Molecular Differentiation of Multi-Drug Resistant *Mycobacterium tuberculosis* Complex isolated from three provinces in Zambia

By

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A thesis submitted to the University of Zambia in fulfilment of the requirements of the degree of Master of Science Degree in Molecular Biology

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DECLARATION

I, **Kapona Otridah,** hereby declare that this thesis represents my work in design and in execution and that it has not to the best of my Knowledge been previously submitted for a degree at this or any other University.

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CERTIFICATE OF APPROVAL

This thesis of Kapona Otridah is approved as fulfilling the requirements for the award of the degree of Master of Science in Molecular Biology by the University of Zambia.

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ABSTRACT

Multidrug-resistant tuberculosis (MDR-TB) is a disease caused by an acid-fast staining pathogenic bacterium, *Mycobacterium tuberculosis* that is resistant to at least isoniazid (INH) and rifampicin (RIF), the two most potent TB drugs. Drug-resistant tuberculosis poses a significant problem for successful treatment of tuberculosis (TB). Worldwide, 4.1 percent of new cases and 19 percent of previously treated cases have been estimated to show MDR-TB and in Zambia, 1.8 percent and 2.3 percent MDR TB among new and previously treated cases respectively have been reported.

This study was conducted to characterize the isolates of *Mycobacterium tuberculosis* complex (MTBC) associated with rifampicin (RIF) and isoniazid (INH) drug resistance and to determine the prevalence of mutations attributed to two specific genes (*rpoB* and *katG*) and the *inhA* promoter region which have been linked to resistance against rifampicin and isoniazid. A panel of 40 INH and/or RIF drug resistant clinical isolates of *Mycobacterium tuberculosis* complex with susceptibility test results from Mycobacteria Growth Indicator Tubes (MGIT) were used. Isolates showing resistance to RIF and INH were analysed by multiplex polymerase chain reaction (PCR) and the amplicons were sequenced using the Big Dye Terminator v3.1 cycle sequencing kit on the ABI Prism 3500***xl*** Genetic Analyser (Life Technologies Corp).

All of the analysed isolates were identified to be *Mycobacterium tuberculosis*. Seventy-five percent (30/40) of these had *katG* mutations at codon 315, 7.5 percent (3/40) had the *katG* mutation alone while 2.5 percent (1/40) had mutations in the *inhA* promoter and *rpoB* in addition to the *katG* 315 mutation. *KatG* S315T mutation and the -18CT *inhA* mutation accounted for only 2.6 percent (1/39) of the INH-resistant isolates.

Of the 40 isolates characterized for *rpoB* mutations, 39 demonstrated mutations with the most common being S450L (48.7 percent), followed by H445T (20.5 percent). In one phenotypically-RIF resistant isolate, no nucleotide changes were detected in the *rpoB* gene. Mutations not previously reported were found at codons 535(CAA-GAA, Q535E) and 545(CTG-CCG, L545R). These results demonstrate varying geographical distribution of mutations in the *rpoB* in distinct regions. One out of 39 (2.6 percent) RIF resistant isolates demonstrated resistance to rifampicin alone which agrees with results from other studies which have reported that rifampicin mono resistance is rare.

The results of the current study are consistent with the results from similar studies in China and Mexico which reported that *katG* S315T and S450L *rpoB* mutations were the most prevalent mutations in clinical isolates. The high percentage of mutations at codon 315 of the *katG* gene (75 percent, n=40) demonstrates the importance of this codon in the development of resistance against isoniazid in *Mycobacterium tuberculosis* species circulating in various provinces of Zambia. Therefore, molecular analysis of these predominant mutations could be useful for the rapid detection of drug resistant tuberculosis.

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ABBREVIATIONS AND ACRONYMS

AFB	Acid Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
AST	Antimicrobial Susceptibility Test
BCG	Bacille Calmette Guerin
bp	Base pair
DNA	Deoxyribonucleic acid
DST	Drug Susceptibility Testing
EMB	Ethambutol
h	hour
HIV	Human immunodeficiency virus
INH	Isoniazid
IS	Insertion Sequence
MDR TB	Multi Drug Resistant Tuberculosis
mg	milligram
ml	mililitre
µl	microlitre
µg	microgram
MS	Microsoft
MOTT	Mycobacterium Other Than Tuberculosis
MTB	*Mycobacterium tuberculosis*
MTBC	Mycobacterium tuberculosis Complex
NHRA	National Health Research Authority
ng	nonogram
NTP	National Tuberculosis and Leprosy Control Program
PCR	Polymerase Chain Reaction
PZ	Pyrazinamide
RIF	Rifampicin
RNA	Ribonucleic acid
STR	Streptomycin
SADCAS	Southern African Development Community Accreditation Services
TB	Tuberculosis

UNZABREC	University of Zambia Biomedical Research Ethics Committee
UTH	University Teaching Hospitals
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.0 OVERVIEW

This chapter establishes the background to the study and outlines a statement of the problem. It states the aim of the study as well as the research objectives and questions. The significance of the study as well as the study hypothesis are explained.

1.1 BACKGROUND

Tuberculosis (TB) continues to be a serious global health problem. In 2017, the WHO estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis* (*MTB*) - the bacterial pathogen that causes tuberculosis (WHO report, 2017). Nine million new cases of active TB and 2-3 million deaths were also reported to occur annually, and out of these 26 percent were estimated to occur in Africa (WHO report, 2014). TB carries high public health importance as every second someone in the world becomes newly infected with TB bacilli. Despite its status as a treatable disease, TB has high morbidity and mortality rates (Global TB report, 2005; Muchemwa *et al*., 2017). In Zambia some of the factors that adversely affect treatment outcomes including exposure to HIV, smoking and occupational hazards have been analyzed (Nanzaluka *et al*., 2019). In 2014, the first ever national tuberculosis survey in Zambia reported 638 incident cases per 100 000 adult population (MOH, 2015). Worldwide, 4.1 percent of new cases and 19 percent of previously treated cases were estimated to have Multi Drug Resistance Tuberculosis (MDR-TB), defined as resistance to at least Rifampicin (RIF) and Isoniazid (INH), as reported by WHO in 2017.

The pathogens that cause tuberculosis in humans and other animals belong to the *Mycobacterium tuberculosis* complex (MTBC). The complex consists of a group of genetically closely-related species of the genus *Mycobacterium*. Members of this group include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. mungi*, *M. suricattae*, *M. orygis*, *M. pinnipedii* and "*M. canetti"* (Yeboah-Manu *et al.*, 2016; Niemann *et al.*, 2002). *M. bovis* comprises *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae*, *M. bovis*-derived BCG vaccine isolate and *M. africanum.* The latter has subtypes I and II while *M. microti* is the sole isolate and "*M. canetti*" may be a mere subspecies of *M. tuberculosis* (Van Soolingen *et al.*, 1997). Although MTBC species are genetically closely-related, they differ in certain ways including phenotypes, pathogenicity, host and geographic distribution. *M. tuberculosis* is the most common species in Europe and America, while *M. africanum* is mostly found in African TB patients of African origin, especially West Africa (Parsons *et al.*, 2002; Chihota *et al*., 2018). *Mycobacterium microti* has been reported to affect voles, (Wells and Oxon, 1937; Frota *et al*., 2004), *M. caprae* is a pathogen of goats and sheep (Aranaz *et al*., 1999) while *M. mungi* and *M. orygis* have been documented to affect mongoose and antelopes respectively (Jakko *et al*., 2012). *M. pinnipedii* is known to affect seals and sea lions (Cousins *et al*., 2003) while *M. bovis* displays the broadest spectrum of host range affecting humans and animals (Cousins *et al*., 2003). *M. tuberculosis* and *M. africanum* are however the main causative agents of TB in humans and are referred to as human adapted MTBC and the remaining seven species are normally described as animal-adapted (Gagneux *et al*., 2007). For many years, it was thought that human tuberculosis evolved from the bovine disease by adaptation of an animal pathogen to the human host (Stead *et al*., 1995). This hypothesis was based on the property of *M. tuberculosis* to be almost exclusively a human pathogen, whereas *M. bovis* has a much broader host range. However, the results from Brosch *et al*., (2002) demonstrated that *M. bovis* has undergone numerous deletion mutations relative to *M. tuberculosis* (Figure 1.1).

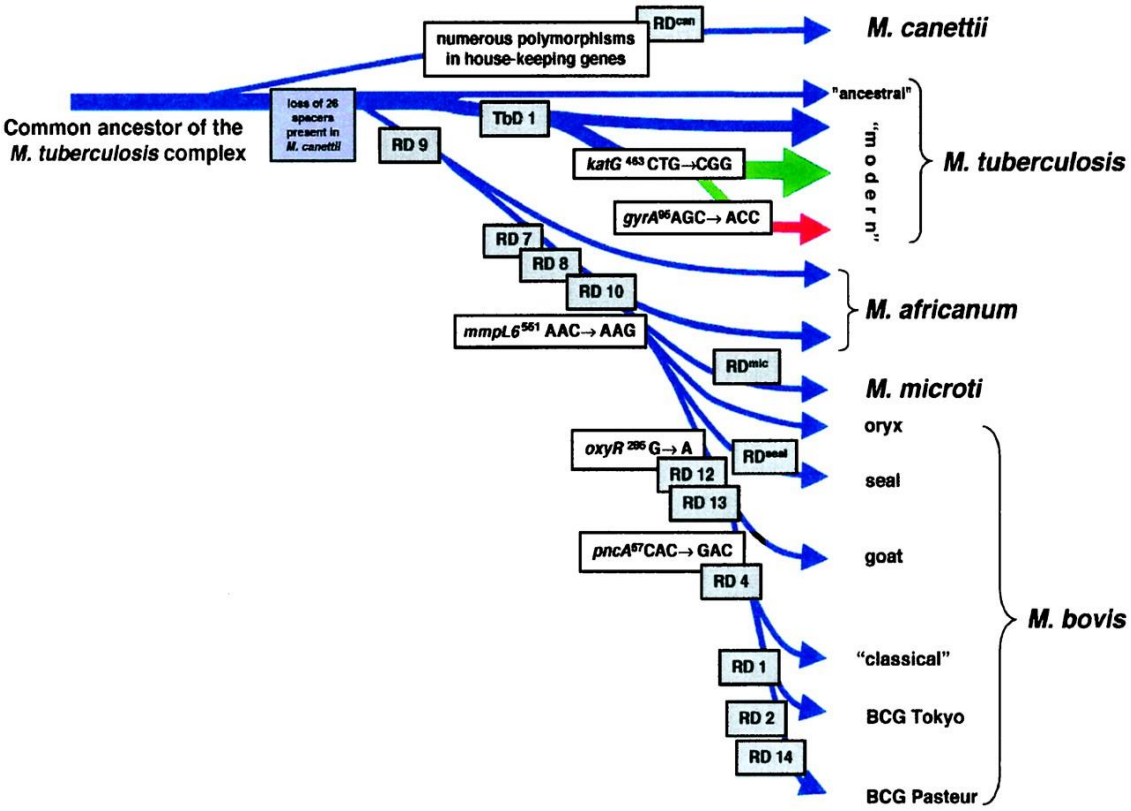

Figure 1.1 Scheme of the proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages (gray boxes). (Brosch *et al.*, 2002)

There was no known anti-TB chemotherapy until the 1940s. In that decade, streptomycin and *para*-aminosalicylic acid (PAS) were introduced as anti-TB drugs (Cavusoglu *et al.*, 2006; Caws *et al.*, 2006). The key steps that heralded the development of modern TB chemotherapy however were the demonstration in clinical trials, in 1947-48, that streptomycin was a viable drug for the disease (reviewed in Caws *et al.*, 2006). Several anti-TB drugs have since been discovered and can be broadly categorized into 2 groups based on clinical uses. These are categorized as first-line and second-line drugs. First-line drugs are used in treatment of new TB cases in patients with low potential risk for resistant TB. Drugs in such cases are usually administered orally. Second-line drugs are used for treatment of TB that is resistant to first-line drugs, and can be further categorized into 4 subgroups, *i.e.* injectable second-line drugs, fluoroquinolones, oral bacteriostatic anti-TB agents and anti-tuberculosis agents with unclear efficacy (Rich *et al.*, 2006). The later are not recommended by WHO for routine use in MDR-TB patients (Drobniewski, 1998; Rich *et al.*, 2006).

