PREVALENCE AND DETECTION OF DRUG RESISTANT MUTATIONS IN
MYCOBACTERIUM TUBERCULOSIS AMONG PATIENTS VISITING SELECTED
HEALTH CENTRES IN NAIROBI, KENYA

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Award of the
Degree of Master of Science (Medical Microbiology) in the School of Pure and Applied
Sciences of Kenyatta University

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DECLARATION

This is my original work and has not been presented for a degree or any other award in any other university or any other institution of higher learning.

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DEDICATION

This work is sincerely dedicated to everyone who supported me in every step of this journey.
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TABLE OF CONTENTS

DECLARATION.......................................................................................... ii
DEDICATION............................................................................................ iii
ACKNOWLEDGMENTS............................................................................... iv
TABLE OF CONTENTS............................................................................. v
LIST OF FIGURES..................................................................................... viii
LIST OF TABLES......................................................................................... ix
LIST OF APPENDICES.............................................................................. x
ACRONYMS AND ABBREVIATIONS........................................................ xi
ABSTRACT.............................................................................................. xiii
CHAPTER ONE .......................................................................................... 1
INTRODUCTION.......................................................................................... 1
1.1 Background Information...................................................................... 1
1.2 Statement of the Problem................................................................. 3
1.3 Research hypotheses.......................................................................... 4
1.4 Objectives.......................................................................................... 4
   1.4.1 General objective........................................................................ 4
   1.4.2 Specific objectives...................................................................... 4
1.5 Justification and Significance of the Study........................................ 5
CHAPTER TWO .......................................................................................... 7
LITERATURE REVIEW............................................................................... 7
2.1 Tuberculosis....................................................................................... 7
2.2 *Mycobacterium tuberculosis*............................................................ 8
2.3 Pathology and pathogenesis of TB.................................................... 10
2.4 Diagnosis of TB................................................................................ 14
   2.4.1 Conventional methods for laboratory diagnosis....................... 14
   2.4.2 Molecular methods for laboratory diagnosis............................. 15
2.5 Treatment of TB................................................................................ 15
2.6 Drug resistance................................................................................ 17
2.7 Mutations......................................................................................... 18
2.8 Mode of Action and Evolution of drug resistance............................ 19
   2.8.1 Resistance to first line drugs..................................................... 19
5.3 RECOMMENDATIONS .................................................................................. 51
REFERENCES ........................................................................................................ 52
APPENDICE ........................................................................................................... 59
LIST OF FIGURES

Figure 2.1: Shows drug targets in the cell wall, cytoplasmic membrane and cytoplasm of Mycobacterium tuberculosis for Isoniazid, Rifampicin and Pyrazinamide…. 20

Table 2.1: Estimated WHO Regional TB statistics for 2016............................... 24

Table 2.2: WHO statistics for TB in “High Burden” African Regions 2016............. 25

Figure 3.1: Map of Kenya showing the Study Area in Nairobi county..................... 29

Figure 4.1: Demographic characteristics of 132 eligible participants....................... 38

Figure 4.2: Notified TB cases by age group and hospital...................................... 39

Figure 4.3: Interpreted DNA strips after hybridization................................. 42

Figure 4.4: Prevalence of mutations in the resistance-determining region (MUT) of the various gene loci (katG, rpoB, gyrA, gyrB, rrs, eis)............................ 44
LIST OF TABLES

Table 2.1: Estimated WHO Regional TB statistics for 2016........................................24

Table 2.2: WHO statistics for TB in “High Burden” African Regions 2016..................25

Table 4.1: Drug susceptibility profile of the M. tuberculosis isolates in the present study....41

Table 4.2: Mutations in rpoB, KatG and rrs genes and the corresponding wild type and
mutation bands according to (Telenti et al., 1993).............................................. 43
LIST OF APPENDICES

Appendix 1: Photos of MTBDRplus DNA strip technology product and interpretation...59
Appendix 2: Photo of MTBDRsl DNA strip technology product..........................61
Appendix 3: The IUATLD scale.................................................................62
Appendix 4: Research approval for the study.............................................63
# ACRONYMS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>AECs</td>
<td>Airway epithelial cells</td>
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<tr>
<td>AFB</td>
<td>Acid Fast Bacilli</td>
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<tr>
<td>AG/CP</td>
<td>Aminoglycosides/Capreomycin</td>
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<td>AMs</td>
<td>Alveolar macrophages</td>
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<tr>
<td>BCG</td>
<td>Bacille-Calmette-Guerin</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DOTS</td>
<td>Directly Observed Treatment, Short Course, Internationally agreed strategy for TB control</td>
</tr>
<tr>
<td>DS</td>
<td>Drug Sensitive</td>
</tr>
<tr>
<td>DST</td>
<td>Drug Susceptibility Test</td>
</tr>
<tr>
<td>EMB</td>
<td>Ethambutol</td>
</tr>
<tr>
<td>GCP</td>
<td>Good Clinical Practice</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HRZE</td>
<td>Rifampicin plus isoniazid plus pyrazinamide plus ethambutol</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>LPAs</td>
<td>Line Probe Assays</td>
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<tr>
<td>MAIT</td>
<td>Mucosal-associated invariant T cells</td>
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<tr>
<td>MDR</td>
<td>Multi-Drug Resistant</td>
</tr>
<tr>
<td>MGIT</td>
<td>Mycobacteria growth indicator tube</td>
</tr>
<tr>
<td>MTB</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>MUT</td>
<td>Mutation</td>
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</table>
NALC  Nalidixic acid

NKs  Natural killer cells

PZA  Pyrazinamide

POA  Pyrazinoic acid

PRRs  Pattern recognition receptors

RIF  Rifampicin

RNA  Ribonucleic acid

STAND  Shortening treatment for advanced novel drugs

STR  Streptomycin

TB  Tuberculosis

TLRs  Toll-like receptors

WHO  World Health Organization

WT  Wild type

XDR  Extensively drug-resistant

ZN  Ziehl-Neelsen
ABSTRACT

Tuberculosis (TB), an ancient scourge of humanity known for several thousands of years, is still a main public health challenge in many countries today even though some progress has been made recently in curbing the disease. Deferred diagnosis, poor treatment regimens and fatality qualify drug-resistant and multi-drug resistant tuberculosis (MDR TB). Unlike other bacterial pathogens, Mycobacterium tuberculosis lacks plasmid mediated mechanisms of resistance. Drug resistance in M. tuberculosis is entirely due to chromosomal changes that occur within the genes, for instance deletions or mutations. These chromosomal modifications influence the drug target or bacterial enzymes that are responsible for activating or customizing the drug. Inadequate or incomplete chemotherapy also enables M. tuberculosis to become drug resistant due to mutants that exist by nature in the mycobacterial community. Since the frequency of mutations vary geographically and limited data is available on the patterns of gene mutation in Kenya, insight of the rates of geographic specific mutations can enable the evolution of internal, PCR-based techniques for targeting mutations relevant in a specific setting. In addition to crafting new molecular biology-based techniques for the examination of MDR tuberculosis and advancement of new drugs, heightened rates of MDR and extremely-drug resistant tuberculosis (XDR TB) worldwide need to be controlled and profiled. Therefore, the main objective of this study was to evaluate the prevalence and detection of drug resistant mutations in Mycobacterium tuberculosis among patients visiting selected health centers in Nairobi, Kenya. The current study employed a cross-sectional study design which involved collecting sputum samples from 132 patients visiting Mbagathi district hospital and Chandaria community hospital. Of the 132 patients, male subjects were seventy-two (54%), while women were 60 (46%), all averaging 35 years and having a range of 18 to 60 years. Overall, 132 sputum samples were collected from patients who were all smear-positive for pulmonary TB and included for processing. Sample processing involved decontamination of collected sputum samples with NALC/NaOH/sodium citrate, thereafter, direct sputum smear microscopy using Ziehl-Neelsen stain for Acid Fast Bacteria (AFB). DNA was then extracted from the positive samples and analyzed by means of Genotype MTBDRplus (Rapid test for rifampicin and/or isoniazid resistance) and Genotype MTBDRsl (Rapid test for fluoroquinolones, aminoglycosides, and low-level kanamycin resistance in addition to confirmation of M. tuberculosis). Data analysis was carried out using chi-square test to analyze the difference in mutations detected. In total, the prevalence for the resistance to first and second-line TB drugs was 1.5% (2/132). Rifampicin resistant strain had the rpoB mutation S531L; isoniazid resistant strains had the mutations katG S315T; AG/CP resistant strains had the rrs mutation G1484T. The molecular analysis indicates confirmation of the transmission of resistant strain. The data suggested that there is homogeneity when it comes to the type of drug resistance and mutation that occurs in the region. This calls for intensified drug resistance surveillance and drug adherence among patients infected with TB. In addition, there is an urgent need for randomized controlled trials to discover the most effective treatment regimen for managing INH resistant TB.
CHAPTER ONE

INTRODUCTION

1.1 Background Information

Although some progress has been made in recent years in controlling tuberculosis (TB) globally, TB has remained a persistent problem in the developing countries of Africa, Asia and South America. In Kenya according to the WHO in 2014, since 2007, a constant drop in figures of TB cases had occurred (116,000 cases), reaching an all-time low of 89,000 in 2013. However, according to recent survey that was carried out from July 2015 to 2016 by the Ministry of Health, results showed that there are 558 people with the disease among 100,000 people revealing a higher TB burden in Kenya than previously thought (Ministry of Health, 2017). TB is among the top three killer infectious diseases, and there is increased TB in the world presently than at any other moment in the past (Todar, 2012). The current TB problem is as a result of the negligence on prompt diagnosis, meager access and framework, indigent patient compliance to medication, inadequate treatment regimens, destitute management of TB control programs, migration, poverty, population growth, and a substantial increment in the number of TB/HIV cross-infection cases (Walley, 1997).

A comprehensive framework that adheres to five key components is essential to the success in treatment of TB patients. The key components are: treatment regimen that are standardized for six to eight months, a regular recording and reporting system, standard continuous supply of all vital TB drugs, incidence detection by sputum smear microscopy, and dedication by the government when it comes to laying out strategies and frameworks to help curb the disease. These five paramount factors are the suggested strategy by the World Health Organization (WHO) to curb TB and referred to as the DOTS approach (Walley, 1997). The entire operation of the directly observed treatment, short course (DOTS) approach is becoming
increasingly laborious in the developing countries of the world that are also struggling to manage the HIV epidemic. Because of indigent treatment adherence, drug resistance is becoming frequent and concern of an epidemic with basically un treatable strains of TB are rising (WHO, 2008).

Unlike other bacterial pathogens, Mycobacterium tuberculosis lacks plasmid-mediated mechanisms of resistance, however, drug resistance is solely acquired because of chromosomal modification such as deletions or mutations (Kassim & Ray, 2004). These alterations affect either the bacterial enzymes modifying or activating the drug or drug target itself (Donald & Van, 2011). Inadequate or incomplete chemotherapy enables M. tuberculosis to become drug resistant due to resistant mutants occurring typically in the mycobacterial population (Chiang, 2010). Mutants resistant to an individual drug develop roughly in every $10^6$ to $10^8$ mycobacterial cells (Donald & Van, 2011). Mono-resistant M. tuberculosis is an isolate resistant to only one drug, whereas multi-drug resistant (MDR) M. tuberculosis is the in-vitro resistance to at least rifampicin (RIF) and isoniazid (INH) which are the two main first line anti-TB drugs. Extensively drug resistant (XDR) on the other hand involves MDR plus resistance to fluoroquinolones and at least one of the three second line injectable drugs (Raviglione, 2006).

