EVALUATION OF NITRATE REDUCTASE ASSAY FOR DETECTION OF MULTI-DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS AMONG PATIENTS AT NATIONAL TUBERCULOSIS REFERENCE LABORATORY ZARIA NGERIA

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MARCH, 2017

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ZARIA

MARCH, 2017

DECLARATION

I declare that the work in this dissertation entitle "**EVALUATION OF NITRATE REDUCTASE ASSAY FOR DETECTION OF MULTI-DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS AMONG PATIENTS AT NATIONAL TUBERCULOSIS REFERENCE LABORATORY ZARIA NIGERIA.**" has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree at this or any other Institution.

_____ _____

Kasimu MAMUDA Date

CERTIFICATION

This dissertation entitled "**EVALUATION OF EFECTIVENESS OF NITRATE REDUCTASE ASSAY AND PROPORTION METHOD FOR DETECTION OF MULTI-DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS AMONG PATIENTS AT NATIONAL TUBERCULOSIS REFERENCE LABORATORY ZARIA.**." By Kasimu MAMUDA meets the regulations governing the award of Master of Science degree of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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.

ABSTRACT

Multi-drug resistant tuberculosis (MDR-TB) remains a major challenge for the control of tuberculosis worldwide. Susceptibility testing of *Mycobacterium tuberculosis* isolates to first line drugs is therefore necessary to ascertain the diagnosis of MDR-TB. A total of 437 re-treatment patients' samples were screened for Acid Fast Bacilli (AFB), 72 were smear positive giving a prevalence of 16.47%. Out of 72 smears positive, 62 were culture positive, 6 were culture negative and 4 were contaminant using Lowenstein Jensen medium. Out of 62 cultures positive, 57 were found to be Mycobacterium tuberculosis complex (MTBC) using immunochromatographic test while 5 were negative which were considered to be NTM (Non-Tuberculosis Mycobacteria). In this study the susceptibility of 57 MTBC isolates to isoniazid (INH), rifampicin (RIF), streptomycin (STR) and Ethambutol (EMB) was determined by Lowenstein Jensen proportion method (LJPM) and Nitrate Reductase Assay (NRA). LJ PM detected 41(71.93%) MDR-TB while NRA detected 44(77.19%) MDR-TB isolates. The occurrence of poly resistance to anti-TB drugs using NRA and LJ PM was 12% and 14% while pan susceptible were of 11% and 14% by LJ PM and NRA respectively. The sensitivities and specificities of NRA compared to those of LJPM were observed to be 98% and 98%, 64% and 68%, 89% and 92%, 80% and 77% for RIF, INH, STR, and EMB respectively. Positive predictive values were 91%, 93%, 87% and 83% for RIF, INH, STR and EMB respectively. Negative values were 80%, 92%, 67% and 90% for RIF, INH, STR and EMB respectively. Good agreement was found in all the tests with κ values of 0.63, 0.61, 0.59, and 0.61for RIF, INH, STR, and EMB respectively. The HIV/TB co-infections and HIV/MDR-TB co-infections were reported to be 9.7% and 7% respectively. Highest MDR-TB among age groups of 21-30 and 31-40 years were detected by NRA and LJPM and higher MDR-TB and TB were observed in male. The NRA has the potential to be a useful tool for rapid detection of MDR-TB in resource-limited settings because of its higher sensitivity and specificity.

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LIST OF ABBREVIATIONS

ACP	Acyl carrier protein
AFB	Acid-fast bacilli
AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
BCG	*Baccile Calmette Guerin*
Bp	Base pair
BSL	Biosafety level
C	Cytosine
CC	Critical Concentration
CFU	Colony Forming Unit
CR	Compliment Receptors
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DR	Drug resistant
DST	Drug Susceptibility Testing
EMB	Ethambutol
G	guanine
HIV	Human immunodeficiency virus
ICT	Immunochromatographic Test
IJTLD	International Journal of Tuberculosis and Lung Disease
INH	Isoniazid
InhA	Enoyl-acyl carrier protein reductase

IPM	Indirect Proportion Method
IUATLD	International Union against Tuberculosis and Lung Disease
KasA	β-ketoacyl-ACP synthase
KatG	Catalase-peroxidase enzyme
KNO_3	Potassium nitrate
LJ	Lowenstein-Jensen
LJPM	Lowenstein-Jensen proportion method
LPA	Line probe assay
MDR-TB	Multidrug resistant tuberculosis
MGIT	Mycobacterium Growth Indicator Tube
MIC	Minimum inhibitory concentration
MODS	Microscopic observation drug susceptibility
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Reduced nicotinamide adenine dinucleotide
NALC	N-acetyl L-cystene
NAOH	Sodium Hydroxide
NRA	Nitrate Reductase assay
NTBLCP	National tuberculosis and leprosy control programme
NTM	Non tuberculosis Mycobacteria
NTRL	National Tuberculosis Reference Laboratory
OADC	Oleic Acid-Albumin-Dextrose-Catalase

PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin
PAS	*p*-aminosalicylic acid
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PG	Pro-Glu
PNB	Para-Nitrobenzoic Acid
PPG	Pro-Pro Glu
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
RLSs	Resource limited settings
RNA	Ribonucleic Acid
RRDR	Rifampicin Resistance Determine Region
sROC	Summary Receiver Operator Characteristic curve
STR	Streptomycin
TAT	Turnaround time
TB	Tuberculosis
VNTRs	Variable number of tandem repeats
WHO	World Health Organization
XDR-TB	Extensively drug resistant tuberculosis
ZN	Ziehl-Neelsen

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Tuberculosis (TB) continues to cause more deaths worldwide than any other infectious disease. The World Health Organization (WHO) estimated that in 2011, there were 8.7 million cases of TB, with nearly 1 million deaths among HIV-negative cases and 0.43 million deaths associated with HIV infection (WHO, 2012). More than 99% of deaths and 95% of new TB cases occur in middle- and low-income countries (Palomino, 2012). In the 2012 Global Tuberculosis Report estimates, expressed in rates per 100,000 populations, were 161 (25-420) for prevalence and 108 (50-186) for incidence. Case detection of all forms stood at 51% (29%-110%). The mortality rate for all forms of TB remains 27 (7-60) per 100,000 populations (46,000 deaths per year) (WHO, 2012). The majority of TB cases worldwide were in the South-East Asia (29%), African (27%) and Western Pacific (19%) regions. India and China alone accounted for 26% and 12% of total cases, respectively (WHO, 2013). Nigeria has one of the highest burdens of TB in the world and remains a major target in the global control of the disease (WHO, 2010). In 2011 an estimated 280,000 cases of TB (68%incident cases) were reported from Nigeria which corresponds to a prevalence rate of 280 per 100,000 populations according the WHO global tuberculosis report of 2012 (Gambo *et al.,* 2013). Based on 2012 survey Nigeria ranked 4th among the highest tuberculosis burden countries in the world. The point estimates of TB prevalence rates are 318 and 524 per 100,000 population (15 years and above) respectively (WHO, 2014).

Multidrug-resistant TB (MDR-TB) is a form of TB that is difficult and expensive to treat because it fails to respond to two important first-line drugs, specifically, rifampicin (RIF) and isoniazid (INH) (Bwanga *et al*., 2010). Data from more than 100 countries collected during the last decade show that 5% of all TB cases have MDR-TB. There were an estimated 500,000 new MDR-TB cases in 2007. Twenty-seven countries accounted for 85% of all MDR-TB cases. The top five countries with the largest number of MDR-TB cases are India, China, the Russian Federation, South Africa and Bangladesh, while extensively drug resistant TB (XDR-TB) has been found only in 58 countries to date (Stop TB partnership, 2009; WHO, 2010) .The number of prevalent cases of MDR-TB in many parts of the world is estimated to be much higher than the number of incident case arising annually. Globally, the median prevalence of MDR-TB is reported to be 3% among the new and 15% among the re-treatment cases (WHO, 2008).Countries and territories in Eastern Europe such as Tajikistan, Uzbekistan and parts of China have the highest MDR-TB prevalence of up to 15% among the new and 60% among the previously treated cases (Wright *et al.,* 2009). In sub Saharan Africa, inadequacy of laboratory services makes it difficult to estimate the actual burden of MDR-TB. However, surveillance data of 2005-7 from Cote d'Ivoire, Ethiopia, Madagascar, Rwanda, and Senegal, reported a prevalence of 1-4% among the new and 4-17% among the previously treated TB cases (WHO, 2008). In WHO TB Report of 2011, the proportion of MDR-TB among TB respondent in Africa is 3.9-5% in new cases and 16.7% in retreatment cases. In the 2008 WHO report, the 27 high MDR-TB burden countries refer to those member states estimated to have at least 4000 MDR-TB cases arising annually and or at least 10% of newly registered TB cases with MDR-TB. According to the Global TB control, WHO Report 2011, the estimated prevalence of MDR-TB

in Nigeria amongst new cases is 2.2% and retreated cases is 9.4% the national survey showed a prevalence of 2.9% among new cases 14.3% for retreatment cases (WHO, 2012).

In low-income settings, TB diagnosis relies on sputum microscopy for acid-fast bacilli (AFB). The few facilities for culture generally use Löwenstein-Jensen (LJ) media, which can take months to give a result; wider use of LJ culture thus has limited potential to accelerate TB diagnosis (Angeby *et al.,* 2003) Liquid media systems with early growth indicators, such as the Mycobacterial Growth Indicator Tube (MGIT), are more rapid and detect more mycobacterial isolates than LJ; (Goloubeva *et al.,* 2001; Bemer *et al.,* 2002; Tortoli *et al.,* 2002). However, morphological examination of colonies alone can no longer be used for species identification, making other rapid methods more important. The Capilia TB (anti-MPB64 monoclonal) assay (TAUNS, Numazu, Japan) uses monoclonal antibodies to detect a secreted mycobacterial protein, MPB64, which can differentiate *M. tuberculosis* complex from non-tuberculous mycobacteria (NTM) (Caviedes, 2000; Woods 2000; Angeby *et al.,* 2002) and shows promise as an easy and rapid tool for identifying *M. tuberculosis* complex in liquid cultures (Hillemann *et al.,* 2005; Bwanga *et al.,* 2009; WHO, 2010)

Determination of resistance to a given drug is performed as an *in-vitro* assay in the Laboratory, a process called drug susceptibility testing (DST). Where resources are limited, the WHO recommends a hierarchy of DST that should include at least RIF and INH the two most efficacious drugs that define MDR-TB (Canetti *et al.,* 1963). For more than 40 years, DST in the developing countries has relied on conventional indirect susceptibility methods on Lowenstein-Jensen (LJ) solid medium (Canetti *et al*., 1963). Indirect testing involves primary isolation of pure colonies of *Mycobacterium tuberculosis*, which are then used as inoculums for DST. In

contrast, direct DST involves inoculation of processed smear positive samples rather than pure MTB colonies. Results of direct testing are much more rapid and help to triage MDR from non MDR-TB patients promptly.

The traditional method for detection of MDR TB with indirect susceptibility testing, involving isolation of the bacterium followed by drug susceptibility testing (DST), has a long turnaround time (TAT) of 10 to 12 weeks(Bwanga *et al.*,2010). Moreover, if the indirect DST is performed on solid medium, the TAT is longer. This long time required by the indirect methods may be a potential threat to patients, health workers, and the community (Golyshevskaia,1996) While the use of liquid systems the Mycobacteria Growth Indicator Tube (MGIT) 960 (Becton Dickinson, Cockeysville, Md.), has improved TAT to about 25–45 days (Bouyer *et al*., 2000; Goloubeva, *et al.*,2001; Bemer *et al*., 2002; Tortoli *et al.,* 2002 Angeby *et al.,* 2003) liquid culture systems require expensive substrates and equipment and are therefore not feasible in resource-poor settings (Bwanga *et al*.,2009). Several rapid methods for MTB diagnosis, such as DNA probes that require sophisticated equipment, have been developed (Bemer *et al*., 2002). The polymerase chain reaction (PCR) is an alternative method that can amplify a small fragment of DNA with high specificity for the diagnosis of infectious diseases (Goloubeva *et al*., 2001). PCR has also been used to detect MTB in respiratory samples (Woods, 2000;Caviedes *et al.*,2000 Bouyer, *et al*.,2000; Angeby, 2002; Tortoli, *et al.,* 2002; Hillemann, *et al.*,2005 Bwanga, *et al*., 2009) Nitrate reductase assay (NRA) is described as a rapid, easily performed and inexpensive method for DST of *Mycobacterium tuberculosis* (MTB), which is based on the capability of MTB to reduce nitrate to nitrite (Bwanga *et al*., 2009) However, since the test is performed indirectly (by using culture isolates of MTB), it takes three to four weeks more for the isolation of the same bacterium.

1.2 Statements of Problems

Global efforts for TB control, especially in resource limited settings, are being challenged by the lack of rapid, reliable and inexpensive techniques for the detection of *M. tuberculosis* (Moore *et al.,* 2006; Shiferaw *et al.,* 2007; Brady *et al.,* 2008; Lazarus *et al.,* 2012).Results from the conventional culture detection methods come too late to influence a timely decision on patient management. Early identification is the key in both patient management and controlling transmission of *M. tuberculosis* (Caviedes *et al*., 2000; Shiferaw *et al*., 2007). Therefore, faster, inexpensive and reliable tests are urgently needed (Moore *et al.,* 2006; Minion *et al.,* 2010; Leung *et al.,* 2012).

There are different methods for detection of TB drug resistance. The proportion method (PM) and other conventional tests, based on the measurement of growth in culture media containing antibiotics, require several weeks to give results. The BACTEC radiometric system has the advantage of being more rapid (5–10 days), but requires the use of radioisotopes and can be costly to be performed routinely. Commercial tests (MGIT, E Test) and molecular tools (LPA) have been proposed, but are expensive and also impractical for routine use (Lemus *et al.,* 2004; Palomino, 2005). For developing countries, it would be useful to have a simple and inexpensive test that could rapidly detect drug-resistant *M. tuberculosis* strains. Several methods have been reported, including colorimetric methods that use redox indicators (MTT and resazurin) and phage amplification technology (Abate *et al*., 1998; Martin *et al*., 2003; Simboli *et al*., 2005).

An alternative method is the nitrate reductase assay (NRA), previously reported as a useful tool for rapid and accurate detection of resistance to first-line antituberculosis drugs (Angeby *et al*., 2002; Panaiotov and Kantardjiev, 2002; Coban *et al*., 2004; Montoro *et al*., 2005).

1.3 Justification

Indirect susceptibility testing on Lowenstein-Jensen (LJ) medium is the most common method for detection of TB drug resistance in Africa. There is an urgent demand for early and proper detection of MDR and XDR-TB cases for the effective management and control of TB. Conventional method (proportion method) (PM) is the gold standard for DST of MTB but has some limitations, such as cumbersomeness and long-turnaround time (TAT). In recent years, a multitude of techniques for rapid DST has been designed and evaluated, such as colorimetric redox method (Franzblau *et al.*, 1998) with this method, results take 2–3 months and during these period patients are given inappropriate drug regimens with poor responses and they continue to spread MDR strains, which might be causing MDR-TB outbreaks (Well *et al.*, 2007). Mycobacterium Growth Indicator Tube (MGIT 960: Becton Dickinson, Sparks, Maryland) and line probe assays **(**HainLifescience. GenoType MTBDRplus 2009**)** allow more rapid detection of resistance, and have been recommended by the WHO (Innogenetics, 2009**;** Albert *et al*., 2010)**.** However, the investment and recurrent costs is an obstacle for the broad implementation of these htechniques in the resource-limited settings (RLSs) of Africa. Therefore, the need for a rapid, affordable, accurate and easy to use test for MDR-TB in RLSs remains a priority (Bwanga *et al.*, 2009).

1.4 Aim and Objectives

1.4.1 Aim

The aim was to detect multi-drug resistant *Mycobacterium tuberculosis* using nitrate reductase assay and proportion method, among patients attending National Tuberculosis Reference Laboratory Zaria.

1.4.2 Objectives

The objectives of the study were:

1 To isolates and characterize *Mycobacterium tuberculosis* from smear positive sputum of re-treatment patients in Zaria.

2 To determine the prevalence of multidrug- resistant *Mycobacterium tuberculosis* using nitrates Reductase assay in comparison with Lowenstein Jensen proportion method.