Zambia has a low incidence of MDR-TB among newly-diagnosed cases than among previously treated cases. A drug resistance survey conducted by a Zambia AIDS Related Tuberculosis (ZAMBART) project reported 1.8 percent and 2.3 percent MDR-TB among newly and previously treated cases respectively (Mulenga *et al*., 2010). Despite the low prevalence of MDR-TB, it is a serious concern because of the huge cost and debilitating side effects associated with its treatment. Additionally, its transmission is the same as that of drug-susceptible *Mycobacterium tuberculosis* (*MTB*), via aerosols. Zambia is ranked among the world's top ten countries with a high burden of tuberculosis caused by *Mycobacterium tuberculosis* (WHO TB report, 2009*)*. Treatment for new cases of TB consists of a six-month fixed dose combination therapy of four first-line drugs: isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZ) combined in a regime consisting of 2–month regime of INH/RIF/PZ/EMB and the last 4 months of INH/RIF (WHO, 2010). The disease is almost always curable if patients are given sufficient uninterrupted therapy. However, despite its being treatable, tuberculosis has proven to be very difficult to eliminate. An increase in the number of drug-resistant cases has in recent years been reported owing to a number of factors including patient non-compliance and interrupted treatment (Bakonyte *et al.*, 2003; Mwanza, 2016).

Global efforts to control the TB pandemic have been undermined by the emergence and spread of isolates that are resistant to the commonly used first-line anti-TB drugs. Tuberculosis strains that are resistant to at least INH and RIF, the two most efficacious TB drugs are referred to as multi drug resistant (MDR) isolates (Hilemann *et al.*, 2001). MDR-TB treatment is complicated as it requires second-line drugs, some of which are only injectables, less efficacious, more toxic and more expensive than the first-line agents (Ramaswamy and Musser, 1998). Treatment with second-line drugs lasts for 18-24 months and only around 50 percent – 60 percent of MDR-TB patients will be cured compared with 95 percent–97 percent cure rate for patients with drug-susceptible isolates treated with first-line TB drugs.

Isoniazid and rifampicin are the most important first-line anti-tuberculosis drugs, and development of resistance to these drugs results in high rates of treatment failure and death (Espinal *et al.*, 2000). Isoniazid acts through a potent anti-

tuberculosis cocktail that attacks *MTB* at multiple target sites including inhibition of both cell wall lipid and nucleic acid synthesis by INH-NAD+ and INH-NAPD products, and respiratory inhibition by INH-derived nitric oxide (Graham *et al.*, 2006). Resistance to INH is complex but most INH-resistant isolates have mutations in the *katG* gene encoding catalase-peroxidase that result in altered enzyme structure. This results in decreased conversion of INH to a biologically active form. Mutations in the *inhA* promoter region and in the *kasA* encoding a *β*-ketoacyl-acyl carrier protein synthase also mediate resistance to INH (Zhang *et al.*, 2005).

RIF is a semi-synthetic, highly-potent derivative of rifamycin that inhibits transcription (Campbell *et al.*, 2001). It achieves this by binding to the *β*-subunit of RNA polymerase of *MTB* thereby inhibiting initiation of *MTB* RNA synthesis and preventing extension of the RNA products beyond a length of 2 – 3 nucleotides (Campbell *et al.*, 2001). The *β*-subunit of the RNA polymerase is encoded by the *rpoB* gene and mutation in this 81base-pair gene mediates resistance to RIF (Ramaswamy and Musser, 1998). During transcription, DNA enters through the jaw side of the RNA polymerase and both the DNA and the new RNA strands get out at the exit channel (Klug, 2001). RIF binds to the exit end of the RNA polymerase in bacterial cells and directly blocks the channel of the elongating RNA when the transcript becomes 2 to 3 nucleotides long (Campbell *et al.*, 2001). This inhibits transcription of DNA to RNA and subsequent translation to proteins (Klug, 2001; Wehrli, 1983). The human RNA polymerase variant is not affected by rifampicin even at 10 times the inhibitory concentration in *Mycobacteria* (Wehrli, 1983). The rifampicin-sensitive RNA polymerase complex in *Mycobacteria* is extremely stable yet experiments have shown that this is not due to any form of covalent linkage (Telenti *et al.*, 1993). It has been hypothesized that hydrogen bonds and *pi* bond interactions between naphthoquinone and the aromatic amino acids (phenylalanine, tryptophan, and tyrosine) are the major stabilizers (Telenti *et al.*, 1993).

Spontaneous point mutations (deletions/substitutions/insertions), occurring in the 81-bp hotspot region of the *rpoB* gene result into replacement of the aromatic with non-aromatic amino acids in the pathogen RNA polymerase. This causes poor bonding between rifampicin and the RNA polymerase (Ramaswamy and Musser,

1998; Telenti *et al.*, 1993) and retention of RNA polymerase activity (transcription), thus explaining the source of resistance to rifampicin in bacteria.

Differentiation of the MTBC into individual species and subspecies is important not only for epidemiological purposes but also for accurate diagnosis and adequate treatment of each patient especially because some of the species in the MTBC are naturally resistant to anti TB drugs. A case in point is that of *M. bovis* which is naturally resistant to PZ (Niemann *et al.*, 2000). Additionally, species differentiation is needed to tell Non-tuberculosis Mycobacteria (NTM) safely apart from MTBC especially in individuals suggestive of having TB as NTM can exhibit clinical and radiographic similaries with MTBC especially in immune compromised individuals (Matveychuk *et al*., 2012; Satyanarayana *et al*., 2011; Taiwo and Glassroth, 2010). A study conducted by Monde *et al*. (2018) in Namwala district in the Southern province of Zambia documented an overall prevalence of 4.6% NTM among patients presenting with TB-like symptoms at local health centers.

The Non-Tuberculous Mycobacteria refer to all Mycobacterium species other than the pathogens of the *M. tuberculosis* complex (MTBC), *M. leprae* and *M. ulcerans*, and rarely cause disease in humans (Chanda-Kapata *et al*., 2015; Malama *et al*., 2014). In Africa, particularly sub-Saharan Africa there is limited available literature on the extent of the burden of pulmonary disease due to NTM. Studies conducted in the later part of the 1950s and early 1960s by Zykovet *et al*. (1967) in some African countries Zambia included, reported the isolation of NTM from both tuberculosis patients (1.1 %) and the general public (19.8 %) (Chanda-Kapata *et al*., 2015). Similar studies conducted in Nigeria and Tanzania suggest NTM infections could be associated with HIV co-infection (Aliyu *et al*., 2013; Crump *et al*., 2009; Henkle and Winthrop, 2014). In studies conducted in Zambia by Buijtels *et al* in 2009 and 2010, clinically relevant infection due to NTM was shown to occur in both HIV-positive and HIV-negative patients in a rural hospital. These studies found that the proportion of NTM-positive sputum samples was significantly higher in the patient group (11 %) than in controls (6 %). Additionally, a study by Chanda-Kapata *et al*., 2015 documented that the problem of NTM in Zambia is substantial with western province having the highest NTM prevalence of 9,824/100,000.

While significant amounts of insights have been gained on MDR-TB from studies elsewhere (Bakonyte *et al*., 2003; Haas *et al*., 1997; Ramaswamy and Musser, 1997; Lee *et al*., 2003) a lot still remains to be done towards the understanding of MDR-TB in Zambia. At the molecular level the variation in TB isolates at the *gyrA/B* locus in Zambian TB isolates was characterized (Mitarai *et al*., 2005). Mutations that have been reported to be associated with resistance to fluoroquinolones such as pyrazinamide (Hameed *et al*., 2018) were isolated in a study conducted in Zambia (Mitarai *et al*., 2005). Changes in the amino acid sequences were observed at residue 74 (A74T) and at residue 88 where glycine was substituted by threonine in some of the isolates. Significant and notable contributions have also been made towards the understanding of TB pathology in the country with focus on aspects of the disease including early and accurate diagnostic methods (Mwanza *et al*., 2017; Mwanza *et al*., 2018) and prevalence of the disease among patients infected with the HIV (Muchemwa *et al*., 2017). In one study (Muchemwa *et al.,* 2017) the prevalence of TB bacteraemia in a cohort of HIV-infected adults was elucidated in the country.

This study was conceived to gain insights into the common MTB species as well as prevalence of resistance-associated mutations in two specific resistance marker genes (*rpoB, katG)* and the *inhA* promoter region of *M. tuberculosis* in isolates archived at the University Teaching Hospitals Tuberculosis Laboratory collected between January 2013 and June 2014. The importance of analyzing the types of mutations in the loci associated with resistance to first-line drugs has been recognized as a means to contribute to the fight against TB in Zambia (Mwanza *et al*., 2017).

The study further compared the frequency of different identified mutations with those in isolates circulating in the other countries such as China that found a high percentage (100 percent) of *katG* mutations at codon 315 (Daoquna *et al.*, 2017), 83 percent in Lithuania (Bakonyte *et al*., 2003), 65.4 percent in Africa (Haas *et al*., 1997), 66.2 percent in Turkey (Aslan *et al*., 2008) and Ramaswamy *et al*. (2008) who reported mutations at codon 450 as the most prevalent *rpoB* gene mutation.

1.2 STATEMENT OF THE PROBLEM

Currently, multi drug resistant TB is routinely diagnosed using conventional liquid and solid pathogen culture methods. These are time-inefficient methods which take 2-8weeks and only show the phenotypic characteristic of the pathogen. The treatment regime for TB in Zambia has been designed to suppress the evolution of resistance through a rifampicin-based combination therapy. In spite of these efforts, drug resistant TB isolates have emerged in the country (WHO, 2008). There are several factors contributing to the emergence of drug resistant TB isolates. These include poor quality TB drugs, inappropriate medical prescription, patient non-compliance and interruption in treatment, lack of standard TB treatment guidelines, and inefficient treatment monitoring (Bakonyte *et al.*, 2003; Mwanza, 2016).

Drug resistant *MTB* isolates exclusively emerge from gene mutations occurring in the *rpoB* for RIF and *katG*, *inhA* promoter, *kasA* plus other unknown genes for INH resistance. In Zambia, until now, the type and frequency of mutations in these genes were not known. Furthermore, the MTBC species that were associated with RIF and INH drug resistance in Zambia were not known and this has resulted in a "shot gun" type of treatment where every case of MTBC is presumptively treated as *MTB*.

1.3 SIGNIFICANCE OF THE STUDY

This study is to the best of my knowledge the first to document the prevalent MTBC species, type and frequency of mutations in TB pathogens associated with RIF and INH resistance in Eastern, Lusaka and Western provinces of Zambia. This has given an insight into the countrywide picture which was not available. Furthermore, the study compared the type of mutations identified with those reported in literature to determine the applicability of commercially available rapid molecular drug susceptibility tests. This is important because the design of molecular drug susceptibility tests is dependent on the type and frequency of mutations occurring in a given population (Heep *et al.*, 2001) and their performance varies by geographic location, depending on the prevalent isolates of *MTB*, type and

frequency of drug resistance-conferring mutations in the population (Morgan *et al.*, 2005; Ling *et al.*, 2008).

The findings of this study could contribute to the efforts of the programmes designed to control drug resistance TB in Lusaka, Western and Eastern provinces. The results will assist policy makers in the Ministry of Health to make evidence-based decisions and policies regarding management of MDR-TB as well as the molecular diagnostic tools that would be ideal in detecting drug resistant TB particularly MDR-TB in Zambia. The study has also generated information that is useful for epidemiology of RIF and INH resistant TB which could help in predicting the transmission patterns of MDR-TB in Zambia's Eastern, Lusaka and Western provinces, and is essential in the control of the disease.

1.4 AIM OF THE STUDY

The aim of the study was to differentiate the MTBC isolates associated with RIF and INH drug resistance and determine the prevalence of resistance-associated mutations in two specific resistance marker genes (*rpoB* and *katG*) and the *inhA* promoter region of *M. tuberculosis* isolated at the University Teaching Hospital Tuberculosis Laboratory between January 2013 and June 2014.

1.5 SPECIFIC OBJECTIVES

The specific objectives of the research were:

a) To identify the MTBC species associated with rifampicin and isoniazid drug resistance in Zambian MDR-TB isolates.

b) To determine the type and frequency of mutations in rifampicin and isoniazid drug resistant *MTB* associated with drug resistance marker genes (*rpoB***,** *katG*) and the *inhA* promoter region.

c) To compare the type of mutations in Zambian isolates with those reported in other countries to determine applicability of commercially available molecular tests for detection of MDR-TB in Zambia.