Mutations in the rpoB gene are linked to RIF resistance and are within an 81-bp region off the gene comprising codons 507 to 533 (Caminero, 2013). A high level of resistance to INH is coupled with mutations in the KatG gene, while low levels of INH resistance are related to mutations in inhA promoter region (Talenti et al., 2001). Mutations linked with resistance to low-level Kanamycin occur in the eis gene (Slayden & Barry, 2000). Resistance to Kanamycin, Amikacin, and Capreomycin is linked to mutations in the 16S rRNA gene rrs, notably at nucleotide positions 1484, 1401 and 1402 (Alangaden et al., 1998). Intervention and prospective analysis to manage dissemination can be exceptionally beneficial in regions with
continuing spread of drug resistant strains. As indicated of late by mathematical modeling, the onus of containing MDR-TB is difficult in the absence of explicit endeavor to minimize transmission (Donald & Van, 2011). In general, detection of drug-resistant MTB based on culture and drug susceptibility testing is strenuous and takes many weeks to months to accomplish. *M. tuberculosis* is a very slow thriving organism and slow diagnosis can be a major cause of MDR-TB. Molecular methods used for the detection of mutations in resistance-inducing genes offers a better channel for rapid screening of *M. tuberculosis* isolates for resistance to antibiotics. Rapid diagnostic assays are therefore essential for controlling drug-resistant TB. The World Health Organization has recommended Genotype MTBDRplus and Gene Xpert MTB/RIF as some of the assays for use in the fast detection of drug resistant TB (WHO, 2008).

According to Hain life Science (n.d), the Genotype MTBDRplus and Genotype MTBDRsl tests rely on a DNA-STRIP technology that grants molecular detection of *M. tuberculosis* complex and resistance to Isoniazid and/or Rifampicin (MTBDRplus) as well as Fluoroquinolones, and Aminoglycosides (MTBDRsl). Although the assays are confined to detection of known mutations, the high accordance rate with ordinary methods and the speedy time to outcome make the MTBDRplus and MTBDRsl assays effective tests for the examination and control of MDR tuberculosis (WHO, 2014).

### 1.2 Statement of the Problem

Tuberculosis (TB) remains one highly communicable disease worldwide. An estimated 9.0 million were detected with TB in 2013, of which 1.5 million perished from the disease and 360 000 of them being HIV infected (WHO, 2015). From these cases, greater than half (56%) were from the South-East Asia and Western Pacific locality. In addition, from the reports a quarter of the total cases were from the African Region, which as compared to population in this region had the topmost rate of cases and deaths (WHO, 2014). With continued increased
morbidity cases, world proportion of newly infected with multidrug-resistant TB (MDR-TB) could increase despite constant reported rates of 3.5 % since 2013. Nevertheless, much increased levels of resistance and inadequate treatment issues are of primary interest in few areas of the world. Throughout the world, 3.5 % of new and 20.5 % of formerly treated TB incidences in 2013 must have had MDR-TB (WHO, 2014). In Kenya since 2007, a constant drop in figures of TB cases has occurred (116,000 cases), reaching an all-time low of 89,000 in 2013 (WHO, 2014). However, data on TB prevalence and mortality are sparse in Kenya considering the last TB prevalence survey conducted was in 1956 (NTLD, 2014).

1.3 Research hypotheses

i. There is a difference in prevalence of first and second line drug resistant mutations in relation to *M. tuberculosis* in Nairobi, Kenya.

ii. There is a difference in detected first and second line drug resistant mutations in relation to *M. tuberculosis* in Nairobi, Kenya.

1.4 Objectives

1.4.1 General objective

Determine the prevalence and detect drug resistant mutations in *M. tuberculosis* among patients in Nairobi, Kenya.

1.4.2 Specific objectives

i. To determine the prevalence of drug resistant *M. tuberculosis* among patients visiting various health facilities in Nairobi.

ii. To ascertain the number of MDR cases in newly infected individuals.

iii. To determine *M. tuberculosis* drug resistant mutations among patients on first and second-line drugs in Nairobi.
1.5 Justification and Significance of the Study

Drug-resistant and MDR-TB is characterized by mortality, inadequate treatment regimens and delayed diagnosis. Implementations of strategies such as the DOTS is essential to establish that all subjects are diagnosed and treated efficaciously to prevent occurrence and spread of resistant strains in the population. However, some of the present strategies have limitations. For instance, sputum smear microscopy is useful to detect the main sources of infection but only helps diagnosis of about half of all TB cases, culture is more sensitive but sluggish to produce results, and six to eight months is still a long time to maintain a patient on regular treatment (Raviglione, 2006). In addition, considering that implementing culture-based DST in areas that are resource-constrained is complex, developing countries may be well suited for a speedy rollout of molecular-based drug resistance testing to undertake the development and spread of drug resistant strains of *M. tuberculosis* (Raviglione, 2006).

A specific and rapid diagnosis is crucial not only for the particular subject but also from the community aspect, considering swift alterations to an efficient drug remedy would minimize the transmission of TB in the population (Sterling *et al*., 2003). From a clinical perspective, patients who are treated with drugs to which they are not susceptible remain infectious since the existent growth-based diagnostics tend to expose too little, too late. On the other hand, patients with HIV and XDR-TB often kick the bucket before diagnosis. Concerning the need to establish novel anti-TB drugs and regarding the aspects of TB transmission, there is need to explore more on drug resistance mutations and their global distribution. Since the frequency of mutations vary geographically, there is limited data available on the patterns of gene mutation in Kenya. Having the know-how in relation to prevalence of geographic specific mutations can allow the establishment of in-house, PCR-based methods for targeting mutations relevant in a specific setting. In addition, data also helps advice in the event of developing new
molecular biology-based techniques that can be used in diagnosing multi-drug resistant tuberculosis and development of novel drugs, something desperately needed, when considering the increasing rates of MDR and XDR-TB worldwide.

Use of rapid techniques to disrupt ongoing transmission assists in systematic surveillance and tracking of drug-resistant TB. This eventually helps in comprehending the overall burden of the disease and hence a need for an improved practice in diagnosis, treatment, and infection control.

The findings of this study will provide a scope of some of the mutations linked with drug resistance to both first line and second line TB drugs. In addition to the drugs that have high resistant levels. Data will be used in TB patients’ management and control in the country, this will help curb and stop development of MDR or XDR cases that may lead to deaths. Subsequently, the data collected can also be used in developing applicable screening tests for patients in the region. For the researcher, the study will help them focus on ways of coming up with new methods to tackle the most common mutations causing drug resistance.
CHAPTER TWO

LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis has a long, rich history that dates to Ancient Egypt, with proof of its existence found in the conserved spines of mummified Egyptians. In the 18th and 19th centuries, before the revelation of the microbial causes of tuberculosis by Robert Koch in 1882, a tuberculosis epidemic stormed throughout North America and Europe (Nerlich et al., 1997). Following his discovery, the evolution of efficient drug treatment and vaccines resulted to the notion that the disease was nearly overcome. Certainly, at a particular stage the United Nations assumed the disease would be eradicated all over the world by the year 2025. However, this was not to be the case, since incidences of TB began to increase again in the United States and worldwide in the mid-80s. This led to declaration by the World Health Organization in 1993 that TB was a worldwide exigence; making it the first time to label a disease as such. Luckily, almost all cases of tuberculosis can be cured with proper treatment. Without it, almost two-thirds of people infected with TB normally perish (McIntosh, 2015).

While most people are able to overcome initial infections with no symptoms, they may from time to time develop a dry cough, fever, and irregularities that may be observed under an X-ray. This is classified as primary pulmonary tuberculosis (Ismail & Ray, 2004). Pulmonary tuberculosis often disappears on its own, but in more than half of the situations, relapse might occur. In a minority of immunocompromised people, Mycobacteria may dissipate via the blood to other body parts. This is known as miliary tuberculosis and symptoms include weakness, fever, loss of weight and appetite. Difficulty in breathing and coughs are rare. These symptoms however, can vary accordingly (Taylor et al., 1994). Lack of treatment may cause the disease to disseminate to other body parts via the bloodstream; joint destruction and spinal pain may occur if TB infects the bones, infection of the brain by
TB can lead to meningitis, infection of the kidneys and liver by the disease can lead to impairment of their waste filtration systems and cause blood to appear in urine, the heart's capability of pumping blood can be impaired if TB infects the heart, leading to a case called cardiac tamponade which may be disastrous (Schaaf et al., 2009).

Tuberculosis is the second largest killer arising from an individual infectious agent all over the world, and in 2012, the disease claimed 1.3 million lives with the number of infected being 8.6 million (WHO, 2014). Two distinct types of tuberculosis infection are known according to doctors: active and latent. In the latter, the Mycobacterium lingers in the body in a dormant state. In latent TB, symptoms are not observed, and the Mycobacterium are not infectious. However, they may become effective. Active TB is characterized by symptoms and is contagious (McIntosh, 2015). It's believed that one third of the world's population possess dormant TB. Chances of lurking TB evolving into active TB is estimated to be at 10%. However, the risk is greater in immunosuppressed people: for instance, those malnourished or living with HIV, or smokers (WHO, 2014). All age groups are affected by TB worldwide. Tuberculosis is however noted to affect mostly young persons, and those staying in underdeveloped countries. In 2012, there were reports of 80% of TB cases occurring in 22 countries alone (WHO, 2014).

2.2 Mycobacterium tuberculosis

Tuberculosis is an illness that arises due to infection from the bacteria “Mycobacterium tuberculosis.” The bacterium is rod shaped, non-endospore forming and is part of the phylum and class Actinobacteria, order Actinomycetales, family Mycobacteriaceae and the genus Mycobacterium (Schaaf, 2009). The mycobacteria have a width of 0.2 to 0.5 μm and a length of 2 to 4 micrometers (μm). There are many species of bacteria among the Mycobacterium genus. Species that are non-causative agents of tuberculosis or leprosy are
included in the Non-Tuberculosis Mycobacteria group (Todar, 2012). These species are often called “environmental mycobacteria” because they are opportunistic pathogens (Todar, 2012). They are usually transmitted to a human through exposure of the environment (no human contact). The most well-known species are *M. xenopi, M. avium, M. chelonae, M. kansasii, M. fortuitum,* and *M. abscessus* (O’Neill et al., 2015).

The pathogenic species are *M. tuberculosis* and *M. leprae. Mycobacterium tuberculosis* is an obligate aerobe and establishes a complex with other higher similar bacteria called the *M. tuberculosis* complex (O’Neill et al., 2015). The complex consists of six mycobacteria: *Mycobacterium africanum* and *Mycobacterium tuberculosis,* known to affect humans; *Mycobacterium bovis,* which affects other mammalian species in addition to humans; *Mycobacterium microti,* which infects vole; *Mycobacterium canettii,* which also infects humans and *M. bovis BCG,* a *Mycobacterium bovis* variant (Ismael & Ray, 2004). Considering this reason, when it comes to tuberculosis, MTB complexes are constantly found in areas well supplied with oxygen, mainly the upper lobes of the lungs. Mycobacteria are facultative intracellular parasites, frequently of macrophages, and have a gradual generation time usually around 15-20 hours. In addition, they possess physiological attributes that can contribute to their virulence (Todar, 2012).

