PREVALENCE OF MULTI-DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* (MDR-TB) IN KADUNA STATE, NIGERIA

BY

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MARCH, 2015PREVALENCE OF MULTI-DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* (MDR-TB) IN KADUNA STATE, NIGERIA

BY

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MARCH, 2015

Declaration

I declare that the work in this dissertation entitled **"PREVALENCE OF MULTI-DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* (MDR-TB) IN KADUNA STATE, NIGERIA"** has been performed by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project dissertation was previously presented for another degree or diploma at this or any other Institution.

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Certification

This dissertation entitled PREVALENCE OF MULTI-DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* (MDR-TB) IN KADUNA STATE, NIGERIA by MUHAMMAD SANI ALIYU meets the regulations governing the award of the degree of Doctor of Philosophy in Microbiology of the Ahmadu Bello University, and is approved for its' contribution to knowledge and literary presentation.

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Dedication

To my beloved parents late Alh. Muhammad Sani Garba and Halima Aliyu and my elder brother late Musa M. Sani, RAHEEMA HUMULLAHU, AMEEN

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All praises be to ALLAH, He that teaches man what he know not and impact unto him the use of the pen. May His peace and benediction be upon the most honourable of His prophets Muhammad (S. A. W.), his household, companions and those that followed their footsteps to the day of reckoning.

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M. S. Aliyu

ABSTRACT

This study determined the prevalence of MDR-TB in Kaduna State, Nigeria using both phenotypic and genotypic methods. A total of 1186 sputum samples were screened for the presence of AFB by Ziehl-Neelsen staining procedure. Two hundred and twenty one (18.6%) of the samples screened were positive for AFB. Of the 221 smear positive sputum samples cultured on Lowenstein Jensen (L-J) medium, 171 (77%) yielded *M. tuberculosis* complex, 22 (10%) were AFB and culture positive but negative for MPT 64 antigen on SD-bioline, these were considered non tuberculous mycobacteria (NTM), 15 (7%) were smear positive but culture negative while 13 (6%) were contaminated. All the MTBC isolates (100%) were further characterized as *M. tuberculosis* genotypically by LPA. Tuberculosis and HIV co-infection was found in 21 (9.5%). One hundred and eighty two (82%) of the subjects were new cases while thirty nine (18%) were retreatment cases. Any drug resistance was found in 21 (52.5%) of all cases; 19 (47.5%) among new cases and 2 (5.0%) were found in retreatment cases. Mono-drug resistance against streptomycin was seen in 9 (22.5%) of the isolates tested. All mono-drug resistant isolates were from the new cases. MDR-TB was detected in 2 (5.0%) of the subjects all of which were found among new cases. Four (10.0%) isolates of all cases were poly-drug resistant, 3 (7.5%) were new cases, only 1 (2.5%) was retreatment case. One isolate each (2.5%) from new case and retreatment case showed poly-drug resistance to SM EMB, 1 (2.5%) was resistant to INH EMB. Poly resistance to SM INH EMB was found in only 1 (2.5%) new case. No poly-drug resistance to SM INH, RIF EMB, RIF SM or RIF SM EMB was observed. One isolate was characterized as MDR with bands at *rpoβ* MUT2A region and *ihn*A MUT2 corresponding to H526Y and A16G mutations respectively. Rifampicin mono resistance with band at *rpoβ* MUT3 corresponding to S531L was found in one isolate. Also, isoniazid mono

resistance was observed in one isolate with *ihn*A MUT2 band corresponding to A16G mutation. The comparison of Geno Type MTBDR*plus* LPA and phenotypic LJ-proportion method showed that one isolate was mono resistant to RIF and one was mono resistant to INH by LPA, one and two MDR-TB isolates respectively were characterized by genotypic and phenotypic methods. The remaining isolates were found to be pan susceptible by both methods. There was association between TB and age (OR=1.72, CI=1.21-2.44, *P*= 0.002), sex (OR=2.10, CI=1.56-2.84, *P*<0.0001), case (OR=21.8, CI=13.35-35.51, *P*<0.0001), contact with TB patient (OR=0.09, CI=0.06-0.14, *P*<0.0001) and HIV (OR=0.22, CI=0.14-0.35, *P*<0.0001). No association was observed between HIV and MDR-TB (OR=6.3, CI=1.64-23.84, *p*=0.007); however, there was association between MDR-TB and sex (OR=0.25, CI=0.07-0.90, *p*=0.034). This study has shown an overall high prevalence of TB and TB drug resistance in Kaduna State. It also demonstrated that the prevalence of MDR-TB is high in the State. These need to be urgently addressed. Laboratory facilities for rapid TB culture and DST are needed in Kaduna State and across Nigeria for early and accurate diagnosis of TB and drug resistant cases. This remains an important step in managing TB drug resistance in Nigeria.

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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	Bacille Calmette-Guérin
BSL	Bio safety level
CC	Critical concentration
CFU	Colony forming units
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DR	Drug resistant
DRS	Drug resistance survey
DST	Drug Susceptibility Testing
EMB	Ethambutol
FIND	Foundation for Innovative New Diagnostics
G+C	Guanine+ Cytosine
GI	Growth Index
HIV	Human immunodeficiency virus
INH	Isoniazid
inhA	Enoyl-acyl carrier protein reductase

IUATLD	International Union Against Tuberculosis and Lung Disease
kasa	β-ketoacyl-ACP synthase
katg	Catalase-peroxidase enzyme
LJ	Lowenstein-Jensen
LPA	Line probe assay
MDR	Multidrug resistant
MGIT	Mycobacterium Growth Indicator Tube
MIC	Minimum inhibitory concentration
MODS	Microscopic observation drug susceptibility
MTBC	*Mycobacterium tuberculosis* complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NRA	Nitrate reductase assay
NTM	Non-tuberculous mycobacteria
PAS	P-aminosalicylic acid
PCR	Polymerase chain reaction
PM	Proportion method
PNB	Para-Nitrobenzoic Acid
PNM	Primer nucleotide mix

PZA	Pyrazinamide
RFLP	Restriction fragment length polymorphism
R	Resistant
RIF	Rifampicin
RLSs	Resource-Limited Settings
RNA	Ribonucleic Acid
rpoβ	β-subunit of DNA dependent RNA polymerase
RR	Resistance ratio
RRDR	Rifampicin resistance determining region
S	Susceptible
SM	Streptomycin
TB	Tuberculosis
TCH	Thiophene-2-carboxylic acid hydrazide
WHO	World Health Organization
XDR-TB	Extensively drug resistant tuberculosis

CHAPTER ONE

1.0 INTRODUCTION

The discovery of anti-tuberculosis drugs in the 1940s followed by combination chemotherapy made tuberculosis a curable disease. In the developed countries, effective treatment and surveillance reduced tuberculosis dramatically with high hopes of total eradication (Raviglione *et al.,* 1992; Raviglione *et al.,* 1995). However, in the 1980s, it was realized that tuberculosis had not only ceased to decline in the developed countries, notably the USA, but was actually increasing, particularly in major cities (Raviglione *et al.,* 1995). It was also soon realized that the disease was out of control and increasing at an alarming rate across most of the poorest regions of the world especially Africa due to HIV/AIDS (Raviglione *et al.,* 1992; WHO, 2009).

Despite aggressive international efforts, tuberculosis remains a leading infectious cause of death, with an estimated 8.6 million incident cases per year. In 2012, an estimated 1.3 million people died from the disease. These death rates, however, only partially depict the global TB threat; more than 80% of TB patients are in the economically productive age of 15 to 49 years (WHO, 2013).

Global tuberculosis control efforts have been threatened by the emergence of multidrug resistant tuberculosis (MDR-TB). MDR-TB is defined as strains of *Mycobacterium tuberculosis* which show high level resistance to both isoniazid and rifampicin, with or without resistance to other anti TB drugs (WHO, 2013). MDR-TB is estimated to cause 4% of new tuberculosis cases in the developing world. Patients infected with MDR strains are not only difficult to cure but also more likely to remain sources of infection for a longer period of time than those with drug susceptible organisms. MDR-TB requires longer duration of treatment (up to 2 years) to achieve cure, in comparison with 6 month treatment for drug susceptible TB, lower cure rates and even higher

default rates. The cost of drugs to treat an MDR-TB case can be up to 100 times more expensive than the cost of treating a drug susceptible TB case (Leimane and Leimans, 2006). Because of its increasing prevalence MDR-TB is now subdivided into basic MDR-TB, with resistance only to rifampicin and isoniazid, and extensively drug resistant TB (XDR-TB), with a similar resistance pattern but with resistance to one or more additional first and/or second line drugs. Various perturbations in the individual drug target genes are responsible for the genesis of anti-TB drugs resistance. Rifampicin resistance has been shown to be caused by a change in the β-subunit of DNA dependent RNA polymerase, which is encoded by the *rpoβ* gene. More than 95% of rifampicin resistant strains are associated with mutations within an 81-base pair region of the *rpoβ* gene, which is termed rifampicin resistance determinant region (Telenti *et al.,* 1993; Traore *et al.,* 2000; Sharma and Mohan, 2006). On the contrary, resistance to isoniazid is due to mutations at one of two main sites, in either the *katG* or *inhA* genes (Zhang *et al.,*1992; Piatek *et al.,* 2000). These mutations are not directly connected, and so separate mutations are required for organisms to change from a drug susceptible isolate to MDR-TB. Furthermore, rifampicin resistance has been considered to be a surrogate marker for checking multidrug resistance in clinical isolates of *M. tuberculosis* since rifampicin resistance is often accompanied by resistance to isoniazid (Traore *et al.,* 2000; Sharma and Mohan, 2006). Drug resistance in *M. tuberculosis* occurs by random, single step, spontaneous mutation at a low but predictable frequency, in large bacterial populations. The accurate diagnosis of MDR-TB requires a positive culture of *M. tuberculosis* and drug susceptibility testing. Previous drug treatment is the largest single risk factor for the presence of MDR-TB. There is a strong suspicion of drug resistance, including MDR-TB, in persons with a history of prior treatment or in treatment failure cases.

The emergence of drug resistance in *M. tuberculosis* has been associated with a variety of management, health provider and patient related factors. These include (i) deficient or deteriorating TB control programmes resulting in inadequate administration of effective treatment; (ii) poor case holding, administration of sub-standard drugs, inadequate or irregular drug supply and lack of supervision; (iii) ignorance of health care workers in epidemiology, treatment and control; (iv) improper prescription of regimens; (v) interruption of chemotherapy due to side effects; (vi) non-adherence of patients to the prescribed drug therapy; (vii) availability of anti-TB drugs across the counter, without prescription; (viii) massive bacillary load; (ix) illiteracy and low socio economic status of the patients; (x) the epidemic of HIV infection; (xi) laboratory delays in identification and susceptibility testing of *M. tuberculosis* isolates; (xii) use of nonstandardized laboratory techniques, poor quality drug powders and lack of quality control measures; and (xiii) use of anti-TB drugs for indications other than tuberculosis (Paramasivan and Venkataraman, 2004).

In most countries, MDR-TB has increased in incidence and interferes with TB control programs, particularly in developing countries, where prevalence rates are as high as 48% (Iseman and Sbarbaro, 1992; Cohn *et al.,* 1997). The high infection and death rates pose an urgent challenge to rapidly detect cases. The extent of the problem of MDR-TB has been examined by the World Health Organization (WHO) in cross sectional surveys of drug resistance in either clinical series or whole country cohorts (Espinal *et al.,* 2001). Cross sectional surveys almost certainly under estimate the burden and number of cases of MDR-TB because they do not take into account the numerical burden of TB in the high burden countries. When the exercise is repeated with a mathematical modelling design using drug resistance estimates and the number of cases of TB, a more accurate picture of the global MDR-TB burden is claimed (Dye *et al.,* 2002b). However,

even this has been criticized as under estimating the global burden for the following reason. The stated number of cases per year from a country often includes up to 20% of cases which are actually on retreatment (i.e. have had a previous course of first line drugs). The prevalence of MDR-TB in retreatment cases is between 30% and 80% depending on the country. In Gujerat, India, for example, where there are about 400,000 new cases annually, if it is assumed that 20% are being retreated and there is an MDR-TB rate of 30 - 80% in retreatment cases, this would include 24,000 - 64,000 cases of MDR-TB [i.e. (400,000 × 0.2 × (0.3 - 0.8)]. The estimate of the global burden obtained by modelling could be wrong by a factor of 2 - 4. In the USA, HIV positive MDR-TB cases initially had a 100% mortality (Small *et al*., 1993), but with greater awareness and early diagnosis an improvement in initial survival rates up to 50% has been reported (Salomon *et al*., 1995). HIV negative cases in the USA have had better response rates of between 56% (Goble *et al*., 1993) and 69% (Telzak *et al*., 1995). Nosocomial outbreaks, often in an HIV setting, are well documented in other countries as well as the USA. An outbreak in Spain between 1991 and 1995 killed 47 of 48 patients infected (Herrera *et al*., 1996), and in two outbreaks in London (Chelsea and Westminster Hospital and St Thomas's Hospital) the mortality was over 50% in HIV positive patients (Breathnach *et al*., 1998).

Understanding the scientific basis of short course 6 month chemotherapy for tuberculosis helps to explain why the loss of sensitivity to both isoniazid and rifampicin, even without resistance to additional drugs, has such major effects on outcome. Numerous controlled trials have shown that a 6 month regimen of rifampicin and isoniazid, supplemented by pyrazinamide and streptomycin or ethambutol for the first 2 months, will provide a cure in > 95% of cases if the medication is taken correctly. Such a regimen also renders infectious cases non infectious in 2 weeks (Ormerod, 1997). Each drug varies in its ability to kill tubercle bacilli (bactericidal ability), to

deal with persistent organisms which are only occasionally metabolically active (sterilizing ability) and to prevent the emergence of drug resistance (Ormerod, 1997). Isoniazid is the best bactericidal drug and if mono resistance to this occurs, treatment with rifampicin and ethambutol has to be extended for 9 - 12 months, in addition to 2 months initial pyrazinamide. Rifampicin is the best sterilizing drug, and mono resistance to this drug requires treatment with isoniazid and ethambutol for 18 months, with 2 months initial pyrazinamide. Therefore loss of response to both the main bactericidal drug and the main sterilizing drug means that patients remain infectious for much longer, both in the community and in hospital, that treatment is required for at least 12 and possibly more than 24 months, and that less effective and more toxic second line drugs have to be used (Joint Tuberculosis Committee of the British Thoracic Society, 1998).

Although some individuals who have not had previous TB treatment are infected by MDR-TB, this is not the case for most patients. Many new cases of MDR-TB are created each year by a combination of physician error and poor patient compliance with treatment, which turn fully susceptible organisms, or those with less complex resistance patterns, into MDR-TB.

1.1 Statement of the problem

The global burden of TB remains enormous. In 2012, there were an estimated 8.6 million incident cases of TB and 1.3 million people died from the disease. Among these deaths there were an estimated 170,000 from MDR-TB. General problem of MDR-TB with an estimated 450,000 incidence cases worldwide annually has been recognized since the first World Health Organization (WHO) global survey on drug resistance in the late 1990s (WHO, 2013). MDR-TB has reached alarming levels worldwide with the emergence of strains that are virtually

untreatable with the existing drugs. Drug-resistant strains, along with HIV/AIDS, are causing the biggest challenge to efficient management and control of TB. The report of an outbreak of extensively drug resistant TB (XDR-TB) in South Africa (Gandhi *et al*., 2006), with its extremely high case fatality rate, has drawn wide attention.

It has been indicated that MDR-TB is likely to be more prevalent in Africa than previous reports indicated. The latest WHO global report on anti-tuberculosis drug resistance in the world was produced in 2008 and published in a 2010 WHO report on MDR-TB epidemic. A systematic literature review of evidence about mortality associated with MDR-TB was commissioned by WHO in 2013. The results have been used to produce global estimates of MDR-TB incidence and mortality in 2012. The estimate of mortality due to MDR-TB is slightly higher than before, while the incidence is similar to the previous estimate (WHO, 2013).

1.2 Justification

Five of 13 countries with the highest incidence rates of TB per capita are in Africa. According to the WHO global report on anti-tuberculosis drug resistance in the world, MDR-TB strains have emerged in all regions of the world (WHO, 2013). The overwhelming burden of MDR-TB is in high burden resource poor countries. The diagnosis depends on confirming the drug susceptibility pattern of isolated organisms, which is often only possible in resource rich settings. Lack of comprehensive national DRS data from all countries in Africa is a barrier to understanding the magnitude of prevalence and incidence of MDR-TB.

WHO (2013) reported Nigeria as the thirteenth in the list of the 22 highest incidence countries on the basis of numbers of new cases of TB. Also, Nigeria is considered as having moderate rate of MDR-TB. According to the National drug resistance survey in 2012, the prevalence rate of

MDR-TB in Nigeria is about 2.9%. However, MDR-TB in Africa, including in Nigeria, is more prevalent than previously reported. Given the limited health care funding and substantial incidence of HIV in Nigeria, even a relatively low but increasing tide of MDR-TB can lead to disastrous consequences for the country. The availability of drugs on the open market and a private sector that delivers drugs to the population in an unregulated fashion in Nigeria could also be factors that might favour development of MDR-TB. There is very little information on the prevalence of MDR-TB in Nigeria and specifically in Kaduna State.

1.3 Research Questions

1. Is there MDR-TB in Kaduna State?

2. If there is, what is the prevalence of MDR-TB in Kaduna State?

3. What socio-demographic factors predispose to MDR-TB in Kaduna State?

1.4 Research Hypothesis

Null hypothesis: MDR-TB is not prevalent in Kaduna State

Alternative hypothesis: MDR-TB is prevalent in Kaduna State

1.5 Aim of the study

The aim of the study is to determine the existence and prevalence of MDR-TB in Kaduna State, Nigeria.

1.6 Objectives

The objectives of the study are:

1. To determine some demographic and risk factors that may be associated with tuberculosis among the study population
2. To screen sputum samples for Acid Fast Bacilli (AFB) using microscopy
3. To isolate and characterize *M. tuberculosis* from smear positive sputum samples
4. To determine the anti-tuberculosis drug susceptibility of the isolates
5. To determine the patterns of mutations in the resistant isolates

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of Tuberculosis

Tuberculosis (TB) has recently re-emerged as a major global health concern. However, TB has been plaguing humanity for centuries before this, with the earliest documentation of TB occurring in Egypt as early as 5000 years ago, based on the isolation of *Mycobacterium tuberculosis* DNA from mummies (Daniel, 2006; Mathema *et al.*, 2006). Earlier names for TB included phthisis, which means consumption or to waste away, and was identified as the most rife disease of the times by Hippocrates (Leão and Portaels, 2007). He also noted that the disease occurred more frequently in individuals between the ages of 18 and 25 years old, and almost always resulted in death. It was Clarissimus Galen, a Greek physician, who described phthisis as an "ulceration of the lungs, chest or throat, accompanied by coughs, low fever, and wasting away of the body because of pus"; and described it as a disease of malnutrition (Pease, 1940).

With the commencement of the 17th century, Europe was struck by a TB epidemic, which lasted for 200 years, and was later known as the "Great White Plague" (Leão and Portaels, 2007). It is believed that overcrowding and poor sanitary conditions that were characteristic of the rapidly growing cities of the Western World provided the necessary milieu for the spread of this airborne pathogen. Due to the exploration and colonization that was typical of this period, the TB epidemic slowly consumed the colonized nations as well (Bates and Stead, 1993). Although it is believed that TB existed in America and Africa before the arrival of the Europeans, the disease was very rare among the indigenous people, but after contact with the Europeans, the mortality rate due to TB rapidly increased within the native populations.

Even though 17th century Europe was plagued with TB disease, it is the century during which scientists began to unravel the mysteries of the disease and its causative agent (Bates and Stead, 1993; Daniel, 2006). It was Franciscus Sylvius de la Böe of Amsterdam who was the first to identify the presence of tubercles in the lungs of TB patients as a characteristic of the disease, and this finding was later corroborated by the English physician Richard Morton (Leão and Portaels, 2007). Both de la Böe and Morton also believed that the disease was hereditary; however, Morton also considered transmission by intimate contact as a possible mechanism. Later it was shown by Gaspard Laurent Bayle that the tubercles noted by de la Böe and Morton were not the products of the disease but the actual cause, which gave rise to the name of the disease used today; Tuberculosis.

During the 18th and 19th centuries, various physicians and epidemiologists made scientific breakthroughs in that they finally discovered that the causative agent of TB was a microorganism, and that it could be transmitted between individuals and between humans and various other mammal species. In 1882, Robert Koch made his famous presentation, in which he showed that the bacterium, *M. tuberculosis*, was the causative agent of tuberculosis disease (Mathema *et al.*, 2006; Leão and Portaels, 2007). Towards the end of the 19th century, Koch announced the isolation of a compound that inhibited the growth of the tubercle bacilli when administered to guinea pigs both pre- and post-exposure. This compound was called 'tuberculin', and soon after its discovery it was used as a therapeutic vaccine in a clinical trial, the results of which were extremely disappointing. However, it was found to be valuable in the diagnosis of TB, and later gave rise to the currently used tuberculin skin test/Mantoux test, which is used for diagnosis of latent TB.

The 20th century brought with it the advent of the first successful vaccine and the use of chemotherapy in the fight against TB. At the beginning of the century, Albert Calmette and Camille Guérin successfully created the attenuated strain of *M. bovis*, Bacille Calmette-Guérin (BCG), which was avirulent in cattle, horses, rabbits and guinea pigs (Daniel, 2006). In 1943, the first antibiotic streptomycin was isolated by Selman A. Waksman and Albert Shatz from *Streptomyces griseus* (Mathema *et al.,* 2006). The drug was effective against *M. tuberculosis in vitro*, and post-infection in guinea pigs. In 1944, the first human was treated with the drug and two clinical studies followed, one in Europe and the other in the United States of America. It was noted that individuals who were treated with streptomycin exhibited a substantial improvement in the outcome of the disease. The findings of these studies were however of a "double-edge sword" nature, in that the investigators also noted that following the first months of treatment, some patients' disease status began to worsen; from which they concluded that the pathogen was able to develop resistance to the drug (Canetti *et al.,* 1964). This was soon followed by the administration of para-aminosalicylic acid (PAS) as an oral therapy (Leão and Portaels, 2007). PAS therapy was successful in treating TB, and unlike streptomycin, it was non-toxic and the bacterium was not able to develop resistance to the drug easily. In the late 40's, researchers noted that when TB patients were treated with both streptomycin and PAS, the disease outcome was much more favorable than when patients were treated with only one of the drugs. The 1950's saw the introduction of isoniazid (INH) as a chemotherapeutic agent against TB. Unfortunately, however, like its predecessor streptomycin, it was found that *M. tuberculosis* was able to easily develop resistance to this drug. On the positive side, treatment with streptomycin, PAS and INH was found to be highly effective in the treatment of TB, and for the first time TB was curable. This lead to a wave of new drugs being developed for the treatment of TB, including rifampicin,

pyrazinamide, ethambutol, cycloserine and ethionamide; all of which are still in use today for the treatment of TB. However, the problem of drug resistance acquisition continues.

TB has had a long history, claiming millions of victims during the ages and taking more lives than any other microbial disease. Due to this, TB has left its mark on humanity; in music, art and literature and playing a major role in the advancement of biomedical sciences and healthcare.

2.2 The genus *Mycobacterium*

The genus *Mycobacterium* is the only genus in the family *Mycobactericeae* and is related to other mycolic acid containing genera. The high G+C content of the DNA of *Mycobacterium* species (61 to 71 mol%, except *M. leprae* [55 mol %]) (Pfyffer and Vincent, 2005) are within the range of those members of the other mycolic acid-containing genera, *Gordonia* (63 to 69mol %), *Tsukamurella* (68 to 74mol %), *Nocardia* (64 to 72mol %) and *Rhodococcus* (63 to 73mol %) (Coville and Witebsky, 2005).

Mycobacteria are aerobic (though some species are able to grow under a reduced O_2 atmosphere), non spore forming, nonmtile, slightly curved or straight rods, 0.2 to 0.6 µm by 1.0 to 10 µm, which may branch. Colony morphology varies among the species, ranging from smooth to rough and from nonpigmented (nonphotochromogens) to pigmented. Colonies of the latter type are regularly or variably yellow, orange or rarely, pink, usually due to carotenoid pigments. Some species require light to form pigment (photochromogens); other species form pigment in either the light or the dark (scotochromogens). Aerial filaments are very rarely formed and never visible without magnification. Filamentous or mycelium-like growth may sometimes occur but, on slight disturbance, easily fragments into rods or coccoid elements (Pfyffer and Vincent, 2005).

The cell wall peptidoglycolipid contains *meso*-diaminopimelic acid, alanine, glutamic acid, glucosamine, muramic acid, arabinose and galactose. Mycolic acids (number of carbon atoms ranging from 70 to 90) together with free lipids (e.g., trehalose-6,6-dimycolate), provide a hydrophobic barrier. Other important fatty acids are waxes, phospholipids and mycoserosic and phthienoic acids. Various patterns of cellular fatty acids (number of carbon atoms ranging from 10 to 20) are found as well, amongst which is tuberculostearic (10-*R*-methyloc-tadecanoic) acid, a unique cell components for a number of members of the *Actinomycetales* (Murray *et al.*, 2007).

The high content of complex lipids of the cell wall prevents access to common aniline dyes. Although not readily stained by Gram's method, mycobacteria are usually considered Gram positive. Once stained with a special procedure, however, mycobacteria are not easily decolourized, even with acid alcohol; i.e., they are acid fast. However, acid fastness can be partly or completely lost at some stage of growth by a proportion of the cells of some species, particularly the rapidly growing ones. A natural division exists between slowly and rapidly growing species of mycobacteria. Slow growers require more than 7 days to produce colonies on solid media from a dilute inoculum under ideal culture conditions. Rapid growers, by definition, require less than 7 days when sub cultured on solid culture media but may take several weeks to appear upon primary culture from clinical specimens (Murray *et al.*, 2007).

2.2.1 Nutritional requirements and growth

Most species adapt readily to growth on relatively simple substrates, using ammonia or amino acids as nitrogen sources and glycerol as carbon source in the presence of mineral salts. A few species are fastidious and require supplements such as mycobactin, hemin or orther iron compounds. To date, *M. leprae* has not been cultured outside living cells. Growth of

mycobacteria is stimulated by carbon dioxide and by fatty acids which may be provided in the form of egg yolk or oleic acid, even though the latter is toxic in higher concentrations (≥1%) and has to be neutralized by albumin (Murray *et al.*, 2007).

