

2.3.6.1 Fluoroquinolones (Ofloxacin)

Fluoroquinolones are currently in use as second-line drugs in the treatment of MDR-TB. Both ciprofloxacin and ofloxacin are synthetic derivatives of the parent compound nalidixic

acid, discovered as a by-product of the antimalarial chloroquine (Anna, 2013). Newer-generation quinolones such as moxifloxacin and gatifloxacin are being evaluated in clinical trials and proposed as first-line antibiotics with the purpose of shortening the length of treatment in TB (Rustomjee *et al*., 2008, Palomino and Martin, 2013). The mode of action of fluoroquinolones is by inhibiting the topoisomerase II (DNA gyrase) and topoisomerase IV, two critical enzymes for bacterial viability. These proteins are encoded by the genes *gyrA, gyrB, parC* and *parE,* respectively (Fàbrega *et al*., 2009). In *M. tuberculosis*, only type II topoisomerase (DNA gyrase) is present and, thus, is the only target of fluoroquinolone activity (Aubry *et al.,* 2004). Type II topoisomerase is a tetramer formed by two α and β subunits, coded by *gyrA* and *gyrB,* respectively, which catalyzes the supercoiling of DNA (Takiff *et al*., 1994; Anna, 2013). The main mechanism of development of fluoroquinolone resistance in *M. tuberculosis* is by chromosomal mutations in the quinolone resistance-determining region (QRDR) of *gyrA* or *gyrB*. The most frequent mutations found are at position 90 and 94 of *gyrA* but mutations at position 74, 88 and 91 have also been reported (Cheng *et al*., 2004, Sun *et al*., 2008).

A recent systematic review of fluoroquinolone-resistance-associated gyrase mutations in *M. tuberculosis* has been published (Maruri *et al*., 2012). One interesting finding in *M. tuberculosis* is the presence of a natural polymorphism at position 95 in *gyrA* that is not related to fluoroquinolone resistance since it is also found in fluoroquinolone-susceptible strains (Musser, 1995; Anna, 2013). Another interesting finding has been the report that the simultaneous occurrence of mutations T80A and A90G in *gyrA* led to hypersusceptibility to several quinolones (Aubry *et al*., 2006). This finding could point out that the problem of fluoroquinolone resistance in *M. tuberculosis* might be more complex than was thought initially. Cross-resistance is assumed to occur between fluoroquinolones although isolated

reports have acknowledged the presence of strains resistant to gatifloxacin and moxifloxacin that were still susceptible to ofloxacin (Von Groll *et al*., 2009). Also, the involvement of efflux mechanisms has been suggested as a possible cause for fluoroquinolone resistance in *M. tuberculosis* (Escribano *et al*., 2007).

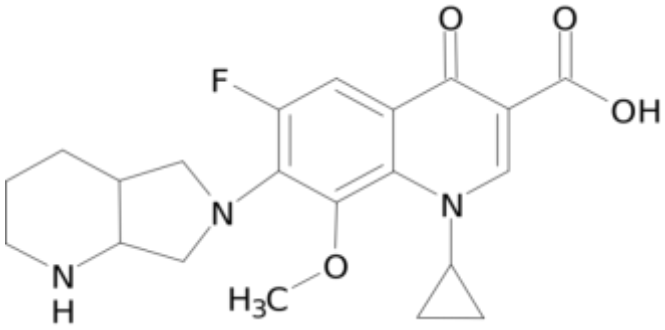


Figure 2.8: Chemical structure of Ofloxacin, Source:**drugbank.ca/drug/DB01165**

2.3.6.2 Aminoglycosides (Kanamycin, Amikacin and Capreomycin).

These four antibiotics have the same mechanism of action by inhibiting protein synthesis, kanamycin and amikacin are aminoglycosides while capreomycin and viomycin are cyclic peptide antibiotics. All four are second-line drugs used in the management of MDR-TB. Kanamycin and amikacin inhibit protein synthesis by alteration at the level of 16S rRNA. The most common mutations found in kanamycin-resistant strains are at position 1400 and 1401 of the *rrs* gene, conferring high-level resistance to kanamycin and amikacin. However, mutations at position 1483 have also been reported (Juan and Anandi, 2014). Full cross-resistance between kanamycin and amikacin is not complete, as previously thought. Some studies have shown variable levels and patterns of resistance suggesting that other mechanisms of resistance might be possible (Krüüner *et al*., 2003). In concordance with this, a low-level resistance to kanamycin has been associated with mutations in the promoter region of the *eis* gene, encoding an aminoglycoside acetyltransferase

(Zaunbrecher *et al*., 2009). Mutations at position −10 and −35 of the *eis* promoter led to an overexpression of the protein and low-level resistance to kanamycin but not to amikacin. These mutations were found in up to 80% of clinical isolates showing low-level resistance to kanamycin (Zaunbrecher *et al*., 2009; Campbell *et al*., 2011).

Capreomycin and viomycin, on the other hand, have a similar structure and bind at the same site in the ribosome, at the interface of the small and large subunits (Stanley *et al*., 2010). They show full cross-resistance as reported in previous studies (Juan and Anandi, 2014). Mutations in the *tlyA* gene have also been associated with resistance to capreomycin and viomycin. TlyA is an rRNA methyltransferase specific for 2'-O-methylation of ribose in rRNA. Mutations in t*lyA* determine the absence of methylation activity (Johansen *et al*., 2006). Although some studies did not find this association, a recent meta-analysis, evaluating the association of genetic mutations and resistance to second-line drugs, has confirmed the presence of *tlyA* mutations in addition to mutations in *rrs* and *eis* (Georghiou *et al*., 2012).

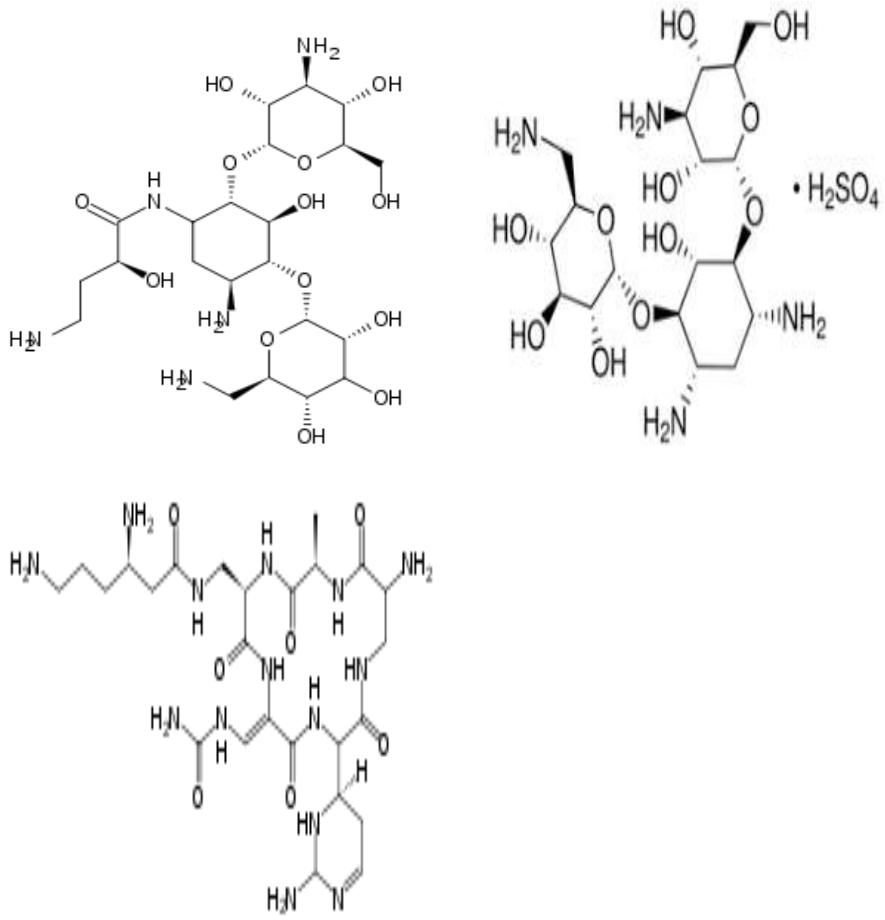


Figure 2.9: Chemical structure of amikacin, kanamycin and capreomycin respectively.Source:**drugbank.ca/drug/DB00479, DB01172 and DB00314 as above.**

2.4 Mycobacterial Culture

Culture is considered the current diagnostic gold standard and is essential for detection of smear microscopy negative cases. The Limit of Detection (LOD) of culture is considered to be 100 bacilli per ml sputum (Tiruviluamala and Reichman, 2002). Due to the particularly slow growth of *M. tuberculosis*, culture is very time-consuming and may take weeks before colonies are obtained. Culture compared to microscopy is an expensive method, and requires specialized laboratories and highly trained personnel (Anna, 2013). Due to the

virulent nature of *M. tuberculosis*, culture isolation is not suitable for laboratories that do not have a proper bio safety level 2 and appropriate equipments. Löwenstein-Jensen (LJ) which is egg-based culture medium is predominantly used for the isolation of *M. tuberculosis* globally (Anna, 2013). However, the agar-based culture medium Middlebrook is also used for culture. Both media require approximately three to six weeks from inoculation to show visible colonies (Naveen and Peerapur, 2012).

2.4.1 Species identification

Differentiation of *M. tuberculosis*complexfrom other species of mycobacteria is important in the diagnosis of tuberculosis. Non tuberculous mycobacteria (NTMs) are increasingly recognized as causative agents of opportunistic infections in humans. *Mycobacterium avium* is the most common and clinically relevant NTM species, especially among HIV-infected persons (Anna, 2013). The probability that the AFB detected by microscopy is actually *M. tuberculosis* depends on the frequency with which the laboratory isolates NTM. Intrinsic resistance to antibiotics is not uncommon among mycobacteria, thus treatment should ideally be guided by species identification and Drug Susceptibility Test (DST) results (van ingen*et al*., 2012). The laboratory approach to bacterial species identification involves Phenotypic and Genotypic characterization.

1. Phenotypic species identification is performed by observing colonial morphologic features, pigment production, growth rate and by employing biochemical tests for studying enzymatic characteristics. Biochemical tests involve for example investigation of reduction of nitrate, niacin, and catalase production. They have successfully been used since the 1950s (Hall and Robert, 2006).

2. High-performance liquid chromatography has been implemented for a more rapid identification of mycobacteria species. The principle is based on the analysis of mycolic acids present in the cell wall (Butler and Gurhertz, 2001).

3. Antigen detection: Immunochromatographic assays based on the reaction of monoclonal antibodies against MPT64, these is a lateral flow assays which detect *M. tuberculosis* antigen specific to members of the MTB complex with exception of sub strain *M. bovis* BCG. BD MGIT TBc identification test, SD Bioline Antigen Detection Test MPT 64, Capilia TB Rapid test that detects MPB64 antigen (Hillemann*et al*., 2005).

4. GenoType *Mycobacterium tuberculosis* complex: rapid nucleic acid amplification tests based on DNA strip technology have been developed to identify different species of *Mycobacterium tuberculosis* complex using the principle of line probe assay (Hain Lifescience GmbH, Nehren, Germany). In addition, the tests GenoType CM and the GenoType AS (Hain Lifescience GmbH) can be used to identify the MTBC and 40 NTMs (Anna, 2013).

2.4.2 Phenotypic drug susceptibility testing (DST).

To control the epidemic of drug-resistant tuberculosis, it is important to detect *M. tuberculosis* and its drug susceptibility pattern especially in settings with high prevalence of drug-resistant tuberculosis, where the current standard TB treatment recommended by the WHO may be ineffective in a very large proportion of the cases (WHO, 2010). Early detection of drug-resistant strains facilitates a timely shift to an effective drug regimen which improves treatment outcomes and reduces the spread of resistant TB in the society. The purpose of DST is to determine whether a particular strain is susceptible or resistant to a certain drug, and is typically achieved by traditional culture-based methods. Conventional

phenotypic DST methods involve culturing bacteria on solid or in liquid media in the presence of drugs under standardized conditions to detect inhibition of growth (Canetti *et al*., 1969; WHO, 2009).

DST testing can be performed directly or indirectly. In the direct test, a set of drug-containing and drug-free media are inoculated directly with a specimen while indirect test involves inoculation of the media with a pure culture and is typically performed with a bacterial suspension made from growth on solid media. Although indirect testing is time-consuming procedure, due to the very slow growth of *M. tuberculosis*, conventional phenotypic DST methods allow detection of drug resistance regardless of resistance mechanism. This is important when the molecular mechanism of drug resistance is unknown and phenotypic methods are not affected by mutations that do not cause drug resistance. Phenotypic DST is most commonly performed on solid culture medium globally. The proportion method, the resistance ratio method, and the absolute-concentration method are phenotypic DST methods on solid medium recommended by the WHO (Canetti *et al*., 1969; WHO, 2009). The proportion method is most commonly used, the percentage of resistant bacteria is reported by comparing the number of colonies on the drug-containing and drug-free media and an isolate is said to be resistant when the percentage is greater or equal to greater than 1% (WHO, 2009).