Tuberculosis predominantly infects the lungs (pulmonary TB). Subjects with active pulmonary TB normally have symptoms which include: a cough, an unusual chest X-ray, and are contagious. Tuberculosis can also be extrapulmonary (occurring in different parts of the body), often in the lymphatic, genitourinary or central nervous systems. It may also occur in the joints and bones (Boshoff et al., 2005). Miliary TB is tuberculosis that occurs scattered throughout the body. Extra pulmonary TB is more typical in immunocompromised individuals and in young children (Frieden et al., 2003).
2.3 Pathology and pathogenesis of TB

Tuberculosis infections usually commence when aerosol droplets consisting of *M. tuberculosis* directly coughed or spat from a person with pulmonary TB are inhaled (Sakamoto, 2012). The infective dose for an individual is estimated to be within 1 and 200 bacilli. Nevertheless, since a single aerosol droplet may have a range of 1 to 400 bacilli, it’s ill-defined what’s thought out to be a biologically appropriate dose (Handzel, 2013).

Once inhaled into the lung the mycobacteria travel along the trachea, bronchus and bronchioles into the alveoli. Respiratory mucosa lines the airway. This is made up of a layer of airway epithelial cells that curb *Mtb* from infecting the tissue by providing a tight barrier and they also consist of many receptors to detect *Mtb*. Airway epithelial cells curb the constitution of airway surface liquid, an entity containing antibodies, anti-microbial peptides, mucus and chemokines/ cytokines. The epithelium is supported by the lamina propria and also known to contain immune cells such as mucosal-associated invariant T cells (MAIT) and macrophages that react to infection. *Mtb* sooner or later reach the alveolae, which are encircled by a rich capillary network that aid in gaseous exchange (Middleton et al., 2002). The alveolus is fundamentally constituted of type I and type II epithelial cells which are located at the cell junctions. Type II epithelial cells secrete a diversity of anti-microbial elements for instance pulmonary surfactant. Dendritic cells (DCs) and alveolar macrophages (AMs) are the initial defense system of the alveolus. These cells are efficient phagocytes and have a scope of inherent anti-microbial capabilities. Further, natural killer cells (NKs) and neutrophils are enlisted from the encircling capillaries to strengthen the host defense (Middleton et al., 2002).

Upon entry in the alveoli, the bacilli are expeditiously phagocytosed by alveolar macrophages. Ligation of toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) agitate these macrophages which eventually bring about proinflammatory chemokines and cytokines. Ultimately propelling the enrollment of more leukocytes to the infected area.
Monocytes and neutrophils appear first, phagocytose extra bacilli, release more chemokines and cytokines, and commence formation of an early granuloma (Barry, 1994). Dendritic cells also engulf Mtb then move to regional lymph nodes where lymphocytes are presented with mycobacterial antigens (Taylor et al., 1994).

A well-formulated granuloma arises ultimately comprising of central, infected macrophages, enclosed by epithelioid macrophages, foam cells, and periodically multinucleated enormous cells of the Langhans type, with incidental fibrous capsule and recruited lymphocytes (Subbian et al., 2011). The structure formed is a well-defined harmony in relation to host supervision of disease and safeguard of Mtb from lymphocytes producing IFN-γ (Middleton et al., 2002). As time elapses, necrosis occurs in the middle of the granuloma, and the immense protein and lipid composition of the lifeless macrophages leads to development of a caseous appearance, both grossly and histologically (Sakamoto, 2012). Due to the persistent presence of immunostimulatory lipids and antigens, a delayed-type hypersensitivity conserves the granuloma. This allows Mtb to exist inside macrophages and extracellularly inside the granuloma (Subbian et al., 2011). The caseum is essentially comprised of host-derived lipids. Recently, gene regulation analysis in the directly neighboring cells display Mtb driven dysregulation of host lipid metabolism (Kim et al., 2010). By utilizing fatty acids in their metabolism, Mtb can adapt to this environment hence reduce active replication, increase cell wall thickness and finally enter an alleged dormant state (Boshoff and Barry, 2005).

The lesion that develops at the principal location of implantation is called the Ghon focus (Kim et al., 2010). Mycobacteria can disseminate before the development of these formulated granulomas through the lymphatics to regional lymph nodes, leading to granulomatous lymphadenitis and lymphangitis, which, besides the Ghon focus, is termed the primary Ranke’s complex (Sakamoto, 2012). Hematogenous spread inside the lung or to other
organs can also develop during the initial stage of infection with TB. Intriguingly, the upper lung lobes of the human support bacillary proliferation considering delayed immune responses and higher oxygen pressure (Mohapatra & Janmeja, 2009).

Despite extensive spreading of \textit{Mtb} during initial infection, most affected, but otherwise healthy, individuals cope with these lesions without having any symptoms (Barry, 1994). Roughly 10\% of infected persons nevertheless end up having serious, life-threatening TB or fundamental gradual TB at this point (Ismail, 2004). This is associated with increase in bacterial load, immunosuppression, rise in bacterial virulence, or genetic susceptibility (Gupta \textit{et al.}, 2012). In many instances however, the primary infection dwindles, and the collagen capsule compresses to create a scar, or the granuloma calcifies. It is captivating to learn that a great number of TB subjects have pulmonary lesions with heterogeneous structure, (Kaplan \textit{et al.}, 2003) implying that every granuloma consists of mycobacteria at various points of inactivity and revival (Fenhalls \textit{et al.}, 2000). Growth of \textit{Mtb} during chronic lesions is mostly in persistent granulomas that have cavitated and entered an airway or in foamy macrophages at the fringe of lesions (Freiden \textit{et al.}, 2003).

Lack of continuous disease in an infected subject may stay without symptoms for many years, even decades, with mycobacteria surviving in a not well comprehended “dormant” state, apparently monitored by the host immune system (Barry, 1994). The capability of \textit{Mtb} to develop an incessant asymptomatic infection, succeeded by revival and spread in years later to new uninfected individuals, contributes to its amazing prosperity as a pathogen. Additional conditions influencing the immune system for instance HIV or alternative infections, malnutrition, old age, immunosuppressive medication and malignant disease may lead to relapse or secondary disease (Verver \textit{et al.}, 2005).

For reasons that are not understood to date, it is noted that around 5\% to 10\% of latently infected individuals at a stage in their life develop secondary disease (Tufariello \textit{et al.}, 2003). In
industrialized countries for instance, these individuals account for 80% of clinical TB cases and almost all cases of transmission (Tufariello et al., 2003). Interestingly, *Mtb* may persistently infect extrapulmonary tissues or extralesional (Neyrolles et al., 2006). It is a well-known fact that almost 15% of TB revival cases takes place at extrapulmonary locations, like the skin, central nervous system, genitourinary tract, and internal organs in absence of occurring lung lesions (De Backer et al., 2006). Occurrence of reactivation, mostly whether “latent” bacilli become propagative or if reactivation is due to bacilli from other sites are questions that remain unanswered.

The commonest form of secondary tuberculosis is generally confined to the lung, and lesions appear as an exudative bronchopneumonia and develop to classical caseous granuloma structure, succeeded by extensive necrosis and cavity formation (Schaff, 2009). Ultimately, the fibrous capsule crumbles and this enables contact with airways. Such incidents enable fast growth of extracellular bacilli and dissemination into airways, resulting in transmission or intrapulmonary dissemination (Handzel, 2013). Analysis of sputum at this stage of TB affirms increase in neutrophils and macrophages (Boshoff & Barry, 2005).

In most cases, symptoms of primary TB are usually elusive and discounted without difficulty. Secondary incidences are defined by localized symptoms, for instance pleuritic pain, hemoptysis, and coughing, in addition to the usual symptoms of night sweats, fever, anorexia, and cachexia (Barry, 1994). The ability of TB to result in cachexia on patients earned it the name *consumption*. In immunocompromised subjects, secondary TB may lead to spread and death in 50% of instances and chronic illness in 25% to 30% of instances (Sakamoto, 2012). If an infected individual can restore immune management over the disease, there is potential for recovery, as is observed in 20% to 25% of cases (Handzel, 2013).
2.4 Diagnosis of TB

2.4.1 Conventional methods for laboratory diagnosis

Microscopy is one of the diagnostic tests used for TB illness and was established more than a century ago (WHO, 2016). Clinical specimens in this case should be representative of the lesion and not be contaminated. Origin of the specimen should mainly be from the respiratory system. After the sample is prepared, for instance through decontamination, staining and microscopy take place. Staining can either be acid-fast (Ziehl-Neelsen) or fluorescent (auramine or rodamine). In this test, specimen is examined under a light microscope to confirm presence of mycobacteria. At least 1 to 9 acid fast bacilli per 100 fields should be present in a sample for a positive result. A positive result will confirm a clinical diagnosis; however, a negative result does not exclude the disease. This technique is non-specific and M. tuberculosis can’t be differentiated from atypical mycobacteria. Atypical bacteria here defined as mycobacteria other than M. tuberculosis complex. Microscopy is nevertheless the fastest and cheapest method of detection and provides information on a patient’s clinical response to treatment. In the present case definitions suggested by the world health organization, a single positive outcome is mandatory for the diagnosis of smear-positive pulmonary TB.

Culture methods are also used for diagnosis and are performed using solid or liquid media. Examples include Lowenstein-Jensen or Middlebrook 7H9 respectively. These methods are known to have high sensitivity and specificity. A positive result in cultures can be observed from week one for rapidly growing mycobacteria, while others may occur five to six weeks later. Final evaluation of cultures is done based on the number of colonies that develop. Use of rapid molecular tests is also allowed.
2.4.2 Molecular methods for laboratory diagnosis

Currently, the Gene Xpert MTB/RIF assay (which concurrently test for TB and resistance to rifampicin) is the main rapid test being widely used and approved for diagnosing TB by the WHO (Cepheid, Sunnyvale USA). It was first endorsed for diagnosis of pulmonary TB in adults in 2010. Subsequently in 2013, it was also approved for diagnosing children and certain forms of extrapulmonary TB. As compared to microscopy and culture methods, the test has much better accuracy (Helb et al., 2010). The current reference standards are microscopy and culture methods. However, results may take up to 12 weeks to acquire and they require more developed laboratory capacity (WHO, 2016).

Other tests are also available for checking TB resistant to first and second-line anti-TB drugs. Such tests include the line probe assays (LPAs) that test for isoniazid and rifampicin resistance (known as first-line LPAs); a rapid line probe assay that tests for resistance to injectable anti-TB drugs and fluoroquinolone resistance (known as a second-line LPA); and sequencing technologies (Helb et al., 2010). The WHO first recommended the use of first-line LPAs in 2008 and years later, approval made for the use of second-line LPA in May 2016. Currently, the gold standard for drug susceptibility testing is the utilization of culture-based approach. Lack of treatment results in high death rates from TB. Data from the natural history of TB illness claim that, lack of treatment with anti-TB drugs (performed before availability of drug treatments) disclosed that roughly 70% of those with sputum smear-positive pulmonary TB perished within 10 years, as did around 20% of those with culture-positive (but smear-negative) pulmonary TB (WHO, 2016).

2.5 Treatment of TB

Development of effective drug treatments commenced in the 1940s. Currently, a 6-month regimen of four first-line drugs: rifampicin, isoniazid, pyrazinamide and ethambutol are
approved treatment for new cases of drug-susceptible TB. Unfortunately, this first line drug therapy often falls short of curing *Mtb* for various reasons. Dissemination of TB and relapse leads to the development of drug resistant mycobacteria. Drug resistant TB is of great worry and needs the use of second-line drugs which are extremely toxic, hard to come by and expensive as opposed to first line drugs. The second-line anti-TB drugs are sub divided into two; Fluoroquinolones (Levofloxacin, Ofloxacin, ciprofloxacin and Moxifloxacin) and injectable anti-TB drugs (Kanamycin, capreomycin and amikacin). Treating rifampicin-resistant TB cases (RR-TB) and multidrug-resistant TB cases (MDR-TB) is lengthy and needs use of second line drugs. The treatment regimens approved by WHO generally persevered for 20 months until early 2016. A standardized shorter MDR-TB regimen of 9–12 months is presently approved for all patients (apart from pregnant women) with pulmonary RR-TB/MDR susceptible to second-line drugs. Current treatment for XDR-TB requires the assembly of a regimen of six drugs, including a six-month phase of treatment that includes injectable drugs, and a further 12-18 months of treatment with five drugs (Tang *et al*., 2015). Some of the drugs used in the treatment of MDR-TB and XDR-TB have serious side-effects, including hearing loss and kidney damage in the case of several injectable drugs. Some drugs used in the regimen are costly and unavailable in some countries, further complicating the assembly of an effective regimen.