Optimum temperature for growth vary widely among species from <30^{o}C to 45^{o}C. Compared to that of other bacteria, the growth of most mycobacterial species is slow, with generation time of up to approximately 20 h on commonly used media. Depending upon the species, visible colonies may appear after a few days to 6 weeks of incubation under optimum conditions (Murray *et al.*, 2007).

2.2.2 Susceptibility to physical and chemical agents

Mycobacteria are able to survive for weeks to months on inanimate objects if protected from sunlight. Members of *M. tuberculosis* complex for instance, survive for several months on surfaces or in soil or cow dung, from which other animals may be infected. Mycobacteria are easily killed by heat (>65^{o}C for at least 30 min) and by UV (sun) light but not by freezing or desiccation. They are more resistant to acids, alkalis and some chemical disinfectants than most other non spore forming bacteria. Quaternary ammonium compounds, hexachlorophene and chlorhexidine are bacteriostatic at best. The concentration of malachite green in egg based media e.g., L-J medium was selected to maximize the growth of mycobacteria while inhibiting that of other microorganisms. Other commonly used sterilants, such as ethylene oxide and formaldehyde vapour, as well as disinfectants such as chlorine compounds, 70% ethanol, 2% alkaline glutaraldehyde, peracetic acid and stabilized hydrogen peroxide are effective in killing *M. tuberculosis* (Best *et al.*, 1990).

2.2.3 Habitats

The genus *Mycobacterium* includes obligate pathogens, opportunistic pathogens and saprophytes. The major ecological niche for *M. tuberculosis* complex and *M. leprae*, incapable of replication in the inanimate environment is the tissues of humans and warm blooded animals. In contrast, the non-tuberculous mycobacteria (NTM) are free living mycobacteria and are usually found in association with watery habitats such as lakes, rivers and wet soil. Some human pathogenic NTM species, however, have yet to be recovered from soil or water (American Thoracic Society, 1997).

2.3 The *Mycobacterium tuberculosis* complex (MTBC)

TB is caused by bacteria from the MTBC, which consists of highly related slow growing, acid-fast, non-motile bacilli belonging to the genus *Mycobacterium* that differs substantially from other bacteria due to the exceptionally thick cell wall and high genomic guanine-cytosine content. The MTBC comprises seven members, *M. tuberculosis*, *M. africanum*, *M canettii* where the natural host are humans and *M. bovis, M. caprae*, *M. microti* and *M. pinnipedii* which usually have animals as their natural hosts. Although the mycobacterial species in MTBC are highly similar to each other on DNA level, MTBC members differ widely in terms of host tropism, phenotype and pathogenicity (Brosch *et al*., 2002; Huard *et al*., 2003; Smith *et al*., 2006). Detection of the different species within the complex has mainly been based on the analysis of phenotypic characteristics such as acid-fast microscopy, colony morphology, growth rate and biochemical tests. Genotyping methods have currently made epidemiological studies and rapid species discrimination more promising, enlarging the understanding of phylogenetic relations and evolutionary origin of the members of the MTBC.

2.3.1 *Mycobacterium tuberculosis*

M. tuberculosis is the principal agent of the disease in humans, first described by Robert Koch in 1882. There has been much debate regarding the origin of the MTBC strains as it was previously thought that *M. tuberculosis* had evolved from *M. bovis* by specific adaptation of an animal pathogen to human host (Stead *et al*., 1995; Brisse *et al*., 2006; Smith *et al*., 2006). However, genomic analysis has shown that *M. bovis* has a smaller genome, suggesting that it is evolutionary younger (Brosch *et al,* 2002). Phenotypically, *M. tuberculosis* can be identified using analysis such as nitrate reductase, production of niacin, resistance to thiophene-2-carboxylic acid hydrazide (TCH) and sensitivity to pyrazinamidase (PZA) (Hoffner *et al*., 1993; Niemann *et al*., 2002). Genotypically, by spoligotyping *M. tuberculosis* has been classified into different phylogenetic lineages (Brudey *et al*., 2006).

2.3.2 *Mycobacterium bovis* and *Mycobacterium bovis* BCG

Mycobacterium bovis is the leading cause of TB in cattle that occasionally affects other species of mammals. This disease is a significant zoonosis spread to humans, typically by the inhalation of aerosols or the ingestion of unpasteurized milk or contaminated meat. Information on human disease due to *M. bovis* in developed and developing countries is scarce. From a review of a number of zoonotic tuberculosis studies, published between 1954 and 1970 and carried out in various countries around the world, it was estimated that the proportion of human cases due to *M. bovis* accounted for 3.1% of all forms of tuberculosis: 2.1% of pulmonary forms and 9.4% of extrapulmonary forms. In developed countries, eradication programs have reduced or eliminated TB in cattle, and human disease is now rare; however, reservoirs in wildlife can make complete eradication difficult. Bovine TB (BTB) is an economical and public health threat in developing countries, including Africa (Ayele *et al*., 2004). In many African settings, domestic animals are

an integral part of human social life and in those cases the risk factors for *M. bovis* infection in both animals and humans are close contact, food hygiene practices and HIV/AIDS infection. Control policies have not been enforced due to cost implications, lack of capacity and infrastructure limitations (Cosivi *et al*., 1998; Ayele *et al*., 2004). Most *M. bovis* infections are extra pulmonary TB cases with rare cases of pulmonary TB. The clinical signs of the disease in humans are indistinguishable from those occurring due to infection with *M. tuberculosis*. *M. bovis* can be distinguished from *M. tuberculosis* on the basis of epidemiology, phenotype and some genetic markers, *M. bovis* does not produce niacin, does not reduce nitrate and is sensitive to TCH but resistant to PZA (Niemann *et al*., 2002). There is also a non-virulent strain of *M. bovis* called Bacillus Calmette Guerin (BCG), which has its origin from a virulent *M. bovis* strain. Calmette and Guerin performed 230 *in vitro* passages of *M. bovis* until the organism lost its virulence. While this strain has been used worldwide as a live attenuated vaccine to immunize people against TB, it may cause disease in some humans.

2.3.3 *Mycobacterium africanum*

Since its first description in 1968, *M. africanum* has been found in several regions of Africa, where it represents up to 60% of clinical isolates obtained from patients with pulmonary TB (Haas *et al*., 1997, Viana-Niero *et al*., 2001). Recent surveys show highly variable prevalence of *M. africanum* in different African regions. For example, *M. africanum* was found in approximately 5% of patients with TB in the Ivory Coast and at least 60% of patients in Guinea Bissau (Källenius *et al*., 1999; Bonard *et al*., 2000; Groenheit *et al*., 2011). In contrast to *M. tuberculosis* and *M. bovis*, *M. africanum* strains show a higher variability of phenotypic attributes, comprising characteristics common to both *M. tuberculosis* and *M. bovis*. This phenotypic heterogeneity of *M. africanum* complicates its unequivocal identification and may

lead to misclassification of clinical strains. According to their biochemical characteristics, two major subgroups of *M. africanum* have been described, corresponding to their geographic origin in western (subtype I) or eastern (subtype II) Africa (Collins *et al*., 1982). *M. africanum* subtype II has been shown to correspond to a particular sublineage of *M. tuberculosis* (Mostowy *et al*., 2002).

2.3.4 *Mycobacterium canettii*

Mycobacterium canettii, a novel rare variant of MTBC with smooth colony morphology was first isolated from a Somali-born patient in 1969 by Canetti (van Soolingen *et al*., 1997). Daffe (Daffe *et al*., 1991) demonstrated that this particular strain differed from the commonly rough strains by having large amounts of lipooligosaccharides. The smooth and glossy colonies produced are highly exceptional for this species. This smooth phenotype is however unstable and can switch to a rough colony morphology (van Soolingen *et al*., 1997).

2.3.5 *Mycobacterium microti*

Mycobacterium microti typically causes disease in voles, wood mice, and shrews, although it was also detected in a limited number of other mammalian species. The causative agent was named *M. tuberculosis* subsp. muris, and later this species was designated *M. microti* and classified as a member of the MTBC. It was first reported in humans in 1998 in immunocompromised patients (van Soolingen *et al*., 1998), although human to human transmission of *M. microti* infection seems to be rare (Xavier *et al*., 2007). *M. microti* differs from other MTBC strains in its S-shaped cell morphology, extremely slow growth *in vitro*, and distinct host-specific pathogenicity for laboratory animals. Based on biochemical properties, this bacterium is difficult to distinguish from *M. tuberculosis*, *M. africanum*, or *M. bovis*, but *M.*

microti strains display characteristic IS6110 banding patterns and spoligotypes, distinct from types previously observed in other MTBC strains (van Soolingen *et al*., 1998).

2.3.6 *Mycobacterium pinnipedii*

In 1993, it was reported for the first time that isolates from seals captured on the coast of Argentina had a characteristic Insertion Sequence (IS) 6110 Restriction Fragment Length Polymorphism (RFLP) pattern (Cousins *et al*., 1993). This seal bacillus was later designated *M. pinnipedii* and appeared to have a unique position in the MTBC (Cousins *et al*., 2003). Later on, reports had described *M. pinnipedii* infections in various marine mammals (Forshaw and Phelps 1991; Thompson *et al*., 1993; Hunter *et al*., 1998). Transmission of *M. pinnipedii* to humans has been reported in individuals who are in close contact with marine mammals (Thompson *et al*., 1993; Kiers *et al*., 2008). *M. pinnipedii* isolates present a distinct spoligotype pattern when compared to other members of the MTBC (Cousins *et al*., 2003).

2.3.7 *Mycobacterium caprae*

Mycobacterium caprae was first isolated from goats in Spain (Aranaz *et al*., 1999), but has since been found in other animals, such as cattle (Prodinger *et al*., 2002; Boniotti *et al*., 2009), pigs, red deer and wild boars (Erler *et al*., 2004). Its isolation from humans has also been described (Kubica *et al*., 2003); often, a contact with livestock has been suggested as a likely means of transmission (Prodinger *et al*., 2002). To our knowledge, this pathogen has never been isolated outside continental Europe, except from a European patient in Australia (Sintchenko *et al*., 2006) and a cow in Algeria (Sahraoui *et al*., 2009). For a long time this species was considered as *M. bovis*, because the biochemical tests results were similar to *M. bovis* and *M. bovis* BCG. By spoligotyping, *M. caprae* species form a homogeneous cluster easily recognizable by the absence

of spacers 1, 3-16, 30-33 and 39-43. The lack of spacers 39-43 has also been described in *M. bovis* and *M. microti* (Aranaz *et al*., 1999; Aranaz *et al*., 2003).

2.4 Pathogenesis and clinical presentations of TB

In humans, the result of *M. tuberculosis* infection can vary between latent TB (no clinical symptoms and no transmission) and active TB disease, which can be pulmonary or extrapulmonary. Ninety per cent (90%) of *M. tuberculosis* infections remain in a latent stage, whereas 10 % will develop into active TB at some point during a person's lifetime (Allix-Beguec *et al.*, 2008).

The most common form is pulmonary tuberculosis where at the site of infection the production of various mediators, the activation and accumulation of alveolar macrophages and lymphocytes can be observed, leading consequently to a helper T lymphocyte/macrophage alveolitis (Schluger and Rom, 1998; Schluger, 2001). The course and progression of disease is dependent on interaction of the pathogen and the various regulatory mechanisms for induction of cytokine production of the host. The cytokine release rate at the site of infection in the lungs, the release rate of T1 helper and T2 helper type cytokines in broncho alveolar lavage (BAL) cells (alveolar macrophages and lymphocytes) and in circulating mononuclear cells correlates with the severity of the disease (Dlugovitzky *et al*., 1997; Somosközi *et al.* 1999; Somosközi *et al*. 2000; Schluger, 2001). In contrast, the secondary immune response function and the expression of molecules (CD58, CD80, CD86 and HLA-DR) in signal transduction system has shown that the activity of alveolar macrophages is not dependent on severity of disease (Somosközi *et al*. 2000).

Transmission of TB occurs by the inhalation of infectious aerosols emitted by active pulmonary TB patients. After inhalation of a droplet containing a few *M. tuberculosis* bacilli, the bacteria

will be phagocytised by alveolar macrophages in the lungs, where the bacilli will settle in the case of pulmonary TB. Occasionally, the bacilli will be further transported to other parts of the human body and will cause extrapulmonary TB. *M. tuberculosis* survival in the host starts with its capacity to persist in alveolar macrophages by preventing the phagosomes maturation (Canetti *et al.*, 1969). In the lungs, the formation of granulomas (or "tubercles", the hallmark of TB) is initiated by the cytokine-mediated recruitment of additional macrophages, dendritic cells and lymphocytes to the site of infection (Kwan and Ernst, 2011). In the granuloma, the bacilli and the immune system initiate a dynamic cross-talk, balancing between infection control and disease (Brites and Gagneux, 2012). The granuloma is first constituted of disorganized macrophages, monocytes, and neutrophils but it becomes more organized when lymphocytes are recruited, with macrophages grouped in the centre and lymphocytes at the periphery. This order of infiltration reflects the successive roles of innate and acquired immunity following *M. tuberculosis* infection (Brites and Gagneux, 2012). The granuloma becomes vascularized, but as it matures, the vessels reduce while the fibrous outer layer thickens (Joint United Nations Programme on HIV/AIDS, 2010). In late stages of the granuloma formation, *M. tuberculosis* can enter a low replicative phase in the hypoxic caseous centre of the granuloma. This low replicative stage corresponds to latent TB. However, there is increasing evidence that the dichotomy between latent and active TB is a too simplified view. In fact, *M. tuberculosis* sub populations in various replicative stages can be found in granulomas, with a spectrum of presentations (sterile tissue, caseous hypoxic lesions or liquefied cavities of replicating bacilli) (Allix-Beguec *et al.*, 2008; Friedlaender, *et al.*, 2008; Kwan and Ernst, 2011). TB can remain in a latent state for decades in equilibrium between *M. tuberculosis* replication and host defence, mediated by the complex interplay of immune cells and inflammatory molecules.

Despite the apparent strong immune response which contains the *M. tuberculosis* infection in the granuloma, the bacilli are yet not fully eliminated. Ultimately, latent TB converts into active disease when cavitary lesions develop and the number of bacilli increases in the caseous centre of the granuloma. Live bacilli can reach the alveoli once the granuloma centre collapses and the patient becomes infectious (Joint United Nations Programme on HIV/AIDS, 2010) (Figure 2.1). Viable and infectious bacilli are freed into the airways, resulting in a productive cough spreading infectious bacilli in the air. The life cycle continues when other persons get infected. It is estimated that a single person with active TB can infect up to 45 other individuals (Stoneking *et al.,* 1990). Active pulmonary TB is characterized by unspecific symptoms, principally prolonged and productive cough (> two weeks), shortness of breath, chest pain, haemoptysis (expectoration of blood), night sweats, weight loss, or fever (Thwaites, *et al.,* 2008). Acute TB, when disease develops directly after infection without previous latent stage or granuloma formation is rare (Canetti *et al.,* 1969). In adults, active TB typically results from the reactivation of existing latent TB, rather than as direct outcome of primary infection. Generally, the immediate onset of disease following infection only occurs in immunocompromised individuals, such as newborns, elderly or HIV infected patients. In fact, the conditions influencing the progression to active TB are not fully understood (Joint United Nations Programme on HIV/AIDS, 2010), although factors such as HIV and diabetes are known to favour a prompter development of active TB (Allix-Beguec *et al.,* 2008; Parwati *et al.,* 2010).

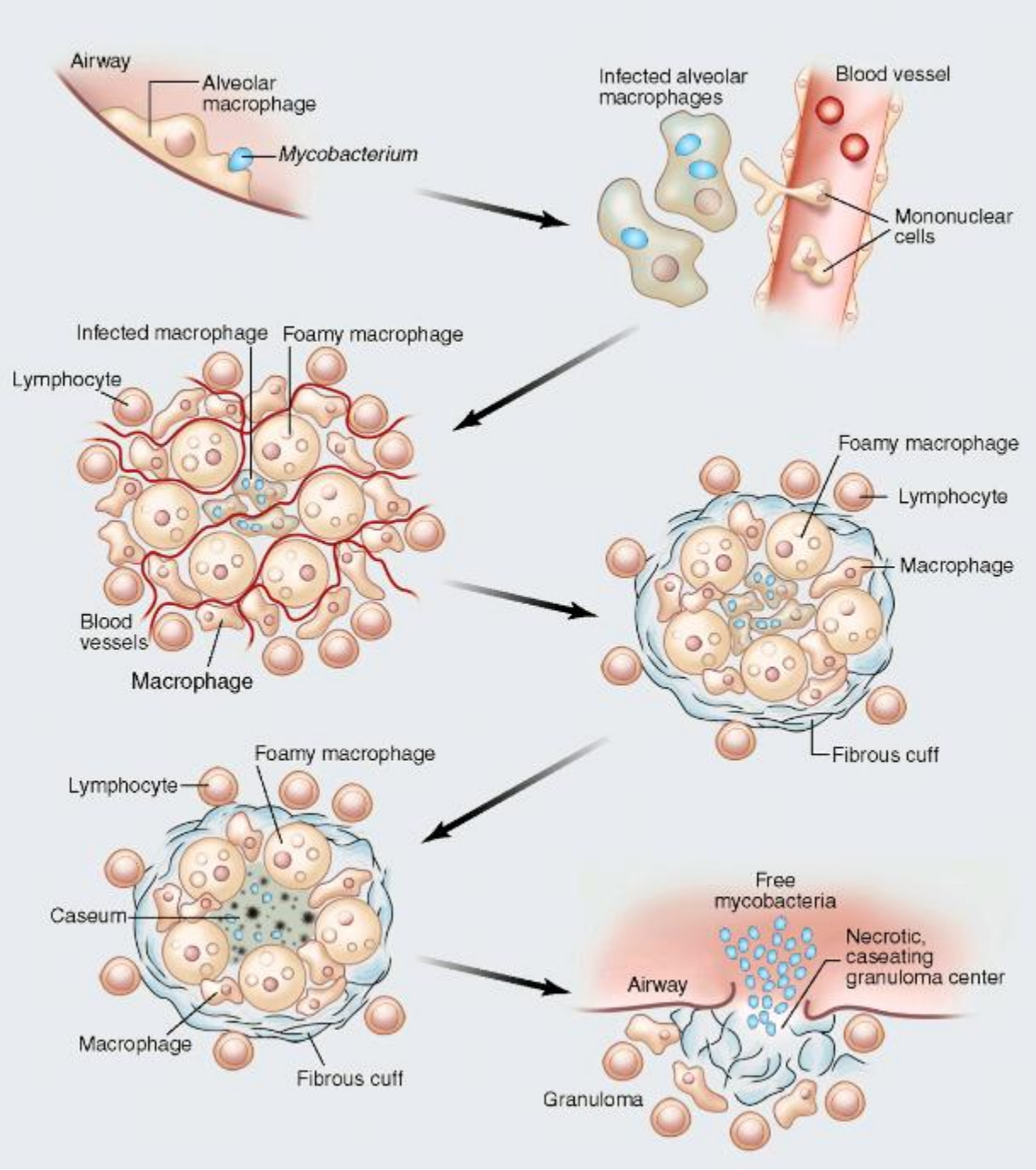


Figure 2. 1: Life cycle of *M. tuberculosis* and granuloma formation (Kwan and Ernst, 2011)

2.5 Global TB burden

In 2012, there were an estimated 8.6 million incident cases of TB and 1.3 million people died from the disease. The incidence rate, prevalence rate and mortality rate due to TB is highest in the WHO Africa region. However, in terms of the absolute number of incident and prevalence cases, South-East Asia Region has the highest TB burden globally contributing 35% to the global TB incidence. There are 22 high burden countries which account for 81% of all estimated incident cases worldwide. The five countries with the largest number of incident cases in 2012 were India (2.0 million-2.4 million), China (0.9 million-1.1 million), South Africa (0.4 million-0.6 million), Indonesia (0.4 million-0.5 million) and Pakistan (0.3 million-0.5 million). India and China alone accounted for 26% and 12% respectively of TB cases worldwide. There were an estimated 12 million prevalent cases of TB in 2012, equivalent to 169 cases per 100 000 population (WHO, 2013). In terms of the percentage of total deaths, TB is ranked 7th in the world accounting for 2.5 per cent of all deaths as per the WHO global burden of disease estimates for the year, 2004.

HIV and TB coexist in many regions and form together a co-epidemic. The proportion of TB cases co-infected with HIV was highest in countries in the African Region (WHO, 2013). TB is the leading cause of death among HIV infected people, accounting for a fourth of HIV deaths worldwide (Kamerbeek *et al.,* 1997). Due to their often impaired immune system, TB patients co-infected with HIV are 21 to 34 times more likely to develop active TB disease than HIV negative individuals (Kamerbeek *et al.,* 1997). About 37% of TB cases were estimated to be co-infected with HIV in Africa, which accounted for 75% of TB cases among people living with HIV worldwide. In parts of southern Africa, more than 50% of TB cases were co-infected with HIV (Kamerbeek *et al.,* 1997; Borrell and Gagneux, 2009). In 2008, South Africa alone

accounted for 24% of all new HIV-TB cases worldwide, even though its estimated population comprised less than 1% of the global population (Supply *et al.*, 2006).

TB is a leading cause of death in HIV infected persons and HIV infection is the most potent risk factor for developing active TB disease from a latent TB infection. TB-HIV co-infection and drug resistant tuberculosis aggravate the TB situation globally. Of the 8.6 million incident TB cases in 2012, 1.1 million (13%) cases were among people living with HIV. Of these HIV positive cases, 75% were in the African region and 39% were in the South-East Asia region (WHO, 2013).

Globally, the emergence of Multi drug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) is a major threat to TB Control. Resistance to anti-TB drugs in populations is a phenomenon that occurs primarily due to poorly managed TB care such as inconsistent or partial treatment, when patients do not take all their medicines regularly for the required period because they start to feel better, because doctors and health workers prescribe the wrong treatment regimens, or because the drug supply is unreliable or erratic. Globally, 3.6% (95% CI: 2.1-5.1%) of new TB cases and 20.2% (95% CI: 13.3-27.2%) of previously treated cases are estimated to have MDR-TB. These estimates are essentially unchanged from 2011. The proportions of new and previously treated TB cases with MDR-TB at the country level for the 27 high MDR-TB burden countries showed that Eastern European and especially central Asian countries have the highest levels of MDR-TB. Among new cases, examples include Azerbaijan (22.3% in 2007), Belarus (34.8% in 2012), Estonia (19.7% in 2012), Kazakhstan (22.9% in 2012), Kyrgyzstan (26.4% in 2011), the Republic of Moldova (23.7% in 2012), the Russian Federation (average: 23.1%, with Yamalo-Nenets Autonomous Area being the highest: 41.9% in 2011) and

Uzbekistan (23.2% in 2011). Among previously treated cases, examples include Azerbaijan (Baku City: 55.8% in 2007), Belarus (68.6% in 2012), Estonia (50.0% in 2012), Kazakhstan (55.0% in 2012), Kyrgyzstan (68.4% in 2012), the Republic of Moldova (62.3% in 2012), Tajikistan (56.0% in 2012) and Uzbekistan (62.0% in 2011). In the Russian Federation, even though the average proportion of cases with MDRTB does not exceed 50%, the proportion is well above 50% in several Federal Subjects (with Ulyanovsk Oblast at the highest level: 74.0% in 2011). Nigeria has MDR-TB prevalence of 2.9% among new cases and 14.3% among retreatment cases (WHO, 2013).

2.6 Drug resistant tuberculosis

Chemotherapy is the most potent weapon available in the fight against tuberculosis. When used properly, available anti-TB drugs are able to reach cure rates above the 85% target recommended by the World Health Organization (WHO). Early in the chemotherapy era, resistance associated with treatment failures emerged and has become a common occurrence worldwide. Of particular concern is the increasing prevalence of organisms resistant to isoniazid and rifampicin, the two drugs that form the backbone of modern short-course therapy. Rifampicin (RIF) resistance occurs mostly in conjunction with INH resistance (90% of cases) and can be used as a surrogate marker for multidrug resistance.

Drug resistance in *M. tuberculosis* occurs as a result of random spontaneous chromosomal mutations during natural cell replication. These mutations are not drug induced and are not linked. The probability of a drug-resistant mutant occurring is directly proportional to the size of the bacterial population. The frequency of primary resistant organisms varies for each drug; however, it is usually between 1 in 10^6 to 1in 10^8.

Spontaneous resistance to isoniazid is estimated to occur once in every 10^6 organisms, and to rifampicin once in every 10^8 organisms. The probability of spontaneous mutants being simultaneously resistant to two or more drugs is the product of the individual mutants. The development of drug resistance is a man made amplification of a naturally occurring phenomenon. Previous treatment for tuberculosis predisposes to the selection of multi drug resistant organisms. Non compliance is a major factor in allowing the resistant organisms to survive. Multi drug therapy is used to prevent the emergence of drug resistant mutants during the long duration of treatment.

Resistance can be defined as single-drug, multi-drug, or poly-drug resistance depending on the number of drugs and/or which drugs are involved (Rieder,1999). Although an unequal global distribution of drug resistance exists between poor and rich countries, the problem is global. The regions where drug-resistant TB is more prevalent lack the resources to implement adequate measures to control even the susceptible types of the disease. Reviews have reported a high prevalence of primary multidrug resistant tuberculosis in Latvia (1998: 9.0%), Estonia (1998, 14.1%), The Dominican Republic (1994-1995: 6.6%), Ivory Coast (1995-1996: 5.3%), Argentina (1994: 4.6%), Russia (Ivanovo Oblast) (1998: 9%), Iran (1998, 5.0%) and Henan, China (1996, 10.8%). South Africa's neighbours Botswana (1995-1996), Lesotho (1994-1995), and Swaziland (1994-1995) have reported encouraging results of 0.2%, 0.9%, and 0.9% respectively. Acquired multidrug resistance of higher than 20% was reported in Guinea (1998: 28.1), Latvia (1996: 54.4%), Mexico (1997: 22.4%) Italy (1999: 33.9%), Russia (Ivanovo Oblast) (1998: 25.9%) Tomsk Oblast (1999: 26.7%), Estonia (1998: 37.8%), Iran (1998: 48.2), Sierra Leone (1997: 23.1%), Argentina (1994: 22.2), and Spain (Barcelona) (1995-1996: 20.5%). Again acquired

MDRTB was low in Botswana (1998: 9.0%), Mozambique (1999: 3.3%), Lesotho (1994-1995: 5.7%), and Swaziland (1994- 1995: 9.1%) (Cohn *et al*., 1997; Espinal *et al*., 2001).