3 To determine the demographic and risk factors associated with *Mycobacterium tuberculosis* among the study population.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mycobacteria

Tuberculosis (TB) is a chronic infectious airborne disease caused by the tubercle bacillus *M. tuberculosis* (Cole *et al.,* 1998). TB infections are characterized by the growth of rod-shaped bundles of the *M. tuberculosis* bacteria which in susceptible animals, including humans produce microscopic "tubercles" consisting of chronic granulomas, some with gaseous necrosis. Lung tissue is frequently infected, but other parts of the body can be involved (Knechel, 2009). In 2009, 9.4 million new TB cases, including the HIV infected population, were recorded. Although one-third of the world's population is infected by *M. tuberculosis*, only approximately 5-10% of the infected population who are HIV uninfected will develop TB at some stage in their life. HIV and TB co-infected populations are more like to develop TB (WHO, 2010). Factors that contribute to the development of tuberculosis disease are complex and not completely understood but suppression of cell-mediated immunity plays a key role (Caws *et al.,* 2008).

2.1.1 Classification of the Genus *Mycobacterium*

Mycobacterium tuberculosis, the causative agent of TB, was first discovered by Robert Koch in 1882 (Grange, 2009). In 1896, Lehmann and Neumann classified the tubercle bacillus under the binomial genus plus species term *Mycobacterium tuberculosis* (Grange, 2009). The *Mycobacterium* genus includes more than 120 different species and is classified under the order of Actinomycetalis, with its own family, Mycobacteriaceae. Within the *Mycobacterium* genus, the pathogens known to cause serious diseases in mammals are those causing tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*) (Ryan *et al.,* 2010). The

genus name is derived from the Greek word, „*myces*-“ which means “fungus” and relates to the mould-like manner in which mycobacteria grow in liquid culture (Kerr, 2004). There are two distinct subgroups in the *Mycobacterium* genus: slow growers and rapid growers (Tortoli, 2003) with the latter being closely related to the *Nocardia* genus. The slow growing populations include members of the *Mycobacterium tuberculosis* Complex (MTBC) which are the causative agents of tuberculosis in animals and humans (Ryan *et al.,* 2010). Species included in the MTBC are; *M. tuberculosis* (the major cause of human tuberculosis), *M. bovis* (cattle, deer), *M. africanum* (human), *M. canetti* (human), *M. caprae* (goats), *M. microti* (vole), *M. pinnipedii* (seal) (Grange, 2009). The MTBC species are grouped by their similarity at the nucleotide level and 16S rRNA sequences but differs largely in their host tropisms, phenotypes and pathogenicity (Brosch *et al.,* 2002). Rapid growers usually have two copies of 16S rRNA, while slow growers have only one copy (Tortoli, 2003).

The clinical significance of *M. tuberculosis* is being selected as the type species of the *Mycobacterium* genus and, with the exception of *M. leprae* and *Mycobacterium ulcerans* the causative agents of Leprosy and Buruli ulcer. Other *Mycobacterium* species are grouped together and are called the non-tuberculous Mycobacteria (NTM). Example includes *Mycobacterium abscessus, Mycobacterium fortuitum,* members of *Mycobacterium avium* complex, *Mycobacterium intracellulare, Mycobacterium gordonae, Mycobacterium terrae* and *Mycobacterium chelonae* (Grange, 2009).

2.1.2 Structure of Mycobacteria

The mycobacteria are mostly straight or curved rods which are non-sporing (Knechel, 2009) and do not have flagellae. The morphology of mycobacteria in clinical specimens varies between species and is affected by exposure to antimicrobial therapy (Grange, 2009). Mycobacteria are

Gram-positive and acid-fast bacilli (AFB) (Brosch *et al.,* 2002). The typical size of mycobacteria is 0.5μm by 3μm and they have a complex cell wall structure which is core to their survival (Knechel, 2009). The cell wall has a high lipid content which includes mycolic acids and a trehalose-mycolic acid component called cord factor which has been identified as an important virulence factor. Below the cell wall is the cell membrane which has two phospholipid layers (Cole *et al.,* 1998). The innermost layer of the cell wall gives the bacterium its rigidity and is made up of a net-like macromolecule comprising peptidoglycan and long polysaccharide chains crossed linked with short four amino acid peptides (Dean *et al.,* 2001). To the peptidoglycan or murein layer, mycolic acids (long chain fatty acids) are attached giving the thick cell wall its rigidity (Tortoli, 2003). This thick lipid wall provides a barrier responsible for the physiological properties of *M. tuberculosis* which enables it to be resistant to antibiotics and resistant to host defence mechanisms (Knechel, 2009). *M. tuberculosis* contains a guanine (G) + cytosine (C) - rich genome (Cole *et al.,* 1998).

2.1.3 Growth and Metabolic Characteristics of Mycobacteria

Most mycobacterial species, both slow and rapid growers are able to grow on simple media due to their undemanding nutritional requirements. Clinical cultivation of mycobacteria in laboratories is usually done on Löwenstein-Jensen (LJ) medium (Ryan, 2004). *M. tuberculosis* grows in small buff-coloured colonies resembling small cauliflowers or bread crumbs. It is a slow grower and takes between 6-8 weeks to produce a full lawn of growth (Kent *et al.*, 1985). The commercial liquid-based mycobacterial culture system such as Becton Dickson BACTEC MGIT 960 system is also used and is preferred as it takes 8-12 day for bacterial growth instead of weeks (Whitelaw *et al.,* 2009).

2.1.4 Mycobacterial Antigens

Mycobacteria have a complex antigenic structure and their antigens can be divided into three main categories: actively secreted, cell-wall bound, and cytoplasmic. The immune system of infected hosts is initially exposed to the first two categories and these play important roles in the activation of host defence mechanisms and in pathogenesis (Grange, 2009). Soluble and cell-bound mycobacterial antigens have been functionally characterized and a few have been used for the development of diagnostic tests involving antibody or cell-mediated host immune responses.

2.1.5 The *M. tuberculosis* Genome

The type strain of *M. tuberculosis*, H37Rv, was isolated in 1905 and is referred to as the TB reference strain. It is used worldwide in biomedical research applications due to its virulence in animal models (Cole *et al.,* 1998). Sequenced in 1998, H37Rv genome consists of $4.4x10^6$ base pairs (bp) with about 4000 genes (Smith, 2003). The large genome is made up of 65% G + C content which is relatively constant throughout indicating that it evolved with little or no other bacterial DNA inclusion from other genera (Grange, 2009). It is rich in repetitive DNA, especially with insertion sequences, new multi gene families and duplicated housekeeping genes (Cole *et al.,* 1998).

In comparison to other bacteria, the majority of the genes are involved in lipid synthesis and metabolism. There are approximately 250 enzymes associated with lipid metabolism of *M. tuberculosis* (Grange, 2009). An unusual feature of the *Mycobacterium tuberculosis* genome is the coding of the Pro-Glu (PG) and Pro-Pro-Glu (PPG) glycine-rich proteins (Cole *et al.,* 1998) whose functions are unknown but are found in the cell membrane and cell wall. It is

hypothesised that these proteins contribute to the antigenic variance of *M. tuberculosis* during infection (Smith, 2003).

2.1.6 The Origins and evolution of *Mycobacterium tuberculosis*

Evidence suggests that tuberculosis would have already infected and caused disease in human ancestors several thousand years (roughly 15000-20000 years) ago (Sreevatsan *et al.,* 1997). Based on evidence of tuberculosis found in early hominids in East Africa during that period, it is suggested that tubercle bacilli emerged in Africa (Gutierrez *et al.,* 2005; Grange, 2009).

The genus *Mycobacterium* is thought to have originated like other aerobic actimomycetes from soil bacteria with some species (*M. tuberculosis* and *M. leprae*) evolving to live in mammals (Smith, 2003). Prior to sequencing, *M. tuberculosis* was thought to have resulted from a species jump from animals to humans (Brosch *et al.,* 2002; Grange, 2009). Domestication of cattle was initially presumed to have allowed the mycobacterium pathogen found in livestock to evolve to humans (Cole, 1998; Smith *et al.,* 2009). Already in 1898 small differences were observed between the tubercle bacilli isolated from humans and cattle. Strains recovered from cattle were referred to as bovine tubercle bacilli (*M. bovis*) (Grange, 2009) and was hypothesised to be the evolutionary precursor of *M. tuberculosis* (Smith *et al.,* 2009). However, detailed genomic sequencing (Cole *et al.,* 1998) shows a different picture. MTBC complex differ in phenotypic characteristics but display extreme genetic homogeneity, 99.95% sequence similarity (Brosch *et al.,* 2002) with a high degree of conservation in their house keeping genes and no significant trace of genetic exchange among species. It is therefore believed that MTBC are the clonal progeny of a single ancestor (Brosch *et al.,* 2002; Gutierrez *et al.,* 2005). Research shows that the ancestral member of the complex, *M. prototuberculosis*" is about 3 million years old. Recent

studies have suggested that *M. bovis* and *M. tuberculosis* have evolved separately around the same time (Sreevatsan *et al.,* 1997).

2.1.7 Epidemiology of tuberculosis

Despite effective treatment, TB remains a major public health threat, causing 2-3 million deaths annually of which 98% are from developing countries (Arnold, 2006). This situation is further exacerbated by the emergence of drug-resistant strains of *M.tuberculosis* requiring prolonged, more expensive, less effective therapy. In 2009, based on infection and disease prevalence surveys, surveillance systems, and death registrations (Dye *et al.,* 2008), an estimated 8.7 million new TB cases were recorded, with a global incidence rate of 125 cases per 100 000 population (WHO, 2011). Globally there were 440 000 new MDR-TB cases (defined as *Mycobacterium tuberculosis* resistant to both INH and RIF) reported with 150 000 deaths in 2008. In 2009, 3.3% of all new cases reported had MDR-TB, with XDR-TB (TB resistant to flouroquinolones and at least one injectable second-line drug) been confirmed in 58 countries (WHO, 2010). The bulk of the global TB burden is from South-East Asia, African and Western Pacific regions In 2009, an estimated 1.1 million (26%) new TB case arose in patients with HIV, with a 53% HIV prevalence among TB patients in the African region (WHO, 2010).

Tuberculosis is a serious public health problem in Nigeria. Under the guidance of the National TB and Leprosy Control Programme (NTBLCP), Nigeria recently finalized it's first TB prevalence survey. This reveals that TB prevalence was two times higher than previously thought (WHO, 2014). Nigeria ranked 3rd globally in terms of the number of new TB cases (behind India and China only). According to the World Health Organization, around 590,000 Nigerians developed TB in 2013, and of these around 140,000 also are infected with HIV.

About 245,000 people die from TB in Nigeria (of those 85,000 with HIV) every year. Tuberculosis therefore accounts for just over 10% of the total deaths in Nigeria annually. Hence, every hour, almost thirty people die of TB in Nigeria, despite effective treatment being available.

The economic case for sustained investment in TB control in Nigeria is compelling. Put simply, TB treatment is low cost and effective, and this combination results in substantial economic return. Moreover, the delivery of high quality TB services can also prevent the spread of the disease to others and slow the emergence of multidrug- resistant TB (MDR-TB), a dangerous and costly form of TB. Investment in TB is also important from a poverty reduction perspective, where the costs of accessing treatment and loss of earnings may force those with TB further into poverty in Nigeria (WHO, 2012).

Drug resistance develops either through acquisition or transmission. Acquired resistance develops spontaneously through poor adherence, inappropriate treatment or poor absorption of drugs. In contrast, primary resistance develops when individuals are infected with an already drug-resistant strain (van Rie *et al.,* 2005). It is important to understand the relative contribution of primary versus acquired drug resistance to the drug resistant TB burden in different communities. A central question is whether MDR-TB strains are transmissible or whether mutations conferring drug resistance affect the fitness of the strain by impairing reproductive function (Mathema *et al.,* 2008). Several MDR and XDR-TB outbreaks have been reported, suggesting that drug resistant TB strains do have the potential for transmission (Sacks *et al.,* 1999; Gandhi *et al.,* 2006).

2.1.8. Pathogenesis and Virulence of Tuberculosis

The majority of tuberculosis infections result from inhalation of airborne droplet nuclei carrying the causative bacteria (Ryan *et al.,* 2010). Infection is established by interactions between *M. tuberculosis* and the host macrophages (Eley *et al.,* 2009). The survival success of MTB, an intracellular pathogen, relies on avoiding the killing action of the host's phagocytes (Ryan *et al.,* 2010).

There are four distinct stages in pulmonary tuberculosis; inhalation of the tubercle bacilli, activation of blood monocytes and other inflammatory cells, cessation of early logarithmic bacillary growth , and dormancy of the infection.

2.1.9 Primary infection

The initial encounter referred to as primary tuberculosis infection typically occurs in the macrophages (Eley *et al.,* 2009). The inhaled pathogenic organism (stage one) deposited in the middle and lower lobes of the alveoli which are recognized by the alveolar macrophage complement receptors (CR1, CR3, CR4). The host's cell-mediated innate immune response is activated and the pathogen is phagocytosed (Ryan *et al.,* 2010). Following phagocytosis by the macrophages, *M. tuberculosis*-specific adaptive cell mediated immunity develops (Eley *et al.,* 2009).

The destruction of mycobacteria is dependent on the host phagocytes‟ intrinsic microbicidal capacity and the ingested mycobacterial virulence factors. Immediate killing of the mycobacteria leads to no infection setting in. Those mycobacteria that escape the initial intracellular killing (Smith, 2003) by interfering with the acidification of the phagosome and affecting lysosomal

enzyme activity, making it less effective (Eley *et al.,* 2009), will multiply and be released from the macrophages. This process will attract blood monocytes and other inflammatory cells to the lung, referred to as stage two of pulmonary tuberculosis (van Crevel *et al.,* 2002). We used to assume that tuberculosis results from a single infection with single *M. tuberculosis* strain. However there is evidence to suggest that mixed infections occur (Warren *et al.,* 2004; van Rie *et al.,* 2005; Cohen *et al.,* 2011).

2.1.10 Reactivation

In tuberculosis, organisms that are not killed during primary infection enter a dormant or stationary phase as a result of oxygen and nutrient deprivation. Latent tuberculosis is present in one third of the world's population with less than 10% developing active disease (Eley *et al.,* 2009; Bezuidenhout *et al.,* 2009). Reactivation of the dormant infection occurs once the host experiences immunosuppression for a variety of reasons (Knechel, 2009) and may lead to the transmission of disease. *M. tuberculosis* reactivates at aerobic sites which favours growth of the aerobe MTB. Even though mycobacterial resuscitation-promoting factors have been identified, the mechanism of reactivation of these dormant foci is still poorly understood (Ryan *et al.,* 2010). In developing countries with large immunosuppressed populations and/or settings of high TB incidence, the risk of re-exposure within the population is high and it is therefore difficult to determine whether recurrent TB disease can be attributed to reactivation or re-infection (Horsburgh, 2008). HIV- infected gold miners are at higher risk of TB recurrence with a substantial portion resulting from re-infection (Charalambous *et al.,* 2008).

In another report from a high tuberculosis incidence area in Cape Town, the age adjusted incidence rate of TB attributable to re infection following successful treatment was four times

that of new TB. These authors challenged the traditional belief that infection with one strain of TB in immune-competent individuals protects against development of disease due to subsequent infection with a different strain. They suggest that patients who have been successfully treated for TB are at higher risk of developing disease from re-infection than the general population and that certain individuals are inherently vulnerable to TB disease (Verver *et al.,* 2005).

2.1.11 Pulmonary tuberculosis

Primary pulmonary TB may be asymptomatic or it may present with clinical systemic symptoms (Ryan, 2010). The principal site of TB is the lungs with presenting clinical features of pulmonary TB being cough, sputum, chest pain, haemoptysis, and uncommonly breathlessness (Hopewell, 2008). Pulmonary TB patients co-infected with HIV experience weight loss and fever more than those patients who are HIV-negative. On the other hand, cough and haemoptysis are less common in HIV-positive patients. Unexplained productive coughing in patients for 2-3 weeks should be screened for TB since the sensitivity of cough as a predictor for TB is very high (Maher, 2009b).

2.1.12 Extra-pulmonary tuberculosis

The predominant location of tuberculosis is the pulmonary system but an increased risk of extra-pulmonary tuberculosis disease occurs in 20% of immune-compromised patients (Knechel, 2009). The high frequency of extra-pulmonary TB among HIV-infected population results from failure of the immune system to contain the infection, thereby assisting haematogenous spread and infection of one or multiple non-pulmonary sites. Patients with extra-pulmonary tuberculosis

present with the same systemic features (Hopewell, 2008) with clinical signs and symptoms related to the pathology at the local site of disease (Maher, 2009b).

The location of extra-pulmonary tuberculosis with the most severe disease is the central nervous system which results in meningitis or space occupying tuberculomas. Tubercular meningitis is fatal in most cases if untreated (Knechel, 2009). The acute dissemination of *M. tuberculosis* into the blood stream is referred to as miliary tuberculosis and the infection can spread throughout the body, leading to multi-organ involvement (Wang *et al.*, 2007). Miliary TB progresses rapidly and can be fatal if not treated early. It is difficult to diagnose due to its systemic and nonspecific signs and symptoms (Knechel, 2009). Tuberculosis lymphadenopathy is the most common extra-pulmonary form of tuberculosis and most commonly presents with cervical lymph node enlargement (Maher, 2009b). Other sites for TB infections include the bone joints, pleura and genitourinary systems (Knechel, 2009).