The first commercial broth-based system for mycobacteria growth detection was introduced in the 1980s. This system, BACTEC 460 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), is a radiometric technique involving monitoring metabolism of bacteria during growth (Siddiq *et al*., 1981; Anna, 2013).The method was later further developed to the nonradiometric BACTEC MGIT 960 system (Becton, Dickinson and Company) (Rusch-Gerdes *et al*., 1999; Anna, 2013). In the automated MGIT 960 system each test

tube, containing liquid medium, has a fluorescence quenching-based oxygen sensor embedded in silicon at the bottom which fluoresces following the oxygen reduction induced by aerobically metabolizing bacteria. The consumption of oxygen during bacterial growth produces fluorescence which is detected by the MGIT 960 machine. The liquid culture and DST methods have shorter turnaround time but are expensive and require specialize equipment (Anna, 2013).

Simpler and inexpensive methods for detecting drug-resistant *M. tuberculosis*, that are non-commercial culture-based methods have been developed and such methods includes colorimetric redox indicator (CRI) assays (Martin *et al*., 2007), nitrate reductase assay (NRA) (Ängeby *et al*., 2002), and the microscopy observation drug-susceptibility (MODS) assay (Moore *et al.,* 2006). The CRI methods are based on the reduction of an indicator dye added to liquid culture medium in a microtitre plate after exposure of strains to anti-TB drugs. NRA is based on the ability of *M. tuberculosis* to reduce nitrate during growth, which is detected by a color change while in MODS assay, growth of *M. tuberculosis* is detected in a sealed microtitre plate containing liquid culture medium by microscopic observation of typical cord formation. These techniques offer an attractive turnaround time, cost, and sensitivity, but there is limited evidence of the performance of the techniques for some first-line anti-TB drugs and for most second-line drugs (Horne *et al*., 2013; Anna, 2013).

2.5 Molecular Detection of Drug-Resistant Tuberculosis

Conventional culture-based diagnostic and DST methods are time and labor-intensive; therefore, there is an urgent need for new rapid TB diagnostic methods. Molecular-based methods are designed to detect chromosomal mutations associated with drug resistance

specifically in *M. tuberculosis*. DNA is a very stable molecule which can be readily detected in clinical specimens from patients who have received anti-TB chemotherapy. Sanger sequencing is the method for determination of the nucleotide order of a given DNA fragment, it has revolutionized the analysis of genomes, and still remains widely used (Sanger *et al*., 1977; Anna, 2013). The polymerase chain reaction (PCR) which amplifies DNA has been since its development in the late 1980s an indispensable technique in both medical and biological research fields (Saiki *et al*., 1985; Anna, 2013).

Molecular test have high clinical and analytical sensitivity and specificity, should be rapid, robust, compliant with decentralized use, inexpensive, have long shelf life, amenable to large-scale production, require minimal laboratory infrastructure and personnel training, and should be possible to apply on all types of specimens, not only sputum. Although a wide range of molecular diagnostic methods have been developed for the detection of drug-resistant *M. tuberculosis*, most are based on PCR amplification of a specific chromosomal region followed by analysis of the PCR product for detection of mutations associated with resistance to a particular drug. The presence or absence of a specific mutation is then regarded as an indication that the investigated isolate is susceptible or resistant to a particular drug. Multiplex PCR employs multiple primer sets in a single reaction mixture to produce amplicons of varying sizes that are specific to different DNA sequences (Anna, 2013). Multiplex PCR, has the potential to decrease cost, time and effort in diagnostics. However, optimization of efficient multiplex PCR requires extensive laborious planning in primer design, nucleotide and primer concentrations, optimal salt and buffer conditions, and cycling temperatures (Markoulatos *et al*., 2002), and is rarely capable of achieving high degrees of multiplexing (Akhras *et al*., 2007).

2.5.1 Line probe assays (LPA)

Molecular line probe assays (LPAs) apply principles of nucleic acid amplification to detect both Mycobacteria species and mutations associated with drug resistance. In these assays, a genomic region is first subjected to PCR with biotinylated primers, followed by hybridization of the PCR product to oligonucleotide probes immobilized on a nitrocellulose strip (Anna, 2013). The presence of a mutation prevents hybridization of the PCR product to wild type probes, and wild type sequence prevents hybridization to the mutant-specific probes. The biotinylated product enables visualization of colored bands by the naked eye. Mutations are detected by lack of hybridization to wild type probes, as well as by hybridization to specific probes designed for the most commonly occurring mutations (Anna, 2013).

The WHO endorsed LPAs for the detection of mutations associated with drug resistance in *M. tuberculosis* in 2008 (WHO, 2008). The predominantly used commercially available LPA assays are INNO-LiPA Rif. TB (Innogenetics NV, Gent, Belgium) (Jureen *et al*., 2004), GenoType MTBDRplus and GenoType MTBDRsl (Hain Lifescience GmbH, Nehren, Germany) (Hillemann *et al.*, 2009). INNO-LiPA Rif TB allows detection of mutations in RRDR associated with RIF resistance (Jureen *et al*., 2004), and the GenoType MTBDRplus assay also detects, RIF resistance, mutations in *katG* and the promoter region of *inhA*, allowing simultaneous detection of INH resistance. While the GenoType MTBDRplus assay enables detection of MDR-TB. The GenoType MTBDRsl assay is developed for detection of FQ resistance by targeting the gene *gyrA* and the injectable drugs AMK, KAN and CAP by detecting the gene *rrs*, enabling detection of XDR-TB. Detection of the gene *embB*, linked to resistance to the first-line drug EMB, is also part of the GenoType MTBDRsl assay. The assays detect MTBC and the GenoType MTBDRplus

assay is also optimized for smear-negative pulmonary clinical specimens (Anna, 2013). The MTBDR*sl* versions 2.0 detects specific mutations in the *gyrB* quinolone resistance-determining region and the *eis* promoter region in addition to version 1.0 that are associated with resistance to the FQs (ofloxacin, moxifloxacin, levofloxacin, and gatifloxacin) and SLIDs (kanamycin, amikacin, and capreomycin) respectively in *M. tuberculosis* complex species (Theoren, 2016).

2.5.2 DNA sequencing

In DNA sequencing, the target genomic region is amplified by PCR, and subjected to a unique sequencing reaction. Sequencing is generally very accurate and robust, and has been widely used for characterizing mutations in *M. tuberculosis*. It's the gold standard for analysis of sequences and mutation detection. Sequencing offers screening of a known as well as novel mutations and it is specific and relatively rapid; however, it requires a high standard sequencing facility (Anna, 2013).

2.5.3 The Sanger sequencing

Sanger sequencing, was developed in the late 1970s (Sanger *et al*., 1977; Anna, 2013), is a chain-terminating sequencing method widely used in medical and research laboratories. In Sanger sequencing, the nucleotide order is determined by preparing a sequencing reaction where regular deoxynucleotides (dNTPs) are combined with terminating dideoxynucleotides (ddNTPs), both which are incorporated during DNA polymerization. These chain-terminating nucleotides lack a 3′-hydroxyl group required for the formation of a phosphodiester bond between two nucleotides, causing the DNA polymerase to cease extension of DNA when a ddNTP is incorporated. The ddNTPs are commonly labeled with

fluorophores, and the nucleotide sequence is analyzed by separating the fragments by capillary electrophoresis. The read length of Sanger sequencing is approximately 800-1000 bp (Anna, 2013).

2.5.4 The Pyrosequencing.

Pyrosequencing, developed 20 years later from when Sangers sequencing was developed (Ronaghi*et al*., 1996; Anna, 2013), employs a different methodological principle: sequencing-by-synthesis. Pyrosequencing differs from Sanger sequencing in that it relies on the detection of pyrophosphate (PPi) release upon nucleotide incorporation, rather than chain termination with ddNTPs. Pyrosequencing renders shorter sequences than Sanger sequencing, typically 50-60 bp, but has a shorter turnaround time and is more easily applicable in a large-scale fashion. In this method, one of the four dNTPs is added separately to the reaction. If the DNA polymerase incorporates the correct complementary dNTPs onto the template, PPi is released (Ronaghi *et al*., 1996; Anna, 2013). The enzyme adenosine 5'-triphosphate (ATP) sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5´ phosphosulfate. This ATP acts as fuel to the luciferase-mediated conversion of luciferin to oxyluciferin which generates visible light in amounts that are proportional to the amount of ATP, i.e. the number of nucleotides incorporated. The light produced in the luciferase-catalyzed reaction is detected by a camera. Unincorporated nucleotides and ATP are degraded by the apyrase, and the reaction can start again with another nucleotide (Anna, 2013).

2.5.5 Spacer oligonucleotide typing (Spoligotyping).

Spacer oligonucleotide typing (spoligotyping) is the most commonly used PCR-based technique for subspeciating *M. tuberculosis* strains (Mathema *et al*., 2006). *M. tuberculosis* complex strains contain a distinct chromosomal region consisting of multiple 36-bp direct repeats (DRs) interspersed by unique spacer DNA sequences (35 to 41 bp). It's driven by transposition of IS*6110*, which is almost invariably present in the DR locus of *M. tuberculosis* complex strains (Van Embden *et al*., 2000). Spoligotyping is based on the detection of 43 interspersed spacer sequences (originally identified in laboratory strain H37Rv and *M. bovis* BCG vaccine strain P3) in the genomic DR region of *M. tuberculosis* complex strains. Additional spacers in this region have been reported (Van *et al*., 2000). Membranes spotted with 43 synthetic oligonucleotides are hybridized with labeled PCR-amplified DR locus of the tested strain, resulting in a pattern that can be detected by chemiluminescence (Mathema *et al*., 2006).

2.6 Treatment of Tuberculosis

M. tuberculosis is a very slow-growing, intracellular organism. Consequently, treatment requires the use of multiple drugs for several months (Goodman and Lipman, 2008). With appropriate antibiotic treatment, TB can be cured in most people. Treatment usually combines several different antibiotic drugs that are given for at least 6 months, sometimes for as long as 12 months. However, many *M. tuberculosis* strains are resistant to one or more of the standard TB drugs, which complicates treatment greatly (NIAID, 2009). Currently, there are 10 drugs approved by the U.S. Food and Drug Administration for the treatment of TB. Of the approved drugs, isoniazid (INH), rifampin (RIF), ethambutol

(EMB), and pyrazinamide (PZA) are considered first-line antituberculosis agents. These four drugs form the foundation of initial courses of therapy.

Multidrug-Resistant Tuberculosis (MDR-TB) defined as an *M. tuberculosis* strain that is resistant to at least the two main first-line anti-TB drugs Rifampicin (RIF) and Isoniazid (INH). Treatment of MDR-TB is complicated; it requires the use of second-line anti-TB drugs which are less effective, more toxic and more costly. Moreover, treatment duration needs to be prolonged to 18-24 months (WHO, 2010). Drug-resistant TB is in many cases the result of sub-optimal antibiotic treatment, poor treatment compliance, lack of high-quality drugs, misuse of drugs, and poor follow-up of patients receiving treatment, lack of initiation of appropriate treatment regimens, and unsupervised usage of drugs obtained without prescription has been highlighted as the most important causes of resistance to anti-TB drugs (Anna, 2013). Extensively Drug-resistant Tuberculosis (XDR-TB) is defined as an MDR-TB strain that is also resistant to any fluoroquinolone (FQ) and at least one of the three injectable drugs amikacin (AMK), kanamycin (KAN), and capreomycin (CAP). Fluoroquinolones (FQs), AMK, KAN and CAP are important second-line drugs for the treatment of MDR-TB. Treatment is even more complicated for XDR-TB, and treatment success is low (Liu *et al*., 2011). A mortality rate of 98% was reported for XDR-TB patients co-infected with HIV, and the majority of the patients died within two weeks of sputum collection (Gandhi *et al*., 2006). These observations called for urgent intervention since XDR-TB severely threatens the success of treatment programmes for TB and HIV.There are six classes of second-line drugs used for the treatment of TB are: aminoglycosides, fluoroquinolones, polypeptides, thioamides, cycloserine, *p*-aminosalicylic acid.

Currently, short course Direct Observation Therapy (DOTS) is a key component of the World Health Organization's campaign to stop TB. DOTS, involves patient case

management by trained health professionals who ensure that the patient is taking his/her TB drugs (Frieden and Sbarbaro, 2007; WHO, 2009). Because TB has such a long course of treatment, many patients stop their medications prematurely. DOTS sends health professionals to the patient to ensure s/he is taking the medication and may also supply the medicine to the patient. In some areas, patients come to the DOT clinic instead of the health worker traveling to them (Frieden and Sbarbaro, 2007; WHO, 2009). Often, DOTS provides enablers or incentives to ensure patients continue their treatment, such as transportation or free meals. DOTS also try to support TB patients (Frieden and Sbarbaro, 2007; WHO, 2009). DOTS has been extremely successful at decreasing the default rates, with cure rates above 80% and default rates less than 10% (Frieden &Sbarbaro, 2007).