Previous treatment for tuberculosis predisposes to the selection of multi drug resistant organisms and non compliance is a major factor in allowing the resistant organisms to survive.

2.6.1 Molecular bases of drug resistance

The molecular bases of mycobacterial drug resistance include:

2.6.2 Resistance to Aminoglycosides

2.6.3 Streptomycin (SM)

Streptomycin is an aminoglycosidic aminocyclitol, the first of this class of antibiotics to be identified. Its clinical introduction marked the beginning of the chemotherapy era of tuberculosis control and most of the clinical trials that defined the principles of anti-tuberculosis chemotherapy used SM. An adverse toxicity profile and the need for parenteral administration have resulted in it being replaced by other agents in first line therapy. However, the emergence of drug resistance has meant that SM is still an important antimycobacterial agent. Other aminoglycoside antibiotics are also being used to treat multidrug resistant cases of tuberculosis and therefore an understanding of the mechanism of resistance to SM has found a new relevance.

Aminoglycosides are broad spectrum antibiotics and their mode of action has been extensively studied in other organisms. SM binds to the 30S ribosomal subunit leading to inhibition of translational initiation and misreading of messenger RNA (Noller, 1984). Resistance to SM and other aminoglycoside in Gram negative organisms is principally due to the acquisition of aminoglycoside modifying enzymes. However, no plasmids or transposons bearing drug resistance genes have been detected in *M. tuberculosis*, and the chromosomally encoded aminoglycoside 2□-*N*-acetyltransferase (*aac*(*2*□)-*Ic*) is apparently unable to acetylate

aminoglycoside, thus differing from its counterpart in *Mycobacterium smegmatis* (Ainsa *et al.* 1997). An *aph*(*3*□□)-*Ic* gene, encoding an aminoglycoside 3□ □-*O*-phosphotransferase which can inactivate SM, has been characterized in *Mycobacterium fortuitum*, but is absent from the genome of *M. tuberculosis* (Ramon-Garcia *et al.,* 2006).

Streptomycin-resistant strains of *Escherichia coli* have been isolated with mutations in two ribosomal components, the S12 protein and the 16S ribosomal RNA. Analysis of these genes in *M. tuberculosis* was therefore a logical first approach to investigating resistance to SM, since mutations at these loci would produce a dominant phenotype, as *M. tuberculosis* possesses only a single copy of these genes (Cole *et al.*, 1998). Various groups in parallel established that mutations in *rpsL*, the gene coding for the S12 ribosomal protein, were associated with resistance (Finken *et al.*, 1993; Nair *et al*., 1993; Honore and Cole, 1994; Cooksey *et al.*, 1996). These mutations were found to occur at codon 43 (Lys43Arg or rarely Lys43Thr), less frequently at codon 88 (Lys88Arg or Lys88Gln), and in isolates exhibiting high-level resistance to SM (MIC of greater than 500µg/mL).

Sequence analysis of the *rrs* gene that codes for the 16S rRNA, from SM resistant clinical isolates also identified a series of single nucleotide substitutions that were in general associated with an intermediate level of resistance and confined to two restricted regions. One group, represented by substitutions at positions 491, 512, 513, or 516 mapped to the 530 loop region of the *E. coli* 16S rRNA, and the other at positions 798, 865, 877, 904, and 906 mapped to the *E. coli* 912 region (Sreevatsan *et al*., 1996). The 530 loop region is one of the most highly conserved regions of the 16S ribosomal RNA, both in sequence and secondary structure, reflecting its importance for some translational function. The 912 region has been implicated in

translational fidelity and is located at the junction of the three major domains of the 16S rRNA. Chemical foot printing experiments using *E. coli* ribosomes have demonstrated that this region is protected by the binding of SM (Moazed and Noller, 1987) and mutations here reduce drug binding, (Leclerc *et al*., 1991; Powers and Noller, 1991) suggesting it may be the primary site of action of SM. Mutations in *rpsL* and *rrs* mutations can only be detected in approximately 50% and 10% of clinical isolates, respectively. The substantial proportion of strains with no detectable ribosomal subunit mutations indicates there are other mechanisms of resistance, although these strains are in general only resistant to low levels of SM (MIC < 50µg/mL). Characterization of the active efflux systems detected in the *M. tuberculosis* genome is not yet very advanced (De Rossi *et al*., 2006), but several have been shown to transport aminoglycosides. The *M. tuberculosis* gene Rv1258c is a homolog of the gene coding for the *M. fortuitum* tap efflux pump, and a member of the major facilitator super family (MFS) of efflux systems (Ainsa *et al*., 1998). Over expression of Rv1258c in *M. bovis* BCG resulted in a modest increase in the MIC for SM, and gene inactivation increased susceptibility, thus suggesting a role in the intrinsic resistance to aminoglycoside. Similar results were obtained with Rv2333c and Rv1410c, two other members of the 16 MFS genes described in the genome of *M. tuberculosis* (Silva *et al.*, 2001; Ramon-Garcia *et al.,* 2007). Whether these or other transport systems are actually responsible for resistance in clinical isolates requires further investigation, but a recent study has provided evidence that mutations in *gidB*, a methyl transferase specific for the 16S rRNA, may be responsible for low level SM resistance in *M. tuberculosis* (Okamoto *et al.,* 2007).

2.6.4 Other aminoglycosides

The emergence of strains resistant to SM and other drugs has necessitated the use of other aminoglycoside to treat individuals infected with such strains. These aminoglycosides have a

range of MICs for *M. tuberculosis*, and unlike SM are made up of a 2-deoxystreptamine ring rather than a streptidine ring. Given this structural difference it is not surprising that these 2-deoxystreptamine aminoglycoside bind to different sites on the ribosome, and appear to be fully active against *M. tuberculosis* strains harbouring SM resistance-associated mutations in their *rrs* and *rpsL* genes (Meier *et al.,* 1996). A structural study of a paromomycin-rRNA complex (Fourmy *et al.,* 1996) indicates that these antibiotics bind to a region encompassing the 30S subunit A site including position 1408, which has been demonstrated to be important for resistance to 2-deoxystreptamine aminoglycosides in *E. coli* (Beauclerk and Cundliffe, 1987). In *M. tuberculosis*, position 1400 of the *rrs* gene (the equivalent of position 1408 of the *E. coli rrs*) was also found to be important in mediating resistance to 2-deoxystreptamine aminoglycoside. Studies have found an A to G substitution at position 1400 of the *rrs* gene in 60% to 76% of kanamycin-resistant clinical isolates of *M. tuberculosis* analyzed (Taniguchi *et al.,* 1997; Alangaden *et al.* 1998; Suzuki *et al.* 1998). Other *rrs* mutations at positions 1401 and 1483 have also been described but these occur rarely, indicating 1400 is the principal site involved in mediating resistance to kanamycin. The equivalent *rrs* gene position 1400 can also confer kanamycin resistance in *M. smegmatis* and other mycobacteria (Sander *et al.,* 1996; Prammananan *et al.,* 1998). As with SM resistance, low-level resistance to kanamycin was not found to be accompanied by mutations in the *rrs* gene, suggesting other pathways to resistance may be involved.

The degree of cross resistance to other aminoglycosides conferred by the *rrs* substitution at position 1400 is not completely known, but appears to confer at least resistance to amikacin (56 Sander *et al.,* 1996; Taniguchi *et al.,* 1997; Alangaden *et al.,* 1998). In various fast growing mycobacteria, a mutation at this position has been shown to convey resistance to five different 2-

deoxystreptamine aminoglycosides (Prammananan *et al.*, 1998), so the A to G substitution at position 1400 is probably a pandeoxystreptamine aminoglycoside resistance conferring mutation in *M. tuberculosis* as well. While mutations at position 1401 appear to cause resistance to both amikacin and kanamycin, the substitutions at position 1483 may result in strains that retain susceptibility to amikacin. Other uncharacterized mutations outside of the *rrs* gene also exist that can confer unique resistance to kanamycin (Kruuner *et al.*, 2003).

Capreomycin is a macrocyclic peptide antibiotic produced by *Saccharothrix mutabolis* subspecies that was originally discovered in the 1960s (Herr and Redstone, 1966). Renewed attention has focused on the mechanism of resistance to this agent to determine if it can be successfully deployed against organisms resistant to other aminoglycoside. Using transposon mutagenesis and insertion site mapping in *M. tuberculosis*, it was recently shown that inactivation of the *tlyA* gene (Rv1694) resulted in resistance to capreomycin, and mutations at this locus have been identified in resistant clinical isolates (Maus *et al.*, 2005a; Maus *et al.*, 2005), *tlyA* was subsequently found to encode a 2□-*O*-methyltransferase that modifies nucleotide C1409 in helix 44 of 16S rRNA and nucleotide C1920 in helix 69 of 23S rRNA, and the positioning of these methylations suggested a site of action for capreomycin (Johansen *et al.*, 2006). The identification of this new resistance mechanism, which can occur in combination with *rrs* mutations, has allowed a rational description of the overlapping resistance profiles of capreomycin and other aminoglycoside (Maus *et al.*, 2005a).

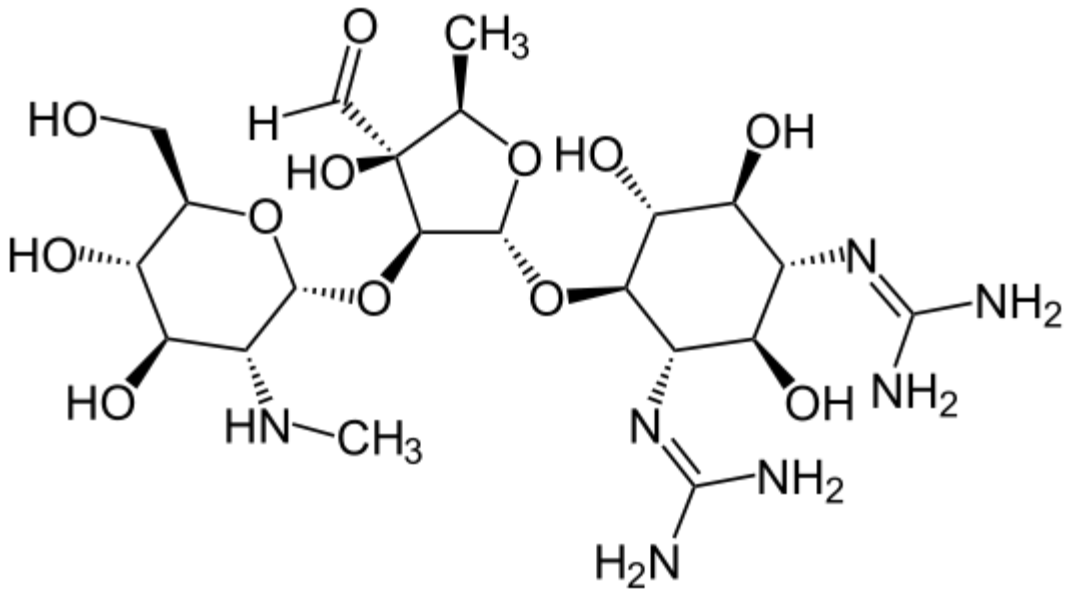


Figure 2. 2: Chemical structure of Streptomycin

2.6.5 Resistance to Rifamycins

2.6.6 Development of Rifamycins

During a systematic search for new antibiotic compounds in the 1950s, workers at the Dow-Lepetit Research Laboratories in Milan observed that crude extracts from the fermentation broths of *Nocardia mediterranei* contained a mixture of microbiologically active agents. These were found to be a group of closely related compounds with an ansa structure (*a*romatic *n*ucleus *s*panned by an *a*liphatic *b*ridge), and were named "Rifamycins", in memory of the then popular French crime story Rifi- about a jewel heist and rival gangs (Sensi *et al.*, 1960). Chemical modification of the rifamycins led to the isolation of rifampin or rifampicin (Maggi *et al.*, 1966), which proved to be a potent anti-tuberculous agent whose clinical introduction enabled the duration of chemotherapy for tuberculosis to be reduced to six months (East African/British Medical Research Council, 1972). Other rifamycin derivatives, rifapentine (RPE) and rifabutin (RBU) have been licensed for the treatment of mycobacterial infections.

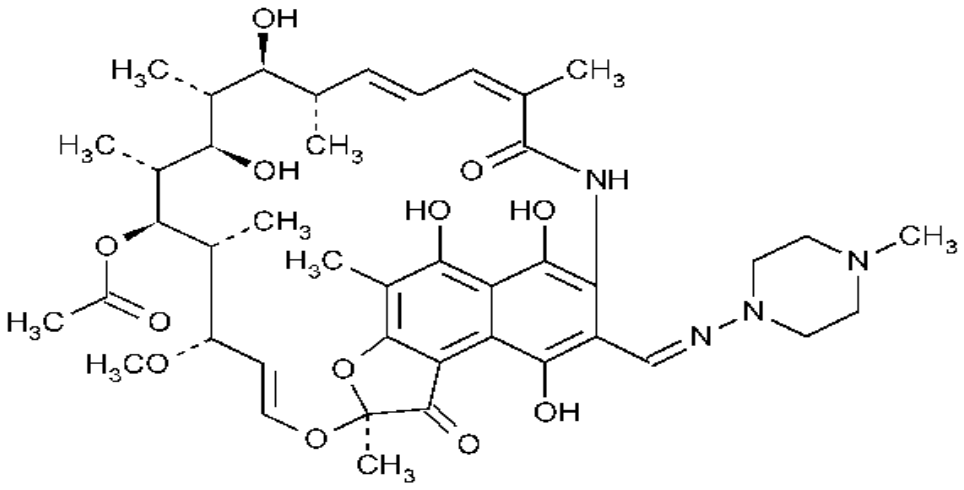


Figure 2. 3: Chemical structure of rifampicin

As was the case with SM, the mode of action and resistance mechanism of RIF had been well characterized in *E. coli*. In *E. coli*, RIF inhibits transcription by targeting DNA-dependent-RNA polymerase (Hartmann *et al.*, 1967), and mutations in several restricted and highly conserved regions of the β subunit, coded for by the *rpoβ* gene, lead to drug resistance (Ovchinnikov *et al.*, 1983). The availability of the *Mycobacterium leprae* , *rpoβ* sequence (Honore *et al.*, 1993) made it possible for two groups to isolate and characterize the *rpoβ* gene from RIF resistant strains of *M. tuberculosis* (Telenti *et al.*, 1993) and *M. leprae* (Honore and Cole, 1993). These studies demonstrated that missense mutations and short in-frame deletions, exclusively associated with RIF resistance, occurred in a central region of the *rpoβ* gene, which corresponded to the region most commonly altered in RIF-resistant *E. coli* strains.

Numerous subsequent studies of RIF-resistant strains of *M. tuberculosis* from globally dispersed sources have confirmed these findings, and in the vast majority of strains analyzed a point mutation or in-frame deletion/insertion can be found within an 81bp region corresponding to codons 507 to 533 of the *rpoβ* gene (Ramaswamy and Musser, 1998). A large number of

mutations have been described, though substitutions at two positions, Ser531 and His526, were found to occur in the majority of strains studied. Mutations at these two positions (Ser531Leu, His526Tyr) and an Asp516Val mutation have been shown to confer resistance when episomal vectors carrying an appropriately mutated *rpoβ* gene were transformed into *M. tuberculosis* (Miller *et al.*, 1994; Williams *et al.*, 1998). The realization that RIF resistance-conferring mutations are confined to a small genetic region has meant that molecular techniques for diagnosing RIF resistance have been relatively straightforward to develop, and numerous different molecular strategies have been successfully employed for detecting them.

Many of these surveys did not sequence the whole 3516 bp *rpoβ* gene, so in the approximately 5% of RIF-resistant *M. tuberculosis* strains that have a wild-type 81bp RIF resistance determining region (RRDR), it is not clear to what extent mutations in other regions of this gene are also involved in mediating RIF resistance.

While several mutations have been reported outside of the RRDR, RIF-resistant strains with a wild-type *rpoβ* gene have also been identified, indicating that RIF resistance can also arise through an *rpoβ* gene independent mechanism (Musser, 1995).

Mycobacterium smegmatis and many strains of the *Mycobacterium avium* complex are innately resistant to RIF despite a drug-sensitive RRDR sequence (Guerrero *et al.*, 1994; Quan *et al.*, 1997). In *M. smegmatis*, there is evidence for ribosylative inactivation of the drug (Quan *et al.*, 1997), but this drug resistance mechanism is absent in *M. tuberculosis*.

2.6.7 Resistance to Isoniazid (INH)

2.6.8 Historical Studies

Unlike SM and RIF, isonicotinic acid hydrazide (isoniazid or INH) is a highly specific antimycobacterial agent, being exquisitely potent against *M. tuberculosis* (and the other members of the *M. tuberculosis* complex: *M. bovis*, *M. microti*, and *M. africanum*), but possessing no or little activity against *M. leprae*, atypical mycobacteria, or other organisms. Investigations into its mode of action were therefore restricted to the inherently difficult to manipulate members of the *M. tuberculosis* complex. Despite this, early studies were able to establish that INH-resistant strains were commonly catalase negative (Middlebrook, 1954) with reduced virulence in animal models (Middlebrook and Cohn, 1953) and that the principal mode of action was likely to be through disruption of the cell wall, probably through inhibition of mycolic acid synthesis (a major cell wall component) (Winder, 1982). Unifying these observations had to wait, though, until the development of the necessary tools to genetically manipulate *M. tuberculosis*.

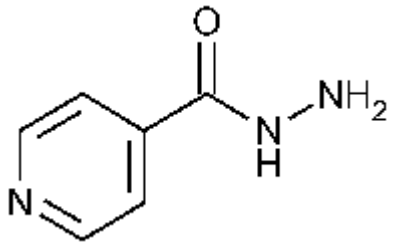


Figure 2. 4: Chemical structure of Isoniazid

2.6.9 Drug Activation

While the role of catalase in the activation of INH to its active form was first proposed in 1958 (Kruger-Thiemer, 1958), it was not until 1992 that the first genetic evidence for this hypothesis became available with the cloning of *katG*, the gene encoding the catalase-peroxidase enzyme of

M. tuberculosis (Zhang *et al.,* 1992). It was shown that over expression of *katG* in catalase negative strains of *E. coli* or in an INH-resistant strain of *M. smegmatis* could render these organisms relatively sensitive to INH. It was further demonstrated that two clinical isolates of *M. tuberculosis* with high-level resistance to INH (MIC > 50µg/mL) had a chromosomal deletion spanning the *katG* gene, and that transformation of these strains with *katG* could restore their INH sensitivity (Zhang *et al.,* 1993). Further characterization of *katG* has demonstrated that it encodes a dimeric, heme-containing enzyme with catalase and peroxidase activity, in keeping with its structural similarity to other eubacterial hydroperoxidase 1 (HPI) enzymes (Johnsson and Schultz 1994; Heym *et al.,* 1995; Johnsson *et al.,* 1997). Confirmation that INH is a prodrug requiring activation by *katG* was provided by demonstrating that *inhA* (a target for INH) is only rapidly inactivated by INH in the presence of *katG* (Johnsson *et al.,* 1997). A mechanism for the oxidation of INH to its bioactive form has also been proposed, in which the drug is converted into a number of highly reactive species capable of either oxidizing or acylating macromolecules (Bertrand *et al.,* 2004; Pierattelli *et al.,* 2004).

A plethora of studies characterized *M. tuberculosis* strains from diverse geographic locations and have shown the majority of clinical isolates resistant to INH have alterations in their *katG* gene (Ramaswamy *et al.,* 2003; Gagneux *et al.,* 2006; Hazbon *et al.,* 2006). While large scale deletions of *katG* have been detected infrequently, missense mutations and small intragenic deletions are the commonest genetic modifications associated with INH resistance. A large number of these mutations have been described, though the serine to threonine mutation at codon 315 is the commonest. The possible explanation for the apparent bias in selection by INH for this mutation over other resistance-conferring mutations in *katG* has been provided by several studies, which have characterized the enzymatic properties of a recombinant *katG* protein

harboring a Ser315Thr mutation (Wengenack *et al.,* 1997; Wengenack *et al.,* 1998; Saint-Joanis *et al.,* 1999). In contrast to other INH resistance-conferring mutations, such as the Thr275Pro mutation which results in concomitant loss of peroxidatic activity and capacity to activate INH, the Ser315Thr mutation results in an enzyme unable to activate INH, leaving at least 50% of its peroxidase and catalase activities intact. Catalase and peroxidase activities are important for protecting *M. tuberculosis* against reactive oxygen species encountered within macrophages (Ng *et al.,* 2004), and are essential for a fully virulent phenotype. Strains with reduced or absent activities are less virulent when assayed in animal models (Jackett *et al.,* 1978; Heym *et al.,* 1997; Ng *et al.,* 2004), and more susceptible to H_2O_2 intracellularly (Manca *et al.,* 1999). A direct comparison of the pathogenicity of isogenic strains harbouring either the Ser315Thr substitution or other *katG* mutations has also been made, and only the 315 mutants retained near normal virulence in mice (Pym *et al.,* 2002). Recently, INH-resistant strains of *M. tuberculosis* with the Ser315Thr substitution have been shown to be more transmissible than strains with other *katG* mutations (Gagneux *et al.,* 2006). It is unfortunate for the control of MDR-TB that *M. tuberculosis* can so successfully balance the competing demands of resistance to oxidative stress and resistance to INH. The elucidation of the crystal structure of *katG*s from *M. tuberculosis* (Bertrand *et al.,* 2004) and two other organisms (Yamada *et al.,* 2002; Carpena *et al.,* 2003) has provided insights into how the S315T substitution could lead to loss of activation of INH but retention of catalase-peroxidase activity. In one model, Ser-315 is located at the periphery of the INH binding pocket, where a threonine substitution would increase the steric bulk at this position, leading to reduced affinity for the drug without fully blocking access to the substrate binding site (Bertrand *et al.,* 2004; Pierattelli *et al.,* 2004).

2.6.10 Drug targets

inhA

Population genetics of INH-resistant clinical isolates have consistently found that a significant proportion of strains possess a wild-type *katG* gene, demonstrating that there are mechanisms of resistance independent of *katG*-mediated INH activation. This suggested that the intracellular targets of activated INH might be involved in mediating resistance. By expressing genomic libraries from two INH-resistant strains of mycobacteria isolated *in vitro* in the fast-growing *M. smegmatis*, it was possible to identify a two-gene operon with homology to proteins involved in fatty acid biosynthesis that could confer INH resistance (Banerjee *et al.*, 1994). Characterization of this operon revealed that only the second gene *inhA* was required for resistance (Banerjee *et al.*, 1998), and sequence analysis of the *inhA* gene from the resistant strains revealed a serine to alanine substitution at position 94 relative to the wild-type gene. Transfer of this mutation to *M. tuberculosis* results in INH resistance (Vilcheze *et al.*, 2006). Biochemical studies revealed that *inhA* codes for a fatty acid enoyl-acyl carrier protein reductase, which is part of a type II dissociated fatty acid biosynthesis pathway (FASII). *inhA* enzymic activity is nicotinamide adenine dinucleotide (NADH) dependent, and reduces the double bond at position two of a growing fatty acid chain linked to an acyl carrier protein ACP, an activity common to all known fatty acid biosynthetic pathways. *inhA* has a marked preference for long-chain substrates which are the precursors of very long alpha-branched fatty acids (C_{40} to C_{60}) known as mycolic acids, a major structural element of the mycobacterial cell wall (Quemard *et al*., 1995).

Structural analysis of the *inhA* protein has characterized the nature of the interaction between INH and *inhA* (Dessen *et al.*, 1995). The observation that *inhA* inhibition by INH requires the presence of NADH and that the Ser94Ala mutant protein has a lower affinity for NADH and

requires higher concentrations of this cofactor before inhibition occurs, suggested that INH may interact with NADH rather than directly with *inhA.* This was elegantly demonstrated by co-crystallization of *inhA* with NADH and INH since the structure showed the activated form of INH covalently linked to NADH within the active site of the enzyme (Rozwarski *et al*., 1998). The INH-NAD adduct has been purified and shown to be a tight-binding inhibitor of *inhA* (Rawat *et al.,* 2003).

The exact mode of interaction of adducts and enzyme has not been defined, but it is now thought that, the adduct is formed in solution before binding to *inhA* (Wilming and Johnsson, 1999; Rawat *et al.,* 2003). This model is consistent with the observation that NADH dehydrogenase defects in *M. smegmatis*, leading to a higher than normal NADH/NAD^+ ratio are associated with a degree of INH resistance (Miesel *et al*., 1998). More recently, *M. bovis* BCG *ndh* (encoding a type II NADH dehydrogenase) mutants have been characterized and found to have aberrant NADH/NAD^+ ratios and reduced INH susceptibility as well (Vilcheze *et al.,* 2005). The unravelling of the interactions of INH and *inhA* has helped spawn the development of new classes of *inhA* inhibitors with promising activity against *M. tuberculosis* and other organisms (Parikh *et al*., 2000; Kuo *et al.,* 2003; Sullivan *et al.,* 2006). This is a good example of how understanding the mechanisms of resistance can assist in rational drug design. Since the original description of the Ser94Ala mutation, other *inhA* gene substitutions have been identified through sequence analysis of INH-resistant clinical isolates (Ramaswamy *et al.,* 2003), confirming that *InhA* substitutions could also be selected for *in vivo*.