Novel TB drugs have begun to materialize, and clinical trials have now begun testing a combination of regimens that include new compounds. One widely used form of treatment is the Bacille-Calmette-Guérin (BCG) vaccine. The BCG vaccine was established near a century ago and has displayed promise in preventing serious forms of TB in children. Nevertheless, there isn’t any vaccine now that is compelling in managing TB disease in adults, be it before or after risk of TB infection. Presently, there are 13 different TB vaccines in Phase I, Phase II or Phase III of clinical trials (WHO, 2016).
2.6 Drug resistance

Drug resistance in TB is interpreted bacteriologically as growth of *M. tuberculosis* on (or in) well-defined artificial nutrient media despite a specific concentration of one of the anti-TB drugs under carefully controlled laboratory conditions (Raviglione, 2006). Thus, drug-resistant TB is diagnosed exclusively in the laboratory. This broad definition does not specify which drug or how many drugs are ineffective at blocking mycobacterial growth in vitro. “Monoresistance” means that exactly one drug is ineffective. “Polyresistance” means that two or more drugs are ineffective (Chiang, 2010). However, neither term indicates which drugs. The term “multidrug-resistance” specifically refers to INH and RIF in the presence or not of other drugs. Thus, MDR-TB may be resistant to “only” two drugs, INH and RIF, or it may be resistant to three, four, or more drugs (Donald & Van, 2011).

Drug resistant mutants are selected when anti-TB drugs are not properly used, for instance, if monotherapy is applied. The factors resulting in the occurrence of drug resistance may be classified under two headings; program factors and clinical mismanagement. Classification of drug resistance can also be primary or secondary. Drug resistance is primary if the patient was never treated before using anti-TB drugs, or treatment occurred for a few months before the specimen was collected (Rattan et al., 1998). Drug resistance is secondary if the patient was treated for one or more months at any time before the specimen was obtained. Programmatically, the implications of these two types of resistance differ.

Primary resistance implies that individuals with infectious, drug-resistant TB remain at large in the community and are transmitting the disease to others in the population. Control measures should focus on finding and curing infectious cases and on preventing transmission in institutions and other high-risk environments. Secondary resistance implies that resistance may have developed during or because of previous treatment (Ismael & Ray, 2004). Control
measures should focus on rapid diagnosis of drug-resistance, appropriate chemotherapy, and diligent case management to assure adherence to treatment.

Acquired resistance means the subject’s isolate was susceptible to a given drug at one point in time and resistant at a later stage during the same course of treatment (Cohen & Murray, 2004). The isolates must also be the same strain, which means, not reflecting a mixed infection or exogenous reinfection. Thus, the diagnosis of acquired resistance requires serial isolates with the same genotype; it implies that resistance developed due to inadequate treatment (Raviglione, 2006).

2.7 Mutations

Antibiotics function by targeting cellular processes that are critical in bacteria by binding their targets at a specific site, generally directly merging with fundamental active residues of the target. The most frequent means of resistance in *M. tuberculosis* is the modification of the targets binding site via the build-up of mutations (Feng *et al*., 2000). The binding affinity of the drug to its target is cutback by these mutations and typically occur in a very defined location of the gene described as the resistance-determining region (Campbell, 2011). The proportion of mutants resistant in a wild-type population of *M. tuberculosis* ranges from $10^{-5}$ to $10^{-7}$ depending on the specific drug and mutation (Donald & Van, 2011).

A drug-resistant strain comes to predominate under the selective action of an anti-TB drug. The drug kills the susceptible bacilli, and the resistant bacilli continue to grow. Unlike many pathogens, mycobacteria do not exchange genetic material horizontally, so mutations are independent. The probability of two simultaneous mutations is vanishingly small, from $10^{-11}$ to $10^{-14}$, far less than the number of bacilli in a patient (Ramaswamy & Musser, 1998). Resistance to two or more drugs arises by serial accumulation of individual mutations, often in more than one patient, over months to years of inadequate treatment (Raviglione, 2006).
2.8 Mode of Action and Evolution of drug resistance

2.8.1 Resistance to first line drugs

Drugs utilized in anti-TB regiment are required to possess an adequate sterilizing action efficient enough in reducing the treatment period. Currently, a four-drug regimen is in use. This comprises of isoniazid (INH), rifampicin (RIF/RMP), ethambutol (EMB) and pyrazinamide (PZA). First line anti-TB drug resistance has been associated with mutations in as few as 10 genes; \textit{inhA}, \textit{katG}, \textit{kasA}, \textit{ndh}, and \textit{ahpC} for INH resistance; \textit{rpoB} for RIF resistance, \textit{embB} for EMB resistance, \textit{pncA} for PZA resistance and \textit{rpsL} and \textit{rrs} for resistance to Streptomycin (STR) (Campbell \textit{et al.}, 2011).

Isoniazid was incorporated in the early 1900s. However, its anti-TB activity was initially observed in 1951 (Heym \textit{et al.}, 1999; Rattan \textit{et al.}, 1998; Slayden & Barry, III, 2000). INH infiltrates the cell as a prodrug that is made active by a catalase peroxidase encoded by \textit{katG}. To turn on INH to a toxic substance in the bacterial cell, peroxidase action of the enzyme is essential (Zhang \textit{et al.}, 1992). Subsequently, the toxic matter influences intracellular targets like mycolic acid biosynthesis, which is a crucial constituent of the cell wall. Absence of mycolic acid synthesis ultimately leads to deprivation of cellular cohesion and the bacteria eventually dies (Barry \textit{et al.}, 1998).

The inception of Rifampicin as an anti-TB drug was in 1972 and it has been frequently utilized as a first-line therapy in association with either drugs for treating TB infections. The basis of short therapy courses is the application of RIF in association with INH or PZA, which eventually cuts back conventional TB treatment from a period of 1 year to 6 months (Telenti \textit{et al.}, 1993). RIF interferes with transcription which is linked to DNA-dependent RNA polymerase. RNA polymerase is comprised of four distinct subunits (\(\alpha\), \(\beta\), \(\beta'\) and \(\sigma\)) encoded by \textit{rpoA}, \textit{rpoB}, \textit{rpoC} and \textit{rpoD} genes respectively. RIF binds to the \(\beta\)-subunit preventing
transcription and eventually eliminating the organism (Rattan et al., 1998). Resistance to RIF occurs because of missense mutations in the gene (Ramaswamy & Musser, 1998).

Figure 2.1: shows drug targets in the cell wall, cytoplasmic membrane and cytoplasm of *Mycobacterium tuberculosis* for Isoniazid, Rifampicin and Pyrazinamide. Adapted from (Somoskovi et al., 2001).

Pyrazinamide (PZA) is a critical first-line drug designed for the primary management of active tuberculosis in children and adults when combined with other anti-TB agents. It contributes significantly to the sterilization of lesions and thus treatment shortening. Pyrazinamide is the pyrazine analogue of nicotinamide, which acts by killing semi dormant bacilli in an acidic environment. Pyrazinamide combined with RIF and INH make up the basis of contemporary TB treatment remedy. Pyrazinamide is a prodrug that is transformed to its active structure pyrazinoic acid (POA) by the mycobacterial enzyme nicotinamidase/pyrazinamidase. Pyrazinamide, fabricated intracellularly, spreads out into the bacilli in a massive way, and pyrazinamidase found within the cell converts it into POA. The
inept efflux system of the mycobacterial cell emits colossal aggregation of POA in the bacterial cytoplasm, resulting in breakdown of membrane capability (Zimhony et al., 2004). In 1996, the \textit{pncA} gene was proved to be greatly linked with PZA resistance in \textit{M. tuberculosis}, and in the subsequent year, researchers ascertained that \textit{pncA} mutations formed the primary mechanism of PZA resistance.

In 2011, through systemic analysis using meta-analysis, a conclusion was drawn by Chang \textit{et al.} that molecular assays relying upon the \textit{pncA} mutations were probably the direction for detecting pyrazinamide resistance in \textit{M. tuberculosis}. However, there is a great variety of \textit{pncA} mutations in various regions, which hinders the establishment and utilization of molecular assays that are usually based on the molecular characterization of gene mutations.

Ethambutol (EMB) is a first-line drug used together with INH, RIF, and PZA to avert the development of drug resistance distinct to \textit{Mycobacterium} (Ramaswamy \textit{et al.}, 2000). EMB is an active bacteriostatic agent for growing bacilli but ineffective to bacilli that are not replicating. EMB affects the cell wall of the mycobacteria via a synthetic mechanism and impedes arabinosyl-transferase, which is vital in cell wall biosynthesis (Lee \textit{et al.}, 2002). Encoded by arabinosyl-transferase and linked to the synthesis of arabinogalactan, arabinosyl transferase has been suggested as the target of EMB activity within \textit{M.t.b} (Mokrousov \textit{et al.}, 2002). Resistance to EMB has been shown in studies to occur as a result of irregular spontaneous genetic mutations developing at a rate of roughly 1 in $10^7$ organisms.

Such mutations frequently lead to rise in production of the enzyme arabinosyl-transferase, eventually overwhelming the inhibitory effects of EMB. Various studies have reported five mutations in codon 306 [(ATG-CTG), (ATG-GTG), (ATG-ATC), (ATG-ATA), and (ATG-ATT)] whose outcome is three distinct amino acid substitutions (Val, Ile and Leu)
in EMB-resistant isolates (Mokrousov et al., 2002). Seventy to ninety percent of all EMB resistant isolates are linked to these five mutations (Ramaswamy and Musser, 1998).

### 2.8.2 Resistance to second line drugs

With regard to the to the WHO, the following drugs can be labelled as second line drugs: aminoglycosides (amikacin and kanamycin) polypeptides (viomycin, enviomycin and capreomycin), fluoroquinolones (gatifloxacin, ciprofloxacin, and ofloxacin), D-cycloserine, and thionamides (prothionamide and ethionamide). However, second-line drugs are intrinsically less effective and more toxic than first-line drugs (WHO, 2001). Second line drugs are usually used to treat MDR-TB and subsequently extend the overall treatment time from six to nine months (Cheng et al., 2004).

Amikacin and kanamycin are aminoglycoside antibiotics, while viomycin and capreomycin are cyclic peptide antibiotics. The four are utilized as second-line drugs in the treatment of MDR-TB. Even though being part of two distinct antibiotic families, all wield their activity at the level of protein translation. The most frequent molecular means of drug resistance has been linked to a mutation in the rrs gene coding for 16S rRNA (Taniguchi et al., 1997). A1401G mutation develops more often in strains with high-level resistance to amikacin and kanamycin (Suzuki et al., 1998). Mutations in the gene tlyA have been related to capreomycin and viomycin resistance. This gene codes an rRNA methyl transferase distinct to 2′-O-methylation of ribose in rRNA. In the event of mutation, it ascertains an absence of methylation activity (Taniguchi et al., 1997).