1.6 STUDY HYPOTHESIS

The study tested the hypothesis that there is no difference in species and gene mutations responsible for INH and RIF resistance in *Mycobacterium tuberculosis* complex isolated at the University Teaching Hospital in Lusaka Zambia between January 2013 and June 2014.

1.7 RESEARCH QUESTIONS

The study was guided by the following research questions:

1. What are the common *MTB* complex species circulating in Zambia isolated at the University Teaching Hospitals in Lusaka Zambia between January 2013 and June 2014?

2. What are the major gene mutations associated with resistance to INH and RIF in MTBC isolated at the University Teaching Hospitals Tuberculosis Laboratory in Lusaka Zambia?

3. What is the frequency of the gene mutations responsible for INH and RIF resistance in MTBC isolated at the University Teaching Hospitals Tuberculosis laboratory in Lusaka Zambia between January 2013 and June 2014?

4. How do the types of mutations identified in Zambian isolates compare with those reported in other countries and how applicable are the commercially available molecular tests for detection of MDR-TB in Zambia?

CHAPTER TWO

LITERATURE REVIEW

2.0 OVERVIEW

This chapter reviews studies undertaken by different scholars and any other authorized literature in related topics to document the current understanding on multidrug resistant TB with reference to the methods, results and gaps in the knowledge.

2.1 GEOGRAPHICAL DISTRIBUTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX SPECIES

The geographical distribution of the MTBC species varies widely. *M. tuberculosis* is the most prevalent human TB pathogen in Europe and America, whereas *M. africanum* is widely distributed among African TB patients of African origin, West Africa in particular (Chihota *et al*., 2018; Niemann *et al.*, 2000). The TB epidemic in Africa is driven by regional epidemics characterized by genetically distinct lineages of *M. tuberculosis.* According to Chihota *et al*. (2018), *M. tuberculosis* in these regions may have been introduced from either Europe or Asia and has spread through pastoralism, mining and war. Lineages of *M. tuberculosis* have been found to exhibit a phylogeographical structure, meaning specific lineages are closely associated with specific geographic regions, and preferentially infect persons originating from these regions (Yeboah-Manu *et al*., 2016). The subspecies of *M. bovis* are reported to infect humans, yet they have a broad host range, including wildlife such as Iberian lynx and wild deer and livestock like cattle and goats (Wedlock *et al.*, 2002).

Although the MTBC species are highly similar to each other at DNA level, MTBC members differ widely in terms of host tropism, *M. bovis* affects humans and livestock (Cousins *et al*., 2003) while *M. mungi* and *M. orygis* have been documented to affect mongoose and antelope respectively (Jakko *et al*., 2012).

They also differ in phenotype and pathogenicity as documented by Brosch *et al.* (2002). Of particular interest is the natural resistance of *M. bovis* subsp. *bovis* to PZ, one of the first-line anti-tuberculous drugs. The vaccine isolate *M. bovis* BCG is more frequently used for bladder cancer immunotherapy and can be detected in human urine specimens from bladder cancer patients (Niemann *et al.*, 2002). Therefore, the isolation of *Mycobacterium tuberculosis* complex in such cases is not necessarily an indication for drug treatment or resistance. Rapid differentiation of MTBC isolates to the species and subspecies levels should therefore be obtained not only for epidemiological purposes but also for adequate treatment of each patient (Brandau and Bohle, 2001).

Efforts aimed at elucidating the genetic structure of the MTBC population and species circulating in certain geographic regions is increasingly becoming important for TB control (Anwar *et al.*, 2015). Geospatial review of various lineages of *M. tuberculosis* has been done and documented (Chihota *et al.*, 2018) (Figure 2.1). Current genomic studies have revealed that substantial strain genetic diversity exists among the different members and genotypes of MTBC, which may have implications for the development and deployment of new TB vaccines and diagnostics (Yeboah-Manu *et al.*, 2016; Hershberg *et al.*, 2008).

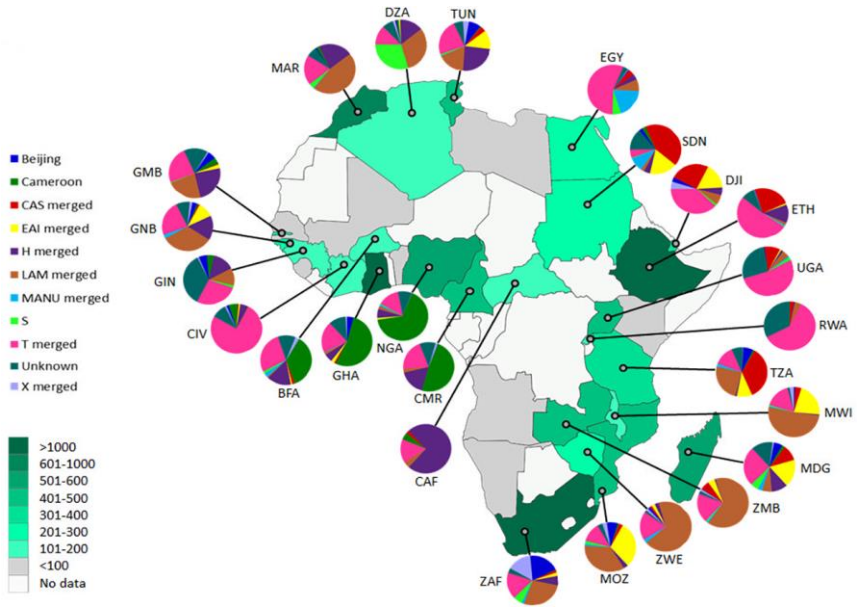

Figure 2.1 Geospatial distribution of *M. tuberculosis* lineages in Africa**.** Each pie chart segment reflects the relative proportion of *M. tuberculosis* isolates belonging to respective major lineages for each country (Chihota *et al.*, 2018).

2.2. IDENTIFICATION AND DIFFERENTIATION METHODS FOR *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

Early diagnosis is important for early patient management and successful patient outcomes in treatment of tuberculosis. There are number of tests available for the diagnosis of TB that range from conventional smear microscopy to culture (gold standard) and nucleic acid amplification techniques (Agrawal *et al*., 2016). Selected growth media and techniques typically used in the study and identification of *M. tuberculosis* together with their strengths and limitations are described below.

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2.2.1 ACID FAST BACILLI (AFB) SMEAR MICROSCOPY

Acid-fast bacilli (AFB) microscopy is the mainstay for TB diagnosis in most low-resource settings due to its simplicity, cost effectiveness and rapidity (Perkins *et al*., 2006; Ritu and Vithal, 2015). It involves microscopic examination of specially stained smears to detect acid-fast organisms such as *Mycobacterium tuberculosis* and nontuberculous mycobacteria (NTM) organisms that do not get decolourized with acid alcohol due to the lipid-rich mycolic acids in the cell wall (I.U.A.T.L.D, 2005). Positive AFB smear results provide a first indication of mycobacterial infection and potential TB disease but should however be accompanied by additional confirmatory diagnosis (Ghosh *et al*., 2016). Although AFB microscopy allows for rapid diagnosis of mycobacteria, its threshold of detection of AFB in sputum samples under optimal conditions is relatively high ranging between 10^4 and 10^5 bacilli per ml (I.U.A.T.L.D, 2005). It has low sensitivity and specificity especially in children and immune compromised individuals who usually present with a pauci-bacillary picture (Khan and Starke, 1995; Ganoza *et al.*, 2005; Karstaedt *et al*., 1998; Siddiqi *et al.*, 2003). Additionally, AFB smear microscopy does not differentiate *M. tuberculosis* from NTM (Chanda-Kapata *et al*., 2015; Taiwo and Glassroth, 2010) and neither does it distinguish between viable and dead organisms (Ghosh *et al*., 2016). Studies by Pokam and Asuquo, (2012) and Ngula Monde *et al.* (2017) have documented that failure to characterize AFB positive NTM lung infections has led to misclassification and incorrect treatment as pulmonary tuberculosis since all diagnosed sputum smear positive patients are placed on TB treatment.

2.2.2 *MYCOBACTERIUM TUBERCULOSIS* CULTURING AND GROWTH

Culturing of pathogenic bacteria is considered the gold standard for sensitivity and specificity for *M. tuberculosis* and is a critical technique for routine diagnosis of tuberculosis that takes at least two weeks (Ghodbane *et al*., 2014). Growth media routinely used in *M. tuberculosis* culturing are prepared either as solid or liquid media. The most commonly used agar-based selective medium for *mycobateria* is the Löwenstein–Jensen (LJ) medium which was developed for selective growth of *Mycobacterium* species (Global Laboratory Initiative, 2014).

In recent years, several new non-radiometric culture systems such as the BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system (Becton Dickinson, Sparks, MD) have been introduced (Lee *et al*., 2003). The MGIT system used for the detection and recovery of *Mycobacteria* contains 7 ml of modified Middlebrook 7h9 broth base supplemented with nutrients (glycerol, oleic acid, albumin and dextrose). An antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (BBL MGIT PANTA) is also added to the MGIT culture tube which contains a fluorescent sensor that detects the concentration of oxygen in the culture medium (Lee *et al*., 2003). After inoculation of each tube with 0.5 ml of the processed specimen, the MGIT culture tubes are incubated at 37 °C in the BACTEC MGIT 960 and monitored automatically every 60 min for increased fluorescence. The level of fluorescence in the MGIT culture tube corresponds to the amount of oxygen consumed by the organisms in the inoculated specimens which is proportional to the number of bacteria present (Lee *et al*., 2003).

When a certain level of fluorescence is reached, the BACTEC MGIT 960 indicates that the tube is positive. The cultures are tested until positive or left to grow for 42 days. Samples found to be positive are removed from the BACTEC MGIT 960 and a smear is prepared from the positive cultured and examined for Acid Fast Bacilli (AFB). If the smear is positive for AFB, the broth is subjected to a purity check by sub culturing on the blood agar (Global Laboratory Initiative, 2014). Broth that passes the purity check is then subjected to a rapid diagnostic test (TAUNS) that distinguishes *Mycobacterium tuberculosis* complex (MTBC) from Mycobacterium Other Than Tuberculosis (MOTT). MTBC positive samples are then subjected to

drug/antimicrobial susceptibility test (AST). This however, has a longer turnaround time (4-8 weeks) and therefore does not provide quick diagnosis. It also requires laboratory biosafety level 2 or 3 as well as highly trained personnel (Hillemann *et al*., 2011).

Another growth and culture medium commonly used for cultivation of the *Mycobacteria* is Middlebrook 7H10 Agar. This is a solid growth media known to reduce the growth of contaminants (Kubica *et al*., 1967). It has a variety of inorganic salts which provide substances essential for the growth of *Mycobacteria*. The sodium citrate, when converted to citric acid, serves to hold certain inorganic cations in solution while glycerol acts as an abundant source of carbon and energy. Oleic acid, as well as other long chain fatty acids plays an important role in the metabolism of *Mycobacteria* while catalase destroys toxic peroxides that may be present in the medium. Albumin protects the tubercle bacilli against toxic agents thereby enhancing their recovery on primary isolation. Partial inhibition of contaminating bacteria is achieved by the presence of the malachite green dye (Finegold and Baron, 1990; Murray *et al*., 2003).

Stonebrink TB medium + PACT is another growth medium used for cultivation of *Mycobacteria*. It is a selective egg-based medium supplemented with antimicrobials; polymyxin B 200,000 units/L, amphotericin B 10 mg/L, carbenicillin 50 mg/L, and trimethoprim 10 mg/L (PACT). The egg yolk is a source of lipids for the metabolism of *Mycobacteria* and partial inhibition of bacteria is achieved by the presence of the malachite green dye. Sodium pyruvate is a growth stimulant as *M. bovis*, *M. africanum* and *M. microti* are unable to use glycerol as a sole carbon source due lack of pyruvate kinase activity. For this reason, pyruvate is routinely added to glycerinated medium to enable growth (Stonebrink, 1958; Wayne and Kubica, 1986).

2.2.3 GENE XPERT MTB/RIF ASSAY (XPERT MTB/RIF)

The Xpert MTB/RIF is an automated cartridge-based real-time PCR used for detection of *Mycobacterium tuberculosis* and tests all positive samples for rifampicin resistance within two hours. It has high sensitivity and specificity with pulmonary samples (Chakravorty *et al*., 2017; Khadka *et al*., 2017; Sharma *et al.*,

2015). Since it is a cartridge-based nucleic acid amplification test (CBNAAT), it does not have any specific prerequisites for its set-up and requires minimal technical training. Additionally, the reagent used for processing is bactericidal and therefore inactivates tubercle bacilli in vitro thereby eliminating biosafety risks and thus enabling its use as a rapid point-of-care diagnostic test (Sharma *et al.*, 2015).