2.1.13 Diagnosis of tuberculosis

2.1.13.1 Mantoux test

The Mantoux test is a widely used test for latent TB. It involves injecting a small amount of purified protein derivative tuberculin into the skin of the fore arm. It's also called the tuberculin skin test (TST).

Those that have a latent TB infection, their skin will be sensitive to Purified Proteins derivatives tuberculin and a small, hard red bump will develop at the site of the injection, usually within 48 to 72 hours of the test.

If there is a very strong skin reaction, there may be need a chest X-ray to confirm whether there is active TB disease or not. If not then a latent infection, the skin won't react to the Mantoux test. However, as TB can take a long time to develop, may need to be screened again at a later stage. Those that had the BCG vaccination may have a mild skin reaction to the Mantoux test. This doesn't necessarily mean they have latent TB (Menzies, 2000).

2.1.13.2 Chest X-ray

Tuberculosis is an infectious disease that causes inflammation, the formation of tubercules and other growths within tissue, and can cause tissue death. The chest x-rays show advanced pulmonary tuberculosis. There are multiple light areas (opacities) of varying size that run together (coalesce). The appearance opacities in the upper area of the lungs is typical for chronic pulmonary tuberculosis but may also occur with chronic pulmonary histiocytosis and chronic pulmonary coccidioidomycosis (Gotway *et al.*, 2010).

Infectious TB disease with the high mortality rate worldwide spreads successfully due to the inability of health systems to detect sufficient number of cases prior to transmission to the uninfected population (Whitelaw *et al.,* 2009). One of the key reasons for poor case detection is that TB is difficult to diagnose. Clinical and radiological findings provide only presumptive evidence in the diagnosis of TB (Somoskövi *et al.,* 1999). The diagnosis of TB relies on the detection of TB bacteria in sputum, aspirates, biopsies or blood (Doherty, 2008). The bacteria can be detected by means of microscopy or culture but the sensitivity of these methods are reliant on the quality of the specimen, concentration of bacteria and the volume of the specimen received (Whitelaw *et al.,* 2009). In addition, MTB grows slowly resulting in longer turnaround times for culture, therefore the need for rapid new molecular diagnostic tests (Afanasev *et al.,* 2007).

2.1.13.3 microscopy

Microscopic detection of *M. tuberculosis* is the most common method used worldwide because it is simple and inexpensive to perform (Doherty, 2008). Mycobacteria are difficult to stain with basic dyes (including those used in gram stain) because of the high lipid content of their cell wall. These acid-fast organisms resist decolorisation by acidified alcohol following prolonged application of a basic fuchsin dye. This property is referred to as acid-fastness and is used in smear microscopy. The sensitivity of smear microscopy is reliant on the quality of the sputum collected (Urbanczik, 2010) and the underlying pulmonary pathology. There are currently two staining methods used: Ziehl-Neelsen (ZN) for light microscopy and Auramine-O fluorochrome staining for fluorescence microscopy. (WHO 1998a). The Auramine method is preferred over ZN as it has an improved sensitivity (by about 10%) and it is easily read, requiring less time to perform (Whitelaw *et al.,* 2009). Overall microscopy only has 50-60% sensitivity (and even lower in HIV-positive population) (Doherty, 2008).

2.1.13.4 Culture

Bacterial culture on solid or liquid media is the gold standard for diagnosis of *M. tuberculosis*. Cultures are also used to distinguish *M. tuberculosis* from other mycobacteria (Doherty, 2008) and to perform drug sensitivity testing (Wood, 2007). Prior to growing the bacteria on culture media, the specimen needs to be digested and decontaminated to remove other faster growing contaminating bacteria or fungi. The most commonly used decontamination and digestion methods are the sodium hydroxide (NaOH) method and the *N*-acetyl-*L*-cysteine-sodium hydroxide (NALC-NaOH) method (Whitelaw *et al.,* 2009). During the decontamination process, some mycobacteria may be killed (WHO, 1998b). The bacterium in the specimen is concentrated

by centrifugation to optimise yield. Once the specimen is decontaminated, it is inoculated onto one or more culture media to achieve growth.

These are different types of media used for the isolation MTBC (Whitelaw *et al.,* 2009);

A Solid media

I. Lowenstein-Jensen (LJ) - an egg-based medium.
II. Middlebrook 7H10 or 7H11 - agar-based medium.
III. Dubos agar-based medium.

B Liquid media

I. Middlebrook 7H9 broth
II. Dubos Tween albumin broth

Although solid media are used widely due to the low cost, there are advantages in using liquid media for culturing TB (Doherty, 2008). One of the key advantages is the rapid growth of the bacteria in liquid media (8-12 days to give a positive growth), compared with solid media (takes 6-8 weeks) (Wood, 2007). The isolation rates of the bacteria from liquid culture are higher especially when using the commercial liquid-based mycobacterial culture system such as Becton Dickson BACTEC MGIT 960 system (Whitelaw *et al.,* 2009). The MGIT 960 tubes contain 7ml of modified 7H9 broth and the system is based on the consumption of oxygen by the growth of MTB which produces fluorescence when illuminated by a UV lamp, (Wood, 2007). The key disadvantage of the commercial liquid-culture systems is the high costs of the reagents and the equipment (Whitelaw *et al.,* 2009) and higher contamination rates than with solid media.

2.1.13.5 M. tuberculosis identification from culture

Once growth is obtained on either solid or liquid media, microscopic examination is performed to confirm the presence of acid fast bacilli and not contaminants (WHO, 1998b). Identification is then confirmed by one of several techniques: There are various techniques used to identify acid-fast bacilli from culture (Whitelaw *et al.,* 2009).

1. Antigen detection: these lateral flow assays detect *M. tuberculosis* antigen specific to members of the MTB complex (examples include Capilia TB Rapid test that detects MPB64 antigen and SD Bioline Antigen Detection Test MPT 64).
2. Phenotypic methods determine the growth rates, pigmentation and growth at different temperatures of mycobacteria which differentiate them from NTM.
3. Genotypic methods are DNA- or RNA-based assays which rapidly identifies MTB with easy interpretation. A large number of nucleic-acid-based tests are available for the detection of MTBC; however, they differentiate between different members of the complex. (WHO, 2008).
4. Mycolic acid analysis can be used to identify mycobacteria and is usually used to describe new mycobacterial species. High-performance liquid chromatography is used for these analyses in research settings only (Butler and Linda, 2001).

2.2 TB Drug Susceptibility Testing

2.2.1 General considerations

Determination of resistance to a given drug is performed as an *in-vitro* assay in the laboratory, a process called drug susceptibility testing (DST). Where resources are limited, the WHO recommends a hierarchy of DST that should include at least R and H the two most efficacious

drugs that define MDR-TB (Rich *et al.,* 2006). For more than 40 years, DST in the developing countries has relied on conventional indirect susceptibility methods on Lowenstein-Jensen (LJ) solid medium (Canetti *et al.,* 1963). Indirect testing involves primary isolation of pure colonies of *Mycobacterium tuberculosis*, which are then used as inoculum for DST. In contrast, direct DST involves inoculation of processed smear positive samples rather than pure MTB colonies.

2.2.2 Conventional susceptibility tests

Three conventional techniques - the proportion method, the absolute concentration and resistance ratio have been standardized, and are widely used in the developing countries (Aziz *et al.,* 2003).

2.2.2.1 Proportion Method

With this method, an equal quantity of a standardized inoculum of *M. tuberculosis* is seeded on a drug-free and drug-containing medium. The drug free medium is seeded with an inoculum that is 100 times diluted compared with that seeded on the drug-containing medium. Distinct, countable colony-forming units (CFU) on the drug-free medium to be compared with the drug-containing medium, only pre-existing resistant mutants are expected to grow. Although the proportion of pre-existing mutants based on a mutation rate of 1 in 10^7 - 10^{10} would be much lower, for ease of interpretation, it is theoretically assumed to be 1%, and this has been determined to predict therapeutic outcome (Canetti *et al.,* 1969). Assuming that 1% of the inoculum on the drug medium are resistant mutants, only these mutants will grow, and by dividing the number of CFU on drug medium by those on drug free medium it is possible to deduce that the isolate is susceptible ($\leq$ 1%) or resistant (>1%). Thus to interpret as susceptible, the number of CFU on the

drug medium must not exceed those on drug free medium. This is the principle underlying the proportional method of DST in MTB (Canetti *et al.,* 1963; Canetti *et al.,* 1969;).

The proportion method can be performed on LJ or Middlebrook agar medium (Makine *et al.,*2006). The LJ medium is recommended by the WHO and the IUATLD for developing countries as it is cheap, easy to read, has low contamination rates and DST results are highly reproducible (WHO/IUATLD, 2008). With the PM, estimation of the inoculums size from the colony forming units (CFU) counts is easy. However, a single CFU could arise from a clump of bacilli rather than from an individual cell, resulting in an inaccurate calculation of the proportion of resistant mutants and thus false results. The LJPM is the DST method commonly used in Nigeria and in many other developing countries.

Critical concentrations of drugs: The critical concentration (CC) is defined as the concentration that inhibits *in-vitro* growth of most MTB cells within the population of wild type strains without appreciably affecting the growth of pre-existing resistant mutants (Canetti *et al.,* 1969). If resistant mutants exceed 1%, the CC may not inhibit growth, and this predicts therapeutic failure.

2.2.2.2 Absolute concentration method

An inoculum of *M. tuberculosis* is added to LJ or 7H10/7H11 agar containing several sequential dilutions of each drug. Resistance is indicated by the lowest concentration of the drug that Inhibits growth, *i.e.* fewer than 20 colonies by the end of 4 weeks (IUTATLD, 1998).

2.2.2.3 Resistance ratio method

The resistance ratio (RR) is the ratio of the Minimum Inhibitory Concentration (MIC) for the patients' strain to the MIC of the drug susceptible reference strain, H37Rv, both tested in the

same experiment (Heifest, 2000). After 4 weeks of incubation, growth on any slope is defined as the presence of 20 or more colonies, and MIC is defined as the lowest drug concentration where the number of colonies is less than 20. A resistance ratio of 2 or less indicates sensitive strain, and a resistance ratio of 8 or more indicates resistant strains (Canetti *et al*., 1969). The RR method is the most expensive of the three conventional methods (Heifets, 2000). Conventional tests have been time tested to offer very reproducible DST results and have been considered as the gold standard tests for TB susceptibility testing. However, when performed on solid medium typical of RLSs, the DST process is very slow (2-3months), necessitating the need for more rapid assays.

2.2.3 New rapid susceptibility tests

2.2.3.1 Solid Media Culture-Based Tests

a. Nitrate reductase assay (NRA***):***

Mycobacteria tuberculosis has nitro reductase enzymes that catabolically reduce nitrate (NO_3) to nitrite (NO_2), in the reaction pathway:

$$KNO_3 + 2e^- + 4H \longrightarrow NO_2 + 2H_2O + K^+$$

In 1879, Griess, a German chemist working at the University of Marburg, described the diazotization reaction, which now forms the basis for the Griess test for the detection of nitrite (Griess *et al.,* 1879). By incorporating 1mg/mL potassium nitrate (KNO_3) in the medium, the reduction of nitrate to nitrite can be detected using the Griess reagent. When Griess reagent is added on the 7^{th}-21^{st} day of incubation, the nitrite in the medium causes a pink-purplish color. In the presence of RIF or INH at the critical concentrations, the appearance of a pink-purple colour

represents resistance to the drug (Angeby *et al*., 2002). Susceptible strains do not grow, as they are inhibited by the antibiotic thus producing a noncoloured reaction. As the NRA uses the detection of nitrate reduction as an indicator of growth, DST results can be obtained faster than by waiting for visual detection of colonies. Progress had been made in the use of the NRA for indirect DST in *M. tuberculosis* showing sensitivity and specificity of 92-100% (Martin *et al.,* 2008). The NRA test is technically easy to set and read, and gives a clear cut answer on susceptibility. When NRA is used for indirect DST, the bio safety and cost is almost similar to the LJPM since it needs a minor modification to perform the test. However, data on the performance of the NRA in RLSs in Africa was limited (Martin *et al.,* 2008)

b Phage-based susceptibility tests.

Phage assays rely on the ability of live and thus resistant *M. tuberculosis* pre-incubated with the test drug to support the growth of an infecting mycobacteriophage - a virus that infects mycobacteria (McNerney *et al*., 2001; Simboli *et al*., 2005). Both the commercially available Fast Plaque TB assay and the in-house versions have been mainly studied for the detection of rifampicin resistance of either *M. tuberculosis* isolates or directly on clinical specimens with good results and rapid time to results of 2-3 days (Albert *et al.,* 2004; Simboli *et al.,* 2005). An evaluation of this assay in Uganda also showed high sensitivity and specificity (Traore *et al.,* 2007).

2.2.3.2 In-House Liquid Media Culture-Based Tests

a. Microscopic-observation drug-susceptibility (MODS) assay

The MODS assay is a broth-based technique for the detection of tuberculosis and multidrug-resistant tuberculosis, indirectly or directly from sputum. The test relies on three principles (Moore *et al*., 2004):

first, *Mycobacterium tuberculosis* grows faster in liquid medium than in solid medium; second, characteristic cord formation occurs and these cords can be visualized microscopically in liquid medium at an early stage; and third, incorporation of drugs permits rapid and direct drug-susceptibility testing concomitantly with the detection of bacterial growth. Resistant strains are detected due to the ability of *M. tuberculosis* to grow with characteristic cord-like structures detected with an inverted microscope. Visualization of cord-like structures in liquid medium containing the tested drug indicates resistance. Recently, the protocol for the MODS assay has been updated to include a well with Para-Nitrobenzoic Acid (PNB) to help identify MTB from atypical mycobacteria. PNB inhibits growth of MTB complex but not atypical mycobacteria (Rastogi *et al.,* 1989). Studies on MODS have shown sensitivities and specificities ranging from 86-100% for rifampicin and isoniazid resistance (Park *et al.,* 2002; Moore *et al.,* 2004). The MODS assay requires minimal training, is easy to set and results are rapid (7-14 days). However, microscopic observation of the cords may be subjective. Being a liquid culture-based test performed on tissue culture plates, concerns have been raised over the bio safety of staff working with this test. However, the main bio safety concern is at the point of sputum processing and inoculation after which the plate is supposedly sealed and never reopened even at microscopic examination. On basis of rapidity (7-14 days), technical ease, and low cost, the MODS assay was thought to be a promising DST method for poor countries, and was therefore listed for further analysis of the accuracy and operational issues in Uganda.

b. Alamar blue, Resazurin and MTT assays

These tests are referred to as colorimetric assays since they involve oxidation-reduction reactions with a colour change (Martin *et al.,* 2007). They all use liquid medium on 96-well micro titre plates although tube assays have been reported (woldeMeskel *et al.,*2005) Supplemented 7H9 broth containing the test drug is inoculated with mycobacteria and incubated for 7 days at 37^{o}C. After addition of Alamar blue or resazurin reagents to wells and if there is bacterial growth, the blue oxidized reagent is reduced to a pink dye visible with the naked eye or with a colorimeter. A change of colour from blue to pink in a drug-containing well indicates presence of growing resistant *M. tuberculosis* (Reis *et al*.,2004). For the MTT assay, detection of resistance is based on the ability of mitochondrial dehydrogenase enzymes from viable mycobacterial cells to cleave the tetrazolium rings of the pale yellow MTT, resulting in formation of violetpurple or dark blue formazan, visible with a naked eye or with a colorimeter (Montoro *et al.,* 2005). After the 7 days incubation the yellow MTT is added to the wells, and the plate incubated for 24 hours to allow the MTT to precipitate in the cytoplasm. A lysing buffer is then added to the wells to lyse the bacterial cell and release the MTT into the medium. Development of a strong violet-purple colour in the drug-containing well indicates presence of resistant mycobacterial strains (Montoro *et al.,* 2005). Each of these colorimetric methods has been assessed in previous studies with reported sensitivity and specificity of 94-100%, and the results obtained within 10 days (Martin *et al.,*2007). These tests use microtitre plates with around 10 samples per plate, thus high throughput, which would be good for TB high-burden settings. However, after 7 days of incubation, the Alamar blue and resazurin plates are opened once while the MTT plate must be opened twice to add the detection reagents. This is not only cumbersome but also carries a serious bio safety risk to the laboratory personnel.