Interestingly, the affinity of the INH-NAD adduct for *inhA* appears not to be affected by some of the *inhA* mutations described in INH-resistant clinical isolates, suggesting the mechanism of

inhibition by the adduct may be through disruption of *inhA* interaction with other components of the FAS II pathway (Rawat *et al.,* 2003). Mutations in the promoter region for *inhA* expression have also been described in INH-resistant clinical isolates, and several of these have been shown, using a gene fusion reporter construct, to confer expression levels from four to eight fold greater than wild-type sequences (Mdluli *et al*., 1996), thus demonstrating that up-regulation of *inhA* is also a resistance mechanism. Population-based surveys have found that *inhA* promoter mutations are the second most frequent INH resistance mutation after the Ser315Thr mutation in *katG*, and structural mutations in *inhA* are rare (Ramaswamy *et al.,* 2003; Gagneux *et al.,* 2006; Hazbon *et al.*,2006).

kasA

A second successful approach used to determine the targets of activated INH has been to examine the early adaptive response of *M. tuberculosis* following a challenge with INH. By examining two-dimensional gel electrophoretic protein profiles after treatment with INH; two up-regulated proteins: an acyl carrier protein (AcpM) and a covalent complex of AcpM and *kasA* (β-ketoacyl-ACP synthase) were identified (Mdluli *et al*., 1998). Labelling after treatment with (^{14}C), INH indicated covalent attachment of INH to this protein complex. These two proteins form part of the FAS type II system. Sequencing of *kasA* in clinical isolates identified several mutations restricted to INH-resistant strains, but these were rare and often occurred in tandem with other INH resistance-conferring mutations (Lee *et al.,* 1999; Piatek *et al*., 2000). These two studies suggest that *kasA* mutations are not clinically important, and recent functional studies have suggested *kasA* may only have a more peripheral role in INH action and resistance than originally proposed (Kremer *et al*., 2003; Argyrou *et al*., 2006; Vilcheze *et al.,* 2006).

2.6.11 Other INH resistance mechanisms

The research focus on *inhA* as the principal target for INH has distracted researchers from investigating more thoroughly the drug's interactions with other intracellular processes. INH is oxidized to a number of highly reactive species capable of either oxidizing or acylating macromolecules (Bertrand *et al.*, 2004; Pierattelli *et al.,* 2004), and can be expected to damage multiple cellular structures. Similar to the interaction with *inhA,* INH has also been shown to form an INH-NADP adduct that inhibits the *M. tuberculosis* dihydrofolate reductase (DHFR), an enzyme essential for nucleic acid synthesis. It is not yet clear whether there is a resistance mechanism to counter this inhibition, but the expression of *M. tuberculosis* DHFR in *M. smegmatis* can protect against growth inhibition caused by INH (Alland *et al*., 2000). Using affinity chromatography, the same group was able to demonstrate high-affinity binding of the INH-NAD/NADP adducts to 16 other proteins in addition to *inhA* and DHFR, broadening the range of putative INH targets still further.

One approach to identifying resistance genes has been to study the transcriptional response to a drug challenge. Using this approach, a three-gene operon *iniABC* (for INH inducible), which is up-regulated following treatment of *M. tuberculosis* with INH and EMB was identified (Alland *et al*., 2000; Colangeli *et al*., 2005). Over expression of one of these genes, *iniA*, in *M. bovis* BCG resulted in increased survival following an INH challenge, and a *M. tuberculosis* strain containing an *iniA* deletion showed increased susceptibility to INH. The INH-resistant phenotype was abolished by the pump inhibitor reserpine suggesting *iniA* could be involved in transport, compatible with its multimeric pore-like secondary structure. However, studies with radio-labelled drug did not show an effect on intracellular INH levels, indicating its mode of action was not through antibiotic efflux. There is more evidence that one of the resistance-nodulation-

cell division (RND) family of transporters encoded in the genome of *M. tuberculosis*, *mmpL7*, is involved directly in INH efflux, as its heterologous expression in *M. smegmatis* is reported to cause INH resistance (Colangeli *et al.,* 2005; De Rossi *et al*., 2006).

2.6.12 Compensatory mutations

Studies using model systems have shown that, although there is a range, most resistance-conferring mutations convey some cost to bacteria in terms of their fitness (Andersson and Levin, 1999). However, in some cases, these costs can at least be partially ameliorated by secondary or compensatory mutations that restore levels of fitness toward those of sensitive strains (Schrag and Perrot, 1996; Bjorkman *et al*., 1998; Maisnier-Patin *et al*., 2002). Given the diverse mechanisms of resistance in *M. tuberculosis*, it is likely that compensatory mutations will be important in maintaining the fitness of MDR and XDR strains. The only compensatory mutations described in *M. tuberculosis* are mutations in the *ahpC* promoter region of *M. tuberculosis*. Initial interest in this gene derived from the observation that an *oxyR* null mutant of *E. coli* (the wild type of this organism is highly resistant to INH) was susceptible to INH (Rosner, 1993) due to down-regulation of *ahpC-ahpF*. This led to characterization of the *oxyR-ahpC* locus from different mycobacterial species (Deretic *et al.,* 1995; Sherman *et al*., 1995). Members of the *M. tuberculosis* complex were found to possess an *oxyR* pseudogene in conjunction with a functional but feebly expressed *ahpC* gene. This led to speculation that the INH susceptibility of *M. tuberculosis* was due to low levels of *ahpC.* However, the over expression of *ahpC* in *M. tuberculosis* did not markedly affect INH sensitivity, but it did confer resistance to hydrogen and cumene peroxide (Sherman *et al*., 1995; Sherman *et al*., 1996). Subsequent analysis of INH resistant isolates identified mutations in the upstream region of *ahpC,* which were associated with enhanced promoter activity and up-regulation of *aphC*

(Sherman *et al*., 1995). These mutations occur almost exclusively in conjunction with *katG* deficiency (Gagneux *et al.,* 2006) and probably enable strains to compensate for the additional oxidative stress imposed by loss of catalase (Sherman *et al*., 1995; Heym *et al.,* 1997; Wilson *et al*., 1995; Wilson *et al*., 1998). There is now evidence that compensatory mutations may also be important in restoring the fitness of RIF-resistant strains, although the mutations have yet to be identified (Gagneux *et al.,* 2006).

2.6.13 Resistance to Pyrazinamide (PZA)

The introduction to the anti-tuberculous pharmacopeia of the nicotinamide analog, PZA, had far reaching consequences, since it enabled the duration of treatment to be shortened from more than a year to six months. Used during the initial intensive phase of short course chemotherapy, PZA, which is more potent at acidic pH, is believed to be particularly active on intracellular *M. tuberculosis*. For instance, the MIC of the drug for a strain grown *in vitro* at pH 7 is >250µg/mL, but can be reduced to 15µg/mL by lowering the pH to 5. It was initially thought that this potentiation effect could be explained by tubercle bacilli residing in acidified phagosomes that concentrated the drug (Mackaness, 1956). However, it was subsequently shown that the pH within these vesicles was neutral (Sturgill-Koszycki *et al.,* 1994). Considerable insight into PZA uptake and resistance mechanisms was provided recently in an elegant series of publications addressing the issues of the remarkable specificity of the drug for *M. tuberculosis* and its relationship with a broad spectrum amidase (Scorpio and Zhang, 1996; Scorpio *et al.,* 1997a; Scorpio *et al.,* 1997; Sreevatsan *et al.,* 1997a; Sun *et al.,* 1997; Sun and Zhang, 1999; Zhang *et al*., 1999).

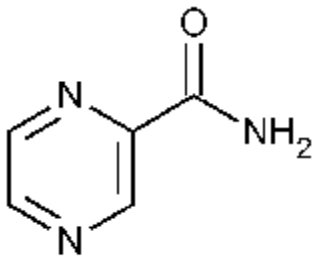


Figure 2. 5: Chemical structure of Pyrazinamide

PZA resistance has long been associated with the loss of activity of pyrazinamidase (PZase), a cytosolic enzyme of 186 amino acids that hydrolyses both PZA and nicotinamide, and which may play a role in pyridine nucleotide metabolism. In a study, Scorpio and Zhang cloned the *pncA* gene encoding PZase from *M. tuberculosis* and demonstrated restoration of drug susceptibility upon its expression in the naturally resistant organism BCG (Scorpio and Zhang, 1996). On further examination, *pncA* mutations that lowered or abolished PZase activity were found in PZA-resistant isolates of *M. tuberculosis* and also in *M. bovis* and BCG (Scorpio *et al.,* 1997). Subsequently, other workers surveyed their strain collections for altered *pncA* genes and found a near perfect association between the presence of mutant alleles and PZA resistance (Sreevatsan *et al.,* 1997a; Hirano *et al.,* 1998; Lemaitre *et al.,* 1999; Marttila *et al.,* 1999). Indeed, detection of resistance by molecular techniques is now preferable to susceptibility testing by microbiological methods, as these are notoriously error prone due to the pH effects discussed above.

Although many mycobacteria contain *pncA* genes and elicit PZase, PZA is most active against *M. tuberculosis*, and its close relatives *M. africanum* and *M. microti*. The natural PZA resistance of *M. bovis,* the other member of the *M. tuberculosis* complex and its descendants stems from the amino acid substitution His57Asp in the *PncA* protein. The *M. tuberculosis* PZase shows 69.9%

and 67.7% identity to those of *Mycobacterium kansasii* and *M. avium*, respectively, and 35.5% identity with the nicotinamidase of *E. coli*. Both of these mycobacterial PZases restored drug susceptibility to levels similar to those conferred by the *M. tuberculosis* enzyme when expressed in a resistant host, such as BCG, although the *M. tuberculosis* protein probably has higher nicotinamidase and PZase activities (Sun *et al.*, 1997; Sun and Zhang. 1999). Nevertheless, both *M. kansasii* and *M. avium* are naturally resistant to the drug and the likely reason for this will be explained below. *M. smegmatis* also produces a highly active PZase, *pza*A, with an apparent molecular weight of 50 kDa. It is quite distinct from the other enzymes, yet hydrolyzes both PZA and nicotinamide. *pza*A probably corresponds to a broad-spectrum amidase capable of hydrolyzing a wide range of substrates (Boshoff and Mizrahi, 1998). Furthermore, *M. smegmatis* also contains a *pncA* homolog. The potential contribution of other amidases to PZA and nicotinamide metabolism has been recognized and discussed by others (Raynaud *et al.*, 1999).

The mutations present in *pncA* from a large number of drug-resistant isolates of *M. tuberculosis* are known. The majority of these (69%) correspond to missense mutations, although frameshifts, insertions, deletions, and nonsense mutations (31%) also occur (Sreevatsan *et al.*, 1997a; Hirano *et al.*, 1998; Lemaitre *et al.*, 1999; Marttila *et al.*, 1999). The higher frequency of missense mutants suggests that the corresponding proteins may retain some residual activity that could confer a competitive advantage. It is of some interest to examine the distribution of the amino acid substitutions resulting from missense mutations. These occur throughout the *pncA* protein, but are generally located in positions that are conserved in all four enzymes. This suggests that these amino acid residues play critical roles in substrate binding and catalysis, but confirmation by biochemical characterization of well defined PZase variants is now required.

The toxicity of PZA results from its conversion to pyrazinoic acid (Konno *et al.*, 1967) and the PZase enzyme from resistant organisms, such as BCG, is unable to catalyze this reaction, which occurs at alkaline, neutral, and acidic pH. However, pyrazinoic acid only accumulates in susceptible tubercle bacilli when the external pH is acidic. In naturally resistant mycobacteria such as *M. smegmatis* or *Mycobacterium vaccae*, efficient conversion of PZA occurs, but pyrazinoic acid is rapidly excreted by a highly active efflux system that can be inhibited by reserpine or valinomycin (Zhang *et al*., 1999).

M. tuberculosis also appears to have a pyrazinoic acid efflux system, but as this is many orders of magnitude less efficient, copious amounts of the acid build up in the cytoplasm. The natural PZA resistance of *M. kansasii* is attributable to the much lower activity of its PZase rather than to efflux mechanisms, since the introduction of the *M. avium* gene into *M. kansasii* results in highly increased PZA susceptibility and pH-dependent pyrazinoic acid accumulation (Sun and Zhang 1999). By contrast, in *M. avium*, which shows lower levels of PZA resistance than *M. kansasii*, an efflux mechanism has been reported whose efficiency is intermediate between those of *M. smegmatis* and *M. tuberculosis* (Sun and Zhang, 1999).

Prior to its interaction with PZase, PZA must cross the cell wall and enter the cytoplasm. It was demonstrated that the radio labelled drug diffused passively through the outer envelope of *M. tuberculosis* and was then actively transported by an ATP-dependent uptake system. This transporter, which was inhibited by arsenate (albeit at very high concentrations) appears to transport nicotinamide (Raynaud *et al.*, 1999). Similar transport systems were also detected in *M. avium* and *M. kansasii*, but not in *M. bovis* BCG. The latter observation was also made by Zhang *et al.*, who found that the drug did not accumulate in PZA-resistant strains belonging to the *M.*

tuberculosis complex (Zhang *et al*., 1999). However, upon introduction of a functional *pncA* gene, PZA uptake and pyrazinoic acid production were restored. This could suggest that the transport system is only expressed when PZase is present. Similar observations regarding nicotinamide uptake and the presence of nicotinamidase have been made in *E. coli* mutants lacking the amidase, and might reflect the existence of regulatory pathways for pyridine nucleotide metabolism (McLaren *et al*., 1973). Alternatively, it is conceivable that PZA also diffuses across the cytoplasmic membrane of mycobacteria in a passive manner and is then converted to pyrazinoic acid by PZase, which is trapped in the cell at low pH in *M. tuberculosis* but excreted by the naturally resistant species. The inhibition of PZA uptake observed in the presence of nicotinamide may simply reflect the fact that as nicotinamide is the preferred substrate, PZase hydrolyses the drug at much lower levels. To summarize, three components appear to be involved in mediating PZA susceptibility in pathogenic mycobacteria: the putative uptake system, PZase, and an efflux pump. The relative contributions of these three factors determine the level of drug susceptibility. Clinically relevant mutations have been described in the *M. tuberculosis* complex that results in loss of PZase activity and concomitantly the absence of pyrazinoic acid. No genes or mutations affecting the PZA transporter (if this exists) or the efflux system have been reported in tubercle bacilli. Over expression of the putative efflux system would lead to increased excretion of pyrazinoic acid. Such mutants might exist and could explain the resistance observed in a small number of clinical isolates with wild-type *pncA* genes.

Since pyrazinoic acid is the active agent, it was logical to test this compound directly, but its bactericidal activity for tubercle bacilli in infected mice was found to be much lower than that of the acid generated endogenously from PZA (Konno *et al.*, 1967). To identify the target for the bioactive form of PZA, attempts were made at isolating laboratory mutants of *M. tuberculosis*

that show increased resistance to pyrazinoic acid. These have been repeatedly unsuccessful (Scorpio *et al.,* 1997). Moreover, in well characterized clinical isolates that display reproducible PZA resistance most strains harboured defective *pncA* genes (Scorpio *et al.,* 1997). These observations suggest that the drug target must be an essential enzyme and current thinking is centered on fatty acid synthase I.

2.6.14 Resistance to Ethambutol (EMB)

Since its introduction in the early 1960s, EMB was thought to act on the mycobacterial envelope, as treatment perturbed the biogenesis of several cell wall components. Through biochemical studies, performed mainly with *M. smegmatis*, it was found that the primary site of action was arabinan synthesis (Takayama and Kilburn, 1989) which, in turn, impacts on arabinogalactan and lipoarabinomannan production (Khoo *et al.,* 1996). It is now clear from work with EMB-resistant mutants and molecular genetics that the main drug target is the arabinosyl transferase(s) involved in the polymerization of arabinan. Using complementary approaches with *M. tuberculosis* (Telenti *et al.,* 1997) and *M. avium* (Belanger *et al.,* 1996), these enzymes were shown to be encoded by linked genes that have evolved by a gene duplication mechanism, controlled by the regulatory gene *embR*. In *M. avium*, *embR* is transcribed divergently from the adjacent *embAB* genes, whereas in *M. tuberculosis*, the *embCAB* operon is situated 1.87 Mb distal to *embR*; *embR* regulation is dependent on phosphorylation by PknH (Belanger *et al.,* 1996).

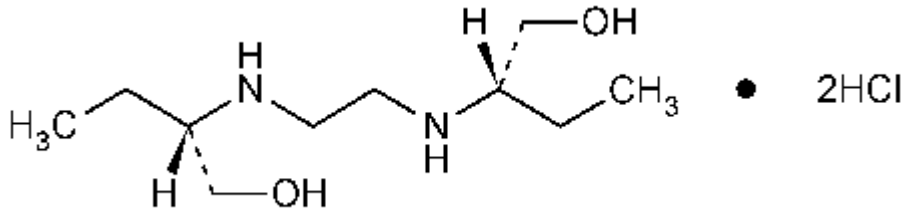


Figure 2. 6: Chemical structure of Ethambutol

The arabinosyltransferases are membrane-bound enzymes (Belanger *et al.,* 1996) with 12 predicted membrane spanning segments and a large extracytoplasmic domain at the COOH-terminus. Missense mutations located in a tetrapeptide at positions 303 to 306 of a putative cytoplasmic loop of e*mbB* have been shown to be responsible for acquired drug resistance in the majority of clinical isolates of *M. tuberculosis* and in laboratory mutants of *M. smegmatis* (Lety *et al.,* 1997; Sreevatsan *et al.,* 1997; Plinke *et al.,* 2006). Mutations affecting Met306 are predominant, and replacement by Leu or Val is associated with higher resistance levels (40 µg/mL) than the substitution Met306Ile (Sreevatsan *et al.,* 1997). High-level resistance has also been described for *M. tuberculosis* strains harbouring *embB* genes with Thr630Ile or Phe330Val mutations although causality has not yet been demonstrated. The natural EMB resistance of a variety of non-tuberculous mycobacteria, such as *M. leprae* , *M. fortuitum*, and *Mycobacterium abscessus*, results from the presence of one or more alterations to the otherwise well-conserved motif at positions 303 to 306 (Alcaide *et al.,* 1997). Yet again, as in the case of INH and PZA, serendipity has played a major role in the susceptibility of the tubercle bacillus to a frontline drug. In roughly 70% of EMB-resistant clinical isolates of *M. tuberculosis*, drug resistance can be explained by alterations to *embB*. However, nothing is known of the mechanism operational in the remaining 30% and these generally display lower levels of resistance (15 µg/mL) (Sreevatsan *et al.,* 1997). It has been proposed that high-level resistance results from a stepwise mutational process in *M. smegmatis* although other workers provide evidence in favour of a single event (Lety *et al.,* 1997). There is a clear indication from heterologous expression of *emb* genes in this host that increased gene dosage confers higher resistance levels (Telenti *et al.,* 1997) and it is conceivable that unlinked mutations leading to over expression of the *M. tuberculosis embCAB* operon may occur in clinical isolates displaying low-level EMB resistance.

A strong candidate locus is *embR*, which is required for transcription of the operon (Belanger *et al.,* 1996) although increased efflux of the drug cannot be excluded.

2.6.15 Resistance to Fluoroquinolones

The discovery that the broad-spectrum bactericidal activity of fluoroquinolones extended to mycobacteria led to their rapid clinical deployment (Tsukamura, 1985; Tsukamura *et al.,* 1985). They have now been used extensively and have established themselves as a key element of therapy for cases of MDR-TB (Iseman, 1993; Iseman, 1999). Their tolerability and oral route of administration make them particularly useful drugs. Most clinical experience has been with ofloxacin and ciprofloxacin, but recently newer fluoroquinolones with lower MICs have become available, of which moxifloxacin and gatifloxacin are currently the most promising. Moxifloxacin has been the most extensively studied in animal models of tuberculosis therapy, and in combination with PZA and RIF has been shown to sterilize mouse lungs more rapidly than conventional anti-tuberculosis regimens (Nuermberger *et al.,* 2004a; Nuermberger *et al.,* 2004b). This has raised the possibility that including a potent fluoroquinolone in first-line treatment could shorten the duration of therapy from six months, and phase III clinical trials are under way to investigate this possibility. Fluoroquinolones target bacterial topoisomerases, so it was expected that mutations in DNA gyrase, a type II DNA topoisomerase, would be the principal mechanism of resistance, as a type IV topoisomerase was not identified in the genome of *M. tuberculosis* (Camus *et al.,* 2002). DNA gyrase is made up of two A and two B subunits encoded by *gyrA* and *gyrB*, and catalyses negative super coiling of DNA (Wang, 1985). Sequencing of the equivalent of the *E. coli* quinolone resistance-determining region (QRDR) of *gyrA* (Yoshida *et al.,* 1990) in fluoroquinolone-resistant clinical isolates showed that a number of distinct amino acid substitutions are associated with resistance and those at codon 94 and 90

occur most frequently (Cambau *et al.*, 1994; Takiff *et al.*, 1994; Alangaden *et al.*, 1995; Xu *et al.*, 1996). Functional analysis of mutant gyrase complexes has shown that fluoroquinolone inhibition of DNA super coiling is reduced by the missense mutations Ala90Val, Ala94Gly, and Ala94His (Aubry *et al*., 2006; Matrat *et al.*, 2006), with larger effects seen with a double mutant. This mirrors the observation that double *gyrA* mutations in clinical isolates are associated with the highest MICs. This study also characterized a *gyrB* mutant confirming that substitutions at this site, which are only rarely encountered clinically (Pitaksajjakul *et al.*, 2005; Aubry *et al*., 2006), can also cause resistance.

There are other pathways to resistance independent of the mutations in the QRDR of *gyrA* and *gyrB*, as fluoroquinolone-resistant clinical isolates that are wild type at these loci have been identified (Sullivan *et al.*, 1995; Xu *et al.*, 1996; Aubry *et al*., 2006). Attention has focused on characterizing the active efflux systems of *M. tuberculosis* because of their proven role in conferring fluoroquinolone resistance in other organisms. *Mycobacterium tuberculosis* possesses representatives of all of the main classes of efflux systems (De Rossi *et al.*, 2002; De Rossi *et al*., 2006), and members of the ATP-binding cassette (ABC) super family and the MFS have been shown to transport fluoroquinolones in mycobacteria (Takiff *et al.*, 1996; Pasca *et al.*, 2004). LfrA, an MFS member identified in the non-pathogenic *M. smegmatis*, can extrude quinolones (Sander *et al.*, 2000; Buroni *et al.*, 2006). However, none of these transporters has yet been implicated in clinical resistance.

Recently, work has suggested that efflux pumps and structural changes to DNA gyrase may not be the only ways *M. tuberculosis* handles the toxicity of fluoroquinolones. Using a genetic screen for fluoroquinolone resistance, Takiff and colleagues were able to identify a gene, *mfpA* that

resulted in low-level resistance when over expressed in *M. smegmatis* (Montero *et al.,* 2001). *M. tuberculosis* contains a 183-amino acid homolog of *mfpA* that encodes a member of the pentapeptide repeat family of proteins (Vetting *et al.,* 2006) in which every fifth residue is a leucine or phenylalanine. Structural characterization found that *mfp*A forms a rod-shaped dimer similar electrostatically and in size to B-form DNA, suggesting the binding and inhibition of DNA gyrase by *mfp*A was through DNA mimicry (Hegde *et al.,* 2005). This would also explain how up-regulation of *mfp*A can block the bactericidal activity of fluoroquinolones, which is dependent on binding irreversibly to the gyrase-DNA complex.

2.6.16 Resistance to New drugs

The scale of the global tuberculosis problem and the continuing expansion of drug resistant strains of *M. tuberculosis* have finally galvanized the search for new antituberculosis drugs. These drug discovery efforts have already produced several new compounds that have entered clinical trials. However, even though these agents have novel modes of action, they are not immune from drug resistance.

R207910 is a diarylquinoline (DARC) with potent antimycobacterial activity that was identified by screening prototypes of different chemical series for growth inhibition of *M. smegmatis* (Andries *et al.,* 2005). It has a low MIC against *M. tuberculosis*, is active against MDR strains including DNA gyrase mutants, and in combination with selected first-line tuberculosis drugs can sterilize lungs of *M. tuberculosis*-infected mice more rapidly that standard regimens, making it an agent with the potential to shorten the duration of chemotherapy (Lounis *et al.,* 2006; Ibrahim *et al.,* 2007). Its mode of action and resistance conferring mutations were ingeniously elucidated by comparing the near-complete genome sequences of R207910-resistant *M.*

tuberculosis and *M. smegmatis* strains selected *in vitro* with their wild-type progenitors. This approach identified single point mutations in *atpE*, which encodes part of the F_0 subunit of ATP synthase. The mutations Asp32Val in *M. smegmatis* and Ala63Pro in *M. tuberculosis* were both located in the membrane spanning domain of the protein and could confer resistance to R207910 when transformed on appropriate constructs into wild-type organisms. Further work has established that the region involved in resistance is conserved across a wide range of mycobacteria except for *M. xenopi*, which unlike most mycobacteria, is naturally resistant to R207910 and has a Met63 genotype rather than the highly conserved Ala63 (Petrella *et al.*, 2006).

The observation that nitroimidazoles, such as metronidazole, have some antimycobacterial toxicity, prompted researchers to identify related compounds with enhanced bactericidal activity against *M. tuberculosis*. Two of these compounds, OPC-67683 (a nitro-dihydro-imidazole) (Matsumoto *et al.*, 2006) and PA-824 (a nitroimidazole-oxazine), (Stover *et al.*, 2000) have already advanced into clinical trials. To date, only the resistance mechanisms of PA-824 have been studied extensively. The intracellular targets have not yet been identified, but PA-824 inhibits protein and cell wall synthesis after reductive activation, which requires a F_{420}-dependent glucose-6-phosphate dehydrogenase (FGD1) encoded by Rv0407. Therefore, it was not surprising that mutations in FGD1 and the genes required for synthesis of cofactor F_{420} (*fbiA*, *fbiB*, and *fbiC*) result in resistance to PA-824 (Choi *et al.*, 2001; Choi *et al.*, 2002). However, a proportion of PA-824-resistant strains can produce FGD1 and F_{420} but are still unable to activate the drug. Barry and colleagues sequenced the genome of these strains and identified mutations in an additional gene (Rv3547), which they propose encodes an essential part of the F_{420}-dependent nitroreductase (Manjunatha *et al.*, 2006). The involvement of at least five genes in PA-824

activation means that the *in vitro* selection rate for resistant mutants is relatively high (Manjunatha *et al*., 2006), and it will be important to determine if resistance to PA-824 emerges rapidly during therapy.

2.7 TB drug susceptibility testing

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 (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 *M. 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. Results of direct testing are much more rapid and help to triage MDR from non MDR-TB patients promptly.

2.7.1 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).

2.7.2 Proportion method (PM)

With this method, an inoculum of 1.0 MacFarland standard of *M. tuberculosis* is prepared. The 1.0 MacFarland standardised suspension was further diluted to 10^{-1}, 10^{-2} and 10^{-3}. The 10^{-1} suspension was subsequently inoculated on the drug-containing medium. Three drug-free LJ slopes were inoculated with 1:10, 1:100 and 1:1000 diluted suspensions of the 1.0 MacFarland standardised inoculum. On 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} 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 (≤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 (PM) can be performed on LJ or Middlebrook agar medium (Makinen *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 (Wanger and Mills, 1994). With this method, estimation of the inoculum size from the 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.