Fluoroquinolones function as bactericidal antibiotics presently used as second-line drugs in treating TB. Ofloxacin and ciprofloxacin are both synthetic derivatives of nalidixic acid, the parent compound discovered in 1965 as a by-product in the purification of the antimalarial drug chloroquine (Maruri et al., 2012). Presently a new generation of
fluoroquinolones, which include gatifloxacin and moxifloxacin, are undergoing clinical assessment and are being suggested as first-line antibiotics with the aim of reducing treatment time of TB (Ginsburg et al., 2003). In M. tuberculosis, the lone target for fluoroquinolone activity is type II topoisomerase. Type II topoisomerase is a tetramer which constitutes two subunits: A and B encrypted by the genes gyrA and gyrB, correspondingly, that catalyzes the supercoiling of DNA (Ginsburg et al., 2003). Missense mutations within the quinolone resistance-determining region (QRDR), along with a preserved region of gyrA (codons 88 to 94) and gyrB (codons 500 to 538), have been determined as the fundamental system conferring resistance towards fluoroquinolones (Maruri et al., 2012).

2.9 Prevalence of TB

Tuberculosis occurs in almost every country yearly. However, the cases reported vary enormously in each country. The figure of new incidences of TB in 2014 was estimated at 9.6 million. China, India and Indonesia had the topmost percentage of TB (WHO, 2015). The percentages were 10%, 23% and 10% of the global total accordingly (WHO, 2015). Since 1998, 22 countries have been considered to be TB “high burden” countries (HBCs). Globally, the highest level of priority has been given to these countries. Apart from China, Indonesia and India, other countries are Bangladesh, Kenya, Nigeria, Ethiopia, Uganda, UR Tanzania, Afghanistan, Mozambique, South Africa, Philippines, Brazil, DR Congo, Myanmar, Vietnam, Thailand, Russian Federation, Pakistan, Cambodia and Zimbabwe (WHO, 2015). These countries accounted for 83% of all predicted cases in 2014 worldwide. Further to the TB high burden countries, Kenya also appears amongst the 30 high TB/HIV and MDR-TB burden regions.
Table 2.1: Estimated WHO Regional TB statistics for 2016

<table>
<thead>
<tr>
<th>Region</th>
<th>TB Mortality</th>
<th>Prevalence</th>
<th>Incidence</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>South-East Asia</td>
<td>652,000</td>
<td>5,400,000</td>
<td>4,670,000</td>
<td>1,946,087,000</td>
</tr>
<tr>
<td>Africa</td>
<td>417,000</td>
<td>3,200,000</td>
<td>2,590,000</td>
<td>1,023,361,000</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>103,000</td>
<td>2,100,000</td>
<td>1,800,000</td>
<td>1,885,184,000</td>
</tr>
<tr>
<td>Eastern Mediterranean</td>
<td>82,000</td>
<td>1,000,000</td>
<td>766,000</td>
<td>669,745,000</td>
</tr>
<tr>
<td>Europe</td>
<td>26,000</td>
<td>440,000</td>
<td>290,000</td>
<td>916,279,000</td>
</tr>
<tr>
<td>Americas</td>
<td>17,000</td>
<td>350,000</td>
<td>274,000</td>
<td>996,613,000</td>
</tr>
<tr>
<td>Global Total</td>
<td>1,297,000</td>
<td>13,000,000</td>
<td>9,600,000</td>
<td>7,437,269,000</td>
</tr>
</tbody>
</table>

In 2015, new tuberculosis cases worldwide were estimated to be 10.4 million, 5.9 million (56%) of which were established among men while 3.5 million (34%) noted among women. New cases among children was estimated at 1.0 million (10%) (WHO, 2016). HIV positive subjects comprised 1.2 million of all new TB occurrences discovered (WHO, 2016). Countries in the WHO African Region had the highest proportion which exceeded 50% in parts of Southern Africa. India, Nigeria, Indonesia, China, South Africa and Pakistan reported 60% of the new cases (WHO, 2017). Nigeria and South Africa each accounted for 4% of the global total. Progress globally is dependent on extensive improvements in the management and concern of TB in these countries. Globally, the percentage of decrease in TB cases stayed at 1.5% from 2014 to 2015 (WHO, 2016).
Table 2.2: WHO statistics for TB in “High Burden” African Regions 2016

<table>
<thead>
<tr>
<th>Country</th>
<th>TB Mortality</th>
<th>Prevalence</th>
<th>Incidence</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kenya</td>
<td>9,400</td>
<td>120,000</td>
<td>110,000</td>
<td>48,164,000</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>32,000</td>
<td>190,000</td>
<td>200,000</td>
<td>101,959,000</td>
</tr>
<tr>
<td>Uganda</td>
<td>4,500</td>
<td>60,000</td>
<td>61,000</td>
<td>37,783,000</td>
</tr>
<tr>
<td>UR Tanzania</td>
<td>30,000</td>
<td>270,000</td>
<td>170,000</td>
<td>56,823,000</td>
</tr>
<tr>
<td>Nigeria</td>
<td>170,000</td>
<td>590,000</td>
<td>570,000</td>
<td>186,476,000</td>
</tr>
<tr>
<td>South Africa</td>
<td>24,000</td>
<td>380,000</td>
<td>450,000</td>
<td>55,969,000</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>2,300</td>
<td>44,000</td>
<td>42,000</td>
<td>16,246,000</td>
</tr>
<tr>
<td>Mozambique</td>
<td>18,000</td>
<td>150,000</td>
<td>150,000</td>
<td>27,216,000</td>
</tr>
</tbody>
</table>

According to the WHO, in 2016, it was estimated that 153,119 cases of multidrug-resistant TB and rifampicin resistant TB (RR-TB) were notified and 129,689 were enrolled in treatment (WHO, 2016). In 2015, those who passed away due to TB was estimated at 1.4 million. In addition, those who were HIV positive and died from TB was estimated at 0.4 HIV (WHO, 2016). Among the causes of death worldwide in 2015, TB remains one of the top 10 as much as the number of deaths from the disease fell by 22% between 2000 and 2016 (WHO, 2017). As compared to 2015, in 2016, 57% of notified TB patients had a documented HIV test result, up from 55% in 2015 and a 19-fold increase since 2004. In the African region where the burden of TB and HIV co-infection is high, 82% of TB patients were HIV positive.
2.10 Limitations of Global TB Estimates

Paul Nunn stated that the WHO surveillance data for DR-TB, MDR and XDR-TB have large confidence limits (Giffin et al., 2009). To enable the WHO to ascertain the prevalence of MDR and XDR TB among all cases of TB, it first estimates the cases that are MDR, and then within those cases, the percentage that are XDR. Several components complicate the WHO estimates. First, limited or no data are available for many locations, especially in sub-Saharan Africa. In addition, in several countries, the availability of diagnostic laboratories is limited; some countries in Africa lack even a single reference laboratory capable of culturing TB and making a diagnosis.

In Kenya for instance, many facilities are used as treatment facilities and are not able to carry out diagnosis due to lack of certain equipment’s such as BACTEC™ MGIT™ 960 TB system that is used for culture and sensitivity. Concerning data, currently there is limited data available on the patterns of Mycobacterium resistance gene mutation or prevalence in Kenya. The survey of TB prevalence was first carried out in Kenya between 1958 to 1959. The second survey was carried out from July 2015 to 2016 by the Ministry of Health where GeneXpert technology was used. The results showed that there are 558 people with the disease among 100,000 people revealing a higher TB burden in Kenya than previously recorded by the WHO. The WHO in 2015 had earlier estimated that there were 233 people with TB among 100,000 people in Kenya. This goes to show the uncertainty of the exact figures in Kenya.

It was also interesting to note that the findings by the Kenya health ministry also highlighted that about 40% of TB cases go unnoticed and untreated. Since one infected and untreated individual can end up spreading infection to 10-15 individuals, these missing TB cases continue to fuel the spread of TB.

Finally, data on mortality are also unreliable since little is known of the long-term effects of MDR cases that are reported. The surveillance data of WHO represent samples of
incident cases in which drug resistance was measured through drug sensitivity testing (Zignol et al., 2006). Two sources of data are applied in this case: those from newly presenting TB patients or retreatment cases. This goes to show that those who develop MDR TB along the course of treatment are not captured, and hence brings about considerable underestimates. Such a degree of underestimation will be based when in the course of an epidemic the data are sampled. When an epidemic is in its early stages, several of those cases develop from individuals who fail therapy and amplify their drug resistance, the estimated numbers will likely be low. Later, when transmission of drug resistance dominates most of those cases, the reported MDR TB rates will be higher.

2.11 Epidemiology of TB in Kenya

Kenya is among the thirty high TB burden countries in the world that accounts for 87% of the global TB burden, WHO (2017). The sheer number of TB cases notified has increased more than tenfold since 1990 while the TB incidence has increased from below 50 per 100,000 population in 1990 to 348 per 100,000 population for all forms of TB in 2017, WHO (2017). The massive increase in the burden of TB in the WHO African region and even in Kenya is mainly contributed to the HIV epidemic. People living with HIV as compared to those who are HIV negative, have a 16-27 times greater risk of developing TB. According to the WHO, the estimated HIV prevalence in new and relapse TB cases in 2016 lies between 20-49 %. Mortality (excluding HIV and TB) lies at a rate of 60 per 100,000 population as compared to 50 per 100,00 population for those inclusive of HIV and TB (WHO, 2017). The percentage of HIV positive people in Kenya (newly enrolled in care) on preventive treatment is at 11%, while that of children (aged <5) household contacts of bacteriologically-confirmed TB cases on preventive treatment lies at 14% (DLTLD, 2016).
To fight the challenges of TB epidemic in Kenya there has been massive scale up of both treatment and diagnostic facilities. However, several problems remain, with as close to 40% of all incident TB cases not being detected. In addition, the significant delays in TB diagnosis facilitates TB transmission and is associated with a higher frequency of the poor sequel of TB and the emerging problem of drug resistant tuberculosis (DLTLD, 2013). The emergence of MDR and XDR-TB poses a serious problem to the treatment of tuberculosis. In Kenya, the estimated MDR/RR-TB cases among notified pulmonary TB cases stands at roughly 1,300 (WHO, 2017). This relates to 1.3% and 9.4% for new and previously treated cases respectively. Laboratory confirmed cases of XDR in Kenya stand at 9, with those who have started treatment being 7 in number (WHO, 2017).

The epidemiology of Kenya can be attributed to certain factors which include but are not limited to economic and environmental factors and the comprehensive TB control strategies that have been implemented by the Government of Kenya, through the health ministry (Sitienei et al., 2013).
CHAPTER THREE
MATERIALS AND METHODS

3.1 Study area

The study was conducted in Dagoretti division in Nairobi Kenya. According to data from DLTLD obtained in 2009, the area had a case notification rate of 585.2 per 100,000 populations (Njau et al, 2012). In addition to an overall population of 260,287 according to the 2009 census. Dagoretti division has approximately twenty-six TB facilities, nevertheless, functional laboratories for conducting TB diagnosis are only found in eight of the facilities while the other facilities were solely used as treatment sites. Only two facilities were included in the present study: Mbagathi district hospital and Chandaria community hospital. In addition, the two facilities were purposively selected based on high patient flow to the health facilities. In Kenya, majority of TB patients visit public health facilities compared to private health facilities. This is basically based on high health care cost demanded by the private facilities. Therefore, in this study, the two facilities were of main focus in order to obtain the required number of patients so as to minimize duration of the study and for effective utilization of resources.