2.2.3.3 Commercial Liquid Media Culture-Based Tests

a. Mycobacteria Growth Indicator Tube**.**

The Mycobacterial Growth Indicator Tube (MGIT; Becton Dickinson, Sparks, Maryland, USA) is based on fluorescence detection of mycobacterial growth in a tube containing a modified Middlebrook 7H9 medium together with fluorescence quenching-based oxygen sensor (a ruthenium pentahydrate substance embedded in silicone rubber) at the bottom of the tube (Rusch-Gerdes *et al.,* 1999; Reisner *et al.,* 2004). As the bacteria grow and consume oxygen, the indicator fluoresces under ultraviolet light, and growth in a tube with the test drug indicates resistance (Reisner *et al.,* 2004). The MGIT system, introduced around 15 years ago, in its manual and now automated versions, is part of the new-generation of rapid tests for detection of drug resistant TB. Studies of both the manual and automated MGIT 960 system have shown very high correlation with conventional DST methods for rapid detection of resistance to the first and second-line anti-TB drugs (Pfyffer *et al.,* 1999; Johansen *et al.,* 2004).

The automated MGIT 960 system has the advantage of high throughput (>900 samples can be tested on one instrument at ago), it is rapid (4-13days), and very easy to interpret results. All these aspects would be suitable for the TB high-burden settings. However, the test has been studied only as an indirect assay mainly in the developed countries. Thus, there is limited data on the technical performance of the test in RLS, and on how the operational issues such as cost, contamination rates, and power failures would impact on the DST results in the low income settings (Reisner *et al.,* 2004).

2.3 Genotypic (Molecular) Methods of Detecting MDR-TB

The molecular mechanisms conferring drug resistance have been described for several of the major first and second line anti-tuberculosis drugs. This understanding has facilitated the recent development of rapid molecular diagnostic tests for identification of drug resistance. Molecular methods or genotypic DST are DNA-based tests which identify mutations associated with drug resistance (Campbell *et al.,* 2011). The major limitation to molecular methods is that for some anti-tuberculosis drugs not all resistance-conferring mutations have been well characterised for some anti-tuberculosis drugs (van Deun *et al.,* 2010). Various in-house molecular tests have been developed and used for the detection of resistance determining mutations (Whitelaw *et al.,* 2009). In 2008, the World health Organisation (WHO) endorsed the use of line probe assays (LPAs) for the rapid detection of MDR-TB (WHO, 2008). LPAs are based on multiplex polymerase chain reaction (PCR) amplification followed by reverse hybridisation for the identification of MTBC and the detection of resistance in the genes associated with the anti-tuberculosis drugs RIF and INH (Albert *et al.,* 2010). The MTBDR*plus* (Hain Lifescience, GmbH) LPA, detects mutations conferring resistance in the *rpoB*, *katG* and *inhA* genes and produces a result within 1 day (Barnard *et al.,* 2007). More recently, in 2010, WHO STAG-TB endorsed the use of the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA), a molecular test that detects *Mycobacterium tuberculosis* as well as rifampicin resistance. WHO recommended GeneXpert MTB/RIF as the initial diagnostic test for TB in individuals suspected of MDR-TB or TB/ HIV-co-infection (WHO, 2011b).

DNA sequencing is considered the gold standard in which the nucleotide sequence of the amplified DNA is visible giving the most accurate result while DNA microarray (DNA biochip)

assays have been developed for the detection of TB drug resistance but they have as yet not been fully evaluated (van Deun *et al.,* 2010).

2.3.1 Genotyping methods used in TB Epidemiology

Molecular methods are also used for epidemiological studies of MTB with many tools available, each with its own positive and negative characteristics (McEvoy *et al.,* 2009). These 26 Molecular tools have been used to give some insight into the genomic evolutionary changes at the DNA level (van der Spuy *et al.,* 2008).

2.3.1.1 IS6110 Restriction fragment length polymorphism (RFLP)

The *M. tuberculosis* genome contains/includes many insertion sequences (IS) which are able to replicate and 'jump' or transpose themselves from one region to another thereby displaying polymorphism in the number of elements per genome (McEvoy *et al.,* 2009). IS*6110* is TB specific and is the most widely used genetic marker with IS*6110* RFLP considered as the 'gold standard' for MTB genotyping (Stavrum *et al.,* 2009).

2.3.1.2 Spoligotyping

All MTBC members have the direct repeat (DR) chromosomal region which is composed of multiple 36-bp directly repeat sequences interspersed by non-repetitive unique spacer regions ranging from 35 to 41 bp in length (van der Spuy *et al.,* 2008; McEvoy *et al.,* 2009).One DR sequence and its spacer is referred to as direct variable repeat (DVR) where polymorphisms occur between MTB isolates (McEvoy *et al.,* 2009). Spoligotyping or spacer oligonucleotide typing is designed to determine the presence or absence of 43 spacer regions in *M.tuberculosis*

isolates (van der Spuy *et al.,* 2008). Spoligotyping has less discriminatory power to distinguish *M. tuberculosis* strains than IS*6110* (RFLP) (Barnes *et al.,* 2003).

2.3.2 Molecular Assays

Molecular methods for MDR-TB detect the common mutations conferring resistance to R and H, rather than the resistance phenotype. The commercially available line probe assays involve DNA extraction, polymerase chain reaction (PCR), and solid phase reverse hybridization of amplified DNA to probes covering the core region of the target gene, immobilized on a nitrocellulose strip. These tests can be applied on MTB isolates or on sputum smear positive sputum (De Beenhouwer 1995; Idigoras *et al.,* 2000).

2.3.2.1 INNO-LiPA Rif TB Assay

(Innogenetics, Ghent, Belgium) detects the common mutations in only the *rpoB* gene for rifampicin resistance (Rossau *et al.,* 1997). Evaluation studies of the INNO-LiPA Rif. TB Assay showed high sensitivity and specificity (Jureen *et al.,* 2004). Rifampicin resistance predicts MDR-TB in over 90% of cases, and may be sufficient for MDR diagnosis. However, isoniazid testing as well may be helpful in the design of second line drug regimens for MDR-TB patients. The GenoType® MTBDR assay (Hain Lifesciences, Nehren, Germany 2009) simultaneously detects the common mutations in the *rpoB* and *katG* gene (Hillemann *et al.,* 2005). The GenoType® MTBDR*plus,* a newer version of the genotype MTBDR detects more of the common mutations in the *rpoB* and *katG* genes, and also mutations in the *inhA* promoter region, making it the most sensitive line probe assay for detection of resistance (Hillemann *et al.,* 2007). Evaluation studies of these assays have reported sensitivity and specificity of 98-100% for rifampicin, and of 70-100% for isoniazid, with results in 1-3 days (Ling DI *et al.,* 2008).

The resistance to anti-tuberculosis drugs occurs naturally by the process of selection of drug-resistant mutants by the treatment agent(s) (Whitelaw *et al.,* 2009) resulting in some bacilli acquiring resistance to drugs used for treatment when they are poorly managed (Campbell *et al.,* 2011). MDR-TB is spreading worldwide requiring more complex and costly treatment regimens with worse outcomes (Whitelaw *et al.,* 2009). The emergence of XDR-TB has highlighted the need for more DST as well as rapid diagnosis of the type of resistance (Campbell *et al.,* 2011).

2.4 Molecular Basis and Mechanisms of Resistance to First-line Drugs

Resistance of *M. tuberculosis* to anti-tuberculous drugs results from a spontaneous genetic event, occurring at a frequency of 10^{-5} to 10^{-8}. Resistance to various drugs arises independently (Pfyffer, 2000). Resistance in *M. tuberculosis* appears to be confined to chromosomal DNA and does not involve transfer of mobile genetic elements (such as plasmids) who occurs in other bacteria and mycobacteria (Pfyffer, 2000).

Resistance to the first-line anti-TB drugs occurs through sequential accumulation of mutations in the genes targeted by the drugs (Johnson *et al.,* 2007). These mutations occur at the drug target region or at the enzymes that are involved in the activation of the drug (Somoskovi *et al.,* 2001). These drug-resistant strains develop when chemotherapy is disrupted or otherwise inadequate (Ramaswamy *et al.,* 1998).

The molecular mechanisms conferring drug resistance have been described for several of the major first and second line anti-tuberculosis drugs. This understanding has facilitated the recent development of rapid molecular diagnostic tests for identification of drug resistance (Zhang *et al.,* 2009). While about 96% of rifampicin resistant isolates have mutations in a defined region of

the *rpoB* gene, resistance to isoniazid is more complicated, involving at least two gene *katG* and *inhA* which together account for approximately 65-75% of resistant strains (Ramaswamy *et al.,* 1998). For other drugs, known drug resistance mutations occur in a smaller proportion of isolates suggesting other mechanisms of resistance (Ramaswamy *et al.,* 1998; Zhang *et al.,* 2009). Several studies have shown that the frequency distribution of mutations among drug resistant isolates shows geographic variability both between and within countries (Ohno *et al.,* 1996; Miotto *et al.,* 2006; Barnard *et al.,* 2008; Green *et al.,* 2008).

2.4.1 Isoniazid

INH is a pro-drug with a simple structure containing a pyridine ring and a hydrazide group requiring activationas shown in figure 1 (Somoskovi *et al,* 2001; da Silva *et al,* 2011). Almost all mycobacteria and prokaryotes other than *M. tuberculosis* are resistant to INH (Ramaswamy *et al,* 1998). INH is activated by the catalase/peroxidase enzyme encoded by *katG* which prevents the synthesis of mycobacterial mycolic acids by inhibiting the NADH-dependent enoyl-ACP reductase coded by *inhA* (da Silva *et al,* 2011). The basis of resistance to INH is more complex and is based on a genetic system involving a large number of gene regions such as *katG, inhA, kasA, aphC, oxyR* and less common *iniA, iniB* and *iniC* genes (Afanas'ev *et al,* 2007; Wei-Wei *et al,* 2007). Studies have shown that *katG* covers a wide range of 50-95% of INH resistance in isolates while *inhA* resistance is present in 20-35% of isolates (Wei-Wei *et al.,* 2007; Aslan *et al,* 2008).

Molecular genetic studies have correlated INH resistance with loss of catalase-peroxidase activity which is controlled by the *katG* gene (Ramaswamy *et al,* 1998). Mutations in the catalase-peroxidase gene, usually at codon 315, prevent the conversion of the INH pro-drug into

its active form (Wei-Wei *et al*, 2007). The promoter region of the *inhA* operon comprises two genes, *fabG1 (mabA)* and *inhA* (Afanas'ev *et al*, 2007; Ramaswamy *et al*, 1998). The metabolic activity of the operon is to code for β-ketoacyl reductase and the fatty-acid enoyl-acyl carrier protein reductase which participate in mycolic acid biosynthesis (Afanas'ev *et al*, 2007). Missense mutations in the promoter region (position -15T or -8C) result in over expression of the target for the active INH radical leading to INH resistance (Afanas'ev *et al*, 2007; Evans *et al.*, 2009). Mutations in the *katG* gene code predominantly for high-level resistance to INH while low-level resistance is determined mainly by mutations present in the 31 *inhA* gene (Barnard *et al*, 2008; van Deun *et al*, 2010). Resistance to the anti-tuberculosis drug ethionamide is also cause by mutations in the *inhA* gene. Further research is required to better understand the mechanisms responsible for INH resistance in the other genes (Afanas'ev *et al*, 2007).

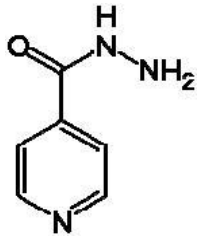


Figure 2.1. Chemical structure of isoniazid (INH)

2.4.2 Rifampicin

RIF is a lipophylic ansamycin with efficient antimicrobial activity and is considered a key drug in the treatment of tuberculosis as shown in figure 2 (da Silva *et al.*, 2011). The activity of RIF involves binding to the β-subunit of ribonucleic (RNA) polymerase resulting in inhibition of transcription (Wei-Wei *et al*, 2007). Resistance to RIF is caused by amino acid substitutions as a

result of missense mutations, or deletions or insertions in this region encoding the β-subunit of the RNA polymerase enzyme (Aslan *et al,* 2008; Ramaswamy *et al,* 1998). Resistance to RIF is largely confined to mutations present in an 81 base pair hotspot region on the *rpoB* gene (Aslan *et al,* 2008; Ramaswamy *et al,* 1998). This drug resistance region is known as the RIF resistance-determining region (RRDR) (Afanas'ev *et al,* 2007; WHO, 2007; Evans *et al.,* 2009). The RRDR spans from codon 507 to 533 of the *rpoB* gene and more than 95% of resistant strains harbour mutations in this region (Afanas'ev *et al,* 2007; Wei-Wei *et al,* 2007; Evans *et al,* 2009).

The characterisation of the *M. tuberculosis rpoB* gene showed a wide variety of distinct alterations in the RRDR in epidemiologically unrelated patient isolates (Ramaswamy *et al.,*1998). Studies have commonly documented missense mutations at codon 531 (Ser), 526 (His) resulting in amino acid replacements amino acid substitutions Ser513Leu and His526Tyr 32 were most common (Afanas'ev *et al,* 2007). Several research groups have investigated the relationship between RIF MIC levels and structural changes found in the *rpoB* gene (Ramaswamy *et al,* 1998). They had found a strong correlation between *rpoB* variants and RIF MICs. High-level drug resistance to rifampicin, rifabutin and rifapentine had amino acid substitutions at codons 513, 526 and 531, whereas, amino acid substitution at positions 514, 521 or 533 conferred low-level resistance to RIF. In RIF resistant isolates that lacked changes in the RRDR, the molecular mechanisms involved are unknown (Ramaswamy *et al,* 1998).

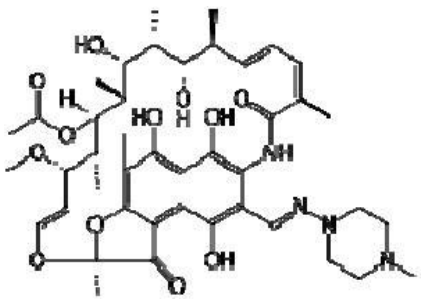


Figure 2.2. Chemical structure of Rifampicin (RIF)

2.4.3 Streptomycin

Streptomycin (STR) is aminocyclitol glycoside antibiotic and was the first antibiotic used in TB treatment (da Silva *et al.*, 2011). STR resistance is associated with the 16s rRNA gene, *rrs* and *rpsL* which encodes for the ribosomal protein S12 (Ramaswamy *et al.,* 1998).

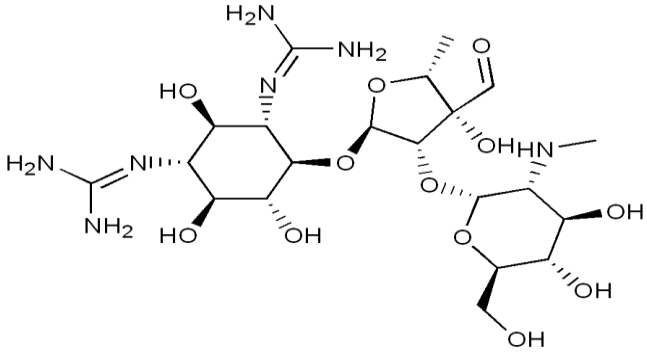


Figure 2.3. Chemical structure of Streptomycin (STR)

2.4.4 Ethambutol

Ethambutol (EMB) ([S,S']-2,2'-[ethylenediimino])di-1-butanol has bactericidal activity and was first used against TB in 1966 (da Silva *et al.*, 2011). It interferes with the biosynthesis of the cell wall arabinogalactan. Mutations in the *embB* operon are responsible for EMB resistance (Ramaswamy *et al.,* 1998).

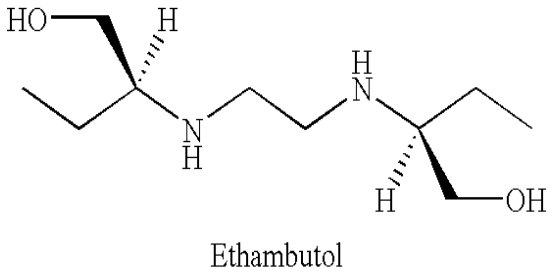


Figure 4.4. Chemical structure of Ethambutol (EMB)

2.5 Treatment, Prevention and Control

2.5.1 Treatment of Tuberculosis

Many antimicrobials have been shown to be ineffective against mycobacteria due their unusually impermeable lipid-rich cell wall. There are a few groups of antimicrobials which are active and are used in the treatment of MTB (Ryan *et al.,* 2010). The primary drugs of choice recommended by the WHO are referred to as the first-line anti-tuberculosis drugs which are administered as a 6-month short course. Pyrazinamide and ethambutol together with INH and RIF are given for the first 2 months during the intensive phase of treatment followed by INH and RIF for the next 4 months (WHO, 2009). The injectable streptomycin may also be used in the initial phase of treatment but its usefulness has decreased over time due to increasing resistance to the drug (Donald *et al.,* 2009). Second-line drugs are reserved for use when there is resistance to first-line drugs, drug intolerance or toxicity (WHO, 2009).