2.7.3 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). Table 1 shows the CC for first line

anti TB drugs for the different tests. If resistant mutants exceed 1%, the CC may not inhibit growth, and this predicts therapeutic failure.

Table 1: Critical concentrations of first line anti TB drugs

Critical concentration µg/ml					
	Indirect LJPM	**Middlebrook agar**		**BACTEC460**	**MGIT 960**
		7H10	**7H11**		
First line drugs					
Isoniazid	0.2	0.2	0.2	0.1	0.1
Rifampicin	40.0	1.0	1.0	2.0	1.0
Ethambutol	2. 0	5.0	7.5	2.5	5.0
Pyrazinamide	NR	NR	NR	100.0	100.0
Streptomycin	4.0	2.0	2.0	2.0	1.0

NR: Not recommended (Christopher, 2012).

2.7.4 The 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 (IUATLD, 1998).

2.7.5 The 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 (Heifets, 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 resource limited settings (RLSs), the DST process is very slow (2-3 months), necessitating the need for more rapid assays.

2.7.6 Rapid Susceptibility Tests

The rapid susceptibility tests include solid media culture-based techniques such as Nitrate Reductase Assay (NRA) (Angeby *et al*., 2002), E test (AB Bio Disk Solna, Sweden) (Wanger and Mills, 1994; Joloba *et al*., 2000), and Phage-based susceptibility tests (Biotec Laboratories Ltd., Ipswich, UK, (Albert *et al*., 2004; Simboli *et al.*, 2005); In-house liquid media culture-based tests such as the microscopic observation drug susceptibility (MODS) assay (Moore *et al*., 2006), Alamar blue (da Silva *et al*., 2006), the MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Mengatto *et al*., 2006), and resazurin assays (Montoro *et al*.,2005); commercial liquid media culture-based tests such as the BACTEC 460 radiometric system (Becton Dickinson, Sparks, Maryland), *Mycobacterium* Growth Indicator Tube MGIT (Becton Dickinson, Sparks, Maryland), and MB BacT/Alert system (bioMe´rieux, Marcy l'Etoile, France) (Woods, 2000; Piersimoni *et al*.,2006); and Molecular tests (Line Probe Assays) such as the INNO-LiPA Rif TB Assay (Innogenetics, Ghent, Belgium) (Jureen *et al*., 2004) and the Genotype® MTBDR and its newer version; the Genotype® MTBDR*plus* (HainLife sciences, Nehren, Germany) (HainLifescience, 2009).

2.7.7 Solid Media Culture-Based Tests

2.7.8 Nitrate reductase assay (NRA)

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

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

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, 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 colour. 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 non coloured 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 resource limited settings (RLSs) in Africa is limited (Martin *et al*., 2008).

2.7.9 E test susceptibility testing

This method uses plastic strips that contain exponential gradients of antibiotics for susceptibility testing of mycobacteria (AB BIODISK, Solna, Sweden). The antibiotic diffuses into the medium and thereby inhibiting growth of susceptible strains. The minimum inhibitory concentration (MIC) is read and the isolate interpreted as resistant or susceptible. Initial studies of the E test

showed high accuracy estimates of close to 100% when compared with the conventional agar proportion and BACTEC radiometric tests (Wanger and Mills, 1994; Joloba *et al*., 2000). However, it is now known that the diffused antibiotics degrade fast amidst the slowly growing mycobacteria resulting in a blurred cut off point for MIC reading. The other disadvantage of the E test is the need for a heavy inoculum i.e. no. 3 MacFarland (MF) equivalent, which may not be achievable with direct DST on sputum sediments, but which also poses a major risk of aerosol generation and inhalation by the staff in the safety level 2 laboratories of developing countries. The E test antibiotic strips are also very expensive (up to USD 30 per strip), which may not be affordable in RLSs.

2.7.10 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, 2001; Simboli *et al.,* 2005). Both the commercially available FastPlaque 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 has shown high sensitivity and specificity (Traore *et al*., 2007). However, the phage assay can be technically complex, labour intensive, and can have high failure rates with inability to have interpretable results.

2.7.11 In house liquid media culture based tests

2.7.12 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*., 2006): first, *M. 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. 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*., 2006). 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 re opened 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.

2.7.13 Alamar blue, Resazurin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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 dehdrogenase enzymes from viable mycobacterial cells to cleave the tetrazolium rings of the pale yellow MTT, resulting in formation of violet purple 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 micro titre 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.

2.7.14 Commercial liquid media culture-based tests

2.7.15 BACTEC 460

The BACTEC 460 (Becton Dickinson, Sparks, Maryland) relies on radiometric detection of $^{14}CO_2$ as an indicator of bacterial growth. The BACTEC vials contain Middlebrook 7H12 medium and fatty acid substrates labelled with ^{14}C. Growing mycobacteria release $^{14}CO_2$ as a metabolic end product. The gas is removed, analyzed and the amount of radioactive ^{14}C is expressed as a numerical value called the Growth Index (GI). When the GI value in the control vial reaches 30, interpretation of drug tube begins on the next day as follows: susceptible, change in GI in the control vial > GI in drug vial; resistant, change in GI in control vial < GI in drug vial and border line, GI in control vial = GI in drug vial. This test is highly sensitive and specific but it uses radioactive carbon whose half life is 5,000 years, which makes it difficult and expensive to dispose.

2.7.16 Mycobacterial 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 (Reisner *et al.*, 1995; Rusch-Gerdes *et al.*, 1999). 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.*, 1995). 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.,* 1997; Idigoras *et al*., 2000; 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, 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 is limited.

2.7.17 The BacT/ALERT ® 3D System

The BacT/ALERT ® 3D System (bioMe´rieux, Marcy Etoile France) is a liquid based automated assay performed in a tube with a liquid emulsion sensor. Growing bacteria produce CO_2, which reacts with the sensor, resulting is a colour change from gray to a lighter colour, detected colorimetrically as growth. Growth in a drug tube indicates resistance. The BacT/ALERT ® 3D System is slightly slow compared with the MGIT 960 (Murray *et al.,* 2007).

2.7.18 Molecular Assays

Molecular methods for MDR-TB detect the common mutations conferring resistance to RIF and INH, 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 smear positive sputum (De Beenhouwer *et al.,* 1995; Rossau *et al.,* 1997).

The INNO-LiPA Rif TB Assay (Innogenetics, Ghent, Belgium) detects the common mutations in only the *rpoβ* 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) simultaneously detects the common mutations in the *rpoβ* 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 *rpoβ* 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 *et al.,* 2008).

Most of these studies were performed in developed countries and there was limited data on the performance of the tests in developing countries (Ling *et al.,* 2008). A major limitation of these assays in developing countries could be the expertise in molecular biology required to perform them correctly, the unidirectional work flow laboratory infrastructure and the cost of molecular assays.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Ethical approval

Ethical approvals were obtained from the ethical committees of the Kaduna State Ministry of Health, Ahmadu Bello University Teaching Hospital, Zaria and National Tuberculosis and Leprosy Training Centre, Saye, Zaria, Nigeria. Consent was obtained from patients, parents or caregivers as the case may be.

3.2 Study design

The study was a descriptive cross sectional survey and was hospital-based.

3.3 Study area

The study was conducted in Kaduna State located in the Northwest Geopolitical Zone of Nigeria. The State lies between latitudes 6° and 11° North and longitude 7° and 44° East and is 608 meters above sea level. It is divided into three Senatorial districts. Kaduna North comprising Kubau, Ikara, Makarfi, Soba, Sabon Gari, Zaria, Lere, Kudan LGs; Kaduna Central with Birnin Gwari, Giwa, Igabi, Kaduna North, Kaduna South, Chikun, Kajuru LGs and Kaduna South comprising Jema'a, Jaba, Kaura, Zangon Kataf, Kaura, Kachia, Kagarko, Sanga LGs. It has distinct wet and dry seasons and is within the Northern Guinea Savannah zone and part of the Sahel Savannah zone of Nigeria. The State shares geographical boundaries with Katsina and Zamfara States to the North, Plateau and Bauchi States to the East, Nasarawa State and the Federal Capital Territory to the South, Niger State to the West, and Kano State to the Northeast. Kaduna State occupies about 48,473.25 sq km, with a human population of over 6,066,562 people according to the census figures of 2006 (Kaduna State Government, 2008).



Figure 3. 1: Map of Kaduna State showing the Senatorial Districts (Nigerian Chamber of Commerce USA, 2014)

3.4 Sample Size

Owing to the difficulty in finding record of any research on the prevalence of MDR-TB in the study area, or any other part of Nigeria, a prevalence of 50% was used to calculate the sample size using the equation:

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

Where:

n = sample size

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

P = Prevalence (50%) (Bland, 1999)

L = allowable error (0.005)

Q = 1-P

Therefore, $n = \frac{(1.96)^2 \times 0.05 \times 0.05}{(0.005)^2} = 384$

The minimum sample size calculated was 384 which was however increased to 1186. This was to avoid attrition, to obtain enough smear positive samples and also due to the population size of the study area.

3.5 Inclusion and exclusion criteria

All AFB positive sputa from consented patients at the hospitals were included, while AFB negative sputum samples and samples from those who did not consent were excluded.

3.6 Sample collection

Stratified sampling technique was used. A total of 1186 sputum samples were collected from patients attending clinics of the selected hospitals (Ahmadu Bello University Teaching Hospital, Zaria, National Tuberculosis and Leprosy Training Centre, Saye, Zaria, Barau Dikko Spacialist, Kaduna, General Hospital Kafanchan) in screw capped, leak proof specimen containers. Demographic and risk information (age, sex, HIV status, TB history, previous treatment, alcoholism, smoking, history of diabetes, history of asthma) were obtained through questionnaires (Appendix X). Samples were transported to mycobacteriology laboratory at the National Tuberculosis and Leprosy Training Centre, Zaria, Nigeria, in insulated cold box with ice packs and analyzed within 24 hours of collection.

3.7 Analyses of samples

3.7.1 Ziehl-Neelsen staining and microscopy

Smears were made by fishing out a loopful of yellowish particle of sputum and spread evenly on the middle of a clean labeled glass slide (approximately 132 cm - 233 cm). The smears were allowed to dry completely at room temperature and fixed by passing over the flame 3 times for about five seconds. The heat-fixed smears were allowed to cool and stained with Ziehl's (1% carbol fuchsin) solution and heated from beneath by spirit lamp until steam came off from the stain. The stain was allowed to act on the smear for 5 minutes and the procedure was repeated 2-3 times. The slides were tilted to remove excess stain and washed gently with stream of running tap water followed by decolorization with 3% hydrochloric acid-ethanol (3% HCl-ethanol) until the solution runs clear. The slides were rinsed with water and tilted to drain off excess water and then counterstained with 0.1% methylene blue for 2-3 seconds. The stain was poured off and the slides washed with gentle stream of running tap water, placed on slide rack. The slides were

dried in the air and examined under the microscope using the oil immersion objectives. Pink coloured rods against blue coloured background were considered positive for Acid Fast Bacilli (AFB) (Fujiki, 2001).

3.7.2 Media preparation

Lowenstein Jensen (LJ) medium base was prepared according to manufacturer's instructions. The culture medium was prepared by weighing 18.65g of the LJ medium base and dissolving in 300 ml of distilled water. Six (6) ml of glycerol (reagent grade) was added. The solution was autoclaved at 121^{o}C for 30 minutes and cooled. Five hundred ml of homogenized whole egg was added and mixed. Then, 6 ml of the medium was dispensed into a 20 x 150 mm screw capped tubes. The culture tubes were inspissated at 85^{o}C for 50 minutes. For sterility checking, prepared culture media were incubated at 37^{o}C for 48 hours and kept in the refrigerator for storage when no bacterial contaminants were detected. All tubes were tightly capped to prevent evaporation during storage (Concepcion *et al*., 2001).

3.7.3 Specimen Digestion and Decontamination

Acid fast bacilli (AFB) positive specimens were processed for isolation of mycobacteria following standard procedures for homogenization, suspension, centrifugation and decontamination. The samples underwent digestion by means of the N-acetyl-L-cysteine and sodium hydroxide was used in order to decontaminate the samples before culturing for TB. Equal volumes of specimen and decontamination reagent (1N NaOH, 0.1N sodium citrate, N-acetyl-L-cysteine) were added to a 50 ml plastic centrifuge tube vortex homogenized and allowed to stand for 15 minutes at room temperature (20^{o}C-25^{o}C). The tube was filled with phosphate buffer (pH 6.8). The mixture was then centrifuged (3000 x g, 15 min) at 4^{o}C, the

supernatant discarded and the pellet re-suspended in 2 ml of phosphate buffer (Concepcion *et al*., 2001).

3.7.4 Isolation

An inoculum of 0.1 ml of the sediment was inoculated into tubes of Lowenstein Jensen (LJ) medium and incubated aerobically at 37^{o}C. The tubes were observed daily for the first week of incubation and weekly thereafter until eight weeks. Caps were opened once a week for a short interval to aerate the cultures and to examine bottles for positive growth. Cultures showing evidence of growth at any time during this period were examined for morphology by making a smear and staining by the Ziehl-Neelsen procedure. All positive cultures were stored at -20 °C for later use (Martin *et al*., 1975; Concepcion *et al*., 2001; Srivastava *et al*., 2008; Gambo *et al*., 2013a).

3.7.5 Characterization

SD BIOLINE TB Ag MPT64 Rapid is a rapid immunochromatographic identification test for the *M. tuberculosis* complex (MTBC) that uses mouse monoclonal anti-MPT64. The kit has sensitivity and specificity of 98.6% and 100% respectively. The test cassette consists of a sample pad, a gold conjugate pad, a nitrocellulose membrane and an absorbent pad. Mouse monoclonal anti-MPT64 was immobilized on the nitrocellulose membrane as the capture material (test line). Another antibody which recognized another epitope of MPT64, conjugate with colloidal gold particles was used for antigen capture and detection in a sandwich type assay.

Four colonies were suspended in 200 µl of the extraction buffer prior to the test. The cassette was removed from the foil pouch and placed on a flat dry surface. One hundred micro litres (100 µl) of the suspended colonies in buffer was added into the sample well. As the test began to

work, a purple colour moved across the result window in the centre of the device. After 15 minutes of sample application, the appearance of two colour bands ("T" test band and "C" control band) within the result window was considered a positive result. Confirmed MTBC isolates were stored at -20^{o}C for further use.

3.7.6 Drug susceptibility testing

Susceptibility to isoniazid (INH), rifampicin (RIF), streptomycin (SM) and ethambutol (EMB) was determined by the proportion method on Lowenstein Jensen egg based slopes containing different concentrations of INH, RIF, SM and EMB (0.2 μg/ml, 40 μg/ml, 4.0 μg/ml and 2.0 μg/ml respectively) (Canetti *et al*., 1969). Standard antibiotic powders (INH, RIF, SM and EMB) were obtained from Sigma-Aldrich (Lot. No. SLBC 3024V; SLBD 2314V; BCBK 3385V; SLBF 2556V respectively).

The inoculum was prepared by directly suspending colonies grown for approximately three weeks on Lowenstein Jensen drug free slopes to a turbidity equivalent to a 1.0 MacFarland standard. The 1.0 MacFarland standardised suspension was further diluted to 10^{-1}, 10^{-2} and 10^{-3}. The 10^{-1} suspension was subsequently inoculated on the drug-containing medium. Three drug-free LJ slopes were inoculated with 1:10, 1:100 and 1:1000 diluted suspensions of a 1.0 MacFarland standardised inoculum. This was done for each sample tested. The drug-susceptible MTB reference strain ATCC 27294 (H37Rv) was used as a susceptible control and known resistant strains (ATCC 35825 H37Rv for INH and SM, ATCC35838 H37Rv for RIF, ATCC 35837 H37Rv for EMB) were used as resistant controls. The slopes were incubated at 37°C and read after 4 and 6 weeks. An isolate was considered resistant if the proportion of bacilli resistant to the critical concentration of a drug exceeded 1% (Canetti *et al*., 1963, Canetti *et al*., 1969).

3.7.7 Genotypic assays

Genotypic assays were carried out by the GenoType® MTBDR*plus* and Genotype MTBC 96 molecular line probe assay according to the manufacturer's specifications.

3.7.8 DNA extraction

Sterile plastic loops (one per sample) were used to suspend colonies from the LJ slants into 2ml micro centrifuge tubes containing 100 µl molecular grade water and centrifuged at 10,000xg for 15 min. The supernatant was discarded and the pellet re-suspended in molecular grade water, to ensure that the suspension of extracted DNA was free of impurities that might inhibit the PCR. The re-suspended solutions were vortexed until they appeared slightly opaque (milky). The tubes were arranged in floating racks, placed in water bath and heated at 95 ^{o}C for 20 min. This was to kill the bacilli and partially lyse the cells, thereby rendering the solution non infectious and to obtained a higher yield of DNA.

The tubes were placed in ultrasonic bath and incubated for 15 min. The powerful ultrasonic shockwaves created by the sonicator disrupt the cell walls of the tubercle bacilli, causing further cell lyses, and releasing the DNA and other cell debris into the molecular grade water. Finally, the tubes were centrifuged at maximum speed (10,000xg) to separate the impure cell debris (containing the cell wall, proteins and other macromolecules) from the DNA. The heavier debris formed the pellet and the lighter DNA (free from impurities) was suspended in the supernatant. The supernatants were transferred into clean labeled micro centrifuge tubes for further use.

3.7.9 PCR amplification of the extracted DNA

The master mix was prepared in clean DNA-free room. The master mix was made of five components with a total volume of 45µl for each PCR reaction (35µl of the primer nucleotide

mix (PNM), 5µl of buffer, 2µl of $MgCl_2$, 3µl of molecular grade H_2O and 0.2 µl of *Taq* polymerase). The master mix was then well mixed and 45µl required for each specimen was transferred into the PCR tubes and 5µl of each sample was added to the corresponding tube containing 45 µl of master mix and then mixed by gently pipetting up and down a few times. The tubes were mixed slightly and spun down for 5 - 10 seconds in a mini-centrifuge before they were placed into the thermal cycler for amplification. A 30 cycle (10 + 20) thermal cycler program was used for the amplification. This involved ten cycles of denaturation at 95 ^{o}C for 30 seconds and elongation at 58 ^{o}C for 120 seconds; followed by an additional 20 cycles of denaturation at 95 ^{o}C for 25 seconds, annealing at 53 ^{o}C for 40 seconds, elongation at 70 ^{o}C for 40 seconds and final extension at 70 ^{o}C for 8 min. The amplicons were used for further analysis.

3.7.10 Denaturation of DNA

The TwinCubator® (shaking water bath) was pre-warmed to 45 ^{o}C and 20µl of denaturation solution (NaOH) was added to each labeled well of the TwinCubator® tray followed by the addition of 20µl of the amplicons respectively. The mixture was mixed gently by pipetting up and down five times and then incubated at room temperature for 5mins.

3.7.11 Hybridization and detection

One (1) ml of the pre-warmed hybridization buffer (HYB) was carefully added to the wells using a pipette. The tray was then carefully tilted back and forth so that the purple denaturation solution and green hybridization buffer were thoroughly mixed. The tray was placed on the TwinCubator® and the labeled strips added to each well ensuring that the strips were completely covered by the liquid. This was incubated at 45^{o}C for 30mins. After incubation, the glass panel lid was opened and the condensate that formed during the incubation wiped off. The HYB buffer

was aspirated completely from each well using a Pasteur pipette. One (1) ml of the pre-warmed red stringent wash buffer (STR) was dispensed into the tray using a multi-channel pipette avoiding the contact of the strips with the tips. After 15 minute incubation at 45 ^{o}C in the TwinCubator®, STR buffer was aspirated and disposed of with a Pasteur pipette. The STR buffer was washed off with 1 ml of Rinse solution (RIN) for 1 minute. One (1) ml of the Conjugate (CON) solution was dispensed into each well and the glass panel lid was closed and incubated for 30 minute on the TwinCubator®. The strips were washed twice with 1 ml of Rinse solution (RIN) for 1 minute in the TwinCubator® to wash off the excess CON solution. One (1) ml of sterile distilled water was added to each strip-containing well, and a 1 minute wash performed on the TwinCubator® to wash off the RIN solution after which the distilled water was completely decanted. One (1) ml of the Substrate solution was dispensed into each well and incubated for 15 minutes on the TwinCubator® after which the Substrate solution was aspirated and the strips washed twice with sterile distilled water. A pair of clean tweezers was used to remove the strips from the TwinCubator® tray and placed onto absorbent paper. The developed strips were partially dried and transferred to the GenoType® MTBDR*plus* or Genotype MTBC 96 score sheet.

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence of TB by microscopy in Kaduna State, Nigeria

A total of one thousand one hundred and eighty six (1186) sputum samples were collected from the three senatorial districts of Kaduna State and screened for the presence of AFB. Two hundred and twenty one (18.6%) of the total samples screened were positive for AFB (Fig. 4.1).

4.2 Prevalence of TB by microscopy in Kaduna State, Nigeria according to age group

Figure 4.2 shows the prevalence of TB in Kaduna State according to age group. Age group 15-24 years had the highest prevalence of 26.0% followed by 25-34 years (21.0%) and 35-44 years (18.5%). The least prevalence (6.8%) was observed among age group 55-64 years. One hundred and seventy three (20.9%) AFB positive samples were collected from age group 15-44 years, while 48 (13.3%) were from age group 45 years and above. There was statistically significant difference in the rate of occurrence of TB among the age groups ($\chi^2 = 15.429$; $p = 0.0087$).

4.3 Prevalence of TB by microscopy in Kaduna State, Nigeria according to sex

The prevalence of tuberculosis in Kaduna State by sex is presented in Figure 4.3. Six hundred and ninety three (693) sputum samples were collected from males of which 155 were AFB positive giving a prevalence of 22.4% while four hundred and ninety three (493) were collected from females out of which 66 (13.4%) were AFB positive. There was statistically significant difference ($\chi^2 = 12.468$; $p=0.0004$) in the rate of occurrence of TB between males and females.

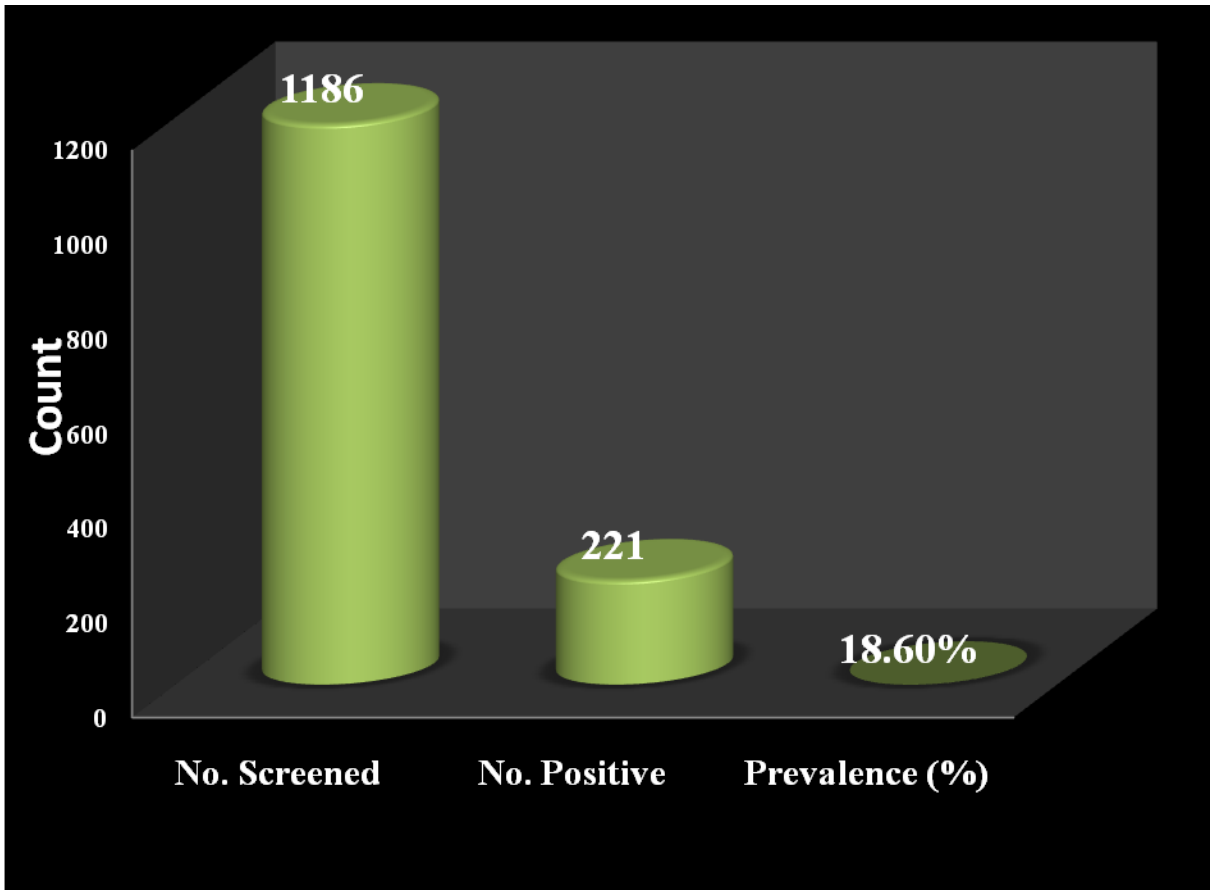


Figure 4. 1: Prevalence of TB by microscopy in Kaduna State, Nigeria

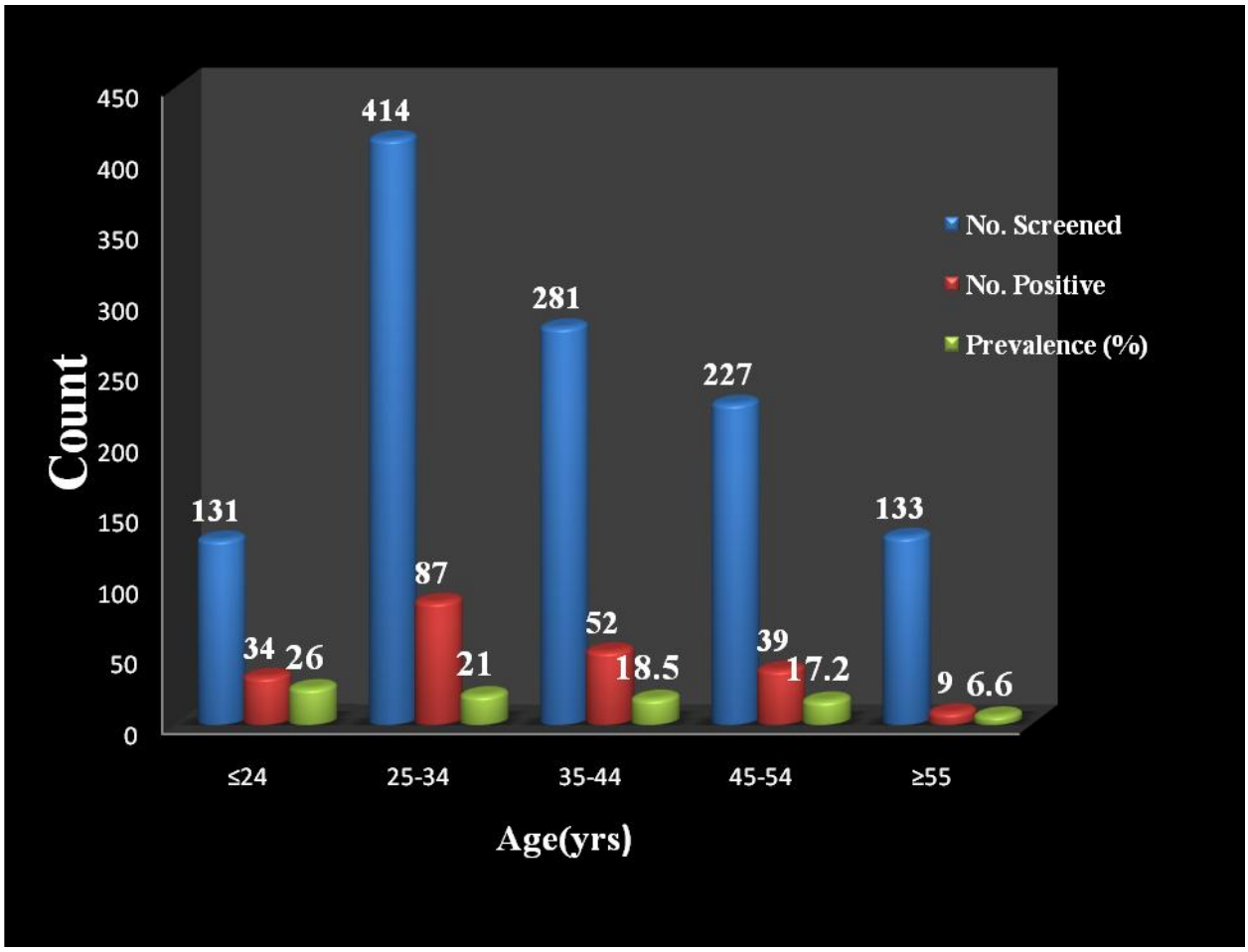


Figure 4. 2: Prevalence of TB by microscopy in Kaduna State, Nigeria according to age group