While Xpert MTB/RIF may be the most preferred technique amongst all molecular diagnostic tests, it has its limitations. Although resistance to RIF is usually taken as a surrogate marker for MDR-TB (Khadka, *et al*., 2017; Vadwai *et al.*, 2012), certain strains may exhibit only mono-resistance to RIF that may not warrant full line MDR therapy. This may lead to unnecessary treatment and over-estimation of the MDR-TB cases. Additionally, Xpert MTB/RIF requires stable electrical power supply and temperature control and its performance is dependent on proper management and annual calibration (Sharma *et al.*, 2015).

2.2.4 LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (TB-LAMP)

The LoopampTM *Mycobacterium tuberculosis* complex (MTBC) detection kit (Eiken Chemical Company, Tokyo, Japan), is a commercial molecular assay that uses loop-mediated isothermal amplification (LAMP), referred to as TB-LAMP (WHO, 2016). It is a manual assay that targets the *gyrB* and *IS* regions of the *Mycobacterium tuberculosis* (MTB) complex genome. Detection of amplified product is based on turbidity visualized either with the naked eye or under ultraviolet (UV) light after 15–60 minutes (Aryan *et al*., 2010; Kaewphinit *et al*., 2013; Mitarai *et al*., 2011; Sethi *et al*., 2013). The advantages of TB-LAMP are that it is relatively high-throughput, does not require sophisticated instruments, and has biosafety requirements similar to those of sputum-smear microscopy. It is simple, rapid and cost-effective with good sensitivity and specificity and better than conventional methods to diagnose extrapulmonary tuberculosis (Ghosh *et al*., 2017). Because of its limited infrastructure requirements and relative ease of use, TB-LAMP is being explored for use as a rapid diagnostic test that can be used as an alternative to smear microscopy in resource-limited settings (Boehme *et al*., 2007; WHO, 2015; WHO, 2016) or as an add-on test to smear microscopy in settings where gene Xpert MTB/RIF is unavailable (WHO, 2016). It can however only be

used as a diagnostic tool and not for monitoring treatment outcome as the presence MTBC DNA does not necessarily mean presence of viable organisms.

2.2.5 POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) sometimes called "molecular photocopying," is a fast and inexpensive technique used to "amplify" - copy - small segments of DNA. It is used in molecular biology to make many copies of a specific DNA segment. Using PCR, copies of DNA sequences are exponentially amplified to generate thousands to millions of more copies of that particular DNA segment. PCR based assays offer high sensitivity by amplification of small amount of DNA, and have been extensively evaluated for the detection of *M. tuberculosis* from clinical samples (Khater *et al*., 2016).

PCR is often used in the diagnosis of tuberculosis by amplification of DNA sequences that code for a 65-kDa antigen known to be present in all mycobacterial species (Brisson-Noel *et al*., 1989; Hance *et al*., 1989; Pao *et al*., 1990) or by amplification of the MPB64 antigen that is thought to be specific for identification of *M. tuberculosis* complex (Shankar *et al*., 1991). It is known to be more sensitive than traditional culture methods for direct detection of *M. tuberculosis* in specimens, for patients known to have tuberculosis (Brisson-Noel *et al., 1989;* Cousins *et al*., 1993; Pao *et al., 1990*). Additionally, PCR offers considerable advantages in speed over the traditional methods of identification and culture of *M. tuberculosis* as specimens could be screened for *M. tuberculosis* in 2 to 3 days and identification of an isolate could be completed within 4 hrs (Cousins *et al*., 1993).

To enhance the convenience of PCR, multiplex PCR, a molecular biology technique for amplification of multiple targets in a single PCR reaction using multiple primers and a temperature-mediated DNA polymerase in a thermal cycle has become a rapid and convenient screening assay in both the clinical and the research laboratory settings (Markoulatos *et al*., 2002). As an extension to the practical use of PCR, this technique is the best method for diagnosis and treatment of MDR-TB as it has the potential to produce considerable savings in time and

effort within the laboratory without compromising on the utility of the experiment (Luke *et al*., 2017; Chae *et al*., 2017).

Multiplex Polymerase Chain Reaction and RD9 based deletion typing are used in characterisation of MTBC species by amplifying specific regions of difference (*cfp32*, RD9 and RD12) that have been reliably identified in multiple distinct MTBC strains, and have been used as gold standard genetic markers for MTBC species and phylogenetic lineage prediction for MTBC classification (Faksri *et al*., 2016; Zufan *et al*., 2017). This however requires skilled training, specialised equipment and like other molecular techniques it is unable to distinguish between DNA from both viable and dead organisms.

2.2.6. IS*6110* REAL TIME PCR

IS*6110* is an insertion sequence that belongs to the IS3 family and is found in MTBC members. Most strains carry 2910 to 15 copies, which are present in a wide variety of chromosomal sites (Negi *et al*., 2007; Sekar *et al*., 2008). Primers and probes used for real time IS*6110*-PCR amplify fragment of IS*6110*, a repeated insertion sequence specific for *M. tuberculosis* complex. Due to its repetitive nature, the IS*6110* insertion sequence is commonly used as a target in the detection of *MTB* in pulmonary and extrapulmonary specimens. Gupta *et al*., 2016 and Negi *et al.*, 2007 documented increased TB positive cases using the IS*6110* target in Pulmonary Samples. IS*6110* real time PCR has been proven to be a simple, quick, sensitive, and specific method to diagnose MTBC (Girish *et al*., 2016). It however does not differentiate between the different known members of the MTBC.

2.2.7 16S RDNA ANALYSIS

16S ribosomal RNA (or 16S rRNA**)** is the component of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence. The genes coding for it are referred to as 16S rDNA gene and are used in reconstructing phylogenies due to the slow rates of evolution of this region of the gene (Rainey and Stackebrandt, 2000). 16S and Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) sequencing are routinely used for identification and comparison of bacteria

or fungi present within a given sample. Although 16S rRNA gene sequencing is highly useful in regards to bacterial classification, it has low phylogenetic power at the species level and poor discriminatory power for some genera (Rainey *et al*., 1996; Roth *et al*., 1998; Woo *et al*., 2008). Over the years, as a result of the widespread use of PCR and DNA sequencing, 16S rDNA sequencing has played a crucial role in the accurate identification of bacterial isolates and the discovery of novel bacteria in clinical microbiology laboratories. For bacterial identification, 16S rDNA sequencing is particularly important in the case of bacteria with unusual phenotypic profiles, rare and slow-growing bacteria and uncultivable bacteria and culture-negative infections (Woo *et al*., 2008). However, one of the greatest challenges in putting 16S rDNA sequencing into routine use in clinical microbiology laboratories is its labour intensiveness (Hall *et al*., 2003; Janda *et al*., 2007). Most of the steps have to be performed manually, although user-friendly commercial kits for some of the steps are available (Woo *et al*., 2008). Additionally, the high degree of sequence conservation among the members of the MTBC has resulted in difficulties for laboratories since commercial DNA probe and amplification assays based on 16S rDNA sequences identical for all MTBC members cannot be used to differentiate members of the complex (Linda *et al*., 2002).

2.2.8 DELETION ANALYSIS

Genetic deletion mapping is a technique used to determine the location of mutation sites within a gene. Insertion or deletion mutations in a gene result in frame-shift changes that alter entire amino acid sequences that follow mutations (Linda *et al*., 2002). Insertions and deletions are usually more harmful than substitution mutations in which only a single amino acid is altered. Deletions often arise from recombination between insertion sequence (IS) elements (Brosch *et al.,* 1999; Fang *et al*., 1999), and the *M. tuberculosis* genome contains greater than 40 ISs and mobile genetic elements that could mediate deletions (Cole *et al*., 1998). Commercially available molecular assays cannot differentiate these organisms because of the genetic identities of their 16S rRNA gene sequences. Comparative genomic analyses with the complete DNA sequence of *M. tuberculosis* H37Rv has provided information on regions of difference (RD 1 to RD 16) deleted in members

of the MTBC other than *M. tuberculosis*. Linda *et al*., (2002) established that the presence of variable regions in members of the MTBC suggests that deletion analysis could be a useful tool for differentiation of members of the MTBC. Deletion analysis provides for a rapid and simple means of differentiating members of MTBC, especially *M. bovis* and *M. tuberculosis*, when it is important to distinguish between zoonotic sources (cattle and unpasteurized dairy products) and human sources of tuberculosis disease (Linda *et al*., 2002) and in generating genetic diversity within the complex (Brosch *et al.*, 2002; Kato-Maeda *et al.*, 2001; Pym and Brosch, 2000).

2.2.9 *CFP32*, RD9 AND RD12 GENETIC REGIONS

In recent years, there has been a remarkable progress in identifying new and potentially useful antigens for diagnosis of both latent and active tuberculosis. Regions of differences (RDs) encoded proteins are among such promising candidate antigens (RD antigens). Some of these antigens are encoded by regions of differences located in the genome of *M. tuberculosis, M. africanum, M. bovis* but are absent in all the Bacillus Calmette Guerin substrains and many of the environmental *mycobacteria* (Parkash *et al*., 2009). Specific Regions of differences (RDs) have been reliably identified in multiple distinct *Mycobacterium tuberculosis* complex (MTBC) strains, and have been used as gold standard genetic markers for MTBC species and phylogenetic lineage prediction for MTBC classification (Faksri *et al*., 2016). The RD9 is only present in *M. tuberculosis* but absent from *M. africanum, M. bovis, M. caprae* and other MTBC and therefore used to differentiate *M. tuberculosis* from other members of the MTBC.

2.3 METHODS FOR DETECTING DRUG RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* SPECIES

Slayden *et al.* (2000) argued that drug susceptibility testing (DST) by the conventional solid medium culture method is highly sensitive and specific but extremely slow due to the slow growth of *M. tuberculosis*. Liquid culture methods reduce the turnaround time but they require specialized instrumentation and reagents and are not feasible in most resource-limited settings. Some of the

methods commonly used for DST in *M. tuberculosis* species are briefly described below.

2.3.1 LIQUID CULTURE DRUG SUSCEPTIBILTY TESTING

The MGIT medium used for DST consists of modified Middlebrook 7H9 broth. It uses an automated MGIT system that requires an instrument called the BACTEC 960 and tubes in this automated system require 7.0 ml of medium. For routine culture of specimens, a growth supplement known as OADC (consisting of oleic acid, albumin, dextrose and catalase) is added to the medium before inoculation to complete the medium. The growth supplement is essential for the growth of many types of mycobacteria, especially those belonging to the *M. tuberculosis* complex. Additionally, it inhibits growth of other bacteria. In addition to the Middlebrook 7H9 liquid medium, the MGIT tube contains an oxygen quenched fluorochrome – tris (4,7-diphenyl- 1,10-phenonthroline) ruthenium chloride pentahydrate – embedded in silicone at the bottom of the tube. During bacterial growth within the tube, the free oxygen is utilized and is replaced by CO_2. The depletion of free oxygen results in fluorescence of the sensor within the MGIT tube when visualized under ultraviolet light. The intensity of fluorescence is directly proportional to the extent of oxygen depletion.

A known concentration of a test agent is added to one of the MGIT tubes and both tubes are incubated. Growth in the tube containing the agent is compared with that in the MGIT tube without the agent (the control tube) (Siddiqi and Rüsch-Gerdes, 2006). If the test agent is active against the *Mycobacteria*, it will inhibit the growth resulting into suppression of fluorescence while growth in the control tube will be uninhibited and fluorescence will increase. Growth is monitored by the MGIT 960 instrument which automatically interprets the difference in Growth Units (GU) values between the control tubes and the tubes containing the agent and results are reported as susceptible or resistant (Siddiqi and Rüsch-Gerdes, 2006). New molecular diagnostic methods present a potentially rapid and sensitive alternative to conventional diagnostics. Rapid determination of the antimicrobial susceptibility pattern in clinical isolates of *M. tuberculosis* is important for the early administration of appropriate therapeutic agents for the prevention of additional resistance development (Nettleman, 2005). In this context, molecular

characterization of drug resistance by identifying mutations in associated genes is an important step in the development of a potential rapid molecular drug susceptibility test as an alternative to conventional methods (Mathuria *et al.,* 2009).