2.7 Prevention

Tuberculosis is a preventable and curable disease, isolation of patients and adequate ventilation are the most important measures to prevent its transmission in the community. World Health Organization recommends that infants and children receive a vaccine called BCG (Bacille Calmette Guerin). BCG is made from live weakened *Mycobacterium bovis*, a bacterium related to *M. tuberculosis*. BCG vaccine prevents *M. tuberculosis* from spreading within the body, thus preventing TB from developing (NIAID, 2009). BCG has drawbacks; it is reasonably effective in preventing development of active disease in children. However, it does not protect against TB in adults (NIAID, 2009). In addition, BCG may interfere with the TB purified protein derivative (PPD) skin test, showing a positive skin test reaction in people who have received the vaccine. BCG is used for children in countries where TB is

endemic. Consequently, much of the population would have a positive TB test regardless of the vaccine (NIAID, 2009).

2.8 The Bacillus Calmette-Guérin (BCG) vaccine

Bacillus Calmette-Guérin (BCG), the only vaccine available against TB, is one of the most widely used vaccines in the world (Ottenhoff and Kaufmann, 2012). The live attenuated vaccine strain was derived from *M. bovis* by Albert Calmette and Camille Guérin between 1906 and 1919. The vaccine, which has a low incidence of major side effects in immunocompetent individuals (Ottenhoff and Kaufmann, 2012), protects children against disseminated forms of TB, such as the severe meningitis (Romano and Huygen, 2012); however, the protection conferred against pulmonary TB in adults, representing the majority of the disease burden, has been highly variable in clinical trials, in fact ranging from 0 to 80% (Andersen and Doherty, 2005). The lowest level of protection has been observed in countries with the highest incidence of TB (Fine, 1995; Anna, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area:

This study was conducted in Zaria Local Government Area (formerly known as Zazzau) and is one of the major cities in Kaduna State in northern Nigeria, lying between latitude 11^0 and 4^0 North and longitude 7^0 and 42^0 East with total area of $563km^2$ (217 sq mi). The city has population of over 408,198 (NPC, 2006), its share boundaries with Sabon Gari, Igabi, Soba and Giwa Local Government Areas. Zaria's economic trade is primarily based on agriculture which includes guineacorn, millet, cotton, groundnuts and tobacco. It is a home for artisans from traditional crafts like leather work, dyeing and cap making, to tinkers, print shops and furniture makers(Kaduna State Government, 2008).

3.2 Study site

The National Tuberculosis and Leprosy Training Centre (NTBLTC) Saye, is located in Zaria, Kaduna State, Nigeria. The Centre has one of the two National Tuberculosis Reference Laboratories (NTRL) in Nigeria, which serve as the largest TB referral laboratory in northern Nigeria. The NTRL is equipped with both biosafety level 2 and 3 (BSL 2 and 3) as well as molecular laboratory.

3.3 Study Population

Presumptive pulmonary tuberculosis patients attending NTBLTC Saye, Zaria, serve as the study population.

3.4 Ethical Approval

Ethical approval was obtained from research ethical committee of the NTBLTC Saye, Zaria.

3.5 Sample Size determination

The sample size of this study was determined using the Kish and Leslie formula (Mshana *et al.,* 2009).

$$n = \frac{Z^2PQ}{L^2}$$

Where

n = sample size

Z = 1.96 at 95% confidence interval.

P = estimated prevalence of the infection (MDR/RR-TB) is 4.3% in Nigeria (WHO, 2016).

Q = 1-P

L = allowable error =0.05

Where by P = 0.043

$$n = \frac{(1.96)^2 \times 0.043 \times (1-0.043)}{(0.05)^2}$$

n =63

In order to increase precision and minimize error, the total number of sample size were rounded up to 100.

3.6 Inclusion Criteria

All Xpert MTB/RIF resistant patients who consented were enrolled.

3.7 Exclusion Criteria

All patients who did not consent were not enrolled.

3.8 Sample Collection

One sputum sample was collected from the patients; the patient was asked to inhale deeply 2-3 times and cough deep from the lungs and spit the sputum carefully into the container to avoid contaminating the outside container. The sputum samples were collected in 50 ml capacity universal container, 3 ml and are mucoid and purulents in appearance (NTBLCP, 2014).

3.9 Analyses of Samples

3.9.1 Xpert MTB/RIF assay

Sample reagents buffer was added to the sputum sample in ratio two to one (2:1),the mixtures were mixed by shaking vigorously 20 times and allowed to stand for 10 minutes; it was then shaken 20 times and allowed it to stand for 5 minutes. Two (2) ml of the mixtures was then inoculated into the cartridge and test was started. The results was obtained in less than 2 hours and after the test, the result were printed as MTB not detected, MTB detected Rifampicin resistance not detected and MTB detected Rifampicin resistance detectedas described by (Panwal *et al.*, 2018).

3.9.2 Media preparation

For the growth of *Mycobacterium tuberculosis*, Lowenstein Jensen media base was prepared and used according to the manufacturer instruction (Difco laboratories, BD

Company). The media was prepared by weighing 37.2g of the powder and dissolved in 600ml of distilled water containing 12 ml glycerol, mixed thoroughly. The solution was then heated with frequent agitation to completely dissolve the powder. The solution was then autoclave at 121ºC for 15 minutes; it was allowed to cool down between 50ºC. One thousand (1000) ml of fresh, homogenized egg was prepared and added aseptically into solution and mixed thoroughly. The media were then dispensed into sterile 15ml falcon tubes, slanted and inspissated at 85ºC for 45minutes, the prepared media was then incubated at 37ºC for 48 hours for sterility check and reference strains of *Mycobacterium tuberculosis* and non tuberculous Mycobacteria were used for performance check as described by (Concepcion *et al*., 2001).

3.9.3 Digestion and decontamination of sputum specimen.

All samples that were Xpert MTB/Rif resistant were processed using NALC-NaOH-sodium citrate solution. The NALC-NaOH-sodium citrate solution was prepared as described by Kent and Kubica, (1985). To the 50ml falcon tube containing 3-5ml of sputum sample, an equal volume of NALC-NaOH- citrate reagent was added and the tubes were vortex for 30 second and allowed to stay for 15 min with vortexing in every 5 minutes interval. Following 15mins of incubation at room temperature, phosphate buffer saline was added to 45ml mark and centrifuged at 3000×gusing refrigerated centrifuge at 4^0C for 15 min, the supernant was discarded and the pellet material were re-suspended with 2 ml phosphate buffer (pH6.8) and mixed by inversion. The pellet served as inoculums as described by (Sharma *et al*., 2012).

3.9.4 Isolation of the Mycobacteria

Lowenstein Jensen media was inoculated with 0.1 ml of the inoculums obtained from the decontaminated sputum. All culture tubes were incubated at 37ºC in slanting position with the loosened caps and observe for the first three days to check for rapidly growing mycobacteria and contaminantss. Caps were then tightened after three days of incubation and tubes were observed macroscopically for growth weekly there after for a period of 8weeks. Tubes showing evidence of growth within the period of incubation were removed and smear was made from the growth and stain using Ziehl Neelsen procedure as described by (Concepcion *et al.*, 2001). Absence of growth at the end of eight (8) weeks were considered as negative culture. All contaminated samples were recorded separately as described by (Sharma *et al*., 2012).

3.9.5 Identification of the isolates.

Mycobacterium tuberculosis complex were identified using rapid identification test, a rapid chromatographic immunoassay for the qualitative detection MPT64 protein fraction that is secreted from MTBc cell during culture. Colonies were harvested from AFB smear-positive culture tube and were suspended, emulsified in 2.0 ml cryovial tubes containing 200 µl of buffer, 100 µl from the emulsify colonies is used as the inoculums. Presence of MPT64 antigen is indicated by producton of pink to red color reaction on both test and control lines and absence of MPT64 antigen in the sample is indicated by the presence of pink to red line color reaction on the control line only while absence of control line was regarded as invalid as described by the manufacturers (Becton Dickson USA, 2015).

3.9.6 Drug Susceptibility Testing by Proportion Method.

3.9.6.1 Preparation of Lowenstein Jensen medium containing drugs.

The second line anti-tuberculosis drugs, which include Amikacin and Ofloxacin were obtained from Molekula while Kanamycin and Capreomycin, were obtained from Sigma Aldrich (St.Lious, Missouri USA). Amikacin, Kanamycin, and Capreomycin were dissolved in sterile distilled water; while Ofloxacin was dissolved in 0.4% NaOH. The final concentration of Amikacin, Kanamycin, Capreomycin, and Ofloxacin were (30µg/ml),(30µg/ml),(40µg/ml) and (2.0µg/ml) respectively, were incorporated into the Lowenstein Jensen medium and inspissated at 85ºC for 45 minutes, then the medium was incubated at 37ºC for 48 hours sterility check (WHO, 2012).

3.9.6.2 Drug susceptibility testing

The drug susceptibility test was carried-out using indirect proportion method on Lowenstein Jensen medium. The *Mycobacterium tuberculosis* colonies from culture were harvested using a sterile loop and suspended in 15 mls falcon tubes containing sterile distilled water and 5 sterile glass beads, vortexed for 30 seconds and allow to stand for 15 minutes for the larger bacteria aggregates to settle down. The homogenous upper part of the supernatant was transferred aseptically into another tube, bacterial suspension was adjusted equal to McFarland standard 1 for visual comparison. The bacteria suspension equivalents to Mcfarland No.1 was diluted into tenfold serial dilution of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. The tubes were arranged in order of C_1, C_2, C_3, Kn, Amk, Cp, and Ofl, representing 10^{-2}, 10^{-3},10^{-4}, kanamycin, amikacin, capreomycin, and ofloxacin respectively. Hundred (100) µl of the bacteria suspension was taken from 10^{-4} dilution and inoculated into C_3, likewise 100µl from 10^{-3} dilution was inoculated into C_2 while 100µl from 10^{-2} dilution was inoculated into C_1 and other tubes containing both first and second line drugs.

Incubation was in a slanting position with loosen caps for 24 hours and caps was tighten and return to upright position at 37ºC for 4-6 weeks. Result were read at 4 weeks and final result was reported after 6weeks, growth on culture was recorded and reported as follows: No growth as Negative, 1-50 colonies as actual count, 50-100 colonies as +, 100-200 colonies as ++ (innumerable colonies) and ≥200 colonies as +++ (confluent growth). The resistance or susceptibility to a given drug was determined using the proportion (Bwanga, 2009). *Mycobacterium tuberculosis* reference strain for second line drugs with the following pattern SRRR which is susceptible to ofloxacin but resistance to kanamycin, amikacin and capreomycin and RSSS resistance to Ofloxacin but susceptible to kanamycin, amikacin and capreomycin serve as controls. All strains were subculture in Lowenstein-Jensen medium for a maximum of 8 weeks (Sagonda *et al*., 2014).

3.9.7 Molecular determination of drug resistant tuberculosis(Line probe assay)

The molecular determination procedure involves three (3) major steps:

3.9.7.1 DNA extraction

DNA was extracted from the sediments (0.5 mls) of the decontaminated specimen and was transferred into 2ml micro centrifuge tube. The tubes were centrifuged for 15 minutes at 10,000xg using micro centrifuge with aerosol tight rotor, the supernant was discarded. One hundred (100) µl of lysis buffer (A-Lys) was added and vortex for 30 seconds, the tube was incubated for 5 min in water bath at 95ºC. After the incubation, 100 µl of neutralizing buffer (A-NB) was added, vortex for 30 second and centrifuged using micro centrifuge for 5 mins at 10,000xg, the supernatant (DNA) was transferred into a clean sterile 2 ml micro centrifuge tube. The DNA was stored at -20ºC.

3.9.7.2 Master Mix preparation

Amplification mix A (AM-A) and Amplification mix B (AM-B) were brought to room temperature. Ten (10) µl of AM-A was added to 35 µl of AM-B in a 0.2ml micro centrifuge tube and was mix gently by inverting 3 times, the master mix was prepared fresh each time and was not allowed to stay for more than 2 hours after reconstitution before it is amplified. Micro centrifuge tubes was labelled, arranged in sequence one tube per sample, including three controls, one for master mix (negative), negative and positive controls. Fourty five (45) µl of the master mix was added to the first tube and other tubes for samples, the first tube was labelled 1 as master mix control and other tubes were close and taken to DNA addition room (Hain lifescience GmbH, 2015).

3.9.7.3 DNA addition.

Tube containing aliquot of master mix and DNA extracted from the respective sample were placed under biosafety cabinet class II, to each of remaining tubes, from tube 2, 5µl of the respectively DNA were added and mix by pipetting up and down. Five µl of molecular grade water was added for the first negative control, while second negative and positive controls were added in the last tube 11 and 12, the tubes were closed and taken to amplification room (Hain lifescience GmbH, 2015).

3.9.7.4 Amplification

Amplification was carried out using thermocycler, the PCR reaction involve heating at various temperatures in order to denature, anneal and elongate the DNA of the specimen. The steps involve denaturation at 95ºC for 15mins, initial denaturation at 95ºC for 30 second, annealing at 65ºC for 2min (10cycles), denaturation at 95ºC for 25 second, annealing at 50ºC for 40 second, extension at 70ºC for 40 second (30cycles), and final extension at 70ºC for 8min. The amplicons were stored at +8 to -20ºC. (Hain lifescience GmbH, 2015).