Figure 3.1: Map of Kenya showing the Study Area in Nairobi county
3.2 Study design

The study design employed was a cross-sectional study design. This involved patient presenting clinical symptoms associated with TB in two different health facilities; Mbagathi and Chandaria hospital both in Nairobi.

3.3 Sampling design

Sample design was purposive/judgment sampling based on non-probability sampling. The sampling procedure was used based on some appropriate characteristic of clinical symptoms associated with TB among individuals being sampled. In addition, the individuals being sampled were those who were already known to be new cases positive for pulmonary TB, hence drug naïve.

3.4 Sample Size Calculation

The prior judgement of the correct value of p was based on data obtained from the WHO report on HIV and TB prevalence within the Kenyan region (WHO, 2014). The sample size of 132 samples for the study with a 90% confidence interval was arrived at using the formula by Magnani (1997):

\[
n = \frac{p(1-p)}{(ME/z)^2}
\]

Where n is the sample size

Z is the Z score, for instance 1.96 for 90% confidence interval.

P is prior judgment of the correct value of p.

ME is the desired margin of error for the sample size

Sample size \( n = \frac{0.022 \times 0.978}{0.0001627} = 132 \)
3.5 Sampling

3.5.1 Inclusion Criteria

i. The study targeted both male and female subjects aged above 18 years.

ii. Patients having body weight equal to or over 30 kg.

iii. Patients diagnosed with drug sensitive (DS) or multi-drug resistant (MDR), smear-positive pulmonary TB (at least 1+ on the IUATLD/WHO scale on smear microscopy).

iv. Signed written consent or witnesses oral consent in case of illiteracy, was required before any project procedure.

3.5.2 Exclusion Criteria

Patient were excluded from participation if they had previous treatment for TB including, but not limited to, gatifloxacin, amikacin, rifabutin, kanamycin, rifapentine, thioacetazone, capreomycin, quinolones, thioamides, and metronidazole.

3.6 Sample collection

Sampling involved collection of an early morning sputum and spot sputum sample from a total of 132 patients. Spontaneous (spot) sputum samples were produced when patient visited TB Clinic while early morning sputum samples were collected by the patient at home and transported to the TB clinic at ambient temperature. Each patient was instructed on how to collect and transport each early morning sample to TB Clinic as early as possible. Specimen label container were completed with subject number, subject initials and sample collection date. Subjects were informed that nasal secretion and saliva are not sputum; the required specimen was to be produced by a deep cough and was thick, mucoid, white-yellow, and constantly blood-tinged; and was from the lower airways and lungs.

Sputum collection into screw cap container involved: rinsing and cleaning their mouth with clean water; standing (if possible) and breathing in and out deeply three times; cough as
hard as possible; place sputum container under their lower lip and collect the sputum; screw the lid on tightly so that it does not leak.

As soon as the spot and early morning samples were acquired in the TB Clinic, the specimens were maintained within the specified temperature range of 2-8°C and transferred to the P3 laboratory for processing. Temperature range of 2-8°C was essential to minimize the growth of any contaminating bacteria present in the sputum sample. Specimen that comprised of saliva or less than 2mL were not processed. This was due to the fact that sputum volume and gross appearance has an impact on smear positivity (Yoon et al., 2012).

3.7 Sample Processing

3.7.1 Sputum Decontamination

Sputum samples were first decontaminated. This involved transferring specimen into a 50-ml centrifuge tube with a screw cap and adding an equivalent volume of NaOH-NALC sodium citrate solution. Samples were then vortexed for 15-30 seconds and then left for 20 minutes to liquefy before adding phosphate buffer saline. Specimen was then centrifuged at a speed of 3000 relative centrifugal force (rcf) for 15 minutes at 4°C before pouring out the supernatant and adding 1 to 2 ml of phosphate buffer saline (pH 6.8) to the sediment. The re-suspended pellet was then used for ZN and inoculation of MGIT tubes (STAND, 2014).

Leftover sediment was stored at 4°C for 10 days until it was confirmed that the inoculated media were not contaminated. In case there was contamination in the MGIT culture within 10 days, the decontamination procedure was repeated with the remaining sediment following the same procedure and new culture inoculated. Quality control was assured by adding a negative tube in the middle of each batch of specimens processed in order to ensure that there was no contamination present in stock solutions and no carry-over of *M. tuberculosis* from one specimen to another. The negative control was treated the same as the patient samples. In case of contamination in the control tube, the results of specimens done in the same batch
were checked to determine whether there was an influence from the contamination. If \textit{M. tuberculosis} was present in the negative control tube, the results of specimens done in the same batch were checked to determine whether false positive culture were present, which might have indicated carry over from one specimen to another.

\textbf{3.7.2 Ziehl-Neelsen (Z-N) Sputum Smear Microscopy}

ZN staining involved placing the slides containing the sputum smears on a staining rack and flooding with carbon fuchsin. The slides were then heated until steaming with a flame and let to stand for 5 minutes. They were then re-flooded with carbon fuchsin and heated again until steaming, then let to stand again for 5 minutes. Distilled water was then used to wash away the carbon fuchsin before flooding the slides with 3 \% acid alcohol. Slides were then let to stand for 9 minutes after which distilled water was used again to wash away the acid alcohol. Malachite green was then used to flood the slides and left to stand for 1 minute before washing away the malachite green using distilled water and letting the slides drain. Examination of the ZN smears followed using microscopy (STAND, 2014).

\textbf{3.7.3 Preparation and Inoculation for Culture}

The BACTEC MGIT 960 is an in vitro diagnostic instrument designed and optimized for the rapid detection of mycobacteria from clinical specimens. Specimens collected from patients are processed and inoculated into the Mycobacteria Growth Indicator Tubes (MGIT) 7ml tubes. Microorganisms present in these specimens metabolize nutrients and oxygen in the culture. The culture vials contain a fluorescent sensor that responds to the concentration of oxygen in the culture medium. The instrument’s photo detectors measure the level of fluorescence, which corresponds to the amount of oxygen consumed by organisms. Instrument detection of the presence of microorganisms growing in the culture medium results from these fluorescence measurements.
After preparation of sediment to be cultured, a vial of MGIT PANTA containing a lyophilized mixture of antimicrobials was reconstituted with 15.0 ml MGIT growth supplement provided. Using a micropipette, 0.8 ml of the mixture was then added to each MGIT tube that was to be inoculated with specimen including the negative control. Using a sterile pipette, 0.5 ml of well mixed processed sample was then added to the corresponding labelled MGIT tubes. The tubes were then closed tightly and inverted three times to allow proper mixture of the components. They were then inserted into the BACTEC machine after scanning each tube (please refer to the BACTEC MGIT 960 instrument manual for details). The instrument maintains a temperature of 37 °C ± 1°C, considering it is the optimum growth temperature for *M. tuberculosis*.

MGIT tubes were incubated until the instrument flagged them positive, as for the negative tubes, they were flagged after a maximum of six weeks when no growth occurred. The tubes that flagged positive were removed and scanned outside the instrument. This followed visual observation of the tube. Mycobacterial growth tends to appear granular, settles at the bottom of the tube, and are rarely turbid as observed with bacterial or fungal contamination.

### 3.7.4 DNA Extraction

DNA extraction using GenoLyse involved centrifuging 1 ml of decontaminated specimen for 15 minutes at 10000×g, after which the supernatant was discarded and 100 μl lysis buffer added to re-suspend the sediment. Sample was then incubated at 95 °C for a period of 5 min before adding 100 μl of neutralization buffer and the mixture vortexed. The final step in the GenoLyse procedure was centrifuging for 5 minutes at full speed. For longer storage, supernatant was stored at -20 °C in a new tube (STAND, 2014).
3.7.5 Amplification, Hybridization and Visualization

To successfully carry out the molecular diagnosis of TB, MDR TB or XDR TB, necessitates 3 simple steps: DNA extraction, DNA amplification, Hybridization and visualization of the amplified products. Multiplex PCR was used to amplify genes liable for drug resistance for instance \textit{inhA}, \textit{rpoB}, \textit{katG}, and \textit{rrs}. Consequently, the biotin-labeled amplicons obtained hybridized to DNA probes bound to membrane probes.

All reagents needed for amplification were included in the Amplification Mixes A and B (AM-A and AM-B) ready for quick use for the test. After extraction of DNA with Genolyse extraction kit, the mixture was as follows; 10 µl AM-A, 35 µl AM-B, and 5 µl DNA solution which adds up to a final volume of 50 µl. The mixture was then loaded into a programmed real-time thermocycler for amplification of the drug resistance-determining region of the target gene, with incorporated biotinylated primers (3’ TGA CCTGAAAAGAC 5’). On the thermocycler, the appropriate program for the amplification procedure was selected and started. The protocol comprised of denaturation at 95°C for 15 min, followed by 10 cycles at 95°C for 30s and annealing at 58°C for 2min, an added 30 cycles at 95°C for 25s, 53°C for 40s, extension at 70°C for 40s, and a final extension at 70°C for 8min. In the hybridization steps of the HAIN tests, probes are embedded in the strips which will complement the correct DNA sequence, if present in the amplicons. Because the primers that were used in the amplification process were biotinylated, when these amplicons are subjected to all conditions as prescribed by the procedure, these complementary sequences are visible as bands on the strips. These bands are further interpreted to define positive diagnosis or absence of susceptible TB, MTBC, NTM, any mono-drug resistant TB, MDR-TB, or XDR-TB as the case might be. The hybridization process was executed manually using a TwinCubator, Memmert-SV1422 (Memmert GmbH & CO. KG, Schwabach, Germany) at 45°C. The DNA strips were then left to dry and elucidated in relation to the instructions given by HAIN life science.
GenoType MTBDRplus and GenoType MTBDRsl assay tests for existence of wild type (WT) and mutant (MUT) probes for each gene. The DNA probes bound to the membrane included eight rpoB wild-type probes, four rpoB mutant probes in positions with H526Y, D516V, S531L, and H526D. One katG wild-type probe, two katG mutant probes with S315T1 and S315T2, two inhA wild-type probes and four inhA mutant probes with A16G, T8A, C15T, and T8C. For detection of FLQ resistance, the GenoType MTBDRsl applies four wild-type probes for gyrA gene (WT1, WT2, WT3) and gyrB gene (WT), and seven mutation probes with A90V, D94G, D94A, D94Y, S91P, D94N and D94H. Two rrs wild-type probes and two rrs mutation probes with A1401G and G1484T. Three eis wild-type probes and one eis mutation probe with C-14T. As recommended by the manufacturer's instructions (Hain Life science, Germany), none resistance to anti-TB drugs was characterized as hybridization to all the wild type probes and no hybridization to the mutant probes.

3.8 Data Analysis

Analysis of 1st and 2nd line drug resistant mutations was done using a template provided for in the GenoType MTBDRplus ver 2.0 and GenoType MTBDRsl ver 2.0 kit based on the various resistant regions on the DNA strip.

Minitab 17 software was used to carry out chi-square test to compare observed frequency of mutations from patient samples obtained from the two public hospitals involved in the study. Quantitative variables in this case were the type of mutations observed and the two hospitals selected. Analysis was done using a significance level of 0.05.