2.3.2 GENOTYPE MTBDR ASSAY

The Genotype MTBDR assay (Hain Lifescience GmbH, Nehren, Germany) is a novel PCR-based reverse hybridization method with membrane strips coated with target-specific oligonucleotides (Hillemann et al., 2005). It is designed to detect mutations within the 81-bp hotspot region of *rpoB* and codon 315 of *katG* genes that confer RIF and INH resistance (Anek-vorapong *et al*., 2010; Cavusoglu *et al*., 2006).

GenoType MTBDR*plus (*Hain Lifescience, Germany) version 1 is an improvement over the MTBDR (Hain Lifescience, Germany) assay with the ability to detect low-level isoniazid resistance (*inhA*). It has high sensitivity and specificity and offers a quicker way to detect MDR-TB. It is however not cost effective especially in resource constrained settings with high TB burden (Ninan *et al*., 2016).

2.3.3 DEOXYRIBONUCLEIC ACID SEQUENCING

Gene or genome sequencing refers to the process of determining the sequence of nucleotide bases in a piece of deoxyribonucleic acid (DNA). Different methods and technologies of DNA sequencing have been developed with the Sanger dideoxy chain termination method (developed by the British biochemist Fred Sanger and his colleagues in 1977) being one of the earliest methods to be developed. Next-generation sequencing (NGS) technologies are newer massively parallel approaches that enable many DNA fragments to be sequenced at once with good performance on throughput and accuracy. These technologies are also cost-effective compared TO the Sanger method (Lin *et al*., 2012). The utility of next-generation technologies was improved significantly by advances in bioinformatics that have allowed for increased data storage and facilitated the analysis and manipulation of very large data sets. The applications of next-generation sequencing technologies are broad due to their low cost and large-scale high-throughput capacity. Using these technologies, scientists have been able to rapidly sequence entire genomes

(whole genome sequencing) of organisms, to discover genes involved in disease, and to better understand genomic structure and diversity among species generally (Lin *et al*., 2012).

Whole genome sequencing (WGS) is the determination of the entire DNA sequence of an organism. It involves the amplification of an entire genome and comparing it with the standard (wild type) genome to identify whether there are any changes (mutations) in a target gene structure. Whole-genome sequencing is increasingly used in clinical diagnosis of tuberculosis and study of *Mycobacterium tuberculosis* complex (MTBC). Many studies have been able to address questions about tuberculosis with WGS, and knowing the sequence of the entire genome, rather than only a few fragments, has greatly increased the precision of molecular epidemiology and contact tracing (Howard and Oscar, 2015; Lin *et al*., 2012). Additionally, mutation, drug resistance, the target of new drugs, and the phylogeny and evolution of the *Mycobacterium tuberculosis* complex bacteria have been made clearer by WGS. However, WGS has not explained differences in transmissibility between strains, difference in virulence and why some strains are more prone to development of multidrug resistance (Howard and Oscar, 2015). The high cost and massive data also remain major challenges for most settings (Lin *et al*., 2012).

2.3.4 MUTATIONAL ANALYSIS

Mutations are commonly identified by polymerase chain reaction (PCR) of DNA followed by direct sequencing of the amplified DNA. Understanding the molecular basis of mutations lays the groundwork for mutational analysis of biochemical, cellular, and developmental pathways (Victor *et al*., 2002). The goal and the challenge are to identify all of the genes that contribute to the process and then to understand the nature of the gene products (usually proteins) and how they contribute and interact in this process or pathway. Genes are identified through their mutant alleles; therefore the genetic approach begins with mutant (Griffiths *et al*., 1999). Mutations are usually identified using sequence alignment that allows for comparing the target sequence to that of the wild type.

2.4 COMMON MUTATIONS IN *MYCOBACTERIUM TUBERCULOSIS* SPECIES

Data collected from different countries have revealed that resistance to RIF in more than 90 percent of cases is due to mutations resulting in an amino acid substitution within the 81-bp core region of the RNA polymerase β-subunit gene (*rpoB*) (RRDR) (Sajduda *et al.*, 2004). This region is sometimes called the RIF resistance-determining region.

Gene mutations at codon 531, 526, 516, and 511, are the most commonly encountered sequence changes in *MTB* isolates worldwide (Cavusoglu *et al.*, 2002; Caws *et al*., 2006). While TTG at codon 531 and CCG at codon 511 are the dominant mutated alleles, codon 526 and codon 516 show large numbers of allelic variations (Cavusoglu *et al.*, 2002). Two postulates were offered by Billington *et al.* (1999) and Mani *et al.* (2001) to explain this situation. On one hand, Billington *et al.* (1999) observed that mutants isolated more frequently in clinical practice have higher mean relative fitness, and the prevalence of each mutant type depends on its ability to survive. This might be the reason for the higher rates of occurrence of the mutation of TCG to TTG at codon 531 resulting in the substitution of serine for leusine and the mutation of CTG to CCG at codon 511(L511I) in isolates worldwide. On the other hand, Mani *et al*. (2001) observed that mutations within codons 526 and 516 have been shown to lead to high-level RIF resistance in *M. tuberculosis* and therefore mutations continue to arise in these codons, probably due to the ability of *M. tuberculosis* to adapt to drug exposure.

Isoniazid resistance-conferring mutations are most frequently detected at *katG*315 locus (Gagneux *et al*., 2006). Mutations at this site are thought to introduce only a slight fitness cost to the bacterium. According to Gagneux *et al*. (2006), this mutation may be more prevalent in mycobacterial isolates of the Euro-American lineage, while the East Asian lineage may be associated with *katG* mutations other than those at codon 315. However, 98.1 percent (159/162 Isolates) of *katG* mutations in the Beijing genotype isolates was reported to have mutations in codon 315 (Gagneux *et al*., 2006). A study by Hu *et al*. (2010) in central China also found a high percentage (100 percent) of *katG* mutations at codon 315 while a similar study by Daoquna *et al.* (2017) in Yunnan China documented 73.3 % of co-

existence mutations at codon 315 and 463. The differences could be explained by geographically-based prevalence and mutation frequency differences associated with different sublineages of *M. tuberculosis* within the East Asian lineage. Another explanation is that there may be a higher selective pressure for *katG*315 mutations in MDR isolates (Iwamoto *et al.*, 2010). Van Soolingen *et al.* (1997) reported that isolates with amino acid substitutions in *katG* 315 are more likely to develop resistance to other drugs. This is consistent with the finding of previous studies (Gagneux *et al*., 2006; Hu *et al.*, 2010; Schilke *et al.*, 1999) that reported substitutions at codon 315 (S315L) of *katG* as being the most common in MDR-TB isolates.

In contrast, INH resistance is mediated by mutations in several genes, most frequently within the *katG* gene, encoding a catalase-peroxidase which transforms INH into its active form (Ramaswamy and Musser, 1998), and in the promoter region of *inhA*, encoding a putative enzyme involved in mycolic acid biosynthesis. Studies conducted in Bangladesh and Nepal have shown that 10 to 34 percent of INH-resistant cases have mutations in the *inhA* promoter region (Nakajima *et al.*, 2012). An upregulation mutation in the *inhA* promoter region that results in the over-expression of *inhA* and confers INH resistance via a titration mechanism has also been reported as another mechanism of drug resistance in *MTB* (Wade and Zhang, 2004).

The most prevalent type of gene mutation is a transversion mutation in *katG* and in the *inhA* promoter region. No deletion or insertion in *katG* has been detected in any isolates (Rouse *et al.*, 1996). It has been postulated that the amino acid substitution in *katG* (S315T) is the most favored by the bacteria because this alteration was elucidated to spoil INH activation and, on the other hand, to retain 30 to 40 percent of the catalase-peroxidase activity necessary for virulence (Rouse *et al.*, 1996).

Hu *et al*. (2010) have reported that the prevalence of the *katG* (S315T) substitution in *M. tuberculosis* isolates around the world varies, especially with regard to the prevalence of TB. A higher prevalence of this substitution has been generally observed in high TB burden regions, often with the predominance of Beijing and MDR *M. tuberculosis* isolates. In regions where the burden of TB is intermediate or low the prevalence of the *katG* (S315T) substitution is low (Hu *et al*., 2010).

A novel mutation in NADH dehydrogenase (*ndh*) has been reported to be associated with INH resistance in isolates from the USA (Vilche`ze *et al.*, 2005). It causes an increase in the NADH/NAD ratio which has been found to cause INH resistance in *M. smegmatis* and *M. bovis* BCG, but not in *M. tuberculosis*, presumably because of the different role that *ndh* plays in *M. bovis* compared with *M. tuberculosis* (Vilche`ze *et al.*, 2005). A study conducted in Brazil detected two *ndh* locus transition mutations (CGT to TGT change in codon 13 and GTG to GCG change in codon 18) (Cardoso *et al.*, 2007)). Mutations in *ndh* appear to be an additional molecular mechanism for isoniazid resistance in *M. tuberculosis* (Lee *et al.*, 2001).

SUMMARY

This chapter presented the literature review. From the literature reviewed, there seems to be a lot of baseline information describing mutations in the *rpoB, KatG* and *inhA* genes of clinical isolates of *M. tuberculosis* in many populations in many studies in different parts of the world. However, there is little or no information on molecular characterisation of mutations responsible for pathogen resistance to rifampicin and isoniazid as well as of *Mycobacterium* species distribution in Zambia.

CHAPTER THREE

MATERIALS AND METHODS

3.0 OVERVIEW

This chapter outlines the different methods, approaches and strategies to execute this study. The methods of the study are presented under the following headings: (1) Study design (2) Study setting (experimental location) (3) Study population (4) Target population (5) Isolate selection (6) Handling of stored isolates (7) Inclusion criteria (8) Exclusion criteria (9) Isolate sample size (10) Sampling method and framework (11) Data collection (12) Sample Selection (13) Genomic DNA extraction (14) *Mycobacterim tuberculosis* complex (MTBC) differentiation multiplex PCR (15) Analysis of PCR products (16) Determination of DNA concentration and integrity (17) Sequencing of the *rpoB* and *katG* genes, and the *inhA* promoter region (18) Sequence analysis (19) Ethical considerations (20) Data processing and analysis.

3.1 STUDY DESIGN

This was a retrospective cross sectional study where all rifampicin and isoniazid drug resistant MTBC isolates archived from routine diagnosis of tuberculosis at the UTH TB laboratory in Lusaka between January 2013 and June 2014 were retrieved for further analysis.

3.2 ISOLATE SOURCE

A record review of laboratory DST records was done to find RIF and/or INH isolates previously tested using phenotypic DST at the UTH TB Laboratory between January 2013 and June 2014. The list of RIF and/or INH isolates generated from the record review was used to locate and retrieve the archived isolates from the laboratory repository. Only the first MDR isolate of each patient was included, excluding multiple isolates from the same patient. All of the available isolates were

retrieved and included in the study. Another record review of "request for examination" forms submitted to the laboratory along with the patient records was done to collect data on province of origin were collected. Other demographic data such as gender and age was incomplete and therefore excluded.

3.3 HANDLING AND STORAGE OF ISOLATES

All work with pathogenic *Mycobacterium tuberculosis* was performed in a Biosafety Level 3 laboratory at the UTH. The pathogenic *Mycobacterium tuberculosis* used in this study were MTBC isolates from routine *mycobacteria* detection and recovery of *mycobacteria* stored in Mycobacteria Growth Indicator Tube (MGIT) at -80 °C.

The MGIT used for the detection and recovery of *mycobacteria* contained 7 ml of modified Middlebrook 7h9 broth base supplemented with nutrients (glycerol, oleic acid, albumin and dextrose). A mixture of five antibiotics (BBL MGIT PANTA) was reconstituted in 15000 µl of deionised distilled water to give final concentrations of the drugs as follows: polymyxin B (0.4 units/ µl), amphotericin B (0.04 µg/µl), nalidixic acid (0.16 µg/µl), trimethoprim (0.04 µg/µl, and azlocillin (0.04 µg/µl) 500 µl of the reconstituted antibiotic mixture was added to each MGIT medium (Lee *et al*., 2003).