2.5.2 Immunity and TB vaccines

Each year millions of infants are vaccinated with the BCG (BacilleCalmette-Guerin) vaccine to prevent severe disease such as meningitis and miliary TB (Dye *et al.,* 2008). BCG is safe and may elicit long-term protective immunity but the escalating TB epidemic worldwide has

occurred even with the widespread use of BCG vaccines. The global increase of TB related mortality shows that the BCG vaccine has failed and the development for new more effective vaccines is required (Hussey *et al.*, 2009)

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 Laboratory Settings for the study

The National Tuberculosis and Leprosy Training Center is the largest TB referral center in northern Nigeria; several hundred patients receive TB/HIV treatment at these facilities. In addition, the NTBLTC serves as the national training center for community health workers and other laboratory health personnel's involved with tuberculosis detection and treatment at the community level. This center is one of the two national TB reference laboratory that is equipped with TB biosafety level 3 (BSL-3) and TB molecular diagnostic laboratories.

3.2 Study population

Patients with suspected new and previously treated TB attending NTBLTC were used for the study.

3.3 Sample size

Sample size was determined using the prevalence rate of MDR-TB of 2.9% (WHO, 2012) and Kish Leisle formula (Mshana *et al.,* 2009) ;

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

Where n=sample size.

Z= 1.96 at 95%confidance interval.

P= known prevalence of the infection 2.9 %(WHO, 2012)

Q=1-P

L= allowable error =0.05

$$n = \frac{(1.96)^2 \times 0.029\ (1- 0.029)}{(0.05)^2}$$

n = 43

A total of 437 sputum sample were screened for AFB in order to increase statistical precision and reduce error.

3.4 Consent of Patients and Ethical Approval

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

3.5 Inclusion criteria

All sample from re-treatment patients who consented to participate in the study and whose sputum samples were smear positive for acid fast bacilli.

3.6 Exclusion criteria

All sample from re-treatment patients who do not consented to participate in the study or those who consented, but whose sputum samples were acid fast bacilli negative.

3.6 Sample collection

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

3.7 Microscopy

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

3.8 Media preparation

Lowenstein Jensen media base was prepared according to manufactures instructions. The culture media was prepared by weighing 37.4g of commercial Lowenstein Jensen medium powder and dissolving in 600ml distilled water and 12ml glycerol was added and mixed thoroughly, the solution was heated with frequent agitation just until the media boils, the solution was autoclaved at 121°C for 30 minutes and cooled, one thousands mls of homogenized eggs were also be added and mixed. The media was then dispensed into 15ml falcon tubes and were inspissated at 85°C for 45 minutes; the media were incubated at 37°C for 48 hours for sterility checks (Concepcious *et al.*, 2001).

3.9 Specimen digestion and decontamination

All specimens that were smeared positive were processed immediately at the NTBLTC. Each specimen was processed with N-acetyl-L-cysteine–NaOH–sodium citrate solution (4% NaoH and 2.9% Na- Citrate). The specimen was then centrifuged at a speed of 3000 g for 15-20 minutes. After centrifugation, tubes were then allowed to sit for 5 minutes to allow aerosols to settle. Then carefully, the supernatant was decanted into a liquid jar containing a mycobactericidal disinfectant. A small quantity of (1-2 ml) phosphate buffer (pH 6.8) was added and the sediment re-suspended with the help of a pipette and vortex mixer. The sediment in each tube was reconstituted with phosphate buffered saline (PBS) to 2 ml, mixed well and then pooled into one tube that served as common inoculum source for all subsequent tests (Kubica,1963).

3.10 Isolation

An inoculum of 0.1ml of the sediments was inoculated into tubes of Lowenstein Jensen (LJ) media and incubated at 37°C in slanting position. The tubes were observed after three (3) day to check for fast growers and contaminant and weekly thereafter till eight weeks. Cultures showing evidence of growth at any time during this period were then checked for morphology by making smear and staining by the Zielh-Neelsen procedure (Martin *et al*., 1975; Concepcious *et al*., 2001; Srivastava *et al*., 2008).

3.11 Identification of Mycobacterial Isolates

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

3.12 Preparation of Lowenstein- Jensen Medium with Drugs

Isoniazid (INH), Streptomycin (STR), Rifampicin (RIF) and Ethambutol (EMB) were obtained as powder from Sigma Aldrich (Bornem, Belgium). Each drug was prepared at a concentration of 10 mg/ml in sterile distilled water with the exception of RIF, which was dissolved in dimethylsulphuroxide (DMSO). Stock solutions were filtered through a mesh of (0.45µm) sterilized and stored at -70°C and used within six months. Different concentration of 0.2µg INH, 2µg EMB, 40µg RIF and 4µg STR, were incorporated into LJ medium and then inspissated at 85°C for 45 minutes. After preparation, the media were incubated for 48 hours at room temperature for sterility check before use (Canetti *et al.,* 1963).

3.13 Preparation of Lowenstein- Jensen Medium with KNO_3 and Drugs

Two grams of potassium nitrate were dissolved in 10mls of sterile distilled water. And 1ml of prepared KNO_3 was added to 200ml of the drug containing and drug free LJ medium, dissolved by stirring, aliquoted and then inspissated for 45 minutes at 85°C. After inspissations, the media was incubated for 48 hours at 37°C for sterility check before use. For NRA, the bottles

containing LJ with antibiotics and KNO_3 were used for the test while LJ with KNO_3 were used as a control. All the tests were conducted in triplicate to reduce the risk of contamination (Canetti *et al.*, 1963).

3.14 Drug Susceptibility Testing by Proportion method

The Indirect Proportion Method (IPM) was performed on LJ medium with the same recommended critical concentrations of antibiotics as mentioned previously. The culture of *M. tuberculosis* strain was harvested and suspended in tube containing sterile distilled water with 5-7 sterile glass beads then vortexed for about 30 seconds to homogenise the bacterial suspension and then allowed to stand for 15-30minutes for large aggrevates of bacteria to settle and turbidity of bacterial suspension was adjusted to match the McFarland turbidity No. 1. The original bacterial suspension was further diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. The tubes were arranged in order of C_1, C_2, C_3, S, I, R and E representing 10^{-2}, 10^{-3} 10^{-4}, streptomycin, Isoniazid, Rifampicin and Ethambutol respectively. One in hundred dilutions (10^{-2}) was inoculated into control (C_1) and four (4) drugs (S I R E), 10^{-3} dilution was inoculated into C_2 and 10^{-4} was inoculated into C_3 and then all tubes were incubated at 37°C for six weeks. Growth was recorded at 28 days and at 42 days as follows: 3+ for confluent growth, 2+ for more than 100 colonies, and 1-100 actual numbers of colonies. Susceptibility or resistance was recorded when the proportion of bacteria in drug-containing medium to that of drug free medium is < 1 or ≥ 1 respectively. MIC is the minimum drug concentration that inhibits the growth of the tested strain. Approximately 100,000 cfu of resistant tuberculosis bacilli were inoculated into the control medium and the medium of a two-fold dilution series of each drug ranging from 0.08 to 100 mg/ml. *M. tuberculosis* reference strains H37Rv (ATCC 27294) sensitive to first-line anti-tuberculosis drugs, RIF-resistant (ATCC

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

3.15 Drug Susceptibility Testing by Nitrate Reductase Assay

Bacterial suspensions was made from a sub-cultured LJ tube by dispensing a loopful of bacteria in 0.5 mL of sterile distilled water in 15mL falcon tube containing a few 3-mm diameter glass beads and vortexed to obtain a uniform solution. To obtain the turbidity of McFarland standard no. 1, approximately 2.5 mL of sterile distilled water was added. The inoculum turbidity was adjusted to a McFarland tube no. 1 and diluted 1: 10 in sterile distilled water. Undiluted suspension (200 ml) was inoculated into tubes of LJ medium with KNO_3 (1 mg/ml) containing each of the drugs at the concentrations described above and 200 ml of the 1 : 10 dilution was inoculated into three control tubes without antibiotics (Appendix II). The assay was developed with reagent mix [50% conc. HCl, 0.2% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride at ratio of 1: 2: 2]. After 7 days of incubation at 37°C a 0.5ml of reagent mix was added to one control tube. When the clear reagent mixture turned pink, the drug-containing tubes were subsequently developed. When there was no colour change, the tubes were re-incubated and the procedure repeated on days 10 and 14. An isolate was considered resistant if the drug-containing tube produced a colour change that was more intense than the drug-free tube. *M. tuberculosis* reference strains H37Rv (ATCC 27294) sensitive to first-line anti-tuberculosis drugs, RIF-resistant (ATCC 35838), INH-resistant (ATCC 35822), EMB-resistant (ATCC 35837) and STR-resistant (ATCC 35820) were used as susceptible and resistant controls. All

strains were sub cultured in Lowenstein–Jensen (LJ) medium for 4 weeks before being studied. (Angeby *et al.*, 2002).

3.16 Data Analysis

The Med Calc software (Med Calc, Belgium) was used to calculate the statistical parameters, such as sensitivity, specificity, and accuracy. The predictive values were calculated using the prevalence of RIF, INH, STR, and EMB resistance in all the TB cases in National tuberculosis and leprosy Training Center Zaria. The agreement between the NRA and the standard PM were determine by the κ-statistic. The κ value, a measure of test reliability, was interpreted as follows: <0.2 poor; 0.21-0.4 fair; 0.41-0.6 moderate; 0.61-0.8 good; and ≥0.81 excellent. The standard error was also estimated to check the reproducibility of the test (Altman, 1999)

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence of TB by Microscopy

Out of the 437 re-treatment tuberculosis suspected samples collected from patients attending National Tuberculosis and Leprosy Training Center, Zaria, 72 were smear positive showing a prevalence of 16.47% (Figure 4.1).

4.2 Culture

Out of all the smear positive samples, 62(86%) were culture positive of which 57 were immunochromatographic test (ICT) positive while only five were negative which were considered to be non-tuberculosis Mycobacteria (NTM),while 6 were culture negative and 4 were contaminated as shown in Figure 4.2.

4.2 Drug susceptibility by LJ PM and NRA

Forty four (44) of the 57 MTBC isolates were found to be MDR-TB by NRA while 41 were found to be MDR-TB by LJPM. The respective prevalence of poly resistance to anti-TB drugs using NRA and LJ PM was 12% and 14% where as pan susceptible isolates had a prevalence of 11% and 14% by NRA and LJ PM respectively as shown in Table 4.1

4.3 Drug susceptibility of *Mycobacterium tuberculosis* complex by Nitrate Reductase Assay

Highest resistance was observed against rifampicin (82.5%) followed by isoniazid resistance (77.2%). Streptomycin and ethambutol resistances were 65% and 51% respectively. Whereas ethambutol had highest number of susceptible isolates of 49%, streptomycin 35%, isoniazid 22.8%and rifampicin (17.5%) with lowest susceptible was recorded as shown in Table 4.2.

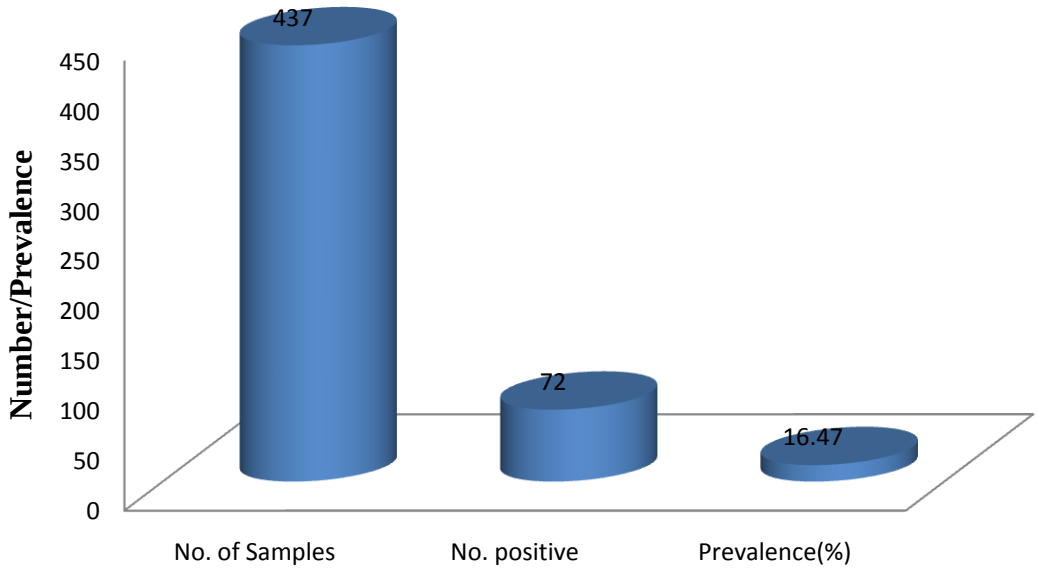


Figure 4. 1: Prevalence of TB by Microscopy

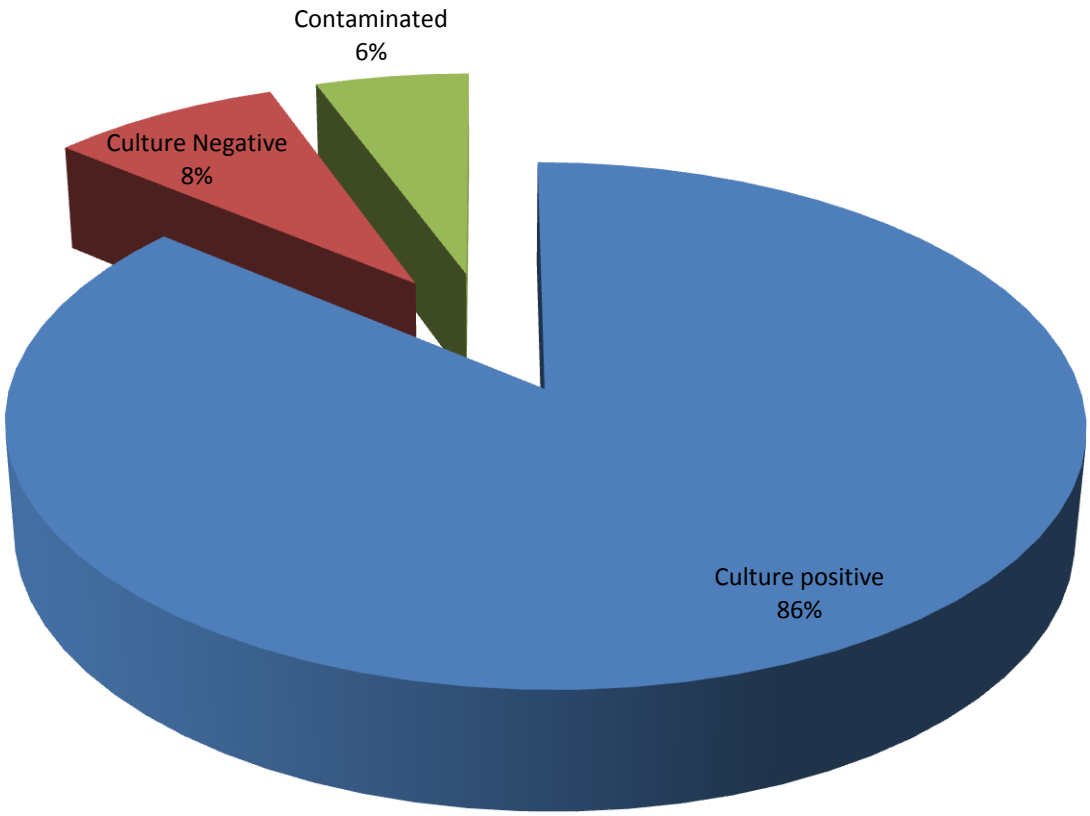


Figure 4. 2: Percentage distribution of culture.

Table 4. 1: Drug Susceptibility by LJPM and NRA

Resistant pattern	NRA	Percentage (%)	LJPM	Percentage (%)
MDR-TB	44	77	41	72
Polyresistance	7	12	8	14
Pan Susceptible	6	11	8	14
Total	57	100	57	100

Key=MDR-TB = multidrug resistant tuberculosis NRA = nitrate reductase assay LJPM = Lowenstein Jensen proportion Method.

Table 4. 2: Drug Susceptibility of Mycobacterium tuberculosis Complex by Nitrate Reductase Assay.