(χ^2 **= 15.429;** ***p*** **= 0.0087**)

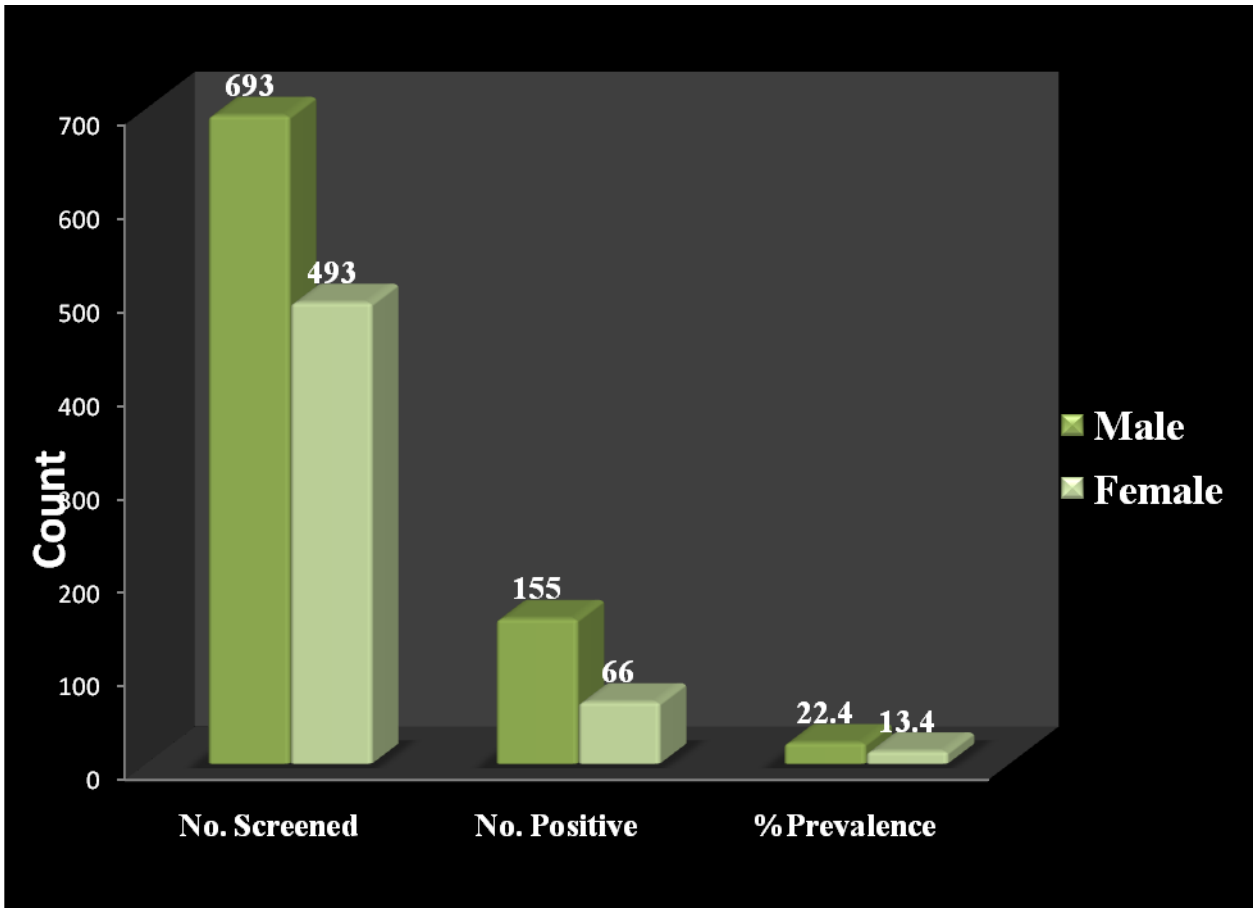


Figure 4. 3: Prevalence of TB by microscopy in Kaduna State, Nigeria according to sex (χ^2 = 12.468; *p* =0.0004)

4.4 Prevalence of Tuberculosis by Senatorial area in Kaduna State, Nigeria

One thousand one hundred and eighty six sputum samples were collected from the three senatorial areas in the State. Fiven hundred ninety seven (597), 250 and 339 samples were screened from the North, Central and Southern Senatorial areas respectively. Number that is positive for each of the area is shown in Table 2. There was no statistical significant difference (χ^2= 0.192; p=0.9085) in the rate of occurrence of TB between the area.

4.5 The distribution of TB by sex across the three Senatorial areas

The prevalence of TB by sex across the three Senatorial areas is presented in Table 3. Of the total samples collected (597) from the Northern part (area A), 363 were from males with a prevalence of 21.8% while 234 samples were from females representing 13.2%. The Central area had 24.0% and 11.6% prevalence for male and female respectively. In the Southern part, males had 22.4% prevalence and females had 15.2%. The highest prevalence for males and females (24.0% and 15.2%) were observed in the Central and Southern parts respectively. There was statistically significant difference in the rate of occurrence of TB between males and females in the Northern and Central parts; however, there was no significant deference in the Southern part (A: χ^2= 5.585, p=0.0181; B: χ^2 =5.413, p=0.02; C: χ^2 =2.184, p=0.1394).

4.6 Mycobacterial culture result

Figure 4.4 shows the mycobacterial culture result. Of the 221 smear positive sputum samples cultured on LJ slants, 171 (77%) yielded *M. tuberculosis* complex, 22 (10%) were AFB and culture positive but negative for MPT 64 antigen on SD-bioline, these were considered non tuberculous mycobacteria (NTM), 15 (7%) were smear positive but culture negative while 13 (6%) were contaminated.

Table 2: Prevalence of Tuberculosis by Senatorial area in Kaduna State, Nigeria

Senatorial area	No. Screened	No. Positive	Prevalence (%)
A (North)	597	110	18.4
B (Central)	250	45	18.0
C (South)	339	66	19.5
Total	1186	221	18.6

(χ^2 = 0.192; *p*=0.9085)

Table 3: Distribution of Tuberculosis by sex across the Senatorial areas in Kaduna State, Nigeria

Senatorial area	Sex	No. Screened	No. Positive	Prevalence (%)	χ^2 (*p*)
A (North)	Male	363	79	21.8	
	Female	234	31	13.2	5.585(0.0181)
	Total	597	110	18.4	
B (Central)	Male	129	31	24.0	
	Female	121	14	11.6	5.413(0.02)
	Total	250	45	18.0	
C (South)	Male	201	45	22.4	
	Female	138	21	15.2	2.184(0.1394)
	Total	339	66	19.5	

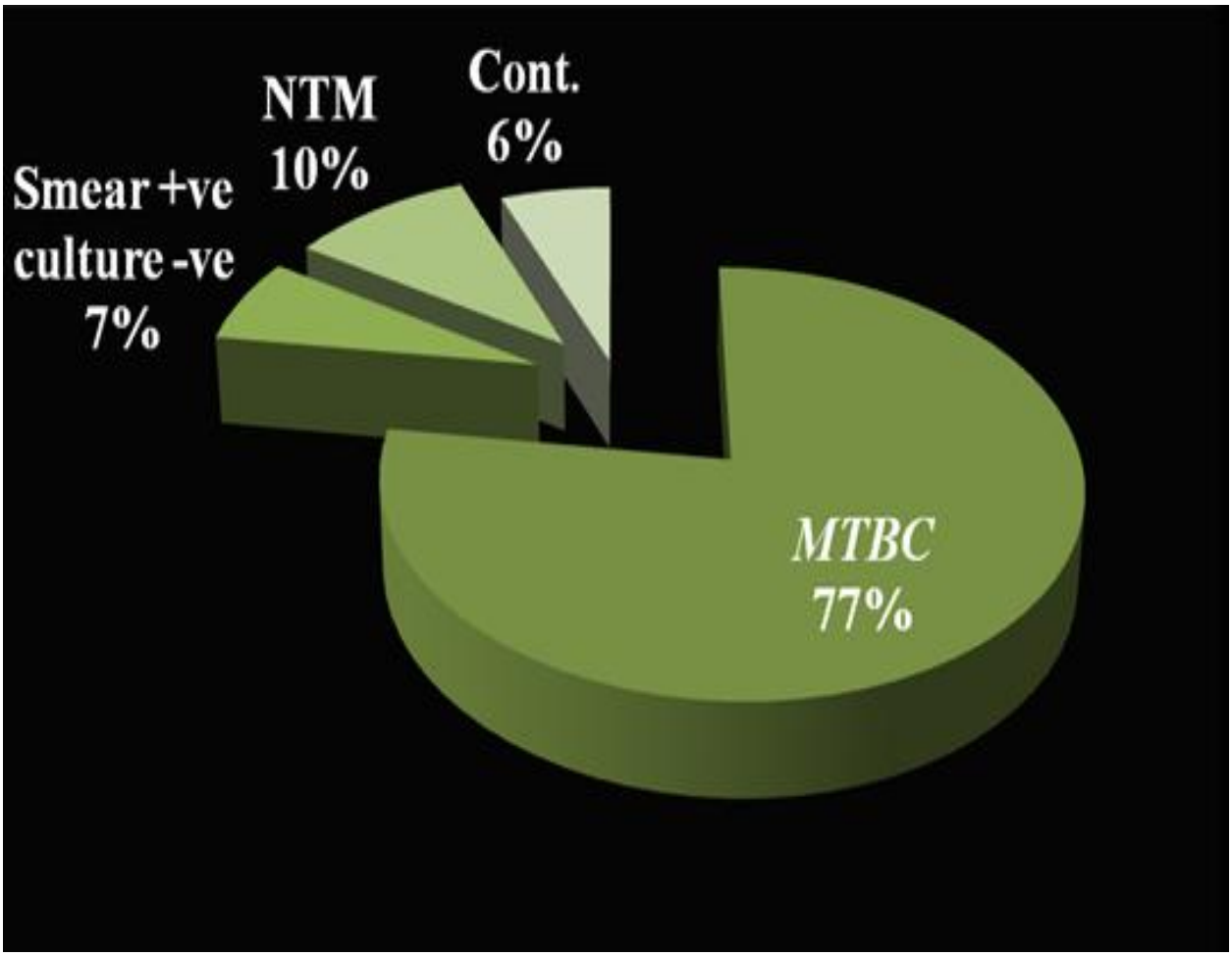


Figure 4. 4: Mycobacterial culture result

MTBC: *M. tuberculosis* complex
NTM: Non tuberculous mycobacteria
Cont: contaminated

4.7 Tuberculosis/HIV co-infection among the study population in Kaduna State, Nigeria

Tuberculosis/HIV co-infection is presented in Figure 4.5. Tuberculosis/HIV co-infection was found in 21 (9.5%) subjects while, 200 (95%) were infected with TB only. Chi-square analysis indicated a significant difference (χ^2= 37.246; p□0.0001) in the rate of occurrence of TB among HIV positive and negative subjects.

4.8 Prevalence of tuberculosis by case definition in Kaduna State, Nigeria

Prevalence of tuberculosis by case definition in Kaduna State is presented in Figure 4. 6. One hundred and eighty two (82%) of the subjects were new cases while thirty nine (18%) were retreatment cases.

4.9 Demographic characteristics and possible risk factors associated with TB

Demographic characteristics and possible risk factors associated with TB are presented in Table 4. One hundred and seventy three (78.3%) respondents were less than or equal to 44 years while 48 (21.7%) were 45 years and above; 138 (62.4%) were males while 83 (37.6%) were females. New cases had a higher prevalence (28%) compared to retreatment cases (18%); 13.5% were HIV positive; 33.3% had contact with TB patients; 18.6% smoke cigarette and 31.2% consume alcohol. None of the respondents has diabetes or asthma.

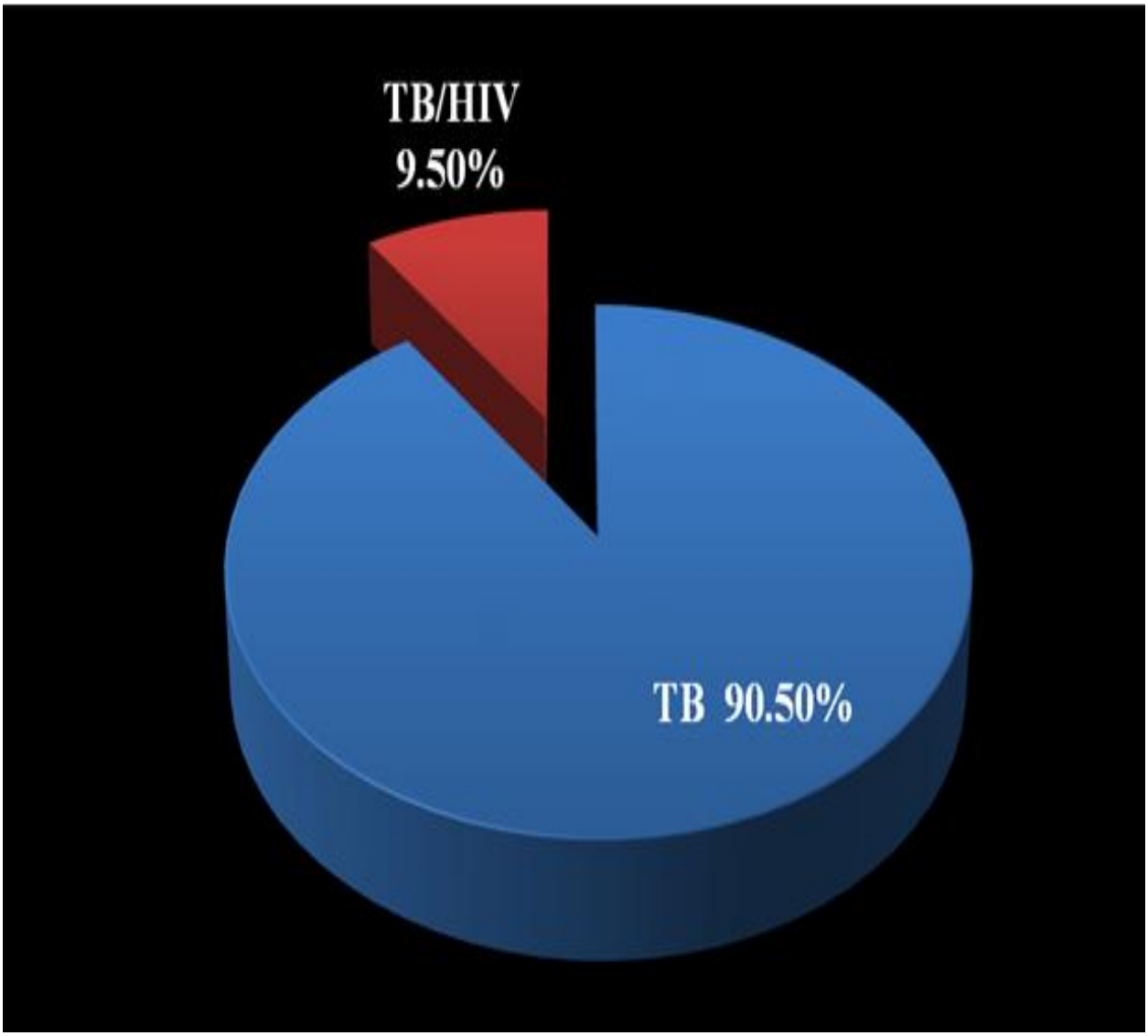


Figure 4. 5: Tuberculosis/HIV co-infection among the study population in Kaduna State, Nigeria

(χ^2= 37.246; *p*□0.0001)

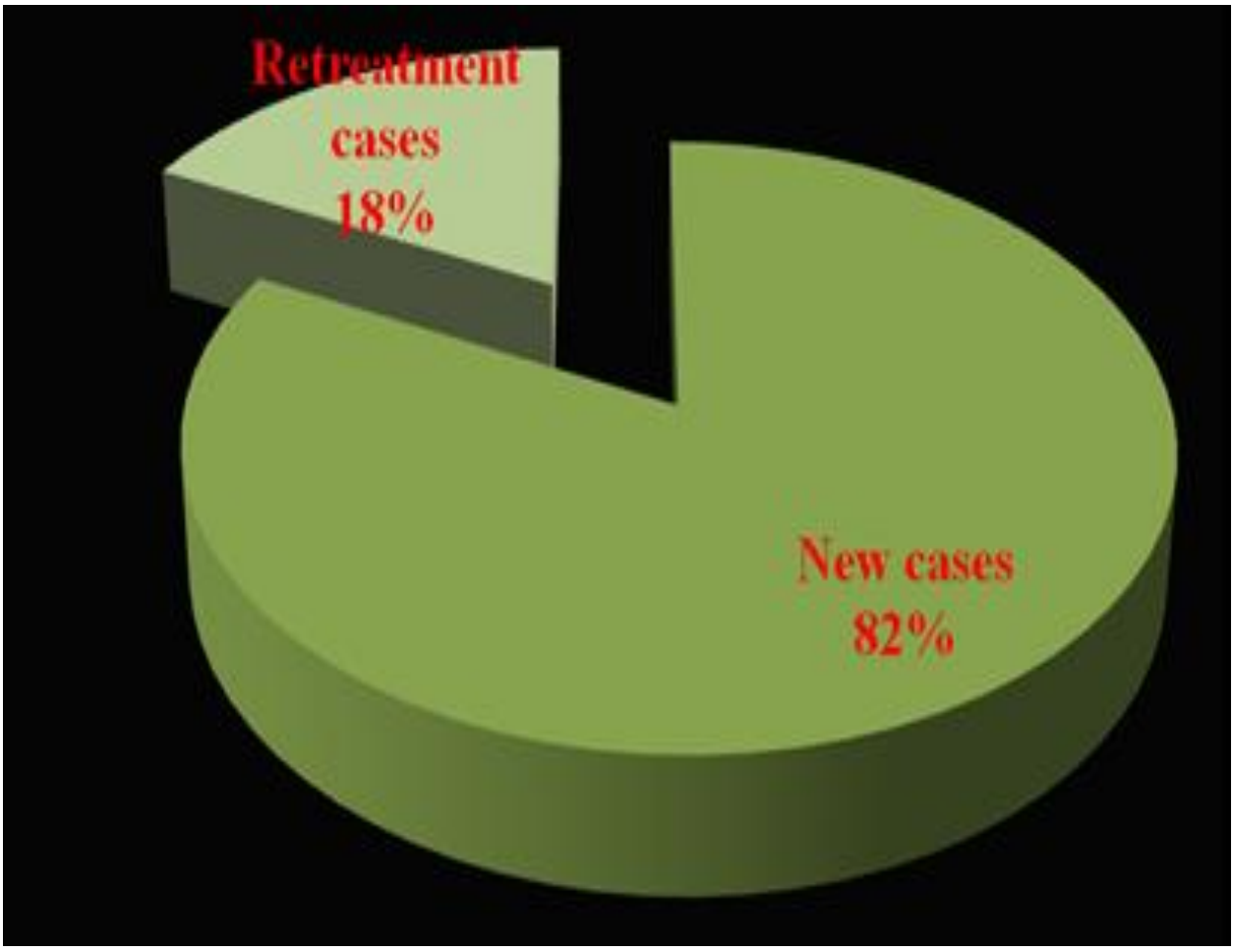


Figure 4. 6: Prevalence of tuberculosis by case definition in Kaduna State, Nigeria

(χ^2 = 185; p □ 0.05)

Table 4: Demographic characteristics and risk factors for infection with *Mycobacterium tuberculosis* among respondents in Kaduna State, Nigeria

Characteristics	No. Positive (n=221)	Per cent age	OR(95%CI)	*P* value
Age (Years)				
15-44	173	78.3	1.72(1.21-2.44)	0.002
≥45	48	21.7		
Sex				
Male	138	62.4	2.10(1.56-2.84)	<0.0001
Female	83	37.6		
Case definition				
New cases	182	82.0	21.8(13.35-35.51)	<0.0001
Retreatment	39	18.0		
Contact with TB patient				
Yes	51	23.1	0.09(0.06-0.14)	<0.0001
No	170	76.9		
HIV infection				
Yes	21	9.5	0.22(0.14-0.35)	<0.0001
No	200	90.5		
Cigarette smoking				
Yes	41	18.6		
No	180	81.4		
Alcohol consumption				
Yes	69	31.2		
No	152	68.8		
History of diabetes				
Yes	00	00		
No	221	100		
History of asthma				
Yes	00	00		
No	221	100		

4.10 Resistance patterns to first-line anti-TB drugs in Kaduna State, Nigeria

Resistance patterns to first-line anti-TB drugs in Kaduna State are presented in Table 5. Any drug resistance was found in 21 (52.5%) of all cases; 19 (47.5%) among new cases and 2 (5.0%) were found in retreatment cases. Mono-drug resistance was seen in 9 (22.5%) of the isolates tested and was against streptomycin. All mono-drug resistance cases were among the new cases. MDR-TB was detected in 2 (5.0%) of the subjects all of which were found among new cases. Four (10.0%) of all cases were poly-drug resistant, 3 (7.5%) were new cases, only 1 (2.5%) was retreatment case. One isolate (2.5%) each from new case and retreatment case showed poly-drug resistance to SM EMB, 1 (2.5%) isolate was resistant to INH EMB. Poly resistance to SM INH EMB was found in only 1 (2.5%) new case. No poly-resistance to RIF EMB, RIF SM or RIF SM EMB was observed.

4.11 Demographic characteristics and risk factors for drug resistance

Statistical analyses revealed no association between HIV and drug resistance (OR=0.25; 95% CI: 0.07-0.90). There was however, association between drug resistance and sex (OR=6.3; 95% CI: 1.64-23.84) as presented in Table 6.

4.12 Banding patterns of mutations associated with rifampicin and isoniazid resistance

The banding patterns of mutations associated with rifampicin and isoniazid resistance in multidrug resistant and mono-resistant strains detected by MTBDR*plus* is shown in Table 7. One isolate was characterized as MDR with bands at *rpoβ* MUT2A region and *ihn*A MUT2 corresponding to H526Yand A16G mutations respectively. Rifampicin mono resistance with band at *rpoβ* MUT3 corresponding to S531L was found in one isolate. Also, isoniazid mono resistance was observed in one isolate with *ihn*A MUT2 band corresponding to A16G mutation.

4.13 Comparison of Geno Type MTBDR*plus* LPA and phenotypic LJ-proportion method

Table 8 shows the comparison of Geno Type MTBDR*plus* LPA and phenotypic LJ-proportion method. The comparison of Geno Type MTBDR*plus* LPA and phenotypic LJ-proportion method showed that one isolate was mono resistant to RIF and one was mono resistant to INH by LPA, one and two MDR-TB isolates respectively were characterized by genotypic and phenotypic methods. The remaining isolates were found to be pan susceptible by both methods.