3.9 Ethical Consideration

Research approval was first obtained from the Kenyatta University graduate school before seeking approval to carry out the research at the Kenya Medical Research Institute. The
samples analyzed in this study were part of STAND (shortening treatment for advancing novel drugs) clinical trial, conducted in collaboration with foreign research institutions that got clearance from the international review board (IRB) as well as the Kenya Medical Research Institute National Ethical Review Committee (KEMRI, ERC).
CHAPTER FOUR

RESULTS

4.1 Demographic and clinical characteristics of the subjects

A total of 132 (100%) patients were culture positive for *M. tuberculosis* and all were included for analysis. Among them, 72 (54%) of the subjects were male while the remaining 60 (46%) were female. All patients averaging 35 years and having a range of 18 to 60 years. Those who were negative for HIV were 116 while those positive were 16. As reflected in the figure below (see Figure 4.1), according to the IUATLD scale for reporting ZN smears, there were 83 recordings showing 1+ (10 to 99 acid-fast bacilli per 100 fields). Thirty-nine recordings showing 2+ (1 to 10 acid-fast bacilli per field in at least 50 fields) and ten recordings showing 3+ (more than 10 acid-fast bacilli per field in at least 20 fields). All the 132 cultures obtained from MGIT were positive, including the positive control (H37Rv) used with all culture batches. None of the negative controls used showed any influence from contamination, hence there was no need for any decontamination repeats.

![Demographic characteristics of 132 eligible participants](image)

**Figure 4.1:** Demographic characteristics of 132 eligible participants
The illustrative figure below (Figure 4.2) shows the notified cases per age group and respective hospital. Both clinics recorded a high level of TB cases among the 18-27 age group, although this number declines as the age group approaches 60 years. Out of the 132 participants, 61 were from Mbagathi hospital while 71 were from Chandaria hospital. In both instances, the number of male diagnosed with TB were higher than that of the women. In Mbagathi for instance, the number of male with TB was 5-fold that of the women. On percentage, this was reflected as 82% for male and 18% for the female. Chandaria hospital on the other hand had 54% of male diagnosed with TB while 46% were female. As seen in figure 4.2, age group 18-27 reported a high number of TB cases (41%) when both health facilities were combined. This was followed by the age group 28-37 which had a percentage notification rate of 29%. The notification rate continued to decrease further down as the age groups increased, age groups 38-47, 48-57 and 60+ giving 16%, 9% and 5% respectively.

![Notified TB cases by age group and hospital](image)

**Figure 4.2:** Notified TB cases by age group and hospital
4.2 Prevalence of drug resistant *M. tuberculosis*

Table 4.1 presents the drug susceptibility profile of *M. tuberculosis* isolates in the present study. As reflected in the table, only one case of mono drug resistance (exactly one drug being ineffective) was observed, this being the resistance to Isoniazid. One case of multi-drug resistance (INH and RIF in the presence or not of other drugs) as well as polyresistance (two or more drugs being ineffective) was observed. In general, in relation to first line drug resistance, two cases of INH were observed as well as one case of RIF resistance. The mono resistance to Isoniazid was identified from a male in Mbagathi hospital while the polyresistance was identified from another male in Chandaria hospital. All the female subjects in the study were susceptible to all the drugs that were tested.

Of the 132 isolates subjected to tests for their susceptibility to second-line drugs, one cross-resistance was detected for AG/CP antibiotics (Kanamycin and amikacin, both AG and capreomycin and viomycin, both CP). This subsequently generated a general prevalence of 1.5% (2/132) among all the subjects that were screened.

On comparing susceptibility between first line and second line drug-sensitivity, it was noted that the MDR-TB case had an additional second-line drug resistance while the mono-resistant case had no additional second-line drug resistance.

4.3 Prevalence of multi drug resistance tuberculosis

Sputum samples of the 132 smear positive tuberculosis patients were tested for MDR-TB by using GenoType MTBDRplus VER 2.0 (A qualitative in vitro test for the identification of the Mycobacterium complex and its resistance to rifampicin and/or isoniazid from pulmonary smear-positive or negative clinical specimens and cultivated samples). The overall prevalence of MDR-TB was 0.8%. The single case was a male who was identified from Chandaria hospital within the age group 18-27 but with no HIV co-infection. According to the WHO recommendation, considering RIF mono resistance as surrogate marker for MDR TB,
prevalence of MDR TB/RIF resistance for this study still stood at 0.8%. This was so, considering no case of RIF mono resistance was detected in the study.

**Table 4.1:** Drug susceptibility profile of the *M. tuberculosis* isolates in the present study.

<table>
<thead>
<tr>
<th>Mono drug resistance</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
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<tr>
<td>Isoniazid</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kanamycin (Low resistance)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Multidrug resistance</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid + Rifampicin</td>
<td>1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Poly-resistance</th>
<th>No</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid/Rifampicin + Fluoroquinolones/Aminoglycosides</td>
<td>1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**4.4 Detection of first and second-line drug-resistant mutations in *M. tuberculosis***

Figure 4.3 shows an evaluation sheet with some of the results from the Hain assay. The conjugate control (CC) in the sheet documents the productivity of conjugate binding and substrate reaction. All the 132 samples tested were able to show a band for the CC. Amplification control (AC) excludes amplification inhibitors and errors during setup and performance of amplification reaction. These bands too were able to be detected for all the samples tested. *M. tuberculosis* complex (TUB) shows a hybridized zone, as known, with amplicons generated from all members of the *M. tuberculosis* complex. If the TUB zone was negative, the tested bacterium did not belong to the *M. tuberculosis* complex and could not be
evaluated by the system. For this, all the samples tested were from the MTB complex and none was recorded as non-tuberculosis mycobacterium (NTM) or Mycobacterium other than tuberculosis (MOTT).

**Figure 4.3:** Interpreted DNA strips after hybridization.

Locus controls (*rpoB*, *KatG* and *inhA*) detect a gene region specific for the respective locus. Test can’t be evaluated if zones do not develop. For this study however, all the Locus
controls were able to be detected for each sample. In the evaluation sheet, sample No.1 (07010011), no signal is displayed for KatG wild type, but signal/band can be seen in KatG MUT1. This is interpreted as positive for mutation. Hence high-level resistance to isoniazid.

Table 4.2: Mutations in rpoB, KatG and rrs genes and the corresponding wild type and mutation bands according to (Telenti et al., 1993).

Table 4.2 presents the mutations in rpoB, KatG and rrs genes and the corresponding wild type and mutation bands. The table 4.2 was used in line with figure 4.4 to analyze the mutations present in the various mutation bands. Figure 4.4 displays the mutations in the resistance-determining region of the various gene loci analyzed in addition to the equivalent
resistance phenotypes. Mutations were noticed in the S315T1 of *katG* in 1.5% INH-resistant isolates, with mutations taking place at codon 315, denoting a change from amino acid Serine to Tyrosine. *KatG* genes are examined for detection of high level resistance to INH. This corresponded with a lack of band being detected at *katG* WT. Not a single of the INH-susceptible isolates showed mutations, hence all *katG* WT probes in the DNA strip stained positive, showing no detectable mutation within the examined region. Additionally, one (0.8%) RIF-resistant isolate with mutation S531L of *rpoB* at codon 530-533 was observed, denoting a change from Serine to Leucine (S to L). *rpoB* genes are examined for detection of resistance to RIF. This corresponded with no band being detected for *rpoB* WT8. All the other wild type bands were detected for those samples that were susceptible to RIF, these were; *rpoB* WT1, *rpoB* WT2, *rpoB* WT3, *rpoB* WT4, *rpoB* WT5, *rpoB* WT6, *rpoB* WT7 and *rpoB* WT8.

---

**Figure 4.4:** Prevalence of mutations in the resistance-determining region (MUT) of the various gene loci (*katG*, *rpoB*, *gyrA*, *gyrB*, *rrs*, *eis*).

Regarding second-line drug resistant isolates, only one mutation (G1484T) was observed for *rrs* at nucleic acid position 1484, denoting a shift of G to T. The *rrs* gene is
examined for detection of cross-resistance to AG/CP antibiotics such as kanamycin (KAN) and amikacin (AMK), both AG, or capreomycin (CAP) and viomycin (VIO), both CP. This was associated with *rrs* WT2 and developing mutation band *rrs* MUT2. Hence, no band was detected in the DNA strip at WT2. From the 132 isolates examined, no mutation was observed for *inhA*, *gyrA*, *gyrB* and *eis*, relating to low level INH resistance, FLQ resistance and low level KAN resistance respectively. This corresponded to detection of bands at *inhA* WT1, *inhA* WT2, *gyrA* WT1, *gyrA* WT2, *gyrA* WT3, *gyrB* WT, *rrs* WT1, *rrs* WT2, *eis* WT1, *eis* WT2 and *eis* WT3.

Results from the hypotheses testing which was carried out using Minitab 17, gave a chi-square statistic value of 2.3 which was smaller than the critical value (5.99). This resulted in rejecting the alternative hypotheses.
CHAPTER FIVE

DISCUSSION, CONCLUSIONS & RECOMMENDATIONS

5.1 Discussion

This study addressed the prevalence and detection of drug resistant mutations in *Mycobacterium tuberculosis* among patients visiting selected health centers in Nairobi, Kenya. This was in addition to the hypotheses that looked to determine if there was a difference in prevalence and detection of first and second line drug resistant mutations in relation to *M. tuberculosis* in Nairobi, Kenya. This was done by investigating the genotypic profiles in the *inhA rpoB, katG, gyrA, gyrB, rrs* and *eis* regions related to drug resistance using the MTBDR*plus* and MTBDR*sl* assay.

Results in this study put the prevalence rate at 1.5% with 1 (0.8%) of the 132 isolates in the current study, presenting MDR-TB (Table 4.1). The 132 isolates represented an area in Nairobi that is highly affected by MTB, hence analysis of these isolates provides a much exhaustive outlook of both prevalence and detection of drug mutations of these isolates. The prevalence found in this study was much expected considering the patients who were used for the study were drug naïve. Similar results were reported in Tanzania (Hoza *et al.*, 2015) and India (Mathuria *et al.*, 2013). Further explanation into this is that the type of resistance in drug naïve and those who have previously received anti-TB therapy is different. The rate of drug resistance in drug naïve patients (primary resistance) is normally low as compared to acquired resistance (that found in patients who have already received anti-TB therapy). In acquired drug resistance there is the aspect of selective pressure of antibiotic use, hence the Mycobacteria tend to mediate further resistance by use of chromosomal mutations. Higher prevalence rates (23%) were however noted in Ethiopia (Seyoum *et al.*, 2014). This may have been due to the
difference in sample size, poor management of TB cases, irregular supply of anti-TB drugs as well as poor treatment compliance.

Delving deep into the data, the isolates presented high level resistance towards isoniazid, rifampicin and a cross-resistance was also noted for KAN, AMK, CP and VIO. These findings are similar to those previously obtained in Mombasa, Kenya (Ombura et al., 2016) where 8 (3.1%) and 1(0.4%) monoresistance to INH and RIF, were reported respectively. However, no resistance was reported for second line drugs. This implies that resistance in clinical MTB isolates is basically ‘multiple’ naturally, the instance it evolves and thus stress the need for continued surveillance and the homogeneity in drug resistance that seems to be occurring in the country (Zhao et al., 2014).

Higher resistance to INH in other countries has also been noted at 5% in Tanzania (Urassa et al., 2008) and 8% in Ethiopia (Bruchfeld et al., 2002). The subsequently high rates of isoniazid resistance in this study as well as in others may be due to the prolonged use of isoniazid ever since its inception as one of the first line drugs for TB treatment. This rational may however be disputed considering new research that considers occurrence of drug resistance due to imperfect drug penetration (Moreno-Gamez et al., 2015). When considering imperfect drug penetration, one must develop a perception about drug combinations that is counterintuitive. Take for instance a combination of two drugs with different targets, suppose drug A reaches its target while drug B does not. You will find the pathogen evolving resistance to drug A and assume that is where the problem is. Instead, it is drug B that isn’t doing its job since it does not reach its intended target and that is the drug that may have to be fixed. However, monoresistance to isoniazid should be well monitored to curb the spread of MDR TB strains in the area.