3.9.7.5 Hybridization

The water bath and twincubator were brought to 45ºC as well as hybridization and stringent solution were brought to 45ºC before use, 20 µl of denaturation solution was dispense into each corner of the well and twenty (20) µl of the amplified sample were added, and mixed by pipetting up and down gently. It was allowed for 5 min incubation in the tray, the strips were taken using tweezers and were placed at the corner of each well, 1ml of the pre-warm hybridization buffer was added and the strip were inserted into the buffer. The tray was shaken until a homogenous color is achieved; the tray was placed in shaking twincubator and incubated for 20 min at 45ºC, completely aspirate the hybridization buffer and add 1ml of stringent wash solution to each well, incubate for 10 min at 45ºC in the shaking twincubator. Stringent washing solution was removed completely and the tray was turn upside down on paper towel. One (1) ml of diluted conjugate was added to each strip and incubated for 20 min on shaking twincubator, diluted conjugate was completely aspirated. Rinse with 1ml rinsing solution to each well and incubated for 1 min at room temperature in twincubator. Completely remove rinse solution and rinse with water for 1min. One ml of the substrate dilution was added to each well and incubated for 5 min at room temperature

in the twincubator. Diluted substrate was completely removed and the reaction was stop by rinsing twice with water 1min in each rinse. Removed DNA strip from the tray, air dry on absorbent paper and dried strip were stick on the worksheet. The chart was use to interpret results (Hain lifescience GmbH, 2015).

3.10 Data Analysis

The Med Calc Software (Med Calc, Belgium) was used to calculate statistical parameters such as Pearson chi-square test and Odd ratio to determine the association between the variables at 95% confidence interval.

CHAPTER FOUR

RESULTS

4.1 Occurence of *Mycobacterium tuberculosis*and rifampicin resistancein patients attending NTBLTC Saye using Xpert MTB/RIF Assay

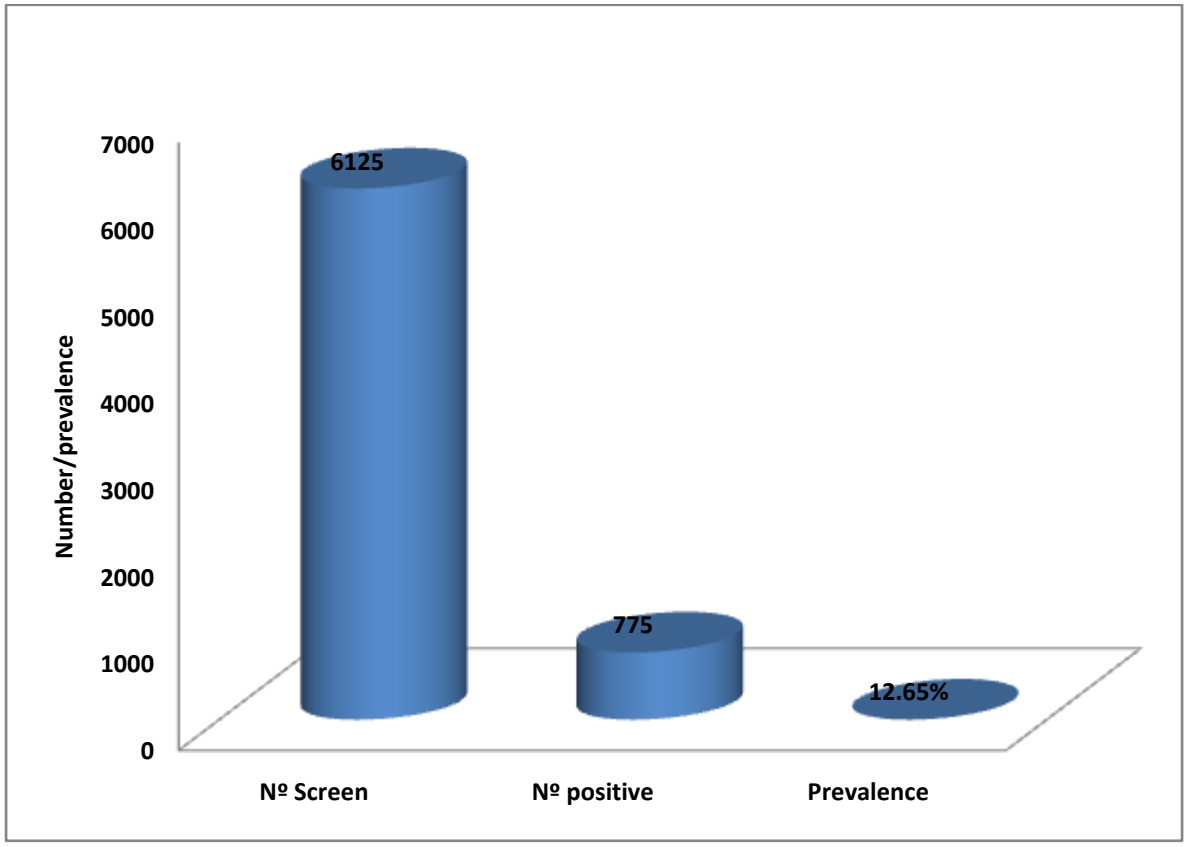
A total of six thousand one hundred and twenty five (6125) samples were screened, out of which 775 (12.65%)were positive by Xpert MTB/RIF assay and out of 775 positive samples, one hundred (100) were resistant to rifampicin with an occurence of 12.9% (Figures 1 and 2).

4.2 Isolation and identification of *Mycobacterium tuberculosis* in patients attending NTBLTC Saye using Culture and rapid immunochromatographic test (TB Ag MPT64)

Out of one hundred (100) samples that were rifampicin resistant, 90 (90%) were culture positive while 7 (7%) were culture negative, 3 (3%) were contaminated. All of the ninety (90) samples that were culture positive were confirmed using rapid immunochromatoghapic test (TB Ag MPT64) as *Mycobacterium tuberculosis* complex (MTBC) (Table 1 and 2).

4.3 Isolation of drug susceptibility of *Mycobacterium tuberculosis*in patients attending NTBLTC Saye using LJ Proportion Method

Twelve (13.3%) out of the ninety *Mycobacterium tuberculosis* isolateswere resistant to ofloxacin, seventy eight (86.7%) were found to be pan susceptible to the second line anti-TB drugs, Eight (9%) were resistant to kanamycin and amikacin while seven (8%) were only resistant to capreomycin by LJPM. Out of the ninety (90) *Mycobacterium tuberculosis* isolates eleven (12.2%) were resistant to fluoroquinolones and seventy nine (87.8%) were pan susceptible to the second line anti-TB drugs while ten (11.1%) were resistant to aminoglycosides and eighty (88.9%) were pan susceptible to the second line anti-TB drugs by MTBDRsl (Table 3 and 4).



Fiqure 4.1. Occurrence of *Mycobacterium tuberculosis*in patients attending NTBLTC Saye using Xpert MTB/RIF Assay

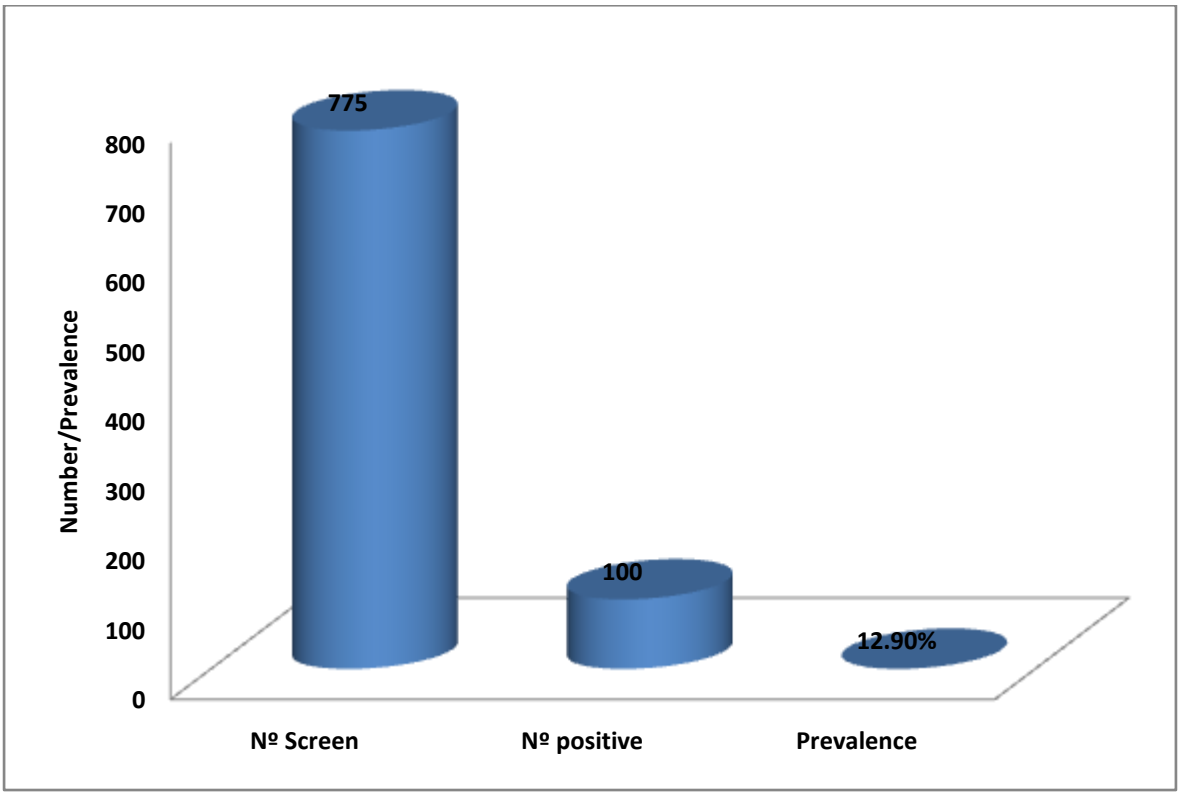


Figure 4.2: Occurrence of rifampicin resistant *Mycobacterium tuberculosis* in patients attending NTBLTC Sayeusing Xpert MTB/RIF Assay

Table 1. Isolation of *Mycobacterium tuberculosis* in patients attending NTBLTC Saye using Culture

Culture	Frequency	Percentage (%)
Positive	90	90
Negative	7	7
Contaminated	3	3
Total	**100**	**100**

Table 2. Identification of *Mycobacterium tuberculosis* complex from culture positive using rapid Immunochromatography test (TB Ag MPT640) in patients attending NTBLTC Saye

Culture	Frequency	Percentage (%)
MTBC	90	100
Non MTBC	0	0
Total	**90**	**100**

Key:

MTBC= Mycobacterium Tuberculosis Complex

Non MTBC= Non Mycobacterium Tuberculosis Complex

Table 3. Isolation of drug susceptibility of *Mycobacterium tuberculosis* using LJ proportion methodin patients attending NTBLTC Saye

Drugs	Susceptible (%)	Resistant (%)	**Total**
Ofloxacin	78(86.7)	12(13.3)	**90**
Kanamycin	82 (91)	8(9)	**90**
Amikacin	82 (91)	8(9)	**90**
Capreomycin	83 (92)	7(8)	**90**

Table 4. Drug susceptibility of *Mycobacterium tuberculosis*in patients attending NTBLTC Saye by MTBDR*sl*

Drugs	Resistance (%)	Susceptible (%)	**Total**
Fluoroquinolones	11 (12.2)	79 (87.8)	**90**
Aminoglycoside	10 (11.1)	80 (88.9)	**90**

4.4 Drug resistance pattern of *Mycobacterium tuberculosis*in patients attending NTBLTC Saye using culture proportion methodand MTBDR*sl*

Pre extensively drug resistant tuberculosis (pre XDR-TB) due to fluoroquinolone (ofloxacin) and aminoglycosides (kanamycin or/and amikacin or/and capreomycin) resistance was observed to be 13.3% and 9% while 77.7% were pan susceptible to the second line anti-TB drugs and XDR-TB was not detected by LJPM. XDR-TB was detected with a prevalence of 1.1%, while pre XDR-TB due to fluoroquinolnes and aminoglycosides resistance were observed to be 12.2% and 11.1% respectively and 75.6% were pan susceptible to the second line anti-TB drugs by MTBDR*sl* (Table 5 and 6).