Table 5: Resistance patterns to first-line anti-TB drugs in Kaduna State, Nigeria

Drug-resistance patterns	All cases N = 40		New cases		Retreatment cases	
	Freq.	%	Freq.	%	Freq.	%
Any drug resistance	21	52.5	19	47.5	2	5.0
SM	13	32.5	12	30	1	2.5
INH	3	7.5	3	7.5	0	0.0
RIF	2	2.5	2	2.5	0	0.0
EMB	3	7.5	2	5.0	1	2.5
Mono-drug resistance	9	22.5	9	22.5	0	0.0
SM	9	22.5	9	22.5	0	0.0
INH	0	0.0	0	0.0	0	0.0
RIF	0	0.0	0	0.0	0	0.0
EMB	0	0.0	0	0.0	0	0.0
MDR-TB	2	5.0	2	5.0	0	0.0
Poly-drug resistance	4	10.0	3	7.5	1	2.5
SM INH	0	0.0	0	0.0	0	0.0
SM EMB	2	5.0	1	2.5	1	2.5
INH EMB	1	2.5	1	2.5	0	0.0
RIF EMB	0	0.0	0	0.0	0	0.0
RIF SM	0	0.0	0	0.0	0	0.0
RIF SM EMB	0	0.0	0	0.0	0	0.0
SM INH EMB	1	2.5	1	2.5	0	0.0

SM: Streptomycin; INH: Isoniazid; RIF: Rifampicin; EMB: Ethambutol

Table 6: Demographic characteristic and risk factor for drug resistance among respondents in Kaduna State, Nigeria

Characteristic	No. of resistance N=40	OR(95%CI)	*P* value
Sex			
Male	15	6.3(1.64-23.84)	0.007
Female	6		
HIV status			
Positive	7	0.25(0.07-0.90)	0.034
Negative	14		

Table 7: Patterns of gene mutations detected by Geno Type MTBDRplus assay in drug resistant *M. tuberculosis* isolates

Gene	Band	Gene region or mutation	MDR strains(n =1)	RIF mono resistant strains(n = 1)	INH mono resistant strains(n = 1)
rpoβ					
	WT1	506-509			
	WT2	510-513			
	WT3	513-517			
	WT4	516-519			
	WT5	518-522			
	WT6	521-525			
	WT7	526-529			
	WT8	530-533			
	MUT1	D516V			
	MUT2A	H526Y	1		
	MUT2B	H526D			
	MUT3	S531L		1	
katG					
	WT	315			
	MUT1	S315T1			
	MUT2	S315T2			
inhA					
	WT1	-15/-16			
	WT2	-8			
	MUT1	C15T			
	MUT2	A16G	1		1
	MUT3A	T8C			
	MUT3B	T8A			

Key: WT= Wild Type, MUT= Mutant, D=Aspatate, V=Valine, H=Histadine, Y=Tyrosine, S=Serine, L=Leucine, T=Threonine, C=Cysteine, A=Alanine, G=Glycine

Table 8: Comparison of Geno Type MTBDRplus LPA and phenotypic LJ-proportion method

Susceptibility	**Geno Type MTBDR*plus***	**phenotypic LJ-proportion**
RIF mono-resistance	1	0
INH mono-resistance	1	0
MDR-TB	1	2
Pan susceptible	37	38
Total	40	40

CHAPTER FIVE

5.0 DISCUSSION

In recent years, there has been an increasing concern on the threat of tuberculosis to public health, especially in developing countries where its alliance with HIV/AIDS is making the situation worse. From this study, a prevalence rate of 18.6% AFB positive was found in Kaduna State. This high rate could be attributable to a combination of high poverty levels, poor socio-economic status, poor education, associated with poor knowledge of TB risks of infection and dissemination as well as poor access to health care facilities. The comparative high HIV prevalence rate (5.1%) among the general population is also fuelling the TB epidemic in the State. The presence of many Federal institutions of higher learning in the State attracts people from within and outside the country to the State which could in part contribute to the high rate of infection in the State. Similar prevalence of 16.83% in Abia (Nwachukwu *et al.,* 2009) and 14.7% in Kano (Imam and Oyeyi, 2008) were reported. However, higher rates were reported in Nasarawa (44.8%) and Lagos (24.8%) by Onubogu *et al.* (2010) and Umeh *et al.* (2007) respectively.

In most instances it cannot be determined why a particular person does or does not develop tuberculosis after becoming infected with tubercle bacilli. However, a multitude of factors have been identified which increase the risk of progression from sub-clinical infection with *M. tuberculosis* to overt tuberculosis (Rieder, 1999).

The prevalence of TB in Kaduna from this study showed that the disease is more prevalent among the economically productive age group (15-44 years) confirming the known fact that TB is more prevalent among this group. About 78.3% of the study population falls within this age group. Exposure to the risk factors like smoking, alcoholism, imprisonment, migration is more

common among these subjects; other co-morbid factors such as HIV also peak within the group. Children are being administered TB vaccine (BCG) at birth and this vaccine is said to have above 80% protection against TB in children for as long as 15years if administered before first exposure (Bannon and Finn, 1999; Crofton *et al.,* 1999). The average life expectancy of Nigerians is 53 years and 55 years for males and females respectively (WHO, 2012). These may in part explain the low rate of TB cases observed in children and in elderly. There was significant association (OR=1.72; 95% CI: 1.21-2.44; *P*=0.002) between TB and age.

Several studies have supported the strong confounding effects of demographic factors such as age and gender on the incidence of TB globally (Holmes *et al.,* 1998; Crampin *et al.*, 2004).

The findings in this study revealed a statistically significant association (OR=2.10; 95% CI: 1.56-2.84; *P*=0.0001) in the rate of occurrence of TB between males and females subjects. The risk ratio of males to females is 1.41 (95% CI: 1.25-1.60; *P*=0.0001). This could be due to differences in access to health care and in health seeking behaviour of people due to stigma associated with TB. Women tend to have a more far reaching psycho-social consequences of stigmatizing ailments and may consequently avoid self exposure inherent in presenting at health facilities. Moreso, majority of women especially in the Northern and Central parts of the State are mostly full time housewives and are thereby hardly exposed to the risk of infection from contact with infected people. Although, most studies are in keeping with this finding, Nwachokor and Thomas (2000), in a 30 year review of tuberculosis in lbadan, Nigeria reported that more females were infected with TB which reached twice the rate of males. It could be because lbadan being in the Southern part of Nigeria, more women are working than the North where the reverse is the case. The social roles of men and cultural habits that influence risk of exposure have also

been implicated as possible reasons. Social factors such as smoking, alcoholism, imprisonment and poor nutrition may also play a role. Several studies have reported higher prevalence of pulmonary TB in males than females (Borgdorff *et al.,* 2000; Al-Hajoj *et al.,* 2013; Gambo *et al.,* 2013a; WHO, 2014).

There was no significant difference ($\chi 2 = 0.192$; p=0.9085) in the rate of occurrence of TB across the three areas. However, the highest prevalence rate was observed in the southern part of the State. High poverty levels and relatively high HIV prevalence rates is fuelling the TB epidemic, especially in the Southern part of the State where the burden of HIV (11.4%) is disproportionately higher than the other two areas (Central = 1.9% and Northern = 0.7%) (Gidado and Ejembi, 2009).

Of the 221 smear positive sputum samples cultured on LJ slants, 7% were smear positive but culture negative. The 4% NaOH used for decontamination of the sample could kill up to 70% of tubercle bacilli this may lower the number thus may not be able to grow when cultured. The samples might have been exposed to sunlight; TB are rapidly destroyed when exposed to sunlight. Moreso, LJ medium does not support the growth of some mycobacterial species such as *M. bovis* which are also AFB, all these may partly explain why some samples were smear positive but culture negative. Contamination rate was found to be 6%. This could be attributed to the nutritionally rich medium (LJ medium) used for the isolation as well as the prolong incubation period. Also, the contaminating microorganisms which are abundant as normal flora in the respiratory tract may cling to food particles commonly found in the sputum thereby evading killing during decontamination. Mycobacteria other than members of the MTBC are abundant in the environmental, this could be responsible for the 10% NTM found in this study.

Similar finding was reported in two different studies (5.7% and 15%) by Gambo *et al.* (2013a; 2013b) in Northern Nigeria, while Al-Hajoj *et al.* (2013) reported 11.5% NTM in Saudi Arabia.

Tuberculosis/HIV co-infection was found in 21 (9.5%) while 200 (95.5%) were infected with TB only. The TB/HIV co-infection rate in this study is lower than the 14.2% rate among tuberculosis patients reported in the National drug-resistant tuberculosis prevalence survey of 2012 (National Drug-resistant TB prevalence survey report, Nigeria, 2012). Similar rates reported in other studies include 9.6% in Edo State (Okodua *et al.,* 2012), 12.0% in Ile-Ife, Osun State (Onipede *et al.,* 1999), 10.5% and 14.9% among children and adults respectively in Sagamu, Oyo State (Daniel *et al.,* 2005), 6.1% among those aged 20-40 years in Jos, Plateau State (Anteyi *et al.,* 1996). According to the United States Embassy in Nigeria, Nigeria Tuberculosis Fact Sheet (2012), about 8% of the HIV-positive persons in Nigeria are tuberculosis positive; this is in keeping with the finding in this study. The low TB/HIV co-infection rate in this study could be as a result of the well established fact that HIV-mediated immunosuppression impairs granuloma formation, resulting in both ineffective containment of *M. tuberculosis* bacilli and diminished formation of pulmonary cavities (Klautau and Kuschnaroff, 2005; Murray, 2005). These effects manifest clinically as frequent extrapulmonary disease (Poprawski *et al.,* 2000) and lower concentrations of bacteria in sputum (Colebunders and Bastian, 2000). Most TB/HIV co-infection patients are smear negative and most of the tuberculosis patients who are HIV positive will have pauci bacillary or smear negative disease. However, the HIV infection rate among TB patients in this study is almost twice that in the general population (5.1%) in the State (Federal Ministry of Health, Nigeria, 2010). This further buttresses the fact of TB/HIV co-morbidity. A higher prevalence of 26.9% was reported in Northern Nigeria by Gambo *et al.* (2013a); a much lower rate of 1.9% was however reported in Saudi Arabia by Al-Hajoj, *et al.* (2013). Chi-square

analysis indicated a significant difference ($\chi 2 = 37.246$; $p \square 0.0001$) in the rate of occurrence of TB among HIV positive and negative subjects.

Our findings showed that the majority (82%) of the TB study participants were newly diagnosed cases whereas the remainder (18%) were previously treated cases. This could be explained by the fact that a pre-existing infection provides some protection against re-infection compared to acquisition of new infection. Thus, if infection occurs, those with pre-existing infection will have, on average, better defense mechanisms than those who have not been previously infected (Rieder, 1999). This is in keeping with previous findings of 82.1% new cases and 17.9% retreatment cases in Nigeria (National Drug-resistant TB prevalence survey report, Nigeria, 2012); and 88.3% new cases and 11.7% previously treated cases in Cameroon (Meriki *et al.,* 2013).

Drug-resistant TB ultimately develops from the inadequate treatment of active pulmonary TB. This may result from poor prescription practices among medical doctors with poor drug selection and insufficient treatment duration (Sharma and Mohan, 2006). Systemic problems, through inadequate public health resources and unpredictable drug supplies or supply of sub standard drugs, also play a role (Mukherjee *et al.,* 2004). Erratic or selective compliance to treatment and default among patients is another key factor as it causes *M. tuberculosis* to be exposed to sub-lethal doses for insufficient durations. These could thus, result in treatment failure and foster emergence of drug-resistant TB (Pablos-M´endez *et al.,* 1997).

The resistance to first line anti-TB drug was found to be 52.5% of all cases of pulmonary TB in Kaduna State. Mono-drug resistance was seen in 9 (22.5%) of the isolates tested and was against streptomycin. The highest resistance to streptomycin was not surprising because it is used for the

treatment of other disease conditions such as brucellosis and cross resistance with other aminoglycosides such as gentamicin which are commonly used in our settings for many years (Houang and Greenwood, 1977). Moreso, similar results of high resistance to streptomycin were previously reported; 30.6% in the national DR survey in Nigeria (National Drug-resistant TB prevalence survey report, Nigeria, 2012), 34% in Sudan (Hassan *et al.,* 2012) 18.8% in Cameroon (Meriki *et al.,* 2013) and 28.1% in India (Sethi *et al.,* 2013).

The most important measure of TB drug resistance is the number of new cases that are MDR-TB (Dye *et al.,* 2002a). According to the Global TB control, WHO report (2012), the estimated prevalence of MDR-TB in Nigeria among new cases is 3.1%. This study showed a prevalence rate of 5.0% (2/40) among new cases. Occurrence of MDR-TB in drug naïve patients is due to the fact that the infecting mycobacteria may not be drug naïve, or acquired resistance might have developed due to natural selection which is a function of ineffective treatment and non compliance to drug prescription by the previous host, when such resistant strains are transferred to a susceptible host, primary drug resistance develops (Johnson *et al.,* 2009). The availability of drugs on the open market and a private sector that delivers drugs to the population in an unregulated fashion in Nigeria could also be factors that might favour development of MDR-TB.

Several biological mechanisms linking drug-resistant TB to HIV infection have been suggested (Dye *et al.,* 2002b). Drug malabsorption in HIV-infected patients, especially rifampicin and ethambutol, can lead to drug resistance and has been shown to lead to treatment failure. Drug-resistant strains may be less virulent and preferentially lead to disease progression in immunocompromised patients, as opposed to immunocompetent individuals. Data supporting this hypothesis has not yet been observed in humans (Anastasis *et al.,* 1997). However, statistical

analysis (OR=0.25; 95% CI: 0.07-0.90; *P*=0.034) in the present study did not reveal any association between HIV infection and anti-TB drug resistance. This is similar to reports from earlier studies in Nigeria. Otu *et al.* (2013) reported no association between HIV infection and anti-TB drug resistance in Calabar; in Abuja, Lawson *et al.* (2010) studied 32 TB culture-positive patients and reported no association between HIV infection and anti-TB drug resistance. Pereira *et al.* (2005) studied a total of 70 *M. tuberculosis* isolates, 30 from HIV seropositive, and 40 from HIV seronegative TB patients in India and found that the prevalence of drug-resistant *M. tuberculosis* isolates among HIV seropositive TB patients was similar to that of HIV seronegative TB patients indicating that HIV infection may not be associated with drug-resistant TB. Three studies in South Africa also found no association between HIV infection and MDR-TB. In a retrospective study in Durban, 2.4% of 42 HIV co-infected and 11.5% of 253 HIV negative patients had MDR-TB (Anastasis *et al.,* 1997). A prospective study of hospitalized TB patients in Cape Town found MDR-TB prevalence of 3.2% in 93 HIV co-infected patients, compared to 2.6% in 115 HIV negative patients (Post and Wood., 1997). In gold miners, the MDR-TB rate was 5.3% among 207 HIV co-infected and 6.5% among 215 HIV negative miners (Murray *et al.,* 2000). From the above, it appears that studies from very high TB burden countries like Nigeria, India, and South Africa have consistently reported no association between HIV and TB drug resistance.

The genotypic drug resistance assay revealed that all the resistant isolates were hetero-resistant; a phenomenon where some cells within a population may remain susceptible to the antibiotic, whereas other cells display varying degrees of drug resistance. This was determined by the simultaneous detection of wild type and mutant molecular susceptibility. In patients infected with a fully susceptible strain, drug resistance can develop gradually during inadequate treatment

due to selection of cells with random mutations in sites associated with drug resistance (i.e. secondary resistance). In this case, as the proportion of susceptible cells decrease and resistant cells increase, a hetero-resistant population of cells will be present. These cells are primarily identical throughout the genome but a proportion of the population differs in sites associated with drug resistance. In contrast, patients infected with fully susceptible strains may develop mixed infections if they are co-infected with another strain that is drug resistant, resulting in a mixed population of two genetically distinct strains, one drug susceptible, and the other drug resistant. Also, hetero-resistance likely represents a natural variation in the population of cells of *M. tuberculosis* (Foundation for innovative new diagnosis, 2012). Prescription of inadequate treatment regimen, irregular drug supply, poor drug quality with low bioavailability, and poor compliance among the study population could be attributed to the development of hetero-resistance observed in this study.

The patterns of mutations associated with rifampicin and isoniazid resistance determines the degree of susceptibility to anti TB drugs. Studies determining MICs have shown that high-level RIF resistance is associated with mutations in codons 526 and 531, whereas alterations in codons 511, 514, 515, 516, 518, 521, 522 and 533 result in low-level RIF resistance. Mutations in *katG* tend to confer high-level resistance to INH, while mutations in *inhA* generally confer low-level INH resistance (FIND, 2012). Thus, identifying and reporting where the specific mutation was detected is important in the selection of treatment regimen. The two RIF resistant isolates in this study have mutations at codons 526 and 531 respectively, suggesting a high-level RIF resistance. A number of published studies have described that the value of the RIF MIC strongly correlates with the position and nature of the amino-acid substitution in *rpoβ* RRDR (Yang *et al.,* 1998; Campbell *et al.,* 2001; Ocheretina *et al.,* 2014).

Discordance between genotypic and phenotypic assays was observed in one isolate with RIF mono resistance. The isolate was classified as MDR by the phenotypic assay. This could be due to silent mutation or synonymous single nucleotide polymorphism (sSNP) at the target site. Silent mutations do not result in structural changes in the *inhA* and so do not interfere with its inhibition by INH. Moreover, findings of silent mutations in *inhA* are not surprising as SNPs occur every 3 kb of MTB genome (Comas *et al.,* 2011). Ando, *et al.* (2014) reported a silent mutation in a significant number of INH resistant *M. tuberculosis* clinical isolates. Mutations conferring INH resistance in other genes such as *mabA* (G609A) (Ando *et al.*, 2014) *aphC* (alkyl hydroperoxide reductase), *kasA* (ß-ketoacyl-ACP synthase) and *nadh* (NADH dehydrogenase) (Cohen *et al.,* 2003; Balasubramanian *et al.,* 2012) not included in Geno Type MTBDR*plus* have also been reported. The critical concentration of INH on LJ medium which is being used for over 50 years is 0.2µg/ml (Jamieson *et al.,* 2014). Lower critical concentrations of 0.0312µg/ml for low-level resistance and 0.125µg/ml for high-level resistance were reported by Gumbo, (2010).

In addition, no mutation was detected by the genotypic assay in a second MDR isolate. For this discrepant isolate, a mutation either outside or in the *rpoβ* RRDR or mutations conferring INH resistance in other genes not included in the probes might have occurred. RIF resistance associated mutations outside the *rpoβ* RRDR V176F (RIF MIC 8 to 32µg/ml) and V146F (RIF MIC 50µg/ml) were reported by Markus *et al.* (2000) and Jamieson *et al.* (2014), or a different mechanism of resistance, may account for the resistance.

Members of the MTBC are highly related mycobacteria exhibiting remarkable nucleotide sequence level homogeneity despite variation in pathogenicity, geographic range and certain physiological features (such as colony morphology as well as profiles of resistance and susceptibility to inhibitors, epidemiology, and host preference). *M. tuberculosis* is the

predominant cause of human tuberculosis. However, each of the MTBC subspecies is known to infect humans, and since most laboratories do not fully identify MTBC isolates, the true cause of tuberculosis in these patients and its source often remain undiscovered. An important health concern is the zoonotic transmission of some MTBC subspecies from animals to humans and vice versa. Of particular significance is the transmission of *M. bovis* to humans from cattle and unpasteurized milk as well as *M. bovis* BCG infection of immunocompromised individuals. *M. bovis* is naturally resistant to pyrazinamide, a first-line antituberculosis drug. Therefore, complete identification of MTBC isolates at the subspecies level is required in order to collect information on their epidemiology and also to enable appropriate patient treatment and public health measures (Huard *et al.*, 2003).

All the isolates (100%) were identified as *M. tuberculosis*. This is in keeping with the well established fact that *M. tuberculosis* is the principal agent of the disease in humans (Huard *et al.*, 2003). Previous studies on characterization of mycobacterial species causing pulmonary tuberculosis in Nigeria and some West African countries indicated the same trend, 92% in Osun State, 94.4% in Northern Nigeria; 93.4% and 73% in Cote d I voire and Ghana respectively (Alli, *et al.*, 2010; Gambo *et al*., 2013b).

5.2 Conclusion

This is the first study to demonstrate the prevalence of MDR-TB by both phenotypic and genotypic methods in Kaduna State, Nigeria to our knowledge. The study showed an overall high prevalence of TB (18.6%) and TB drug resistance (52.5% any drug resistance; 12.5% poly-drug resistance and 22.5% Mono-drug resistance). It also demonstrated that the prevalence of MDR-TB (5.0%) is high in the State. There was association between TB and age, sex, case, contact with TB patient and HIV. No association was observed between HIV and MDR-TB; however, there was association between MDR-TB and sex. One (1) isolate was mono resistant to RIF by LPA, one (1) was mono resistant to INH by LPA, one (1) and two (2) MDR-TB isolates respectively were characterized by genotypic and phenotypic methods. Also, the remaining isolates were found to be pan susceptible by both methods. All the isolates (100%) were found to be *M. tuberculosis*.

5.3 Limitations

1. Difficulty in obtaining ethical approval delayed the early completion of the work
2. The hospitals (Ahmadu Bello University Teaching Hospital, Zaria, National Tuberculosis and Leprosy Training Centre, Saye, Zaria, Barau Dikko Spacialist, Kaduna, General Hospital Kafanchan) selected for the study might not be representative enough; they were selected based on the large number of attendees, their centrality and high status compared to other medical facilities in the study area
3. Sequencing was not done due to limited financial support
4. The accuracy of the responses generated depended on the accuracy of information given by the respondents

5.4 Recommendation

1. Laboratory facilities for rapid TB culture and DST are urgently needed in selected hospitals in the State and across Nigeria for early and accurate diagnosis of TB and drug resistant cases
2. Use of robust molecular techniques such as DNA sequencing employed for the detection of occult cases with low-level resistance and other resistance not included in the Geno Type *MTBDRplus* kit is recommended
3. Regular supply of standard drugs as well as strict adherence to the DOTS strategy should be ensured by the authorities concerned
4. Appropriate measures should be taken by the relevant authorities to prevent the indiscriminate and sub-therapeutic use of anti-TB drugs to avert the emergence of resistant strains
5. Further studies to describe the molecular epidemiology of MTBC in Nigeria; and to identify the predominant genotypes responsible for TB transmission and prevalence should be conducted
6. Patients recruited in the study were those presenting at the clinics, thus, further studies should be conducted to include active recruitment method for those in communities
7. Collaboration between academic institutions and health care institutions should be encouraged
8. Improved health care facilities, standard of living as well as proper education of the public on the knowledge of TB and associated risks of infection and dissemination are also recommended