Before inoculation into the MGIT, sputum was decontaminated using a decontaminant consisting of 4 percent sodium hydroxide (NaOH), *N*-acetyl-L-cysteine (NALC) and sodium citrate in order to digest the mucous components and kill contaminating normal flora to facilitate the growth of *Mycobacteria*. NaOH acts as an emulsifier and a decontaminant that breaks down mucoid material and inhibits the growth of contaminants. NALC combined with NaOH, facilitates decontamination by further digesting mucopurulent specimens which allows the NaOH to penetrate. Sodium citrate, aids in the liquification by binding heavy metals, thus stabilizing NALC and allowing it to work properly (Jorgensen *et al*., 2015).

3.4 STUDY SETTING

The study was carried out at the University Teaching Hospitals (UTH) Tuberculosis (TB) Laboratory in Lusaka which is the TB Reference Laboratory for the Southern region of Zambia, (Figure 3.1). It is a referral TB culture facility for Lusaka, Eastern and Western Provinces and also provides TB diagnostic services for UTH and offers an External Quality Assurance Scheme for TB smear microscopy to diagnostic sites in the above provinces.

UTH TB Laboratory is also enrolled with the South African National Institute for Communicable Diseases in TB smear microscopy External Quality Assessment (EQA) and Centre for Disease Control and Prevention (CDC) in GeneXpert EQA. The laboratory recently underwent the Southern African Development Community Accreditation Services (SADCAS) audit and has since been recommended for international accreditation.

3.5 STUDY POPULATION

The study population was tuberculosis (TB) patients whose *Mycobacterium tuberculosis* complex isolates exhibited resistance to INH and/or RIF through phenotypic drug sensitivity testing done using an automated Mycobacteria Growth Indicator Tube (MGIT) at the UTH TB Laboratory between January 2013 and June 2014.

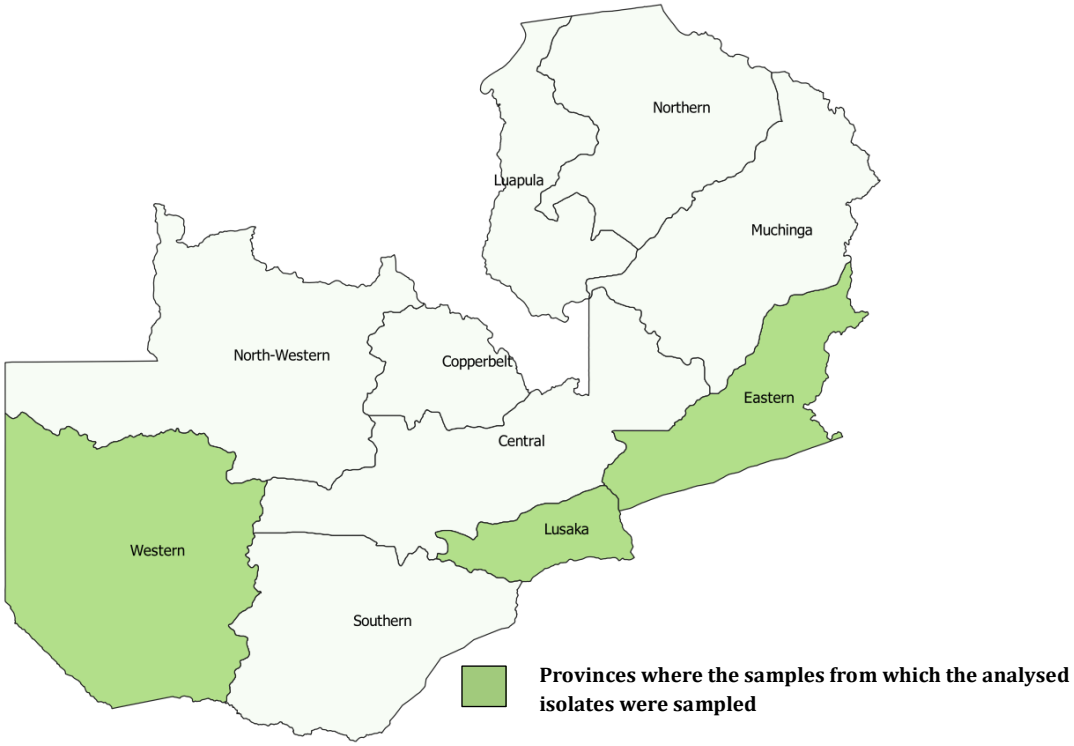


Figure 3.1: Map of Zambia showing the 3 provinces where the patient samples from which the analysed isolates were purposefully sampled.

3.6 TARGET POPULATION

The target population was composed of tuberculosis patients in the study population based on the criterion of having bacterial pathogen isolates resistant to either RIF or INH or both (i.e. MDR TB) as outlined in the inclusion and exclusion criteria.

3.7 INCLUSION CRITERION

All TB patients with bacterial pathogen isolates resistant to RIF and/or INH.

3.8 EXCLUSION CRITERIA

All TB patients with isolates of Mycobacterium Other Than Tuberculosis (MOTT) and TB patients with isolates of MTBC resistant to streptomycin and ethambutol.

3.9 ISOLATE SAMPLE SIZE

Forty rifampicin and isoniazid resistant sample isolates were used in this study. These were selected from all *Mycobacterium tuberculosis* complex (MTBC) isolated at the University Teaching Hospitals Tuberculosis laboratory between January 2013 and June 2014. Four of the forty patients from whose sputum the study isolates were recovered were repeat treatments (had been previously treated with the standard first-line TB drugs) and therefore likely had Acquired or Secondary Resistance: drug resistance occurring in patients who have a history of previous TB treatment while thirty six of the forty isolates likely had Initial Resistance (drug resistance in a newly diagnosed patient who has never received anti-TB drugs that occurs when a patient develops tuberculosis after being infected by another person who has resistant tubercle bacilli) (MOH, 1986).

3.10 SAMPLING METHOD AND FRAME

Samples were selected from all isolates deemed to qualify as MTBC that were resistant to first-line TB drugs archived at UTH TB Laboratory between January 2013 and June 2014. From these isolates, using a purposive sampling method, RIF and INH resistant samples were selected. A purposive sampling method was chosen so as to include all RIF and/or INH resistant isolates in order to determine the type and frequency of mutations in the isolates.

3.11 DATA COLLECTION

The data collected through a record review of laboratory DST records were recorded in a laboratory worksheet and finally entered into an Excel database as shown in Table 3.1.

Table 3.1. Data collection sheet with selected entries.

Lab ID.	Type of Specimen	Place/Town of origin	Drug Susceptibility Pattern				Species identification by MTCD-MPCR
			RIF	INH	STR	EMB	
Zut0089	Sputum	Mongu	R	R	S	S	*Mycobacterium tuberculosis*
Zut0062	Sputum	Lusaka	R	R	S	S	*M. tuberculosis*
Zut001	Sputum	Katete	R	R	S	R	*M. tuberculosis*
Zut0017	Sputum	Lusaka	R	S	S	S	*M. tuberculosis*
Zut0029	Sputum	Sioma	R	R	S	R	*M. tuberculosis*
Zut0032	Sputum	Chipata	R	R	S	S	*M. tuberculosis*
Zut0016	Sputum	Senanga	R	R	S	R	*M. tuberculosis*
Zut0101	Sputum	Lusaka	R	R	S	S	*M. tuberculosis*

The place/town of origin was extracted from the laboratory database since the types of gene mutations vary depending on the geographical location as reported by Morgan *et al*. (2005) and Ling *et al*. (2008). The type of specimen was also considered to ascertain possibility of disseminated drug resistance TB.

3.12 SAMPLE PROCESSING

3.12.1 GENOMIC DNA EXTRACTION

Approximately 5ml of the broth from each MGIT containing pathogen isolates were transferred to a labelled 50 ml Falcon tube (Thermo Fisher Scientific, Waltham, MA, USA). Samples were centrifuged at 3,000*xg* in a Tomy LX-141 refrigerated microcentrifuge (Tomy Kogyo.co. Tokyo Japan) for 15 minutes at 4°C to concentrate pathogen suspension cultures after discarding the excess supernatant. 500µl of the centrifuged bacterial pathogen were transferred from the bottom of the Falcon tubes into 2 ml microcentrifuge tubes. DNA was extracted from the cell pellet by heating at 95°C for 20 minutes on a heating block. The resulting supernatant of each isolate containing DNA was transferred to a fresh labelled 1.5 ml microcentrifuge tube and stored at -20°C.

The concentration and integrity of the extracted DNA were assessed using a nanodrop spectrophotometer and by agarose gel electrophoresis. Approximately 1µl of elution buffer was placed into the sample hole on the nanodrop spectrophotometer followed by a command to read the blank. The sample hole was then wiped using paper towel and the procedure was repeated with 1µl of extracted sample DNA followed by a command to read the sample and result printed and recorded in a table. This was done for all the extracted genomic DNA. DNA concentration ranged between 23.9 ng/µl and 60.2 ng/µl.

3.12.2 *MYCOBACTERIUM TUBERCULOSIS* COMPLEX (MTBC) DIFFERENTIATION MULTIPLEX PCR

To differentiate the isolates included in the study, pathogen genomic DNA was analysed by multiplex PCR with primer pairs as shown in Table 3.2, designed to amplify three genetic regions *cfp32* (Rv0577F 5'-ATGCCCAAGAGAAGCGAATACAGGCAA-3'; Rv0577R 5'-CTATTGCTGCGGTGCGGGCTTCAA-3'), RD9 (Rv2073cF 5'-TCGCCGCTGCCAGATGAG-3'; Rv2073cR 5'-TTTGGGAGCCGCCGGTGGTGATGA-3'), and RD12 (Rv3120F 5'-GTCGGCGATAGACCATGAGTCCGTCTCCAT-3'; Rv3120R 5'-GCGAAAAGTGGGCGGATGCCAG-3'). These have been authenticated in multiple distinct *Mycobacterium tuberculosis* complex (MTBC) strains in previous studies and have been used as gold standard genetic markers for MTC species. Additionally, these have been used for phylogenetic lineage prediction and classification of MTBC (Huard *et al.*, 2010; Nakajima *et al.*, 2010; Lee *et al.*, 2003). To conduct the PCR, 2 µl of the genomic DNA was transferred to each separate PCR tube. PCR reaction mixes were prepared by transferring 1 µl of $MgCl_2$ (25mM), 1 µl dNTP mix (10 mM), 4 µl 5X Go *Taq* buffer green, 2 µl betain (5M), and 2.6 µl primers mixes for *cfp32*, RD9, and RD12 in the ratio 2:1:1 of both forward and reverse primers respectively. 7.4 µl of molecular grade nuclease free water was added bringing the final reaction volume to 20 µl. The PCR thermal cycle profiles were preceded by an initial template denaturation at 96 ºC for 5 minutes followed by 35 cycles of 30 seconds denaturation at 96 ºC, 30 seconds, annealing at 60 ºC, for 60 seconds and extension at 72 ºC. A final extension at 72

ºC for 5 minutes was included in the amplification step. All subsequent PCRs were conducted using similar thermal cycling conditions. For quality control, *M. tuberculosis* H37Rv was used as a positive control and molecular grade water as a negative control in every PCR run. PCR products were analysed on 1 percent horizontal agarose gel electrophoresis in tris acetate EDTA (TAE) buffer stained with 0.5 picograms/ml ethidium bromide.

The PCR reagents including oligonucleotides, dNTP solution mix and *Taq* DNA polymerase were procured from Inqaba Biotechnology Industries (Pty) Ltd, South Africa.

Table 3.2. Primers for MTBC multiplex PCR.

Target locus	**Primer**	**Primer sequence** 5'-3'	**Location**	**PCR product Size (bp)**
cfp32	Rv0577F	ATGCCCAAGAGAAGCGAATACAGGCAA	671166-192	786
	Rv0577R	CTATTGCTGCGGTGCGGGCTTCAA	671951-928	
RD9	Rv2073cF	TCGCCGCTGCCAGATGAGTC	2330579-598	600
	Rv2073cR	TTTGGGAGCCGCCGGTGGTGATGA	2331173-150	
RD12	Rv3120F	GTCGGCGATAGACCATGAGTCCGTCTCCAT	3485558-587	404
	Rv3120R (390-369)	GCGAAAAGTGGGCGGATGCCAG	3485961-940	

3.12.3 ANALYSIS OF PCR PRODUCTS

The PCR products were analysed on 1 percent horizontal agarose gel slabs in tries acetate EDTA (TAE) buffer stained with 0.5 picograms/ml ethidium bromide. Three DNA molecular weight markers capable of distinguishing the three expected PCR product size ranges of 786bp, 600bp and 404bp were used.