Table 5: Drug resistance pattern of *Mycobacterium tuberculosis* in patients attending NTBLTC Saye using Lowenstein Jensen proportion method

Resistance pattern	LJ Proportion method	Percentage (%)
Pan susceptible	70	77.7
Pre XDR-TB (Ofloxacin)	12	13.3
Pre XDR-TB (Kanamycin/Amikacin/Capreomycin)	8	9
XDR-TB	0	0
Total	**90**	**100**

Key:

XDR-TB: Extensively drug resistant tuberculosis

Pre XDR-TB**:** pre extensively drug resistant tuberculosis

Table 6: Drug resistance pattern of *Mycobacterium tuberculosis* in patients attending NTBLTC Saye using MTBDRsl

Resistance pattern	MTBDRsl	Percentage (%)
Pan susceptible	68	75.6
Pre XDR-TB (Fluoroquinolones)	11	12.2
Pre XDR-TB (Aminoglycosides)	10	11.1
XDR-TB	1	1.1
Total	**90**	**100**

Key:

XDR-TB: Extensively drug resistant tuberculosis

Pre XDR-TB**:** pre extensively drug resistant tuberculosis

MTBDRsl: *Mycobacterium tuberculosis* drug resistance second line

LPA: Line probe assay

4.5 Occurrence of pre XDR-TB and XDR-TB among patients attending NTBLTC Saye based on Age groups

The age distribution among the respondents showed that no XDR-TB was detected between the age groups. However, resistance due to ofloxacin or aminoglucosides (kanamycin or/and amikacin or/and capreomycin) was observed to be high between the patients age groups 31-45 and 16-30 while no pre XDR-TB was detected among the patients age groups 0-15 and >60 by LJPM, the differences observed were not statistically significant $p > 0.05$ (Table 7).

4.6 Occurrence of pre XDR-TB and XDR-TB TB among patients attending NTBLTC Saye based on Gender

XDR-TB was not detected among male and female participant, while pre XDR-TB was detected more among male 13 (65%) than their female 7 (35%) counterpart by LJPM and the differences observed were not statistically significant $p > 0.05$ (Table 7).

4.7 Occurrence of pre XDR-TB and XDR-TB TB among patients attending NTBLTC Saye based on residence

Twelve (60%) of the urban settlers have more pre XDR-TB compared to their counterpart living in the rural settings 8 (40%) by LJPM. The differences observed were not statistically significant $p > 0.05$ (Table 7).

4.8 Occurrence of pre XDR-TB and XDR-TB TB among patients attending NTBLTC Saye based on HIV status

The highest prevalence of pre XDR-TB obtained in this study was among non reactive patients 12 (60%) while 7 (35%) were among those whose HIV status were unknown but 1 (5%) was HIV sero positive patients and no XDR-TB was detected by LJPM. The differences observed were not statistically significant $p > 0.05$ between HIV and drug resistance (Table 7).

4.9 Occurrence of pre XDR-TB and XDR-TB TB among patients attending NTBLTC Saye based on alcohol consumption

A prevalence rate of 90% was obtained from those without history of alcoholism and lower prevalence of 10% was observed among those that are alcoholic by LJPM. The differences observed were not statistically significant $p > 0.05$ (Table 7).

4.10 Occurrence of pre XDR-TB and XDR-TB TB among patients attending NTBLTC Saye based on previous treated TB

The highest prevalence of 85% was obtained from those that were not previously exposed to TB treatment with anti-TB drugs and lower prevalence was obtained among those that have taken TB drugs (15%) by LJPM. The differences observed were not statistically significant $p > 0.05$ (Table 7).

Table 7. Socio-demographic and risk factors to drug resistant *Mycobacterium tuberculosis* among patients attending NTBLTC Saye by LJ proportion method

Demographic and risk factors	No. positive (Pre XDR-TB)	Percentage (%)	No. positive (XDR-TB)	Odd ratio (95% C.I)	χ^2	Df	P value
Age (Years)							
0-15	0	0	0		3.184	4	0.5275
16-30	7	35	0				
31-45	12	60	0				
46-60	1	5	0				
>60	0	0	0				
Gender							
Male	13	65	0	0.69 (0.24-2.0)			0.4839
Female	7	35	0				
Residence							
Rural	8	40	0	1.06 (0.38-2.93)			0.8875
Urban	12	60	0				
HIV status							
Reactive	1	5	0		1.046	2	0.5928
Non reactive	12	60	0				
Unknown	7	35	0				
Alcohol Consumption							
Yes	2	10	0	2.48 (0.39-15.99)			0.3349
No	18	90	0				
Previous treated TB							
Yes	3	15	0	0.71 (0.18-2.75)			0.6468
No	17	85	0				

Key:

Pre XDR-TB= pre extensively drug resistant *Mycobacterium tuberculosis*

XDR-TB= extensively drug resistant *Mycobacterium tuberculosis*

4.11Occurrence of pre XDR-TB and XDR-TB among patients attending NTBLTC Saye based on Age groups

One XDR-TB was detected among the age group between 31-45 years. The high prevalence of pre XDR-TB was observed among the patients age group 31-45 and 16-30 while no pre XDR-TB was detected among the patients age groups 0-15 and >60 by MTBDR*sl*. The differences observed were not statistically significant $p > 0.05$ (Table 8).

4.12Occurrence of pre XDR-TB and XDR-TB among patients attending NTBLTC Saye based on Gender

One XDR-TB was detected among male, while pre XDR-TB was detected more among male (66.7%) than their female (33.3%) counterpart by MTBDR*sl*. The differences observed were not statistically significant $p > 0.05$ (Table 8).

4.13Occurrence of pre XDR-TB and XDR-TB among patients attending NTBLTC Saye based on residence

One XDR-TB was obtained among those living in the urban, twelve (57%) of the urban settlers have more pre XDR-TB compared to their counterpart living in the rural settings (43%) by MTBDR*sl*. The differences observed were not statistically significant $p > 0.05$ (Table 8).

4.14 Occurrence of pre XDR-TB and XDR-TB among patients attending NTBLTC Saye based on HIV status

The highest prevalence of pre XDR-TB obtained in this study was among non reactive (62%) while 33.3% were among those whose HIV status were unknown but one (4.7%) was HIV sero positive and XDR-TB was detected among HIV sero-positive by MTBDR*sl*. The differences observed were not statistically significant $p>0.05$ between HIV and drug resistance (Table 8).

4.15 Occurrence of pre XDR-TB and XDR-TB among patients attending NTBLTC Saye based on alcohol consumption

A prevalence rate of 95% was obtained from those without history of alcohol consumption and lower prevalence of 5% was observed among those that are alcoholic and XDR-TB was obtained among those without history of alcohol consumption by MTBDR*sl*. The differences observed were not statistically significant $p > 0.05$ (Table 8).

4.16 Occurrence of pre XDR-TB and XDR-TB among patients attending NTBLTC Saye based on previous treated TB

The highest prevalence of 81% was obtained from those that were not previously exposed to TB treatment with anti-TB drugs and lower prevalence was obtained among those that have taken TB drugs (19%) by MTBDR*sl*. The differences observed were not statistically significant $p > 0.05$ (Table 8).

Table 8. Socio-demographic and risk factors to drug resistant *Mycobacterium tuberculosis* among patients attending NTBLTC Saye by MTBDR*sl* assay

Demographic and risk factors	No. positive (Pre XDR-TB)	Percentage (%)	No. positive (XDR-TB)	Odd ratio (95% C.I)	χ^2	df	P value
Age (Years)							
0-15	0	0	0		3.857	4	0.4257
16-30	7	33.3	0				
31-45	13	62	1				
46-60	1	4.7	0				
>60	0	0	0				
Gender							
Male	14	66.7	1	0.76 (0.27-2.17)			0.6078
Female	7	33.3	0				
Residence							
Rural	9	43	0	1.24 (0.46-3.34)			0.6701
Urban	12	57	1				
HIV status							
Reactive	1	4.7	1		0.833	2	0.6595
Non reactive	13	62	0				
Unknown	7	33.3	0				
Alcohol Consumption							
Yes	1	5	0	0.81 (0.09-7.69)			0.8561
No	20	95	1				
Previous treated TB							
Yes	4	19	0	0.78 (0.23-2.65)			0.6894
No	17	81	1				

Key:

Pre XDR-TB= pre extensively drug resistant *Mycobacterium tuberculosis*

XDR-TB= extensively drug resistant *Mycobacterium tuberculosis*

CHAPTER FIVE

DISCUSSION

The occurrence of *Mycobacterium tuberculosis* was found to be 12.65% by Xpert MTB/RIF assay this could be due to the method of detection that has high sensitivity (92.5%) and specificity (98%) for *Mycobacterium tuberculosis*, presence of higher institutions of learning in the study area could in part contribute to high rate of infection, endemicity of tuberculosis in different study population and category of TB patients studied. The findings in this study were lower than 22.9% reported by Ikuebe and Ebueyi (2018) in Bayelsa and Panwal *et al*., (2018) who reported 27.8% in Kaduna.Resistance due to rifampicin by Xpert MTB/RIF assay was found to be 12.9%, this could be attributed to the method of detection that has high sensitivity (98%) and specificity (99%) for *Mycobacterium tuberculosis* and its resistance to rifampicin, presence of higher institutions of learning in the study area could in part contribute to high rate of infection, endemicity of tuberculosis in different study population and category of TB patients studied. Resistance to rifampicin obtained in this study is lower than14.7% reported by Ikuebe and Ebueyi (2018) in Bayelsa and 16.2% reported by Panwal *et al*., (2018) in Kaduna but higher than the 8.9% reported by Akanbi *et al.,* (2017) in Plateau State and 4.2% reported by Fadeyi *et al*., (2017) in Kano.

Ninety (90%) of the isolate were confirmed using rapid immunochromatographic test (TB Ag MPT64) as MTBC, this may be due to primary detection method (Xpert MTB/RIF) which has high sensitivity and specificity to *Mycobacterium tuberculosis* and its resistance to rifampicin, the use of moderate decontaminant (4% NaOH) which allow more recovery

of Mycobacteria in sputum sample and nutritional rich of the medium (LJ containing egg) used in the isolation of MTB, this is similar with the finding of Chihota *et al*., (2010) who reported 88.9% in South Africa and that of Mamuda *et al*., (2017) who reported 86% in Kaduna State but higher than 79.5% obtained by Molina-moya *et al*., (2018) in Abuja . Seven (7%) were culture negative, this could be due to presence of dead bacilli, longer time of exposure of the viable organism to the action of sodium hydroxide (NaOH) used during decontamination or low number of viable tuberculosis (TB) bacilli that cannot be detected by LJ media, this wasalmost in agreement with the findings of Mamuda *et al*., (2017) who reported 8% in Kaduna State but lower than 8.6% reported by Molina-moya *et al*., (2018) in Abuja but higher than 4.9% reported by Chihota *et al*., (2010) in South Africa. While contamination rate was found to be 3% which is within the acceptable limit of World Health Organization of 3-5%; this could be due to high quality standard of the laboratory used for the study and this could also be due to the number of samples used in this study, this contamination rate is lower than 9.3% reported by Chihota *et al*., (2010) in South Africa, 6% reported by Mamuda *et al*., (2017) in Kaduna and 6% as reported by Aliyu M.S. (2015) (Unpublished thesis) in Kaduna state as well as 11.9% reported by Molina-moya *et al*., (2018) in Abuja.

The occurrence of Ofloxacin resistance was found to be 13.3% by LJPM while resistance due to fluoroquinolones (FLQ) was observed to be 12.2% by MTBDR*sl*, this might be due inappropriate usage of these drugs especially FLQs including ofloxacin due to its broad spectrum of activity against gram-negative and gram-positive organisms most commonly prescribed and used in the treatment of respiratory tract infections and other infections other than tuberculosis and are available in our pharmacies without prescription in an unregulated

fashion with easy access and inappropriate use of these drugs increase the risk for drug resistant TB emergence. The findings in this study is similar to Tasnim *et al*., (2018) in Bangladesh who reported 13.24% and Hu *et al*., (2013) who reported 12.6% in China but higher than 11.3% reported by Kim *et al*., (2010)in South Korea, 6% reported by Adam*et al*., (2017) in Sudan and 7% reported by Tuelo *et al*., (2019) in Botswana but the findings were lower than 17.9%, 22.2%, 26.3% and 55.94% reported by Hoa *et al*., (2016) in Viet Nam, Oudghiri *et al*., (2018) in Morocco, Jain *et al*., (2012) in India and Adwani *et al*., (2016) in India respectively. Occurrence due to kanamycin (KM), amikacin (AM) and capreomycin (CM) were found to be 9%, 9% and 8% respectively by LJPM, in this study all TB cases that were resistant to KM were also resistant to AM and CM with the exception of one capreomycin and occurrence due to aminoglycosides was observed to be 11.1% by MTBDRsl.

The findings in this study is lower than 13.5% reported by Jain *et al*., (2012) in India, Hu *et al*., (2013) in China reported 15% for kanamycin, 11.6% for amikacin and 10.8% for Capreomycin, but similar to the study by Kim *et al*., (2010) who reported 8.3% in South Korea. This findings were higher than the 2.22% for kanamycin, 3.33% for amikacin and 1.11% for capreomycin reported by Oudghiri *et al*., (2018) and 2.94% reported by Tasnim *et al*., (2018) in Bangladesh and that of Hoa *et a*l., (2016) who reported 6.0% in Vietnam.