Drugs	Streptomycin	Isoniazid	Rifampicin	Ethambutol
Resistance	37(65%)	44(77.2%)	47(82.5%)	29(59%)
Susceptible	20(35%)	13(22.8%)	10(17.5%)	28(49%)

4.4 Drug Susceptibility of *Mycobacterium tuberculosis* Complex by LJ Proportion Method.

Among fifty seven (57) isolates, 33(58%) isolates were found to be resistant to streptomycin. Forty seven (82.5%) and 44 (77%) were found be resistant to isoniazid and rifampicin respectively. While 35 (51%) isolates were found to be resistant to ethambutol. Isoniazid recorded the lowest susceptible of 17.5%, followed by Rifampicin 22.8% and streptomycin 42%. Ethambutol (39%) had the highest susceptibility (Table 4.3).

4.5 Evaluation of drug susceptibility of *Mycobacterium tuberculosis* using Nitrate Reductase Assay and LJ proportion Method.

The sensitivity and specificity of NRA compared to that of LJPM were observed to be 98% and 89%, 98% and 92%, 64% and 80%, 68% and 77% for RIF, INH, STR, and EMB respectively. Positive predictive values were 91%, 93%, 87% and 83% for RIF, INH, STR and EMB respectively. Negative predictive values were 80%, 92%, 67% and 90% for RIF, INH, STR and EMB respectively .Overall, the sensitivity, specificity ,positive predictive value and negative predictive value of NRA in detecting MDR-TB were 90%,82%,85%and 73% respectively. Good agreement was found in all the tests with κ values of 0.63, 0.61, 0.61 and 0.62for RIF, INH, EMB and MDR-TB respectively only STR shows moderate agreements with k value of 0.59 (Table 4.4).

Table 4. 3: Drug susceptibility of Mycobacterium tuberculosis complex by LJ Proportion method

Drugs	Streptomycin	Isoniazid	Rifampicin	Ethambutol
Resistance	33(58%)	47(82.5%)	44(77.2%)	35(51%)
Susceptible	24(42%)	10(%)	13(22.8%)	22(49%)

Table 4. 4: Evaluation of drug susceptibility testing of *Mycobacterium tuberculosis* using Nitrate Reductase Assay and LJ proportion Method

Drugs	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	κ-statistic
Streptomycin	64	80	87	67	0.59
Isoniazid	98	92	93	92	0.61
Rifampicin	98	89	91	80	0.63
Ethambutol	68	77	83	90	0.61
MDR-TB	90	82	85	73	0.62

Key: PPV = positive predictive value NPV = negative predictive value κ = kappa

4.6 Prevalence of MDR-TB in Relation to Age Group

The age distributions showed that the age groups of 21-30 and 31-40 years had the highest MDR-TB detected by NRA and LJPM. However, lower MDR-TB were detected between the age of 10-20 and 51-60 years by LJ PM and NRA (Figure 4.3). The differences observed were not statistically significant $p \leq 0.05$.

4.7 Prevalence of MDR-TB in Relation to Gender

Out of the 41 male patients screened, 28(62%) and 29(66%) MDR-TB were detected by LJ PM and NRA respectively. While out of 16 female screened 13(38%) detected by LJ PM and 15(34%) detected NRA (Figure 4.4). The differences observed were not statistically significant $p \leq 0.05$.

4.8 MDR-TB/HIV co-infection based on Age Group

The highest prevalence (13%) of HIV among MDR-TB patients was observed among those between the age 41-50 years and the least 3.6% was between ages 21 and 30 years, however, MDR-TB patients between the ages of 51-60 years and 10-20 years were seronegative (Figure 4.5). The differences observed were not statistically significant at $p \leq 0.05$.

4.9 MDR-TB/HIV Co-infections based on Gender

A 12.5% prevalence rate was obtained from females and lower prevalence of 4.8% was recorded for male patients (Figure 4.6). The differences observed were not statistically significant $p \leq 0.05$.

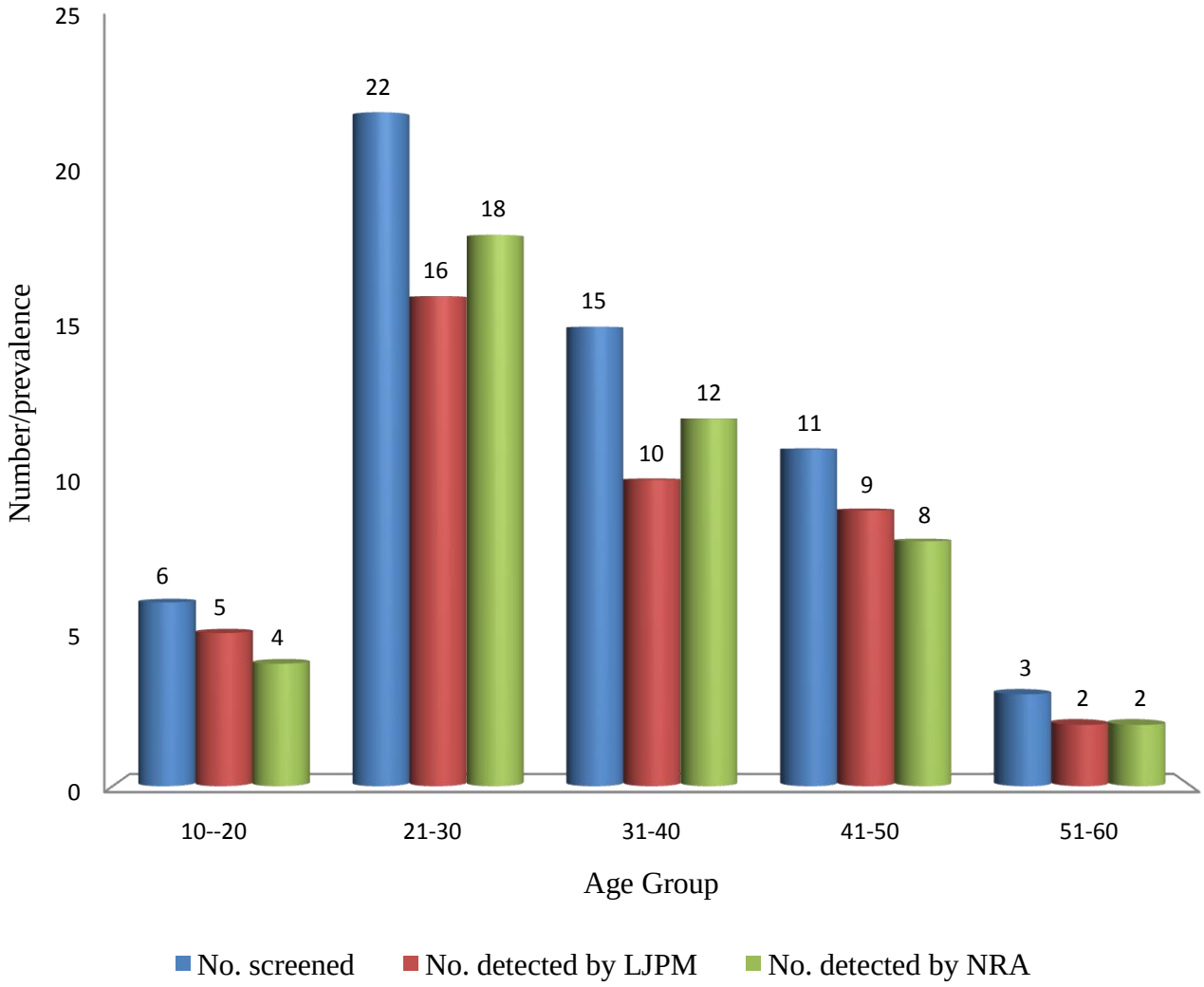


Figure 4. 3: Comparison of MDR-TB detection by Lowenstein Jensen Proportion Method and Nitrate Reductase Assay based on Age group.

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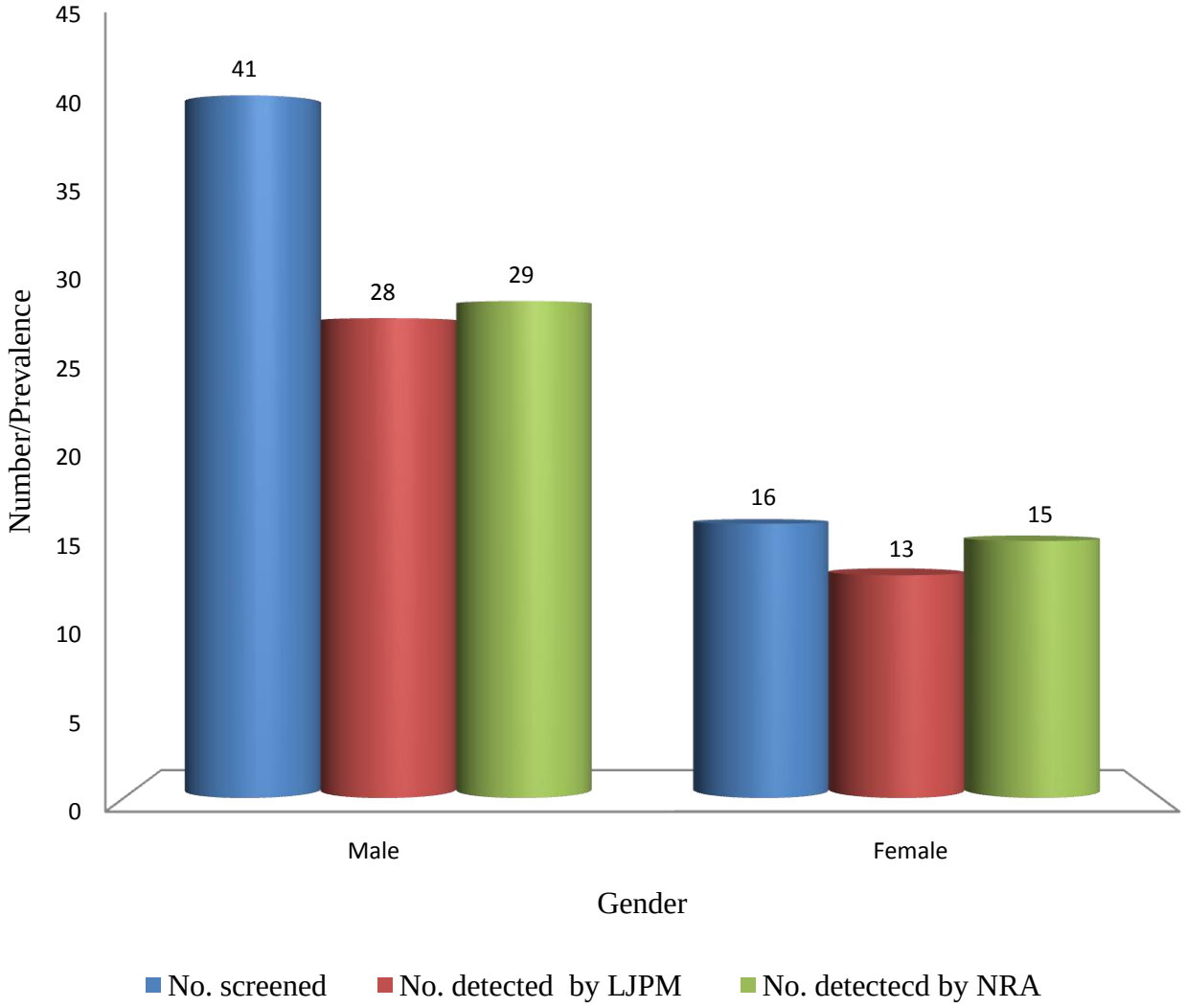


Figure 4. 4: Comparison of MDR-TB detection by Lowenstein Jensen Proportion Method and Nitrate Reductase Assay based on Gender.

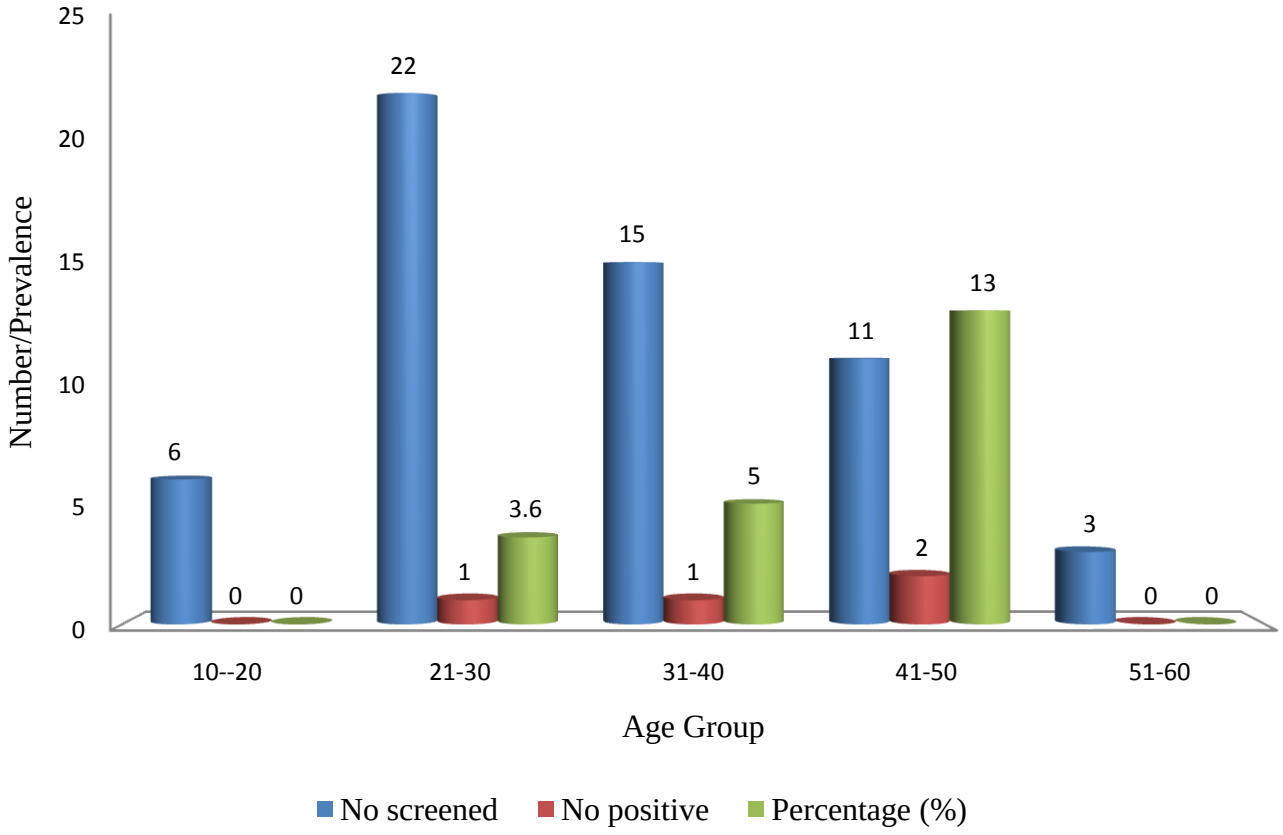


Figure 4. 5: Prevalence of HIV among MDR-TB Patients based on Age Group.

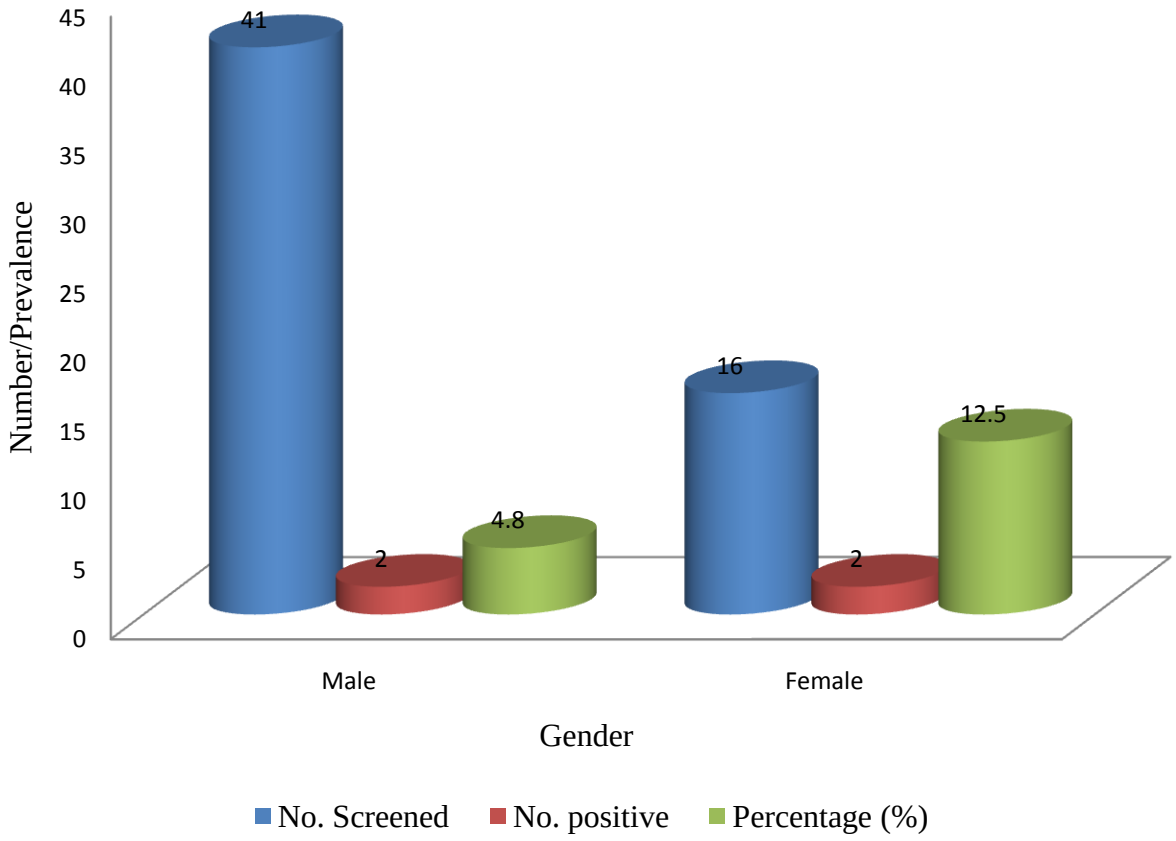


Figure 4. 6: Prevalence of HIV among MDR-TB Patients based on Gender.