3.12.4 SEQUENCING OF THE *RPOB* AND *KATG* GENES AND THE *INHA* PROMOTER REGION

To determine variation among pathogen isolates with reference to gene sequences associated with drug resistance, three resistance marker genes were amplified in three separate polymerase chain reactions using the primers TB *rpoB* S and TB *rpoB* AS; TB *katG* S and TB *katG* AS; TB *inhA* S and TB *inhA* AS (Table 3.3) for *rpoB* gene**,** *katG* gene, and *inhA* promoter region respectively. The target region where mutations occur in the *rpoB* locus is between 1276 and 1353, *katG* (823-1140) and *inhA* (-50 to-1) as illustrated in Table 3.3. PCR products were extracted from the 1 percent agarose gel by cutting out the bands on the gel with a blade under UV and transferring the gel pieces into individual eppendorf tubes which were left in a freezer overnight. Eppendorf tubes with frozen pieces of gel were centrifuged at 4°C at 15,000 *xg* for 10 minutes. Supernatants were transferred to fresh tubes and the concentration of the amplicon DNA was determined using a NanoDrop UV-Vis spectrophotometer with an inbuilt computer system. The concentrations of the amplicon DNA as determined by the spectrophotometer were entered into the table and used to compute the correct concentration of amplicon DNA for sequencing. The amplicon DNA concentrations ranged from 1.9 ng/µl to 21.8 ng/µl.

Amplicon DNA was sequenced using the respective primers by Big Dye Terminator v3.1 cycle sequencing kit on the ABI Prism 3500*xl*Genetic Analyser (Life Technologies Corp).

Table 3.3. Primers for amplification and sequencing of bacterial pathogen gene loci associated with resistance to first-line TB drugs.

Locus	Primer	Nucleotide sequence (5′–3′)	Target region	Expected product size (bp)
rpoB	TB *rpoB* S	5'-CAGGACGTGGAGGCGATCAC-3'	1276-1353	278
	TB *rpoB* AS	5'-GAGCCGATCAGACCGATGTTGG-3'		
katG	TB *katG* S	5'-ATGGCCATGAACGACGTCGAAAC-3'	823–1140	392
	TB *katG* AS	5'-CGCAGCGAGAGGTCAGTGGCCAG-3'		
inhA	TB *inhA* S	5'-TCACACCGACAAACGTCACGAGC-3'	−50 to −1	231
	TB *inhA* AS	5'-AGCCAGCCGCTGTGCGATCGCCA-3'		

3.13 ETHICAL CONSIDERATION

This was a laboratory-based study in which only pathogenic bacteria were handled and there was no direct contact with human participants. Laboratory serial numbers and town of residence were only used to identify the sources of the pathogen isolates. No personal identification details of any patient who provided the sample was collected in this study.

There was no direct benefit to the patients whose samples were included in the study but the findings of this study will be disseminated to relevant stakeholders

and will be used to inform policy makers in the National Tuberculosis and Leprosy Control Programme (NTP) for effective management and control of future drug resistant TB. Permission was sought from the University Teaching Hospitals Tuberculosis Laboratory through the Head of Pathology and Microbiology Department's office to use the archived samples for this study. Ethical clearance for the study was obtained from the the University of Zambia Biomedical Research Ethics Committee (UNZABREC) I.R.B. No. 00001131, F.W.A. No. 00000338 under approval ***Reference number: 010-08-16*** and authority to conduct the study was granted by the National Health Research Authority (NHRA).

3.14 DATA PROCESSING AND ANALYSIS

The data collected on each individual isolate were recorded in a laboratory worksheet and analysed in Microsoft (MS) Excel. The place/town of origin was extracted from the laboratory database as the types of gene mutations vary depending on the geographical location (Morgan *et al*., 2005; and Ling *et al*., 2008). The type of specimen was also considered to ascertain possibility of disseminated drug resistance TB. The MTBC differentiation was achieved based on the pattern observed in the results of the agarose gel electrophoresis. Mutations at the 3 loci were determined by aligning the resulting sequences of each gene with the wild-type sequence of *MTB* H37Rv control strain using Bio-edit software version 7.03. After the alignment, single nucleotide polymorphisms were highlighted and used to determine the type of gene mutations prevalent in the study population. The identified mutations were then compared with those identified in other countries using the TBDReaMDB databases (Sandgren *et al*., 2009).

SUMMARY

This chapter explained the materials and methods that were used to conduct this study. The various techniques used in the study as well as the experiments carried out were also described.

CHAPTER FOUR

RESULTS

4.0 OVERVIEW

This chapter presents the findings on the molecular differentiation of *Mycobacterium tuberculosis* complex isolated at the University Teaching Hospitals TB Laboratory using multiplex polymerase chain reaction to determine the most common species of pathogenic *Mycobacteria* and the type and frequency of gene mutations responsible for INH and RIF resistance in the isolates.

4.1 DETERMINATION OF DNA CONCENTRATION

The concentrations of genomic DNA samples as determined spectrophotometrically were entered into the Table 4.1 and used to compute the correct template concentration for the PCR and sequencing. The DNA concentrations ranged from 1.9 ng/µl to 21.8 ng/µl.

Table 4.1. Concentration of selected *katG* bacterial amplicon DNA as determined by nanodrop spectrophotometry.

Sample ID	**Nucleic Acid Conc. (**ng/µl)
katG 27	3.1
katG 98	8.8
katG 15	21.8
katG 13	20.0
katG 23	1.9
katG 68	13.9
katG 82	4.8
katG 38	10.0
katG 109	6.5
katG 64	5.0
katG 89	5.5
katG 29	12.2
katG 2	4.4
katG 22	2.0
katG 21	4.8
katG 127	6.6

4.2 DIFFERENTIATION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX (MTC) BY MULTIPLEX PCR

Genomic DNA from forty clinical isolates of *M. tuberculosis* complex resistant to INH and/RIF isolated from sputum was amplified by multiplex PCR using specific primers. Three fragments were observed in the multiplex PCR amplification of all the 40 isolates and were estimated to be 786 bp, 600 bp and 404 bp long as shown in Figure 4.1 for seven randomly selected isolates 2, 7, 8,15,16,18 and 21.

While the 786 bp fragment indicative of the *cfp32* region is present in all *Mycobacteria* genera, RD9 is only present in *M. tuberculosis* but absent from *M. africanum, M. bovis, M. caprae* and other MTBC. RD12 on the other hand is present in *M. tuberculosis* and *M. africanum* but absent in *M. bovis* and *M. caprae*.

Based on the results in Figure 4.1 and those presented in Table 4.2 from previous studies, it was inferred that all of the isolates included in this study were *Mycobacterium tuberculosis* species only and not any other species that have been identified to belong to the complex.

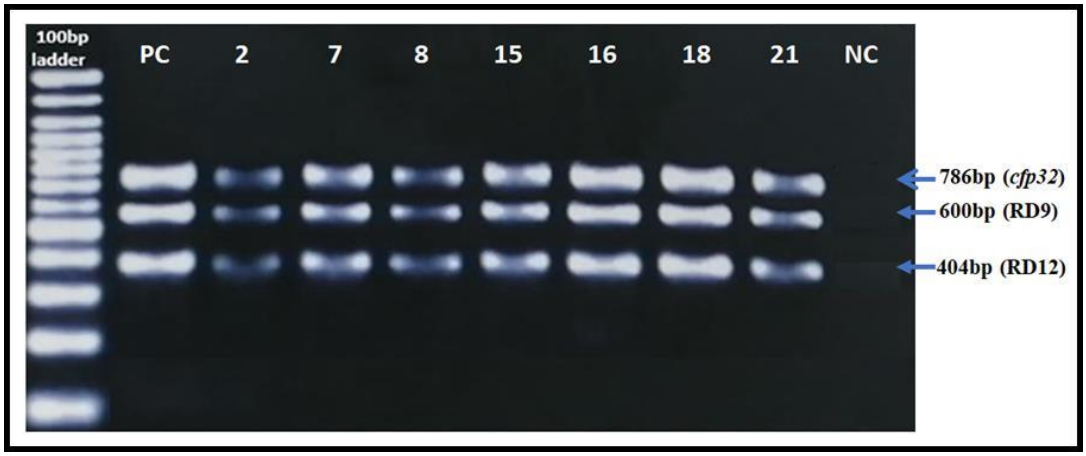


Figure 4.1.: Agarose gel electrophoresis analysis of PCR amplification of regions of differentiation of seven randomly selected bacterial genomic DNA extracted from the MTBC isolates. L=100bp molecular weight marker; PC= positive control using genomic DNA from *Mycobacterium tuberculosis* strain H37RV; NC is negative control in which all reagents were added to the PCR tube except template DNA which was replaced with molecular grade nuclease-free water; All forty isolates included in this study showed similar band migration profile. The experiment was repeated three times with similar results being observed.

The migration distance of the amplified DNA relative to the 100 base pair molecular DNA ladder was approximately 400 bp for RD12, 600bp for RD9 and 786 base pairs for *cfp32*. The MTBC species associated with RIF and/or INH resistance were differentiated using the presence and absence of bands as shown in Table 4.2.

Table 4.2. Expected DNA fragment size profiles for differentiating MTBC species.

Species	***cfp32*, 786bp**	**RD9, 600bp**	**RD12, 404bp**
M. tuberculosis	Band Present	Band Present	Band present
M. africanum or other MTBC*	Band Present	Band Absent	Band Present
M. bovis or *M. caprae*	Band Present	Band Absent	Band Absent
other bacteria	Band Absent	Band Absent	Band Absent

*MTBC other than MTB, *M. caprae* or *M. bovis*

4.3 ANALYSIS OF *RPOB, KATG AND INHA* PCR RESULTS

The results of the PCR demonstrated that all the amplified genes were intact and had fragment sizes of 278 bp (*rpoB*), 231 bp (*inhA)* and 392 bp (*katG*). As shown in Figure 4.2, all isolates were positive for *rpoB, katG and inhA* locus giving a fragment in each case of *katG* 392 bp (Figure 4.2A), *rpoB* 278 bp (Figure 4.2B) and *inhA* 231 bp (Fig 4.2C).

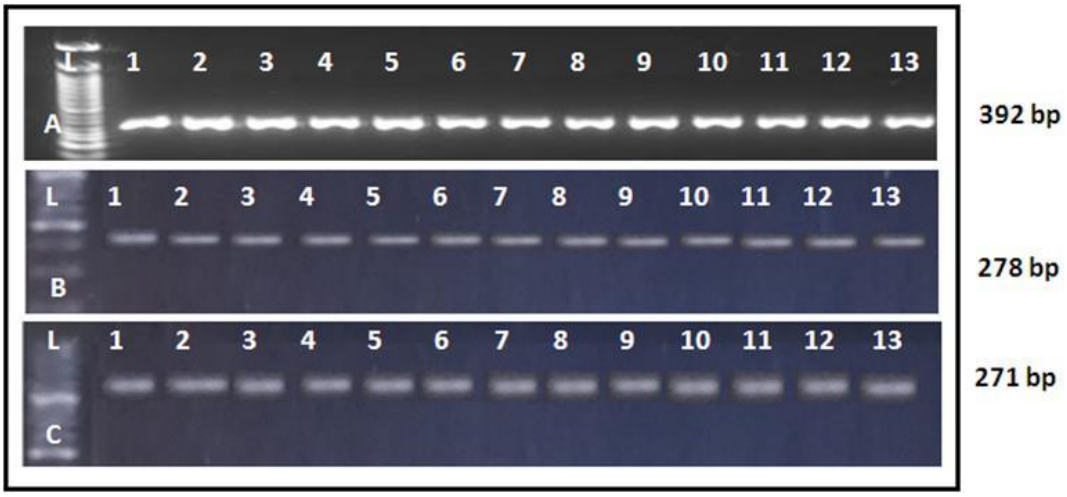


Figure 4.2: Gel electrophoresis analysis of *katG* gene (A), *rpoB* (B) and *inhA* (C) loci. PCR products were analyzed on 1% agarose and visualized with 0.01% ethidium bromide. Primers were designed to produce a 392 bp fragment (*katG*), 278 bp (*rpoB*), and 271 bp (*inhA*); NC, negative control. Only thirteen of the 40 reaction products are shown and no other bands were observed in all the three individual PC reactions. Experiments were repeated three times with similar results being observed.