Our findings revealed that no XDR-TB was detected in this study by LJPM, a high proportion of pre XDR-TB due to ofloxacin resistance was observed to be 13.3% while pre XDR-TB due to kanamycin, amikacin and capreomycin resistance was observed to be 9% by LJPM. One (1.1%) XDR-TB was detected by MTBDR*sl*, resistance due to fluoroquinolones and aminoglycosides were found to be 12.2% and 11.1% by

MTBDR*sl*,this may results from poor prescription practices among medical doctors, the exposure of rifampicin or multidrug resistant TB patients to fluoroquinolones such as ciprofloxacin, ofloxacin and levofloxacin is common because these drugs are available in our pharmacies for the treatment of other respiratory infections. The indiscriminate use of these antibiotics may have contributed to the evolution of resistant pre-XDR-TB cases found in this study and it could be due to inadequate treatment by health providers, drugs may be of poor quality and low compliance to full therapy by TB patients and some mutation that were expressed by this organism does not confer any resistance and in some cases the mutations identified are synonymous and are not always related to the acquisition of resistance. The findings in this study was higher than that of Daniel *et al*., (2013) in Nigeria who reported 16.7%, 17.9% reported by Hoa *et al*., (2016) in Vietnam and 19.2% reported by of Gallo *et al*., (2018) in Brazil, but lower than 27%, 39.5% and 42.3% reported by Sagonda *et al*., (2014) in Zimbabwe, Rao *et al*., (2015) in Karachi and Jain *et al*., (2012) in India respectively. There is an increase in the rate of pre XDR-TB reported globally (WHO, 2017). A large proportion of RR-TB isolates 77.7% were susceptible to second line anti-TB drugs by LJPM and 75.6% are susceptible to second line anti-TB drugs by MTBDR*sl*, this is in agreement with 73% reported by Sagonda *et al.*, (2014) in Zimbabwe.

Occurrence of pre XDR-TB was high among age groups 31-45 and 16-30 years, the probable cause of the higher numbers of drug resistant TB in this active groups, this may be attributed due to their frequent movement/interaction, greater exposure to the environment, coming in contact with more people outdoors and higher case notification due to greater health awareness and concern among young adults and usually good quality sputum are

obtained within these active groups, this agrees with the study by Murase *et al*., (2010) who reported between 21-40 years in Japan, 20-39 years reported by Tuelo *et al*., (2019) in Botswana but slightly differ from the study conducted in India by Adwani *et al*., (2016) and Daniel *et al*., (2013) in Nigeria who found higher numbers of pre XDR-TB cases among the young adult group, with ages ranging from 18-25 years and 15-29 years respectively and that of Tasnim *et al*., (2018) in Bangladesh who reported higher number of pre XDR-TB cases in the age group of 21 - 30 years . While age groups between 0-15 and ≥60 show no pre XDR-TB was detected by both methods, this could be due to the less exposure of these individuals that belong to those age groups and most of them were unable to produce good quality sputum sample which could result to low bacterial load in the sample and might be due to less number of participants among these group.

XDR-TB was observed to be 1.1% among men, this could be attributed to the role of menand cultural habit that influence the risk of exposure have been implicated as possible reasons. Social factors such as smoking, alcoholism, imprisonment, poor nutrition and usually male abuse drugs than there female counterpart, this findings are lower than 6.0% reported by Hoa *et al*., (2016) in Vietnam among male, while no XDR-TB was detected by LJ proportion method, this could be as a result of some mutation that were expressed by this organism detected using MTBDR*sl* v.2 but does not confer any resistance and in some cases the mutations identified are synonoumous and are not always related to the acquisition of resistance. Pre XDR-TB was high among male (65%) compared to the female (35%), this agree with the findings of Mirza *et al*., (2013) in Pakistan who reported 66.6% for male and 33.3% for female but higher than the study reported by Adwani *et al*., (2016)

in India who reported 55.1% and 44.9% among male and female respectively and lower than 78% for male and 22% for female reported by Hoa *et al*., (2016) in Vietnam, this was thought to be related to alcohol and smoking dependency, imprisonment status where more male than female are involved and it could also be due to less number of female participants in this study.

The occurrence of pre XDR-TB was observed to be slightly higher (60%) among urban resident than those living in rural setting (40%), this could be as a results of congestion and overcrowding commonly found inurban market places, higher institutions of learning, mosque, church, and football watching centers, there is more awareness of the TB disease in urban than rural areas, it could also be attributed to the lesser number of patients from rural setting than urban setting in this study, there's no association between pre XDR-TB and type of residency.

The occurrence of pre XDR-TB in this study was observed to be among non-reactive HIV patients (60%) by LJ proportion and (62%) by MTBDR*sl*, this could be related to the fact that HIV does not cause drug resistance but rather predispose individuals to TB and drug resistant TB infection or disease progression, the findings agree with the study reported by Hoa *et al*., (2016) in Vietnam who reported high prevalence of drug resistance (pre/XDR-TB) among HIV sero-negative but disagree with the findings by Tuelo *et al*., (2019) who reported high occurrence among HIV sero-positive patients. Statistical analysis showed no association between HIV and drug resistance(pre XDR-TB/XDR-TB) with $p > 0.05$.

The occurrence of pre XDR-TB in relation to alcohol consumption was high among those that have not taken alcohol before (90%) compared to those that have consume alcohol before (10%) by LJ while high prevalence by MTBDR*sl* was observed to be 95% among those that have not taken alcohol compared to those that have taken alcohol (5%). Statistical analysis revealed no association between alcohol consumption and drug resistance (pre XDR-TB/XDR-TB) $p > 0.05$; this could be due to the lesser number of participant that have taken alcohol in this study.

The occurrence of pre XDR-TB was observed among those with new cases were 85% and 81% by LJ and MTBDR*sl*, this could be due to the transmission of this infectious organism directly from patients who have drug resistant strains to another person and could be due to the sampling, by which majority (90%) of the participant in this study were newly diagnosed case. Statistical analysis showed that no association exist between previous TB and drug resistance, our findings agrees with the study by Hoa *et a*l., (2016) in Vietnam who reported high prevalence of pre XDR-TB among new cases (19.5%) than in previously treated cases (16.3%).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

This study showed an occurrence of *mycobacterium tuberculosis* (12.65%) while rifampicin resistant*mycobacterium tuberculosis* (13%) by Xpert MTB/RIF assay.

Ninety 90 (90%) samples turns out to be culture positive, 7 (7%) were culture negative while 3 (3%) were contaminated. Ninety 90 (100%) isolates from culture positive were confirmed as *Mycobacterium tuberculosis* complex (MTBC) using Rapid immunochromatographic test (TB Ag MPT64).

The findings in this showed that no XDR-TB was identified by LJ proportion method, because none of the isolate meets such resistance patterns of XDR-TB. XDR-TB strains are defined as MDR-TB that is resistant to a fluoroquinolone and any second line injectable anti-TB drugs. The drugs evaluated in this study, were ofloxacin, kanamycin, amikacin and capreomycin. Therefore, in our study an XDR-TB is resistant to either ofloxacin and kanamycin, ofloxacin and amikacin or ofloxacin and capreomycin.The overall occurrence of pre XDR-TB by LJ was found to be 22.3% while 77.7% were pan susceptible to the second line drugs, resistance due to fluoroquinolones and aminoglycosides were found to be 13.3% and 9% respectively. The prevalence XDR-TB and pre XDR-TB by 2^{nd} line MTBDR*sl* (LPA) was found to be 1.1% and 23.3% respectively, while 75.6% were pan

susceptible, resistance due to fluoroquinolones and aminoglycosides were observed to be 12.2% and 11.1%.

The only XDR-TB detected by MTBDR*sl* was among male and between the age group of 31-45 years who are living in urban area. Pre XDR-TB was high among male and between the age groups of 31-45 years who are sero negative (HIV) and living in urban areas. No statistically significant association was found between the pre extensively drug resistant TB and XDR-TB with age, gender, residency, HIV status, alcohol consumption and previous TB. This study has shown an overall high occurrence of rifampicin drug resistant TB and pre-XDR-TB among the study population. Therefore, there is urgent need to increase case detection across the Nigeria using rapid molecular test for early and accurate diagnosis of drug resistant tuberculosis.

6.2 Recommendations

1. There is need for more awareness to the general public on tuberculosis and its risk factors
2. There is a need to monitor over the counter sales of some of the drugs used in the treatment of TB patients specifically quinolones in our pharmacies and medical stores for sales without prescription.
3. Patient recruited in the study were those presenting at the clinic, therefore, further studies should be conductedthat will recruite from those in the communities.
4. Collaboration between the health and academic institutions should be encouraged.
5. There is a need for close monitoring of the TB patients for proper treatment and compliance to prevent the emergence of drug resistant tuberculosis and its subsequent transmission/spread in to the community.

6. MTBDR*sl* thus represents a better screening tool for the rapid detection of resistance to second-line drugs, and it should be recommended in countries or settings with a high burden/high reported cases of RR/MDR-TB.

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Appendix I: Msc. Research Questionnaire (Confidential).

Department of Microbiology,

Faculty of Life Sciences,

Ahmadu Bello University, Zaria.

Research Topic: DETECTION OF EXTENSIVE DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS AMONG PATIENTS ATTENDING NATIONAL TUBERCULOSIS REFERENCE LABORATORY SAYE ZARIA, KADUNA STATE, NIGERIA.

Serial no:……

Introduction:I'm Suleman Mikailu M.Sc. student of Ahmadu Bello University Zaria, carrying out research work on the above topic, i will be interviewing people about their lifestyle and related health condition. When completed, this research work will help policy makers and other stakeholders in TB management in Nigeria and will go a long way in validating other drugs susceptibility testing method. I therefore need your acceptance to participate in this study by providing your sample (sputum) and other related information for the achievement of aim and objectives of the study. All the responses provided in this questionnaire will be used only for study purpose and will be kept confidential.

Accept: [ ] Decline: [ ]

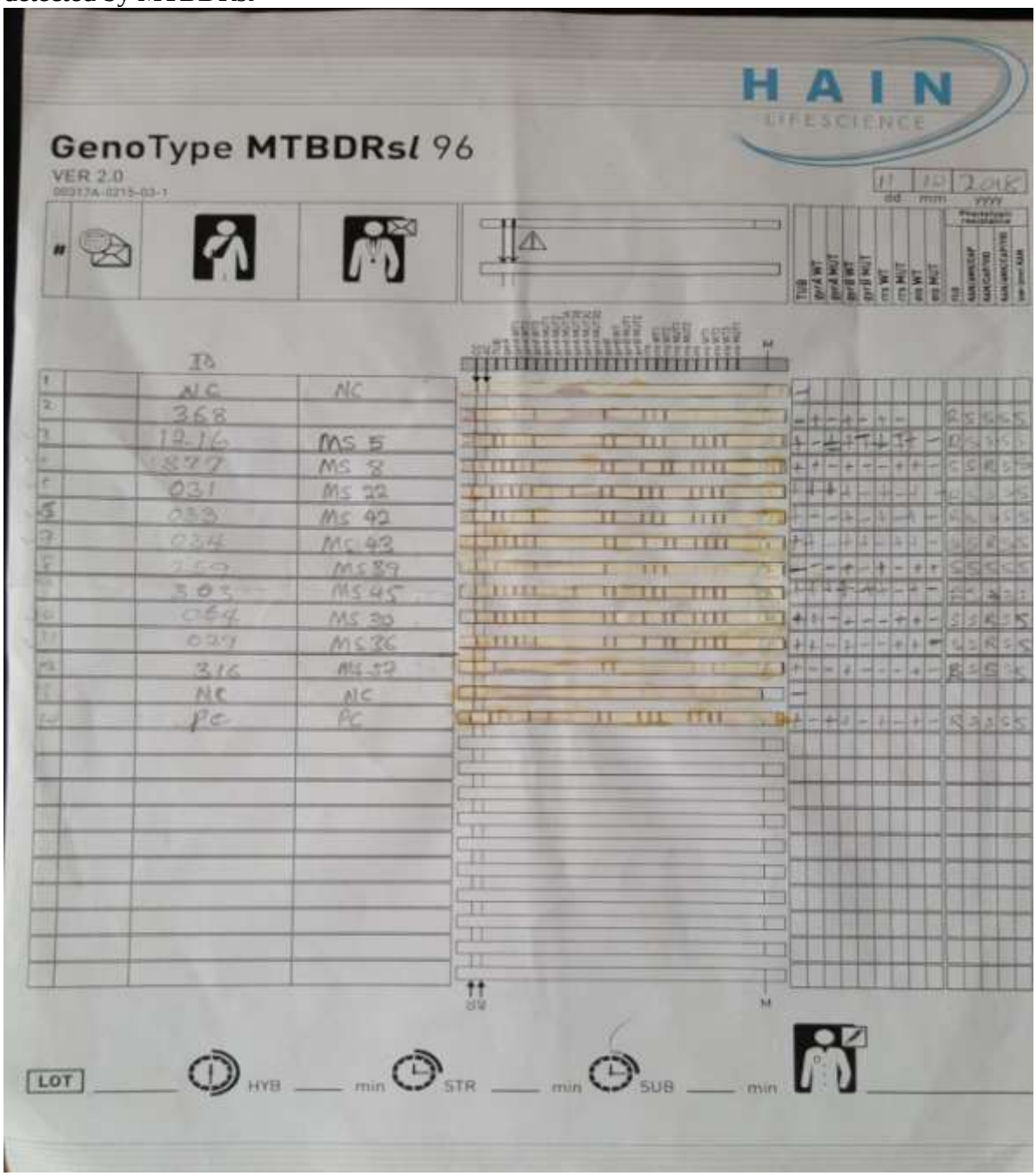
Bio data:

1. Gender: Male [ ], Female [ ].
2. Age: 0-15 [ ], 16-30yrs [ ], 31-45yrs [ ], 45-60 [ ], above 60yr [ ].
3. Type of residence: Urban [ ], Rural [ ].