4.10 TB/HIV Co-infections patients based on age group

The highest prevalence (33.3%) of HIV among TB patients was observed among those between the ages 51-60 years and the least prevalence (3.6%) was between ages 41 and 50 years, however, the overall prevalence of HIV among TB patient was found to be 9.7% (Figure 4.7). The differences observed were not statistically significant $p \leq 0.05$.

4.11 TB/HIV Co-infections patients based on gender

Out of the 72 smear positive samples, 56 were from male while 16 were from female. TB/HIV co-infection was found in 4(7.1%) of the 56 male patients. While 3(18.8%) out of the 16 female patients had TB-HIV co-infection. The differences observed were not statistically significant $p \leq 0.05$.

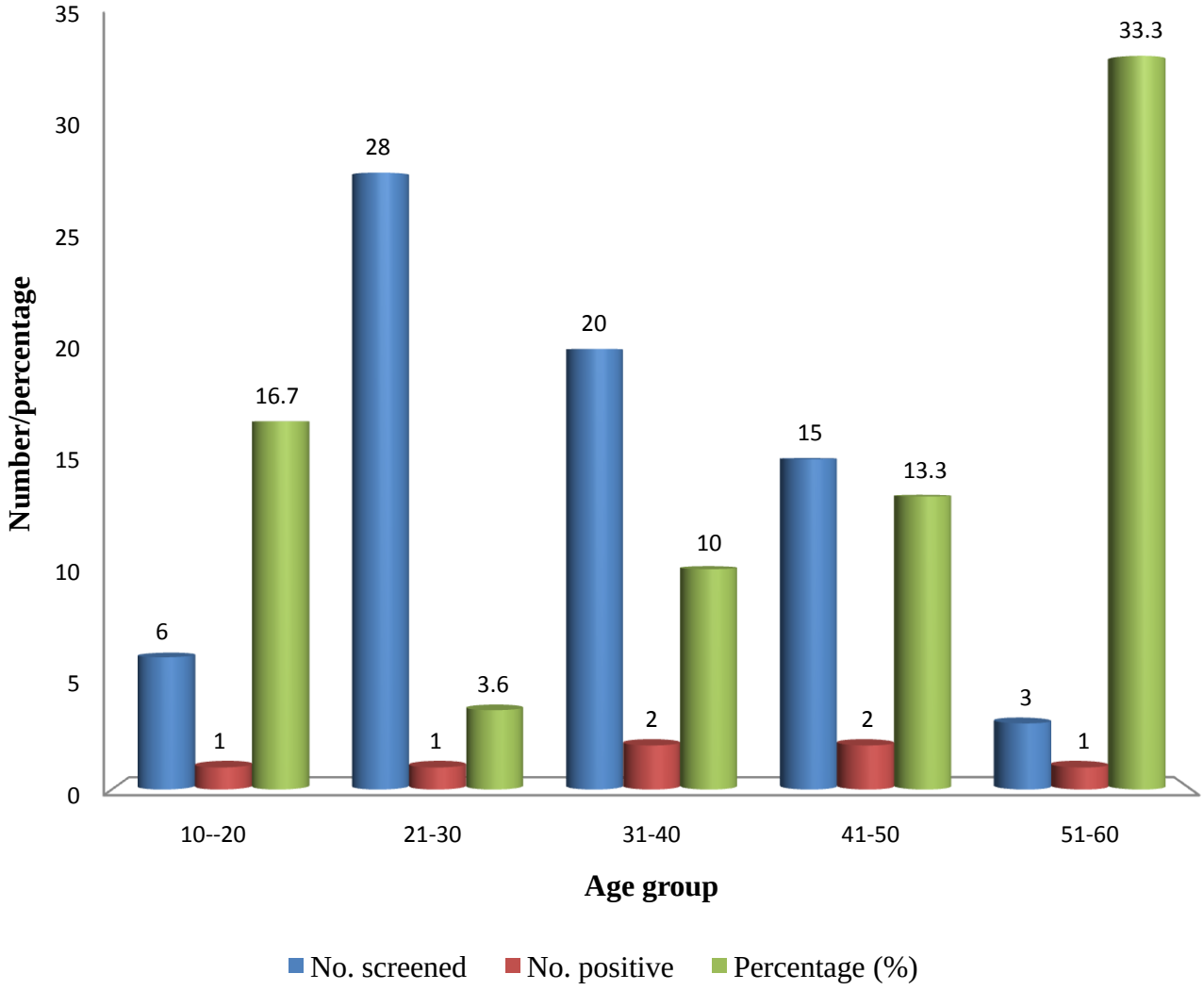


Figure 4. 7: Prevalence of HIV among TB Patients based on Age group.

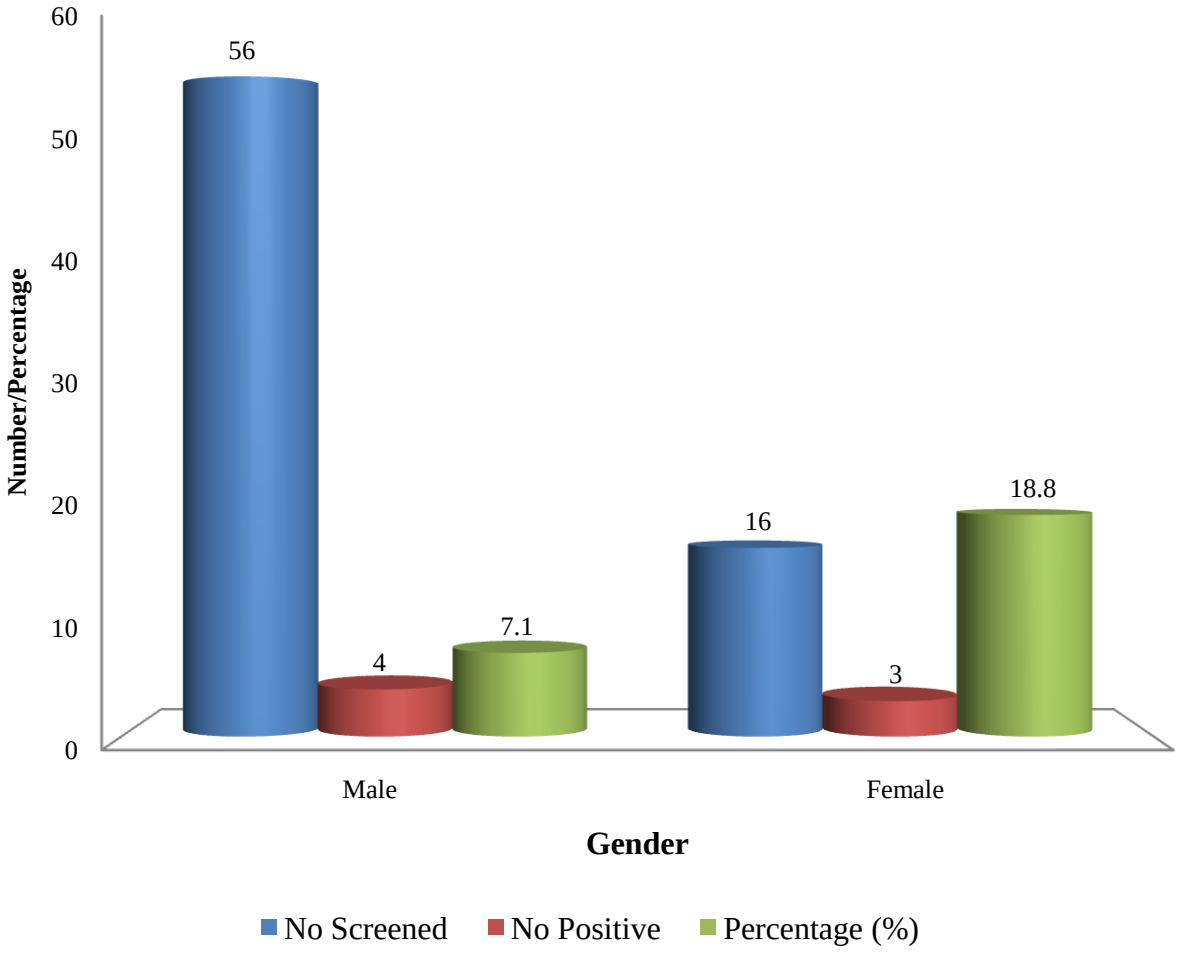


Figure 4. 8: Prevalence of HIV among TB Patients based on Gender.

CHAPTER FIVE

5.0 DISCUSSION

The overall prevalence of TB by microscopy among patients attending NTBRL, Zaria was found to be 16.47% (72/437). Five (7%) of the isolates were non-tuberculosis Mycobacteria. this percentage of NTM found may be due to environmental Mycobacteria, that is abundant in soil and water, exposure to Harmattan dust. This is similar to the result obtained in northern Nigeria and America by Gambo *et al.* (2013) and Sharan *et al* (2013) where they reported 5.7% and 4.3% of NTM respectively. Contamination rate was observed to be 6% which could be due to incomplete digestion, use of contaminated reagent, media or equipments. This percentage (6%) is lower than 14.7% contamination reported by Gambo *et al.* (2013) in northern Nigeria. Smear positive culture negative sample were found to be 8%, this is could be attributed to the dead Acid Fast Bacilli, less number of viable bacilli that LJ media cannot detect or could be due to action of NaOH used for decontamination.

The higher detection rate of MDR-TB by NRA could be as a result of borderline that exhibit color change lower than that obtained in the control tube and therefore this borderline isolate are frequently associated with problems of interpretation of DST by NRA. Bacterial contamination may cause false positive results and low inoculation in the media could also result in false positive result (Kent and Kubica, 1985). More than half of MDR-TB isolates were resistant to all four first-line anti tuberculosis drugs tested. Re-treatment cases were significantly more likely to have MDR-TB than non-MDR-TB. Previous studies have shown that resistance to rifampicin is a significant predictor of resistance to isoniazid and streptomycin; isolates resistant to rifampicin were also resistant to isoniazid and streptomycin (Mdivani *et al.*, 2008).

The results of drug susceptibility testing by NRA and LJPM indicate that the resistance rate was higher as against of isoniazid and rifampicin, which are the most important first line drugs used for the treatment of tuberculosis. This could be as a result of overuse of these drugs for the treatment of TB. This is in agreement with the work of Mahadev *et al.* (2004) and Iqbal *et al.* (2000) who reported higher resistance to rifampicin and isoniazid.

When the results of NRA and LJ PM were compared, higher agreement was observed for RIF and INH, however there were disagreements in relation to the antibiotics EMB and STR. The factors which led to lower specificity and sensitivity values of LJ could be as a result of degradation of the antibiotics in LJ medium. The higher rate of detection of RIF resistance by NRA method is the reason for the use of NRA for the screening of MDR strains, as resistance to RIF is a marker for MDR detection. In a multicentre study, the NRA was evaluated in different settings to determine the susceptibility of *M. tuberculosis* to first-line anti-tuberculosis drugs. Higher accuracy of 96.6% was observed for INH and RIF, while that for STR and EMB were lower (85.3%) (Martin *et al*., 2005a). Percentage agreement of 98.3%, 98.3%, 90.8% and 93.3% by NRA were observed for INH, RIF, STR and EMB respectively by Shikama *et al.* (2009). However, similar lower sensitivity and specificity for STR and EMB have also been reported by Lemus *et al.* (2006) and Montoro *et al.* (2005) as also reported in this study.

This study showed that MDR-TB is prevalent among patients within the age group 21-30 and 31-40. Member of these age groups are economically and reproductively active and this may account for the higher prevalence observed. However this result is in disagreements with that of National Drug Resistant Tuberculosis survey which reported that MDR-TB peaks among the age groups 45-64 (WHO, 2012).

A higher prevalence of MDR-TB was observed in male 29(66%) compared to female 15(34%).While the observation made from this study could be as a result of poor knowledge about TB as well as "male ego" that is common with males make them to seek alternative local herbs and generally male abuse drugs Males predominate among TB cases in most countries (WHO, 2010b), the variation in the effect of gender in harboring MDR-TB is multi-factorial.

A relatively higher MDR-TB among males was also reported by Okodua *et al.* (2012) to be (19.7%) compare to females (14.3%). Also, reports from South Africa show a higher number of MDR-TB cases in males (4826 cases) than in females (4615 cases) (WHO, 2010b). The higher MDR-TB among males in this study is at variance with report from China where a higher MDR-TB was recorded among females (19.0%) than in male (15.8%) (Shao *et al.*, 2011). Reports from Australia, the Netherlands and United State of America showed that female TB patient's harbor MDR-TB strains than male (WHO, 2010b).

The overall prevalence of HIV among TB patient was found to be 9.7%. This can be attributed to decline in the immune system of the patients which means tuberculosis advances HIV and causes other opportunistic infections to set in to the patients (Agbaji *et al.*, 2013). This high prevalence rate in this study suggests strong association between TB and HIV infection in this environment. Factors associated among them are the demographic position of the patient, their socioeconomic status. The fact that endogenous reactivation of TB occurs most commonly among persons with HIV (Jawetz, 2006) .This could also be the reason for the high prevalence rate of TB among HIV patients.

The prevalence of TB-HIV co-infection was reported to be 9.6% in Jos (Agbaji *et al.*, 2013). Globally the prevalence of TB-HIV co-infection is reported to be 0.18% (Dye *et al.*, 1999) which

is less than the result obtained in this study. NTBLCP (2011) also reported 25% TB-HIV co-infection in Nigeria which is higher than the result obtained. This result is similar with some reports from other parts of this country and beyond. The prevalence of HIV among TB patients was 5.3% in Lagos (Idigbe *et al.,* 1994), Plateau state 30% (FMH, 2000), Tanzania 19.3% (Kano *et al.,* 2000), Guinea 20% (Sobesk *et al.,* 2000), Lagos 12.6% (Salami and Oluoyo, 1999).

Female had higher prevalence of TB/HIV co-infection rate of 18.8%. This may be due to the less access to health care by females and therefore more unreported TB cases, also higher prevalence of HIV among female may be the reason because the paucibacillary nature of pulmonary TB that is common in higher HIV prevalence setting leading to increase proportion of microscopy negative result. This agrees with previous reports indicating higher prevalence in males than females (Homes *et al.,* 1988; Abeld, 2000). Moreover, a complex biological interplay occurs between *M. tuberculosis* and HIV in the co-infected host that results in the worsening of both pathologies. HIV promotes progression of latent *M. tuberculosis* infection to disease and, in turn, *M. tuberculosis* enhances HIV replication, accelerating the natural evolution of HIV infection (Xu *et al.,* 2006).

The overall prevalence of MDR-TB/HIV co-infection in the study was 7.0%.This can be attributed to the emergence of resistant strains of TB. Factors associated are default among patients, supply of sub-standard drugs among others and lowered immunity of HIV patients. This is in agreement with National Drug resistant TB survey done in Nigeria where 6.8% was reported (WHO, 2012). This prevalence is similar to 5.8% prevalence rate reported in Abeokuta (Oluwalase *et al*., 2013) and 6.0% reported by Beatriz *et al.* (2009) in Southwestern Columbia. The prevalence is however lower than that reported by Van Pie *et al.* (2006) in a community in Cape Town, South Africa. This could be as a result of higher prevalence of HIV among Female

than Male. In setting where HIV infection is linked to socioeconomically vulnerable population, poor treatment adherence and lack of access to proper treatment may contribute to the development of drug resistance.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1CONCLUSION

Mycobacterium tuberculosis was isolated from smear positive sputum giving a prevalence of 86% (62/72). Culture negative smear positive samples and contamination rate gave a prevalence of 8% (6/72) and 6% (4/72) respectively.