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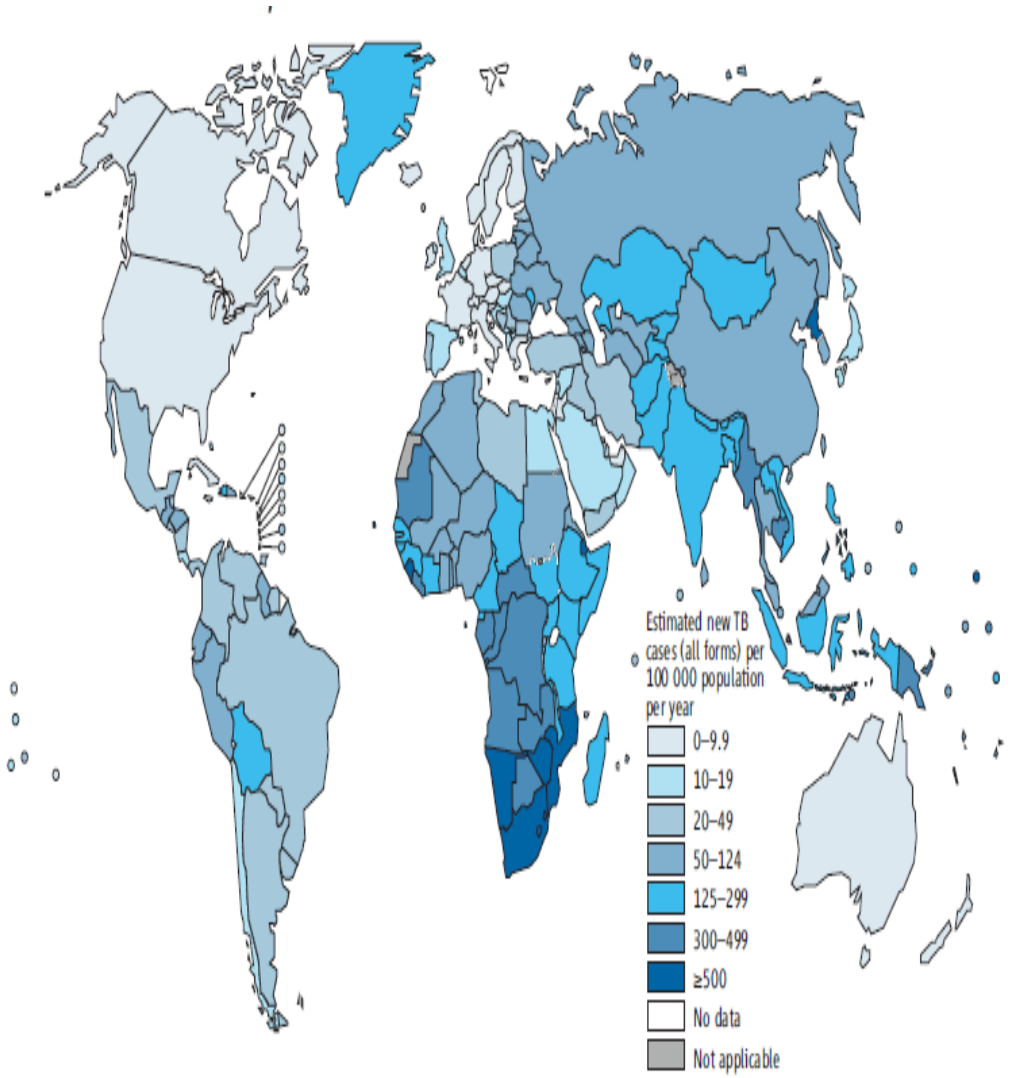
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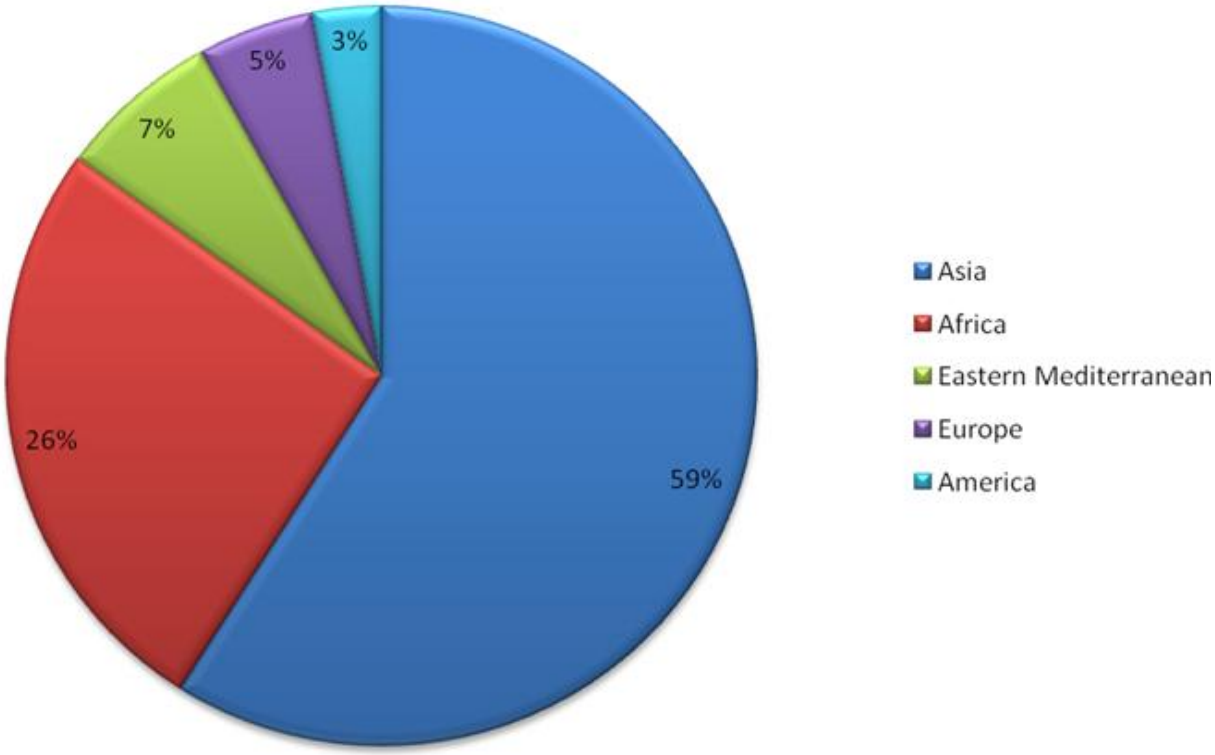
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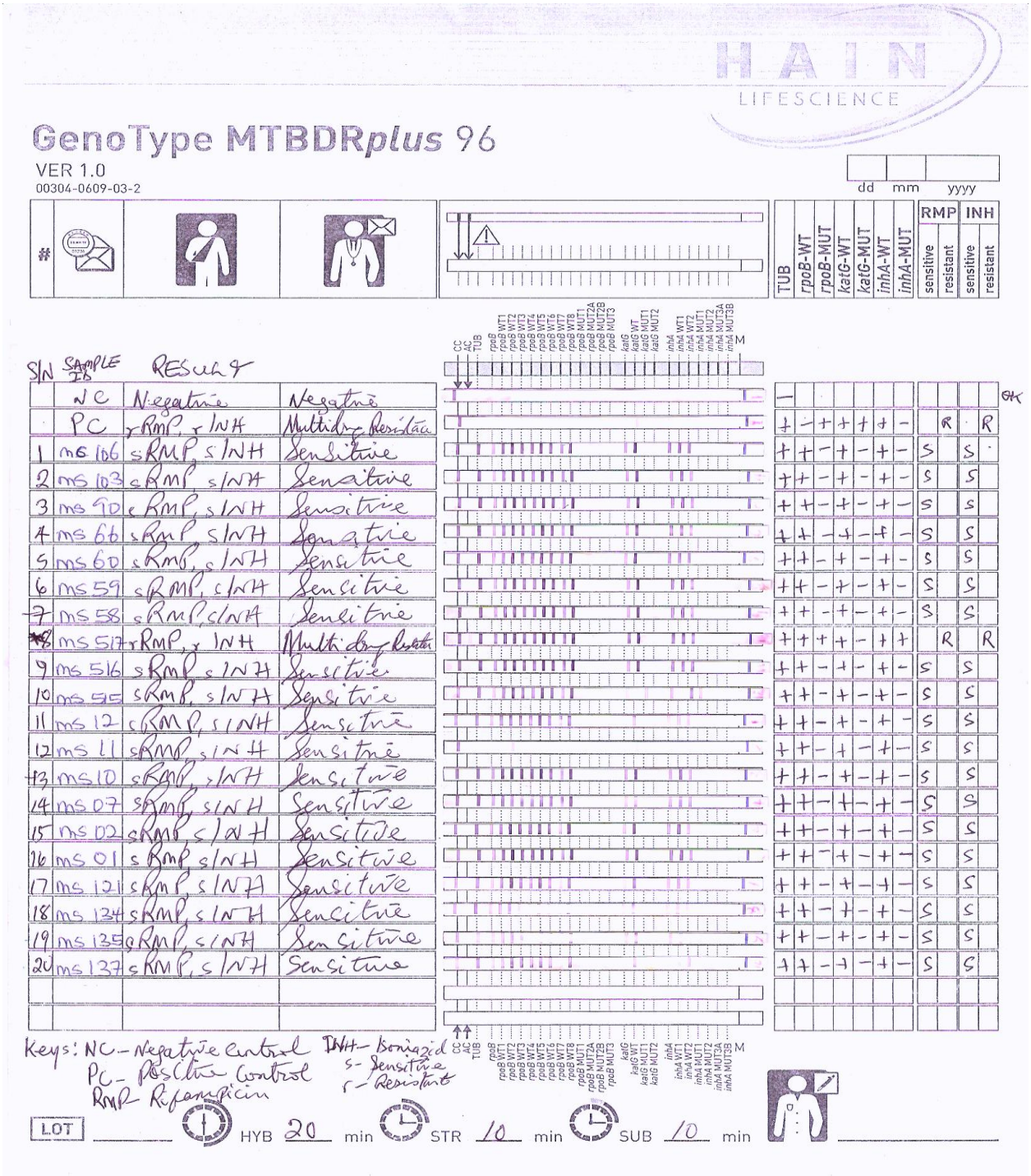
Appendix I: The estimated rate of TB incidence by country in 2010



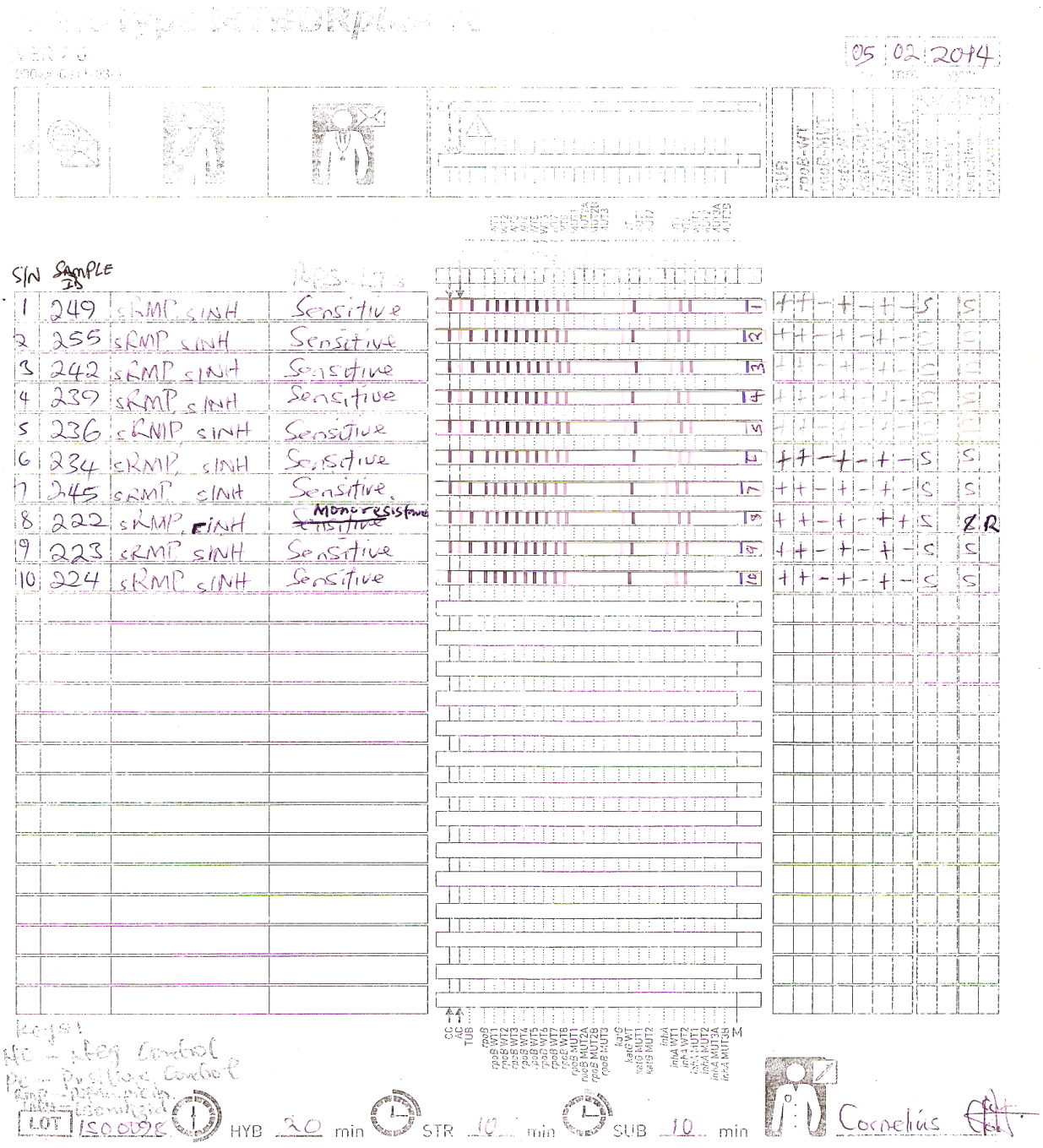
Appendix II: Global TB Burden by region



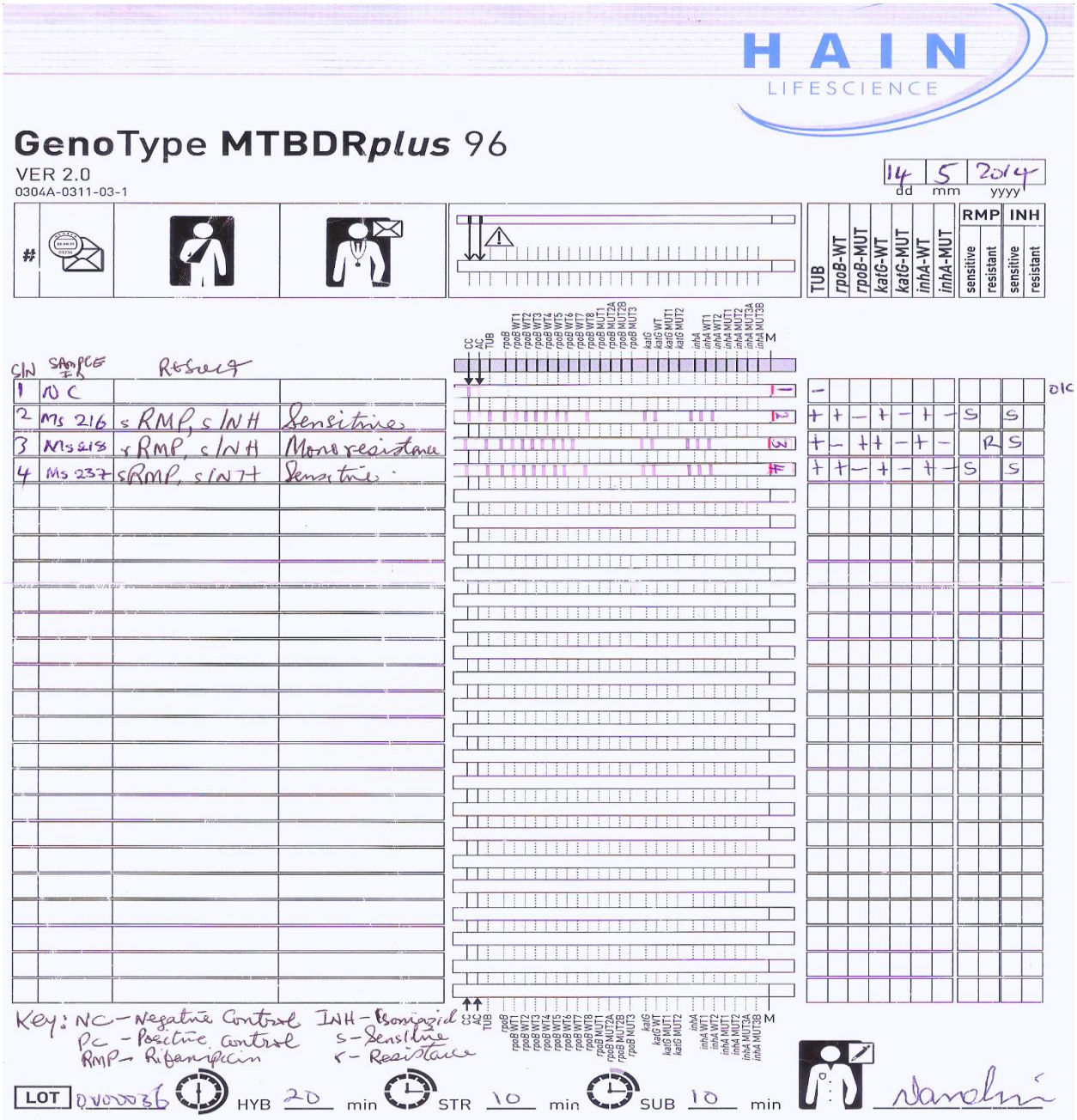
Appendix III: Patterns of mutations associated with rifampicin and isoniazid resistance in multidrug resistant strains detected by MTBDRplus



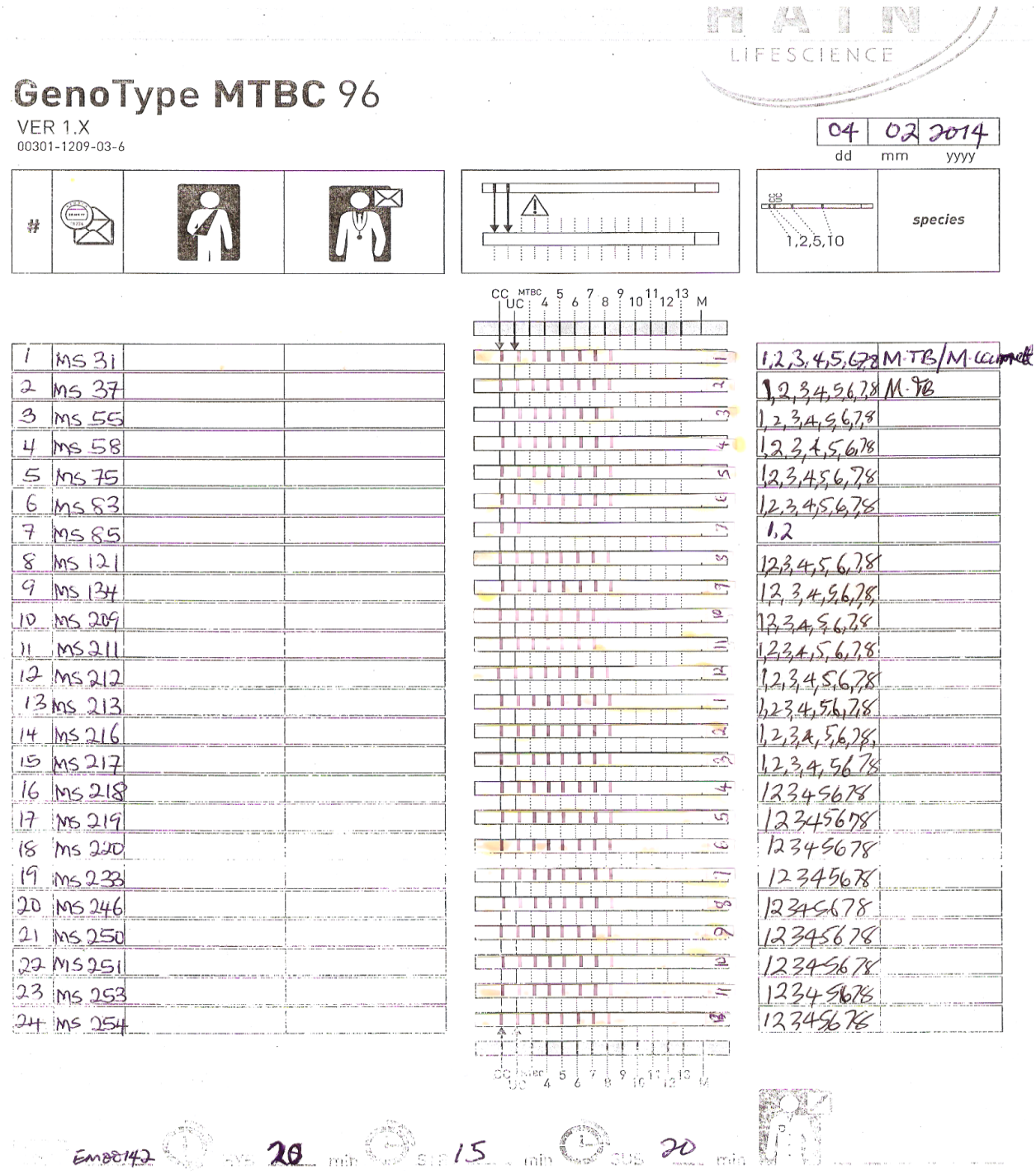
Appendix IV: Patterns of mutations associated with isoniazid mono-resistant strains detected by MTBDRplus



Appendix V: Patterns of mutations associated with rifampicin mono-resistant strains detected by MTBDRplus



Appendix VI: Patterns obtained with the Genotype MTBC assay of species of MTBC



Appendix VII: Ethical approval by Kaduna State Ministry of Health

MINISTRY OF HEALTH, KADUNA STATE

All Communications to be Addressed to:
THE HON. COMMISSIONER
Quoting Reference and Data
Tel: (062) 248084
(062) 248252

Independence Way,
P.M.B. 2014,
Kaduna,
Kaduna State, Nigeria.

MOH/ADM/744/VOL.I **17th April, 2012**

The Head of Department
Microbiology,
Ahmadu Bello University
Zaria.

RE: APPLICATION FOR ETHICAL APPROVAL

I have been directed to convey the Honourable Commissioner's approval to **Muhammad Sani Aliyu**, a Ph.D student of the department of Microbiology A.B.U. Zaria to carry out a research on the topic **"Prevelance of Multi-Drug Resistant Mycobaterium tuberculosis (MDR-TB) in Kaduna State, Nigeria".**

2. A copy of his finding(s) is to be submitted to the Ministry please.

F.A. KURAH
Secretary, Ethical Committee.

CC:
The Medical Directors,
Barau Dikko Specialist Hospital, Kaduna

Hajiya Gambo Sawaba General Hospital, Zaria

General Hospital Kafanchan.

F.A. KURAH
Secretary, Ethical Committee.

Appendix VIII: Ethical approval by Ahmadu Bello University Teaching Hospital, Zaria

Ahmadu Bello University Teaching Hospital

P.M.B. 06, Shika - Zaria, Kaduna State, Nigeria. ☎:069-550098, 551399
Fax: 069-555001, E-Mail: macoabuth@yahoo.com

Chief Medical Director: PROF. ABDULMUMINI H. RAFINDADI, *MBBS, FMC-Path.*
Chairman, Medical Advisory Committee: DR. LAWAL KHALID, *MBBS, FMCS, FWACS, FRSC(ED), mni*
Director of Administration: BARR. ISHAK BELLO, *LL.B, BL, LL.M, PGDM, AHAN, FCAI*

Our Ref:

ABUTH/HREC/TRG/36

30th April, 2012.

Muhammad Sani Aliyu
Dept of Microbiology
,ABU Teaching Hospital
Zaria

ETHICAL CLEARANCE

Your application for ethical clearance on the research proposal titled**"PREVALENCE OF MULTI-DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS (MDR-TB) IN KADUNA STATE, NIGERIA. "** refers.

This is to convey ethical approval for you to commence the study. However, note that the committee has the prerogative to monitor the research process at any time from inception to conclusion.

Prof. J. U. Okpapi
Chairman, ABUTH HREC

Appendix IX: Research Ethics Clearance School of Post Graduate studies A.B.U., Zaria

SCHOOL OF POSTGRADUATE STUDIES
AHMADU BELLO UNIVERSITY ZARIA, NIGERIA

Vice-Chancellor: PROFESSOR ABDULLAHI MUSTAPHA, B.Sc. (Hons) Pharm. (A.B.U), Ph.D. (London), FPSN

Dean: PROFESSOR ADEBAYO A. JOSHUA, B.A., M.A., Ph.D. (ABU)

25th April 2011

The Principal
National Tuberculosis and Leprosy
Training Centre,
Saye, Zaria.

RESEARCH ETHICS COMMITTEE CLEARANCE FOR 'PREVALENCE OF MULTIDRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* IN KADUNA STATE

This is to certify that the Research topic titled 'Prevalence of Multidrug Resistant *Mycobacterium tuberculosis* in Kaduna State' by Muhammad Sani Aliyu (PhD. /Scie/01740/08-09) has been evaluated by the School of Postgraduate Studies Research Ethics Committee and has satisfied all the requirements.

The aforestated research is therefore approved for the PhD study by Mohammed Sale Aliyu (PhD. /Scie/01740/08-09)

Thanking you in anticipation of granting him all the necessary assistance.

Sincere Regards.

Prof. Zoaka A. HASSAN
(Chairman, SPGS Research Committee)

Telephone:069-891355; 891360 email: dean-pgschool@abu.edu.ng

Appendix X: Research questionnaire

RESEARCH QUESTIONNAIRE

(STRICTLY CONFIDENTIAL)
PREVALENCE OF MULTI-DRUG RESISTANT *MYCOBACTERIUM TUBERCULOSIS* (MDR-TB) IN KADUNA STATE, NIGERIA

QUESTIONNAIRE IDENTIFICATION NUMBER.....

Bio data:
Age ----------

Sex: M [ ] F [ ]

Level of education: None [ ] Primary [ ] Junior Secondary [ ] Senior Secondary [ ] Certificate/Diploma [ ] University [ ] Others [ ]

Ethnic group Hausa [ ] Fulani [ ] Yoruba [ ] Igbo [ ]

Other specify [ ]

Marital status: Married [ ] Single [ ] Divorced [ ]

Occupation ----------

Economic status High [ ] Medium [ ] Low [ ]

Have you ever smoked cigarettes in your life? Yes [ ] No [ ]

Have you ever drunk alcohol in your life? Yes [ ] No [ ]

Have you ever been diagnosed having any of the following diseases?

Diabetes Yes [ ] No [ ]

Hypertension Yes [ ] No [ ]

Asthma Yes [ ] No [ ]

Other specify [ ]

TB History:

Is there any TB patient in your family, place of work etc? Yes [ ] No [ ]

When were you diagnosed having TB? ----------Years, ---------- Months, ----------Wks

Have you ever had a BCG immunization? Yes [ ] No [ ]

Treatment:

Never treated Yes [ ] No [ ]

Treated previously Yes [ ] No [ ]

Completed treatment Yes [ ] No [ ]

If No why----------

Currently on treatment Yes [ ] No [ ]

HIV Status:

HIV positive Yes [ ] No [ ]

On ARV Yes [ ] No [ ]

Appendix XI: Informed Consent form (ICF)

DEPARTMENT OF MICROBIOLOGY
SCHOOL OF POSTGRADUATE STUDIES
AHMADU BELLO UNIVERSITY ZARIA NIGERIA

Head of Department: **Prof O.S. Olanitola ;** B.Sc, M.Sc, PhD

E-mail: microbiology@abu.edu.ng

INFORMED CONSENT FORM (ICF)

This Informed Consent Form is for patients who attend chest (TB) clinics in Kaduna state, Nigeria. We are inviting you to participate in this research work titled "Prevalence of Multidrug resistant *Mycobacteria tuberculosis* in Kaduna state, Nigeria".

Principal Investigator:	Muhammad Sani Aliyu
Collaborating Investigators:	Prof. O.S. Olanitola Prof. J.U. Okpapi Prof. I.A. Abdulkadir Dr. E.D. Jatau
Name of Organization:	Department of Microbiology Ahmadu Bello University, Zaria - Nigeria
Name of Sponsor:	Self
Name of Proposal:	PhD 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 **Muhammad Sani Aliyu** a postgraduate student of the Department of Microbiology Ahmadu Bello University, Zaria, carrying out a research work under the supervision of Prof O.S. Olanitola, Prof. J.U. Okpapi, Prof. I.A. Abdulkadir and Dr. E.D. Jatau. We are doing a research work on the Prevalence of Multidrug resistant *Mycobacteria tuberculosis* in Kaduna state, Nigeria. I am going to give you information and invite you to be part of this research. You do not have to decide 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 stop as we go through the information and I will take time to explain. If you have questions later, you can ask them of me, the study doctor or the staff.

Purpose of the research

The aim of the study is to determine the prevalence of MDR-TB in Kaduna state.

Participant selection

We are inviting all AFB positive sputum at the hospitals chest clinics.

.

- *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 cervical cancer, and we will tell you more about it later. You may change your mind later and stop participating even if you agreed earlier.

UNIFORM CONSENT

- *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

When you have agreed to participate in this research, we will take sputum from you.

Description of the Process

During the research you will make at least three visits to the clinic.

In the visits, a small amount of sputum will be collected from you in a container. 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 TB.

Duration

The research will take place over a period of twelve months. During that time, it will be necessary for you to come to the clinic at least three times.

- *How many times are we asking you to come to the hospital to participate in the research?.....*
- *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 tested for MDRTB.
- ✓ 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.

- *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 number on it instead 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.

INFORMED CONSENT

- *Do 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 community meetings 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 them now or later, even after the study has started. If you wish to ask questions later, you may contact any of the following:

Muhammad Sani Aliyu 08022360774

This proposal has been reviewed and approved by Scientific Ethical Committee (SEC) of the Ahmadu Bello University Teaching Hospital, 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, ABUTH, 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 language that I understand. I have also talked it over with the doctor 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:

1. *Sputum sample will be taken*

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

INFORMED CONSENT