Medical history

4. Patient category: New case [ ], retreatment case [ ].
5. Have you taken TB treatment before? Yes [ ], No [ ].
6. HIV status: Positive [ ], Negative [ ], Unknown [ ].
7. Have you ever drunk alcohol in your life? Yes [ ], No [ ]

Appendix II: Pattern of mutation associated with Fluoroquinolones and Aminoglycosides detected by MTBDR*sl*




HAIN
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Appendix III. Preparation of working solution of Antibiotics

Conc. Stock sol. µg/ml	**Dilution 1**			**Dilution 2**			**Amount of dilution in 200ml LJ**	**Final working Antiboiticcs conc. µg/ml**
	ml stock sol.	ml**steril e dist. Water**	**Conc. µg/ml**	**ml of dilutio n 1**	**ml sterile dist. water**	**Conc. µg/ml**		
INH 10, 000 µg/ml	**1.0**	9.0	1000	1.0	9.0	100	**0.4**	0.2
RMP 10, 000 µg/ml	**-**	-	-	-	-	-	**0.8**	40.0
EMB 10, 000 µg/ml	**1.0**	9.0	1000	-	-	-	**0.4**	2.0
AMK 10, 000 µg/ml	**-**	-	-	-	-	-	**0.6**	30.0
KAN 10, 000 µg/ml	**-**	-	-	-	-	-	**0.6**	30.0
CAP10, 000 µg/ml	**-**	-	-	-	-	-	**0.8**	40.0
OFL 10, 000 µg/ml	**1.0**	9.0	1000	-	-	-	**0.4**	2.0

Appendix IV: Informed consent form

DEPARTMENT OF MICROBIOLOGY

SCHOOL OF POSTGRADUATE STUDIES

AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

Head of Department: **Prof I.O. Abdullahi; B.Sc**, M.Sc, Ph.D, MBA (ABU) E-mail: microbiology@abu.edu.ng

INFORMED CONSENT FORM (ICF)

This Informed Consent Form is for patients attending National Tuberculosis Reference Laboratory Zaria, Kaduna State. We are inviting you to participate in this research work titled ".Detection of extensively drug resistant mycobacterium tuberculosis among patients attending national tuberculosis reference laboratory Zaria, Kaduna State"

Principal Investigator:	Suleman Mikailu
Collaborating Investigators:	Prof. S.A. Ado
	Dr. M.S. Aliyu
Name of Organization:	Department of Microbiology
	Ahmadu Bello University, Zaria - Nigeria
Name of Sponsor:	Parents
Name of Proposal:	Postgraduate Research Proposal

This Informed Consent Form has two parts:

- Information Sheet (to share information about the research with you)
- Certificate of Consent (for signatures if you agree to take part)

You will be given a copy of the full Informed Consent Form

PART I: INFORMATION SHEET

Introduction

I am Suleman Mikailu. A postgraduate student of the Department of Microbiology, Ahmadu Bello University, Zaria, carrying out a research work under the supervision of Prof. S.A.Ado and Dr. M.S. Aliyu

We are conducting a research work in Zaria, Kaduna State. I will give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

There may be some words that you do not understand. Please ask me to pause as we go through the information and I will take time to explain. If you have questions, you can ask me, the study doctor or the staff.

Purpose of the research

The purpose of this research is to detection of extensively-drug resistant mycobacterium tuberculosis among patients attendingnational tuberculosis reference laboratory Zaria, Kaduna State.

Participant selection

We are inviting all patients that have been confirmed to be Xpert MTB/RIF resistant, so that we can detect the extensively drug resistant Mycobacterium Tuberculosis attending National Tuberculosis and Leprosy Training Centre Saye, Zaria.

Do you know why we are asking you to take part in this study? YES.....NO……..

Do you know what the study is about? YES..... NO. .....

Voluntary Participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. Whether you choose to participate or not, all the services you receive at this hospital will continue and nothing will change. If you choose not to participate in this research project, you will be offered the treatment that is routinely offered in this hospital/clinic for tuberculosis, and we will tell you more about it later. You may change your mind later and stop participating even if you agreed earlier.

If you decide not to take part in this research study, do you know what your options are? YES…… NO……

Do you know that you do not have to take part in this research study, if you do not wish to? YES…… NO ……. Do you have any questions? YES……. NO. ……

Procedures and Protocol

Sputum sample would be collected from patients that have been confirmed to be Xpert MTB/RIF resistant attending NTBLTC Saye, according to NTBLTCP guidelines.

Description of the Process

During the research you will make just one visit to the Hospital/clinic. One specimen from each patient would be collected into well-labeled wide-mouth screw cap containers and then covered with lids. They would then be transported to the laboratory for processing.All specimens that are Xpert MTB/RIF resistant would be processed immediately at the NTBRL, Saye. Each specimen would be processed with N-acetyl-L-cysteine–NaOH–sodium citrate solution. XDR-TB will be detected using Proportion Method and Line Probe Assay.We will also ask you a few questions about your general health and other measures so as to investigate the epidemiologic risk factors associated with the occurrence of XDR-TB infections.

Duration

The research will take place over a period of months. During that time, it will be necessary for you to come to the clinic just once. After that, the research will be concluded.

How many times are we asking you to come to the hospital to participate in the research?.....

Can you tell me the number of time you will give us your sputum sample?.....

Do you have any questions?.....

Do you want me to go through the procedures again?.....

Risks

There is a risk that you may share some personal or confidential information by chance, or that you may feel uncomfortable talking about some of the topics. However, we do not wish for this to happen. You do not have to answer any question or take part in the research if you feel the question(s) are too personal or if talking about them makes you uncomfortable.

Benefits

If you participate in this research, you will have the following benefits:

- ✓ You will have the opportunity of having been screened for infection with XDR-TB.
- ✓ Your sputum sample will be screened for XDR-TB.
- ✓ Early diagnosis will prevent, or delay the transmission of XDR-TB in the community.
- ✓ There may not be immediate benefit to the society at this stage of the research, but there will be more awareness and future generations are going to benefit more.
- ✓ Apart from these few benefits listed above, your participation is likely to help us find answer to the research questions.

Reimbursements

You will not be provided with any special incentive or travel allowance for you to take part in the research. But we advise that your visit to the Hospital/clinic will have to be on your normal schedule clinic days. However, you will have the opportunity of been screened for HIV.

Can you tell me if you have understood correctly the benefits that you will have if you take part in the study?.....

Do you know that the study will not pay for your travel costs?.....

Do you know the kind of benefit you can get by participating in this research?.....

Do you have any other questions?.....

Confidentiality

With this research, it is possible that if others in the community are aware that you are participating, they may ask you questions. We will not be sharing the identity of those participating in the research.

The information that we collect from this research project will be kept confidential. Information about you that will be collected during the research will be put away and no-one but the researchers will be able to see it. Any information about you will have a study number on it in place of your name. Only the principal investigator will know what your number is and we will lock that information up with a lock and key. It will not be shared with or given to anyone except the principal investigator.

Did you understand the procedures that we will be using to make sure that any information that we as researchers collect about you will remain confidential?.....

Do you have any questions about them?.....

Sharing the Results

The knowledge that we get from doing this research will be shared with you through clinic meetings with your health care provider before it is made widely available to the public. Confidential information will not be shared. There will be small meetings in the community and these will be announced. After these meetings, we will publish the results in order that other interested people may learn from our research.

Right to Refuse or Withdraw

You do not have to take part in this research if you do not wish to do so, refusing to participate will not affect your treatment at this clinic in any way. You will still have all the benefits that you would otherwise have at this clinic. You may stop participating in the research at any time that you wish without losing any of your rights as a patient here. Your treatment at this clinic will not be affected in any way.

Who to Contact

If you have any questions you may ask your health care provider now or later even after the study has begun. If you wish to ask questions later, you may contact any of the following:

Suleman Mikailu

Kasimu Mamuda.

This proposal has been reviewed and approved by Research Ethical Committee of National Tuberculosis and Leprosy Training Centre Zaria, which is a committee whose task is to make sure that research participants are protected from harm. If you wish to find out more about the Research Ethical Committee, contact Research Ethical Committee of National Tuberculosis and Leprosy Training Centre (NTBLTC) Zaria.

Do you know that you do not have to take part in this study if you do not wish to? ……
Do you know that you can ask me questions later, if you wish to? ……
Do you know that I have given the contact details of the person who can give you more information about the study?.....
You can ask me any more questions about any part of the research study, if you wish to. Do you have any questions?.....…

PART II: CERTIFICATE OF CONSENT

I have read the foregoing information, or it has been read and translated to me in a language that I understand. I have also talked it over with my health care provider to my satisfaction. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I understand that my participation is voluntary. I know enough about the purpose, methods, risks and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I have received a copy of this consent form and additional sheet to keep for myself. I therefore consent voluntarily to participate as a participant in this research.

Name of Participant:

.....

Signature of Participant: .....

Date: .....

Day/month/year

Statement by Witness

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness: ..... AND Thumb print of participant

Signature of witness: _____

Date _____

Day/month/year

Statement by the Researcher/Person Taking Consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

One (l) Sputum sample will be collected

I confirm that sufficient information, including about risks and benefits, to make an informed decision have been fully explained to the participant. The participant was given an opportunity to ask questions about the study, and all the questions asked by participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

Name of Researcher/person taking the consent: .....

Signature of Researcher /person taking the consent: _____

Date _____ Day/month/year

Appendix V: Ethical Approval by National TB and Leprosy Training Centre Saye, Zaria, Kaduna State.

FEDERAL MINISTRY OF HEALTH

NATIONAL TUBERCULOSIS AND LEPROSY TRAINING CENTRE
AND REFERRAL HOSPITAL

DEPARTMENT OF PUBLIC HEALTH

Saye Village, Old Zaria - Kaduna Road,
P. M. B. 1089 Zaria, Kaduna State, Nigeria.

OFFICE
Telephone: 08037043838
08099926347
e-mail: drlabaran@yahoo.com
Web Site: www.ntbltc.org

Your Ref: NTBL/TRG/ZA/182/Vol.IV
Our Ref: 30/03/2018.
Date:

Head of Department,

Department of Microbiology,

Ahmadu Bello University,

Kaduna State.

ETHICAL APPROVAL

ATTENTION: SULEMAN MIKAILU (P15SCMC8002)

your research proposal titled:"DETECTION OF EXTENSIVE DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS USING GENOTYPE MTBDRsl ver. 2. 0. ASSAY AMONG PATIENTS ATTENDING NATIONAL TUBERCULOSIS REFERENCE LABORATORY, SAYE, ZARIA, KADUNA STATE, NIGERIA" Refers:

This is to convey the approval for ethical clearance for the commencement of the research and the use of the National Tuberculosis Training and Referral Hospital, Zaria for collection of samples

You are required to notify the committee of the progress being made and any protocol amendment(s), serious or unexpected outcome related to the conduct of this study or termination for any reason.