It also seems that resistance to the other drugs occurs based on resistance to the first-line drugs of rifampicin and isoniazid, since resistance to other agents without involving at
least one of these two drugs was exceedingly uncommon (none out of 132 isolates) and that 99.2% of isolates remained susceptible to each of the other three test drugs (FQ, AG and CP). This circumstance is probably associated with the sequential use of distinct anti-tuberculosis drugs for treatment, as well as spread of the MDR strains (Chiang et al., 2010).

In this study, only one case of MDR TB was detected. This accounted for 0.8% of the samples. This agrees with other studies in various countries which encountered the same low levels, countries such as Ethiopia with 1.1% (Seyoum et al., 2014) as well as India recording the same percentage (Sharma et al., 2011). These findings however do not concur with other reports from other countries where the prevalence of MDR TB was higher. Case in point being countries such as Mozambique with 5.8% (Nunes et al., 2003) and Swaziland reporting 7.7% (Sanchez-Padilla et al., 2012). The divergence may be due to variations in sample size, population studied, effectiveness of DOTS strategy and access to health care facilities. A recent study however shows that the rise is due to the quality of care in both private and public sectors, which has fallen short of international standards and urgently needs improvement. (Cazabon et al., 2017).

Various mutations were detected in this study, implying that the scope of mutations that confer resistance to MTB may be much more comprehensive than those proclaimed in the research. In a meager region of amino acids situated between position numbers 507-533 of the rpoB gene, a bulk of mutations that confer resistance to RIF (73-100%) are consistently present (Hu Y et al., 2013). The proclaimed epidemiological information implies the RIF resistant strains are extensively disseminated in many regions worldwide. In the current study, the RIF resistant isolate had the mutation S531L; the most often recorded resistance mutation in various countries (Ritu et al., 2013).

KatG gene is the most common targeted region with a bulk of mutations occurring in codon 315 in 30%-90% of INH resistant strains, however, this relies upon graphical distribution
(Chan et al., 2007). A great number of reports imply that resistance of *M. tuberculosis* to INH show mutation at codon 315 (Yi Hu et al., 2013). Findings of this study were complementary, displaying mutations at S315T associated with elevated level of drug resistance to INH. Comparable mutation trends on S315T have been confirmed at *KatG* gene and have been reported in other studies including Ethiopia (Biadglegne et al., 2013), Uganda (Lukoye et al., 2013). This indicates possible use of the same drug analogs in the regions. Different drug analogs used in different regions has been shown to select for different drug resistant mutations (Sandgren et al., 2009). Mutations that are observed from the use of the different drug analogs may shed some light on some of the reported geographical differences in drug efficacy.

Nevertheless, in this study, gene mutation associated with low level drug resistance induced by the mutations in the promoter region of *inhA* gene were not detected. Comparable verdicts were reported from research conducted in Ethiopia (Biadglegne et al., 2013). As opposed to the current study, low and high-level frequency of *inhA* mutation have been reported in Canada 26% and Tunisia 36.1% (Boloton et al., 2009). This highlights the importance of surveillance and the heterogeneity in drug resistance that may occur within Kenya.

In the present study, all the gene mutations among strains that are resistant to *rrs* by Genotype MTBDRsl assay was detected at nucleic acid position 1484 resulting in the mutation G1484T. Mutation G1484T has also been reported to be dominant in various regions (Courtney et al., 2005). Mutations observed among isolates of *M. tuberculosis* in this study also exhibited cross-resistance to AG/CP and were further resistant to both INH and RIF, suggesting an MDR strain of *M. tuberculosis*. Similar drug resistance trends can be found in other MDR cases in other areas, however the second line drug /antibiotic resistance may vary (Courtney et al., 2005). Mutations related to second line drug resistance in this study were minimal mainly since the patients were drug naïve and had no history of second line drug treatment. This is unlike cases involving patients who have undergone retreatment failure and had longer history of
second line drug treatment and are more likely to exhibit isolates with mutations linked with resistance to the drugs.

The findings in this study carry some important implications. First, the high incidence of resistance to isoniazid implies that precursors of isoniazid resistance are accumulating in the study setting which can increase the likelihood of MDR TB if rifampicin resistance rises. Secondly, the prevalence of MDR-TB in drug naïve patients is still at an all-time low over the years, this reflects the success of DOTS in effective treatment of drug-susceptible TB and preventing the emergence of MDR-TB. In addition, since MDR-TB is rare among new TB cases, all new cases of pulmonary tuberculosis can be treated with empirical category I regimen without the risk of treatment failures or aggravation of drug-resistance. Finally, mutations that are observed from the use of the different drug analogs may shed some light on some of the reported geographical differences in drug efficacy.

In this study, Genotype MTBDRplus and MTBDRsl assays detect the resistance originating from the katG, rpoB, inhA, gyrA, gyrB, rrs and eis regions as examined. As recommended by the manufacturers’ instructions for usage, a considerable disadvantage of the assay is that few of the mutations that confer resistance to RIF and INH anti-TB drugs are proven. Additionally, the sample size was limited to ongoing studies and therefore limited in power to detect other mutations within the population. It would be of interest if the study was conducted with a larger sample size.

5.2 CONCLUSIONS

1. There is no difference in prevalence and detection of first and second line drug resistant mutations in relation to M. tuberculosis in Nairobi, Kenya.
2. There is homogeneity when it comes to the type of drug resistance and mutation that occurs in the region, this call for an urgent need for randomized controlled trials to discover the most effective treatment regimen for managing INH resistant TB.

3. The detected prevalence of 1.5% resistance to first and second line drug resistant mutations of *M. tuberculosis* in this study calls for intensified drug resistance surveillance and drug adherence among patients infected with TB.

### 5.3 RECOMMENDATIONS

1. The continued rise in the prevalence of the mutations associated with MDR and the prevalence of those linked with mono-resistance indicates that a continued drug pressure, from the first and second line drug use is preventing the restoration of susceptible *M. tuberculosis* in Nairobi. Hence the need for a more judicious use of anti-TB drugs.

2. More research needs to be done on the impact of the mutations on the efficacy of isoniazid (INH).

3. There is need for more focused clinical and molecular studies on the *in vitro M. tuberculosis* susceptibility to first line TB drugs in this region.

4. It would be of interest to study the correlation between second line drug resistant mutations in association with INH and RIF mutations.
REFERENCES


with multiple episodes of anti-tuberculosis treatment. *BMC Infectious Diseases, 16* (2), 3.


APPENDICE
Appendix 1: Photos of MTBDRplus DNA strip technology product and interpretation.
<table>
<thead>
<tr>
<th>Sample</th>
<th>RIF WT</th>
<th>RIF MUT</th>
<th>RAF WT</th>
<th>RAF MUT</th>
<th>AMK WT</th>
<th>AMK MUT</th>
<th>RMP WT</th>
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</table>
Figure: Shows interpreted DNA strips after hybridization. Conjugate control (CC)- documents the efficiency of conjugate binding and substrate reaction. Amplification control (AC)- excludes amplification inhibitors and mistakes during setup and performance of amplification reaction. M. tuberculosis complex (TUB)- this zone hybridizes, as known, with amplicons generated from all members of the Mycobacterium tuberculosis complex. If the TUB zone is negative, the tested bacterium does not belong to the M. tuberculosis complex and can’t be evaluated by this system. Locus controls (gyrA, gyrB, rrs and eis) detect a gene region specific for the respective locus. Test can’t be evaluated if zones do not develop.
The IUATLD scale proposes five groups for reporting the results of reading smears for acid-fast bacilli. They should be recorded as follows:

**Appendix 3: The IUATLD scale**

<table>
<thead>
<tr>
<th>FINDING</th>
<th>RECORDING</th>
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</thead>
<tbody>
<tr>
<td>No acid-fast bacilli found in at least 100 fields</td>
<td>negative</td>
</tr>
<tr>
<td>1 to 9 acid-fast bacilli per 100 fields</td>
<td>Scanty positive</td>
</tr>
<tr>
<td>10 to 99 acid-fast bacilli per 100 fields</td>
<td>+</td>
</tr>
<tr>
<td>1 to 10 acid-fast bacilli per field in at least 50 fields</td>
<td>++</td>
</tr>
<tr>
<td>More than 10 acid-fast bacilli per field in at least 20 fields</td>
<td>+++</td>
</tr>
</tbody>
</table>

Appendix 4: Research approval for the study

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
kubps@yahoo.com
Website: www.ku.ac.ke

FROM: Dean, Graduate School

TO: Ogari Collins Otieno
c/o Microbiology Department.

DATE: 4th May 2016

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

We acknowledge receipt of your revised Research Proposal as per our recommendations raised by the Graduate School Board of 13th April 2016 entitled “Prevalence and Genetic Characterization of Drug Resistance Genes in mycobacterium tuberculosis among Patients Visiting Selected Health Centres in Nairobi, Kenya”.

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology and Innovation and Kenyatta University Ethical Clearance Committee.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking Forms per semester. The form has been developed to replace the Progress Report Forms. The Supervision Tracking Forms are available at the University’s Website under Graduate School webpage downloads.

Thank you.

ANNBELL MWANIKI
FOR: DEAN, GRADUATE SCHOOL

CC: Chairman, Microbiology Department

Supervisors:

1. Dr. Anthony Kebire
   C/o Microbiology Department
   Kenyatta University

2. Dr. James Nohor
   C/Microbiology Department
   Kenyatta University

AM/16
KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 8710001 Ext. 87530

Our Ref: 156/27317/14

DATE: 4th May 2016

Director General,
National Commission for Science, Technology
& Innovation
P.O Box 36023-00100
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION FOR OGARI COLLINS OTIENO – REG. NO. 156/27317/14

I write to introduce Mr. Ogari Collins Otieno who is a Postgraduate Student of this University. He is registered for M.Sc degree programme in the Department of Microbiology.

Mr. Ogari intends to conduct research for a M.Sc. Proposal entitled, “Prevalence and Genetic Characterization of Drug Resistance Genes in mycobacterium tuberculosis among Patients Visiting Selected Health Centres in Nairobi, Kenya”.

Any assistance given will be highly appreciated.

Yours faithfully,

MRS. LUCY N. MBAABU
FOR: DEAN, GRADUATE SCHOOL
KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 5440-00100, NAIROBI, Kenya
Tel (254) (0)20 2722541, 2713349, 0722-205901, 6733-400033; Fax: (254) (0)20 2722030
E-mail: director@kemri.org info@kemri.org Website: www.kemri.org

KEMRI/RES/7/3/1

March 16, 2015

TO: DR. EVANS AMUKOYE (PRINCIPAL INVESTIGATOR)
THE DIRECTOR, CRDR
NAIROBI

Dear Sir,


Reference is made to your letter dated 26th February, 2015 and received at the KEMRI/Scientific and Ethics Review Unit (SERU) on 2nd of March, 2015.

This is to inform you that the Committee notes that the issues raised at the 235th meeting of the KEMRI/Ethics Review Unit (ERC) held on 20th January 2015 have been adequately addressed. Consequently, the study is granted approval for implementation effective this 18th March, 2015 for a period of one year. Please note that authorization to conduct this study will automatically expire on March 15, 2016.

If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by February 2, 2016. The regulations require continuing review even though the research activity may not have begun until sometime after SERU approval.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

In Search of Better Health
However, please note that work on this project may begin only after approval from the Expert Committee on Clinical Trials (ECCT).

Yours faithfully,

PROF. ELIZABETH BUKUSI,  
ACTING HEAD,  
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT  

Cc. Secretary, ECCT