The prevalence of MDR-TB among patients with retreatment cases as detected by NRA and LJPM were 77% and 72% respectively. MDR-TB was more prevalent among patients within the age group of 21-40. So also it was more prevalent among male patients.

The prevalence of HIV among the MDR-TB cases was 7%. HIV was more common among patients with MDR-TB within the age group of 41-50years. HIV/MDR-TB co-infection cases were found more in females.

From the result of the study, the NRA may be of great importance in the context of emergence of MDR-TB because it showed higher sensitivity and specificity for the rapid detection of rifampicin and isoniazid resistance, the two most important drugs for tuberculosis treatment. However ethambutol has shown greater potency among all the drugs.

On the basis of the findings, NRA as opposed to LJPM has the potential to be a useful tool for accurate detection of MDR-TB in the study area.

6.3 RECOMMENDATIONS

1. The Nitrate Reductase Assay should be further evaluated for second-line drugs.
2 For STR and EMB testing, further studies are required to optimize preparation of adequate inoculum and drug concentration in order to avoid resistance strain misclassification.
3 The NRA should be further evaluated for detection of MDR-TB directly from smear positive clinical samples.
4 There should be a continuation of public awareness and good health education on how tuberculosis is transmitted.
5 All drug resistance cases should be detected and treated with appropriate drugs, combinations or dosages and they should always adhere to their treatments without Interruption or irregular treatments and incomplete treatments duration.

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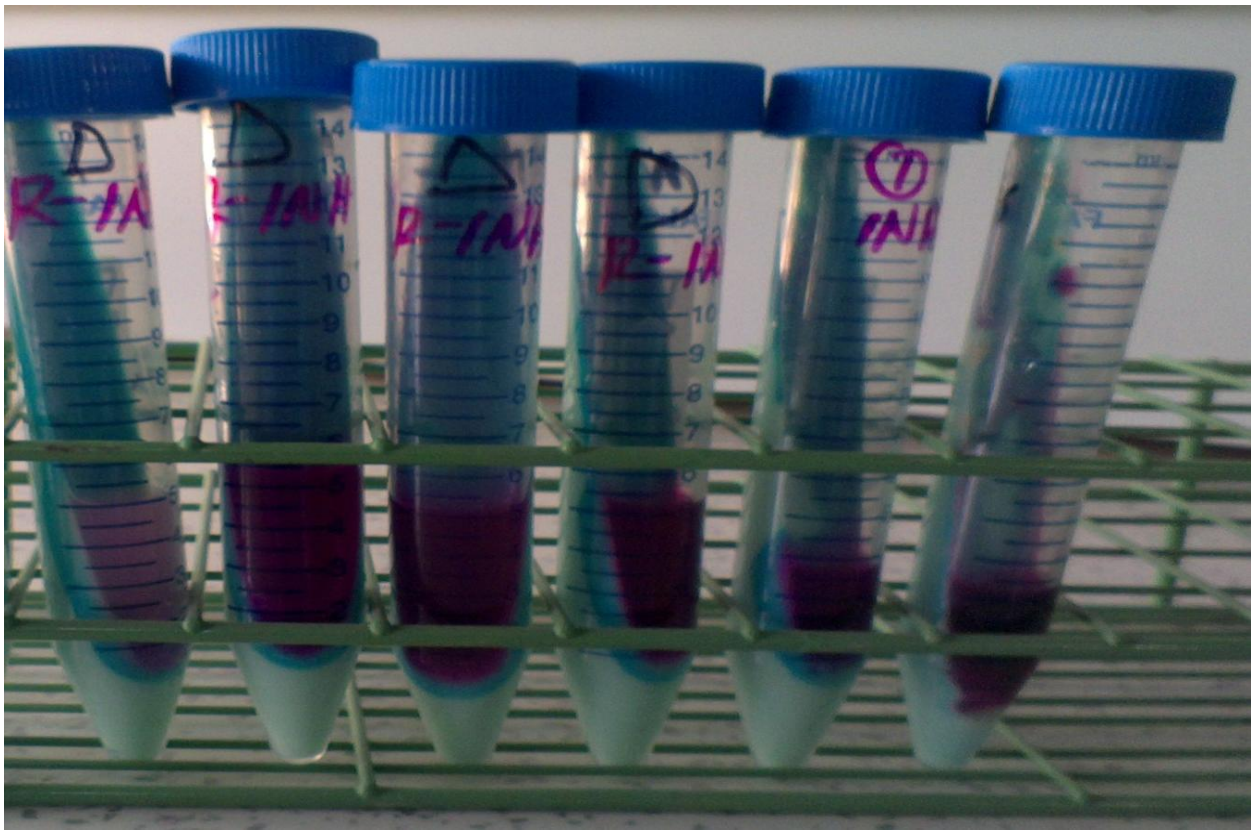
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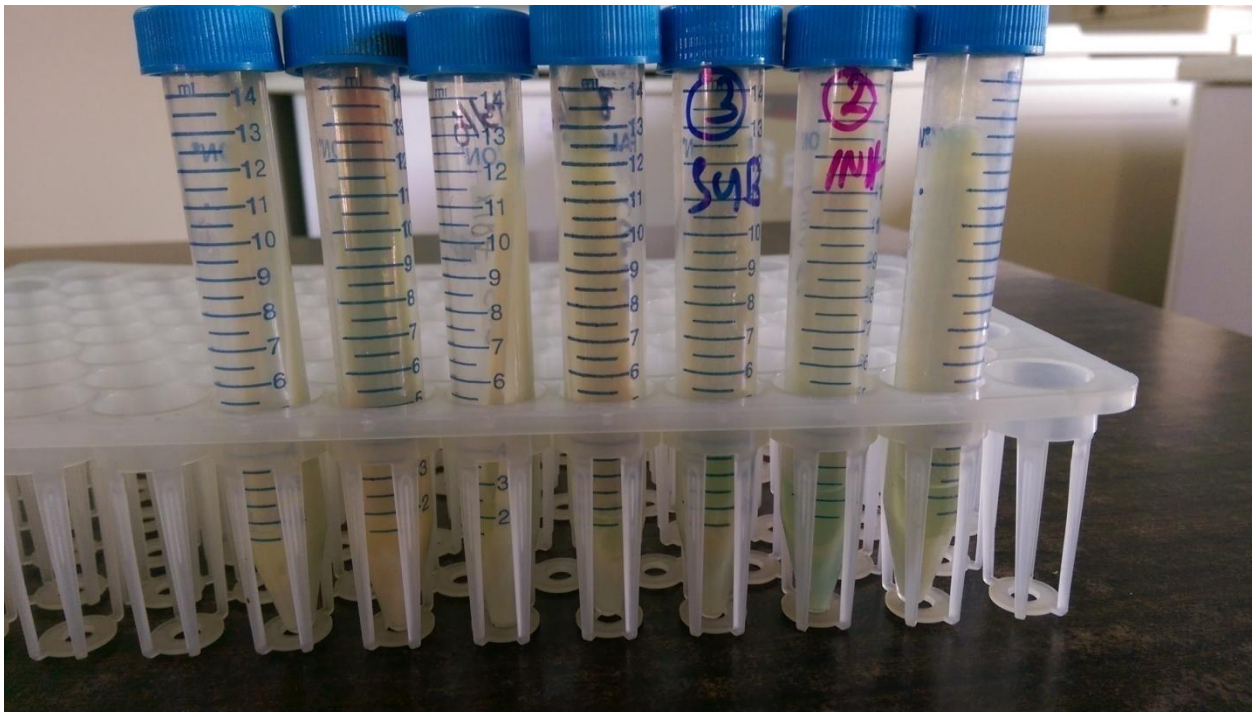
APPENDICES



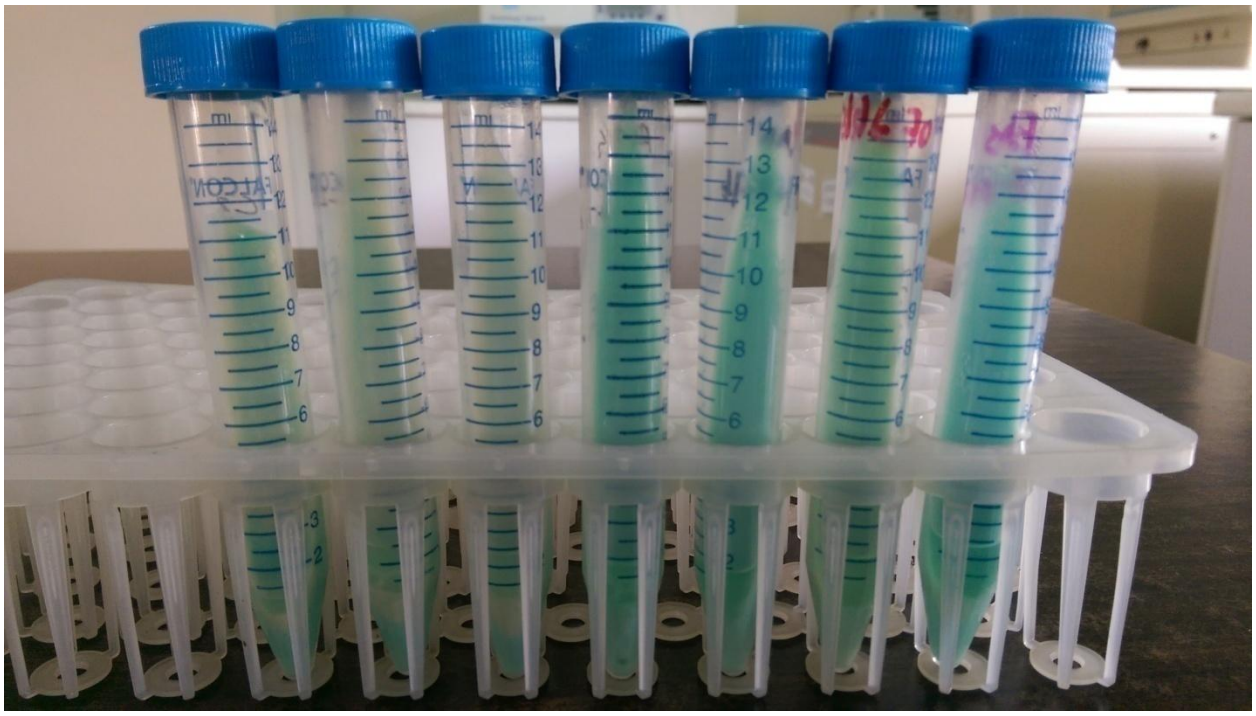
Appendix I : Result of Nitrate Reductase Assay showing susceptible isolates.



Appendix II: Nitrate Reductase test result showing Resistant isolates



Appendix III: LJPM result for resistant isolates.



Appendix IV: LJPM result for susceptible isolates.

PREPARATION OF WORKING SOLUTION OF ANTIBIOTICS

(200mls of LJ or 20 tubes)

Conc. Stock sol. µg/ml	**Dilution 1**			**Dilution 2**			**Amount of dilution in 200 ml L-J**	**Final working Antibiotic conc. µg/ml**
	ml Stock sol.	**ml sterile H20**	**Conc. µg/ml**	**ml of dilution 1**	**ml sterile H20**	**Conc. µg/ml**		
INH 10,000 µg/ml	**1.0**	9.0	1000	**1.0**	9.0	100	**0.4**	0.2
RMP10,000 µg/ml	**-**	-	-	**-**	-	-	**0.8**	40.0
EMB10,000 µg/ml	**1.0**	9.0	1000	**-**	-	-	**0.4**	2.0
STR 10,000 µg/ml	**1.0**	9.0	1000	**-**	-	-	**0.8**	4.0

Appendix V: Preparation of Working Solution of Antibiotics

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

M. Sc. Research Questionnaire (strictly confidential)

Research Topic: "**DETECTION OF MULTI- DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS USING NITRATE REDUCTASE ASSAY AND PROPORTION METHOD AMONG PATIENTS ATTENDING NATIONAL TUBERCULOSIS REFERENCE LABORATORY, SAYE, ZARIA."**

Instruction: Please tick as appropriate and provide answers where necessary.

Personal Data

1. Age …….
2. Gender : Male [ ] Female [ ]
3. Marital Status: Single [ ] married [ ] widow [ ]
4. Type of sample: urine [ ] sputum [ ] others [ ].
5. Educational Status: None [ ] Primary [ ] Secondary [ ]Tertiary [ ]
6. Type of Occupation: Farming [ ] Civil Servant [ ] Self Employed [ ] Others [ ]
7. Type of residence: Urban [[Rural [ ]
8. State of Origin/Resident……
9. Have you ever have TB before? Yes [ ] No [ ] Can't remember [ ]
10. Have you ever taken TB treatment? Yes [ ] NO [ ]
11. HIV status; Positive [ ] Negative [ ] Unknown [ ]
12. Have you smoke cigarette in your life time? Yes [ ] No [ ]
13. Have you drink alcohol in your life time? Yes [ ] No [ ].
14. Have you being diagnosed with Diabetes? Yes [ ] No [ ].
15. Do you have TB patient in your family? Yes [ ] No [ ].
16. Patient TB category New case [ ] DR suspect [ ]

Appendix VI: Questionnaire

DEPARTMENT OF MICROBIOLOGY

SCHOOL OF POSTGRADUATE STUDIES
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

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 multi-drug resistant mycobacterium tuberculosis using nitrate reductase assay and proportion method among patients attending national tuberculosis reference laboratory Zaria, Kaduna State"

Principal Investigator: Kasimu Mamuda

Collaborating Investigators: Prof.O.S Olonitola

Prof.E.D Jatau

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 Kasimu Mamuda. A postgraduate student of the Department of Microbiology, Ahmadu Bello University, Zaria, carrying out a research work under the supervision of Prof.O.S Olonitola and Prof.E.D Jatau

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 multi-drug resistant mycobacterium tuberculosis using nitrate reductase assay and proportion method among patients attending national tuberculosis reference laboratory Zaria, Kaduna State

Participant selection

We are inviting all patients that has been confirmed positive and has enrolled at NTBLTC to confirm the presence of multi-drug resistant *Mycobacterium tuberculosis*.

- *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 clinic 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 clinic/hospital for MDR-TB , 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 the patient attending NTBLTC following World Health Organization (WHO) tuberculosis guidelines.

Description of the Process

During the research you will make just two visits to the clinic.

Two specimens 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. Each specimen would be smeared, air dried, heat fix and stain with Ziehl- Neelsen (Z-N) reagents. All specimens that are smeared positive would be processed immediately at the NTBLTC. Each specimen would be processed with N-acetyl-L-cysteine–NaOH–sodium citrate solution.MDR-TB will be detected using Nitrate Reductase Assay and Proportion Method 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 HPV 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 we will take your blood 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 MDR-TB.
- ✓ Your sputum will be screened for MDR-TB.
- ✓ Early diagnosis will prevent, or delay the progression of MDR-TB.
- ✓ There may not be any benefit to the society at this stage of the research, but future generations are likely to benefit.
- ✓ 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 clinic will have to be on your normal schedule clinic days. However, you will have the opportunity of been screened for high risk Tuberculosis.

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:

Nicholas Ezati

Kasimu Mamuda.

This proposal has been reviewed and approved by Scientific Ethical Committee (SEC) of National Tuberculosis Reference Laboratory, 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 SEC, contact the office of the Assistant Dean, School of postgraduate studies, ABU, 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:

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 VII: Informed consent form

FEDERAL MINISTRY OF HEALTH

DEPARTMENT OF PUBLIC HEALTH
NATIONAL TUBERCULOSIS AND LEPROSY TRAINING CENTRE
Saye Village Old Zaria - Kaduna Road,
P.M.B. 1089 Zaria, Kaduna State, Nigeria.
Tel: 069-332726, Telefax: 069-333205
E-mail: ntbltc@hotmail.com

NTBL/TRG/ZA/182/Vol.IV
Ref No: .....
Date: 4/06/2015

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

LETTER OF APPROVAL

ATTENTION: **MAMUDA KASIMU (P13SCMC8033)**

Your research proposal titled: **"DETECTION OF MULTI-DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS USING NITRATE REDUCTASE ASSAY AND PROPORTION METHOD AMONG PATIENTS ATTENDING NATIONAL TUBERCULOSIS REFERENCE LABORATORY ZARIA"** refers.

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

Yours Faithfully,

Dr Kenneth O. Adagba
Chair, Ethical Committee