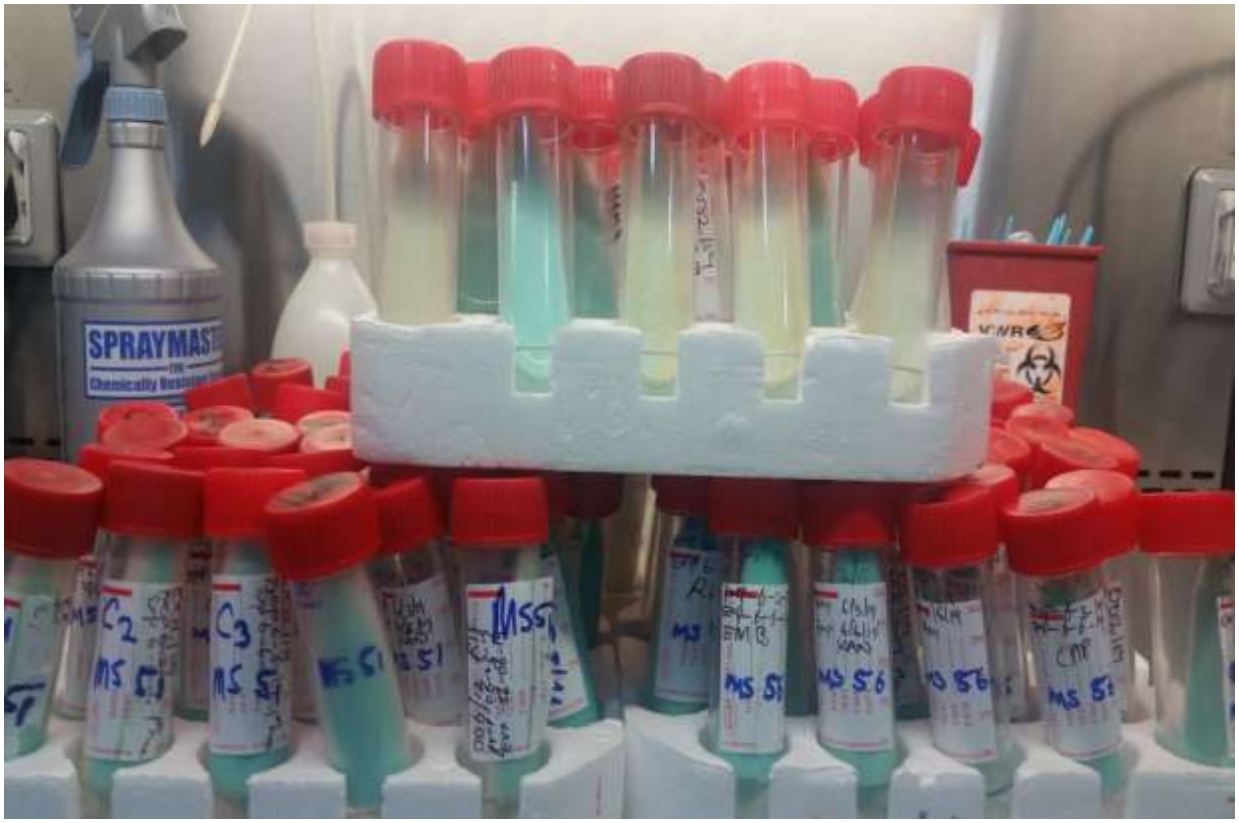
Also note that you will have to share findings of the study in both hard and soft copies upon completion.

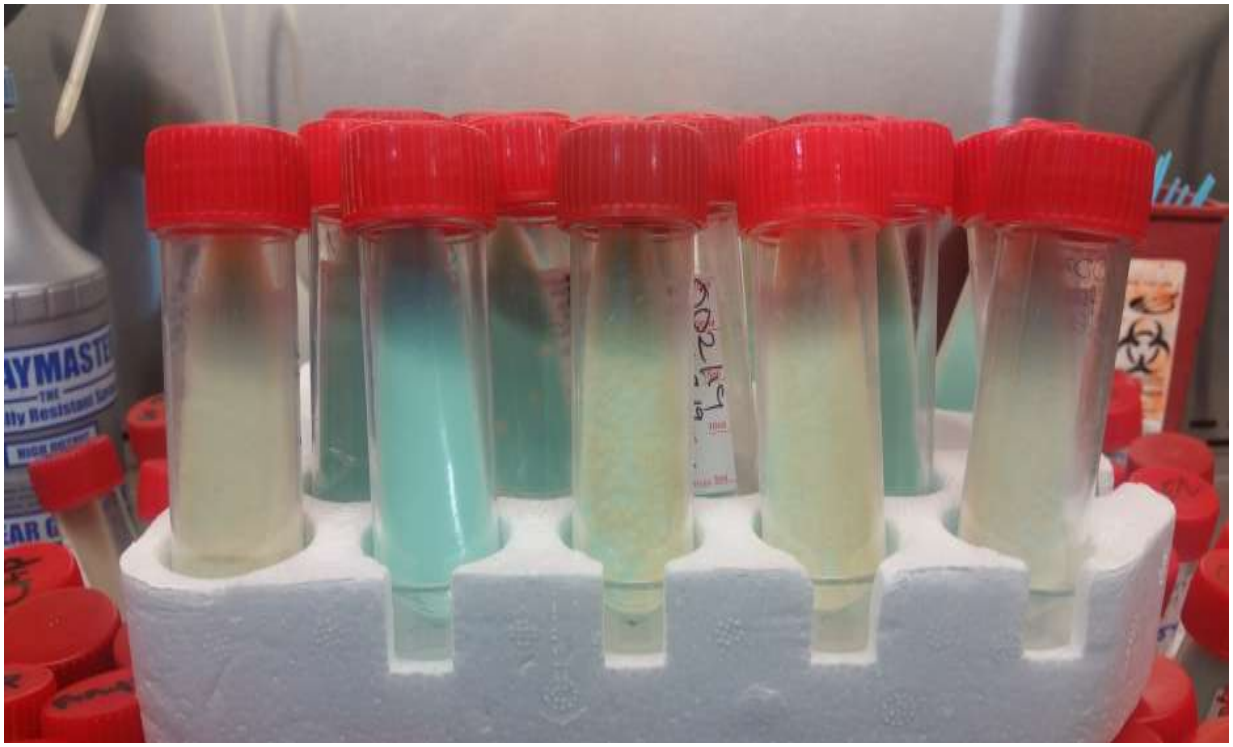
The NTBLTC takes this opportunity to thank you for choosing this institution and wishes you the best in your endeavors.

Yours faithfully,

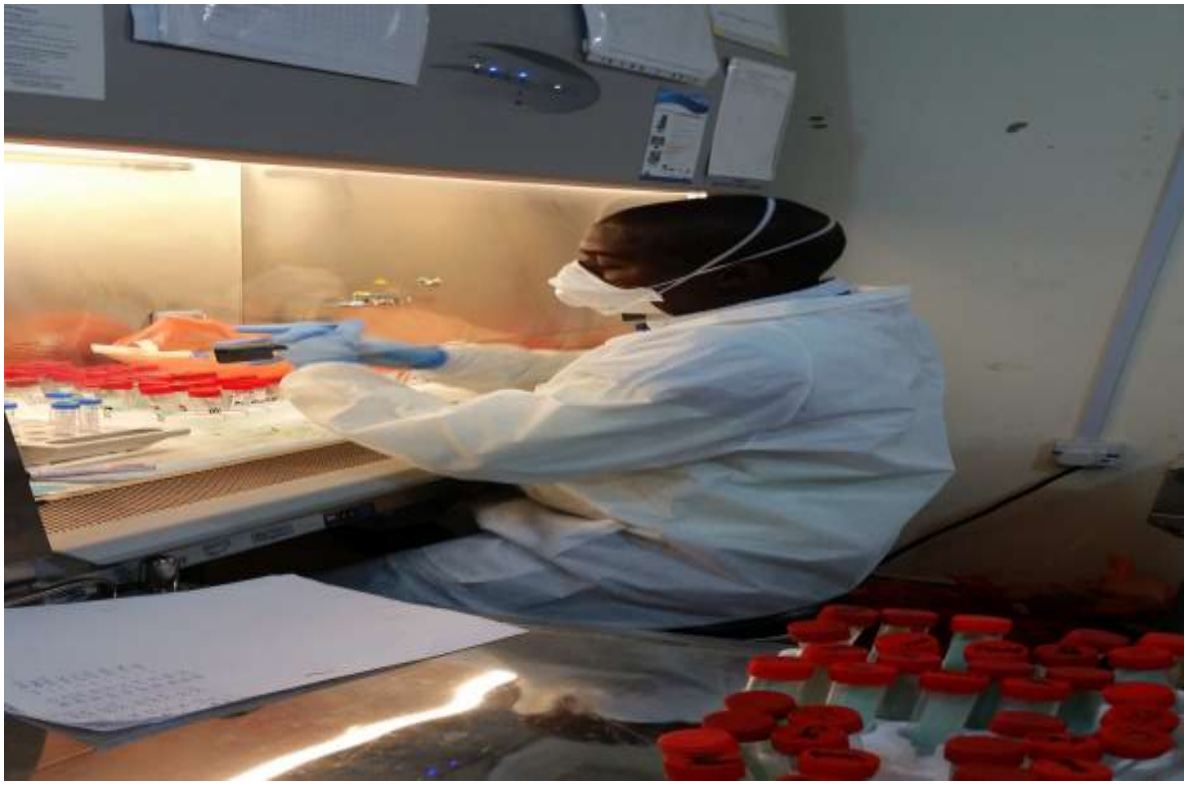
Chairman,
HREC

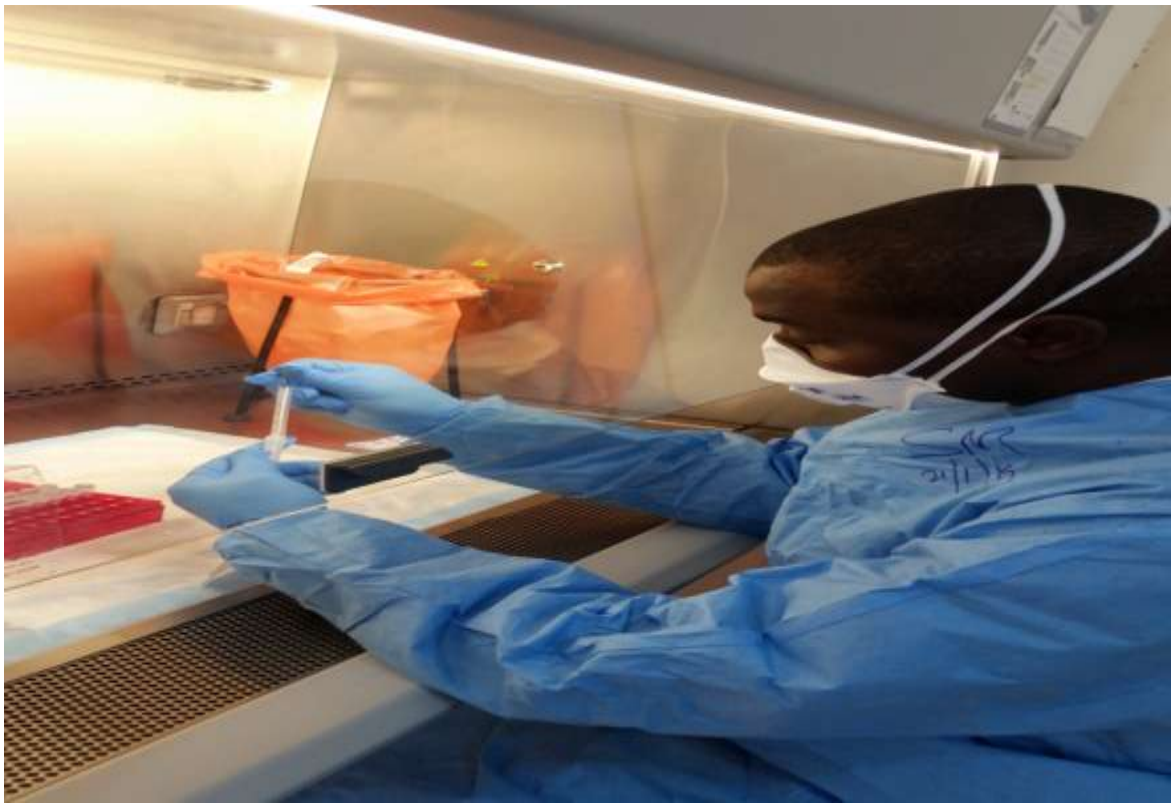
Appendix VI: Lowenstein Jensen media showing colonies of MTB after cultures





Appendix VII: Some pictures from the research work





Appendix VIII: Research Publication

Suleman *et al.*, 2020 Nigerian Journal of Microbiology, 34(1): - 4987 - 4997

Detection of Second Line Drug Resistant *Mycobacterium tuberculosis* among Patients Attending National Tuberculosis and Leprosy Training Center Zaria, Nigeria

Suleman, M[1,2]., Ado, S.A[1]. and Aliyu, M.S[1].

[1] Department of Microbiology, Ahmadu Bello University, Zaria.

[2] National Tuberculosis and Leprosy Training Center, Zaria.

*Correspondence author: mikhasulemada@yahoo.com

Abstract: Tuberculosis (TB) is an infectious disease cause by *Mycobacterium tuberculosis* and it remain one of the major public health problem. This study was to detect resistance to second line anti-tuberculosis among patient attending national tuberculosis and leprosy training centre Zaria, Nigeria using Lowenstein Jensen proportion (phenotypic) methods. A total 6125 patients were recruited, out of which 775 (12.6%) were MTB positive and 100 out of 775 were resistant to rifampicin by Xpert MTB/RIF with a prevalence of 13%. Out of 100, (90%) were culture positive while 7 (7%) were culture negative and 3 (3%) were contaminated. All of the ninety (90) samples that were culture positive were confirmed as *Mycobacterium tuberculosis* complex using immunochromatoghapic test. Seventy (77.7%) isolates were found to be pan susceptible while twelve (13.3%) and eight (9%) were resistant to Fluoroquinolones and Aminoglycoside respectively. Resistance of *Mycobacterium tuberculosis* to second line anti-TB drugs in this study was observed to be high among age groups 31-45 and 16-30 years who are male living in urban setting. It was