4.4. MUTATIONAL ANALYSIS OF *RPOB* SEQUENCE ALIGNMENT

To determine the nature of nucleotide changes that may be attributed to an MDR phenotype, amplified DNA fragments were sequenced by the Big Dye terminator. The nucleic acid and translated sequences of the *rpoB* gene were analysed by Bio-edit software version 7.03 and presented in Figure 4.3A and 4.3B.

A number of significant mutations that have been hypothesized to be directly linked to drug resistance were discovered in the sequence alignment results.
Thirty-nine out of the forty isolates analysed at the *rpoB* locus had various types of substitution mutations with the commonest mutation being S450L (48.7 %), followed by H445T (20.5 %) as demonstrated in Table 4.5. In one phenotypically-RIF resistant isolate, no substitution or deletion mutations in the *rpoB* gene were observed. Mutations not previously reported were found at codons 535 where CAA coding for glutamine was changed to GAA coding for glutamic acid (Q535E) and 545 where CTG coding for leucine was changed to CCG coding for Arginine (L545R). One out of 39 (2.6 %) RIF resistant isolates demonstrated resistance to rifampicin alone.

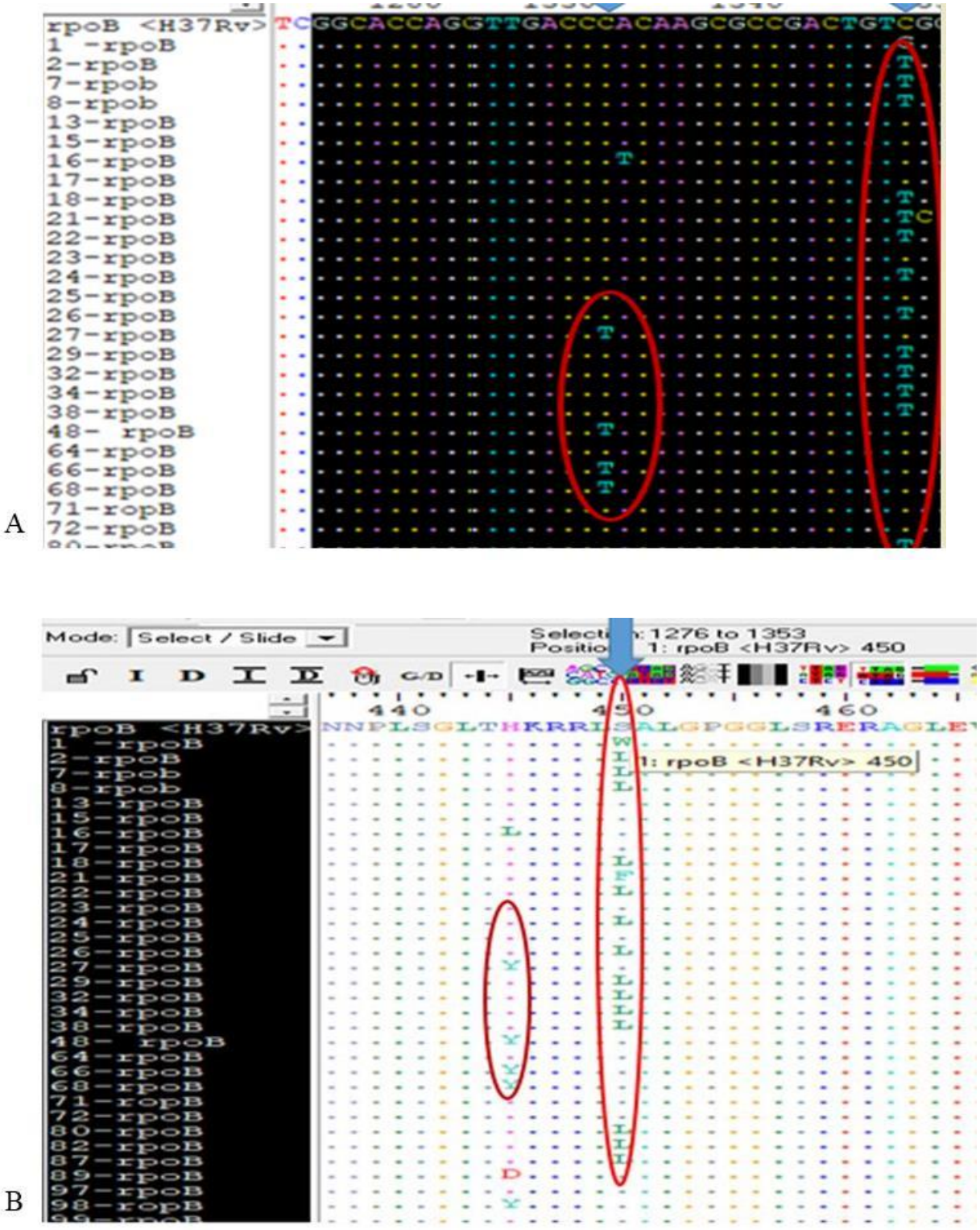


Figure 4.3: Nucleic Acid sequence (A) and amino acid sequence (B) alignment analyses of the *rpoB* locus showing sequence variations between selected isolates and the wild type strain of *M. tuberculosis* H37Rv.

4.5 FREQUENCY OF MUTATIONS IN *RPOB* GENE REGION

To determine the frequency of mutations in *rpoB*, the nucleotide changes at specific positions were analysed and results presented in Table 4.3.

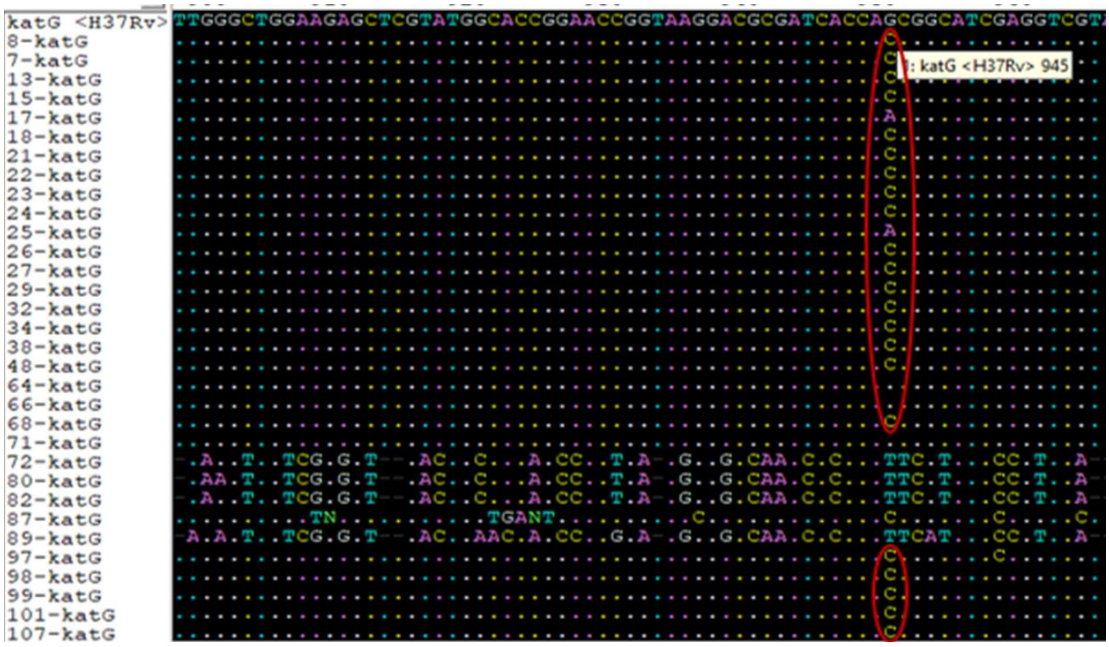
Table 4.3. Frequency of mutations in *rpoB* gene region associated with RIF resistance detected by sequence alignment.

Location of Mutation	**Nucleotide changes**	**Amino acid changes**	**No. (%) of Isolates N=39**
Codon 450	TCG-TTG	Ser – Leu (S450L)	19 (48.7)
Codon 445	CAC-TAC	His - Tyr (H445Y)	8 (20.5)
Codon 516	GAC-GTC	Asp -Val (D516V)	5 (12.8)
Codon 545	CTG-CCG	Leu - Pro(L545P)	2 (5.1)
Codon 535	CAA-GAA	Gln - Glu (Q535E)	2 (5.1)
Codon 524	TTC-CTC	Phe– Leu (F524L)	1 (2.5)

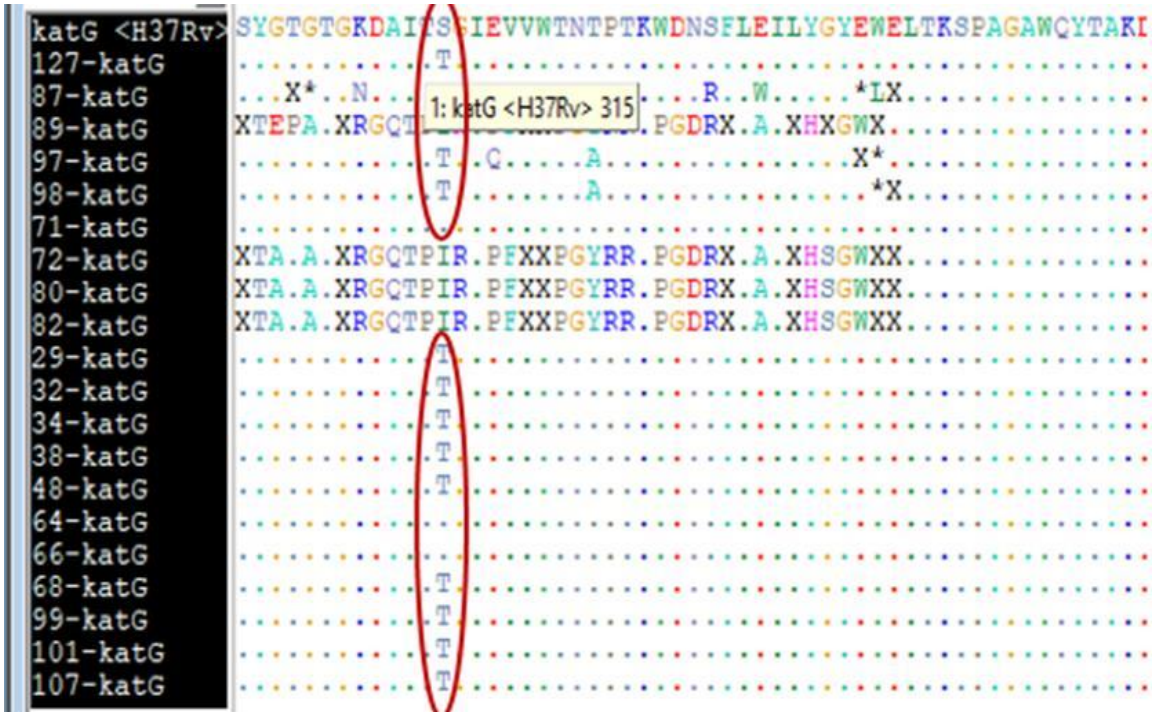
The most frequent *rpoB* mutation was a transition mutation occurring at codon 450 in which serine was substituted for leucine (S450L). The second most frequent mutation occurred at codon 445, also a transition mutation in which histidine was substituted for tyrosine (H445Y) as demonstrated in Table 4.3.

4.6 MUTATIONAL ANALYSIS OF *KATG* AND *INHA* PROMOTER SEQUENCE ALIGNMENT

To determine the intra sequence variation between isolates and a wild type strain of *M. tuberculosis,* the sequenced loci and their translated equivalents were compared by sequence alignment (Figures 4.4A and 4.4B). The *katG* sequence alignment reviewed mutations common at codon 315. The most commonly observed mutations at this position were transversion mutations with a few isolates demonstrating transition mutations (isolates 17 and 25). In most of the transversion mutations, the most frequent was a substitution mutation in which serine has been replaced by threonine.



A



B

Figure 4.4: Nucleic Acid sequence (A) and amino acid sequence (B) alignment analyses of the *katG* locus showing sequence variations between selected isolates and the wild type strain of *M. tuberculosis* H37Rv.

Seventy percent (30/40) of the isolates had *katG* mutations at codon 315, 7.5 % (3/40) had the *katG* mutation alone while 2.5 % (1/40) had mutations in the *inhA* promoter and *rpoB* in addition to the *katG* 315 mutation. The S315T mutations in

the *katG* mutation and the -10 GA *inhA* mutation accounted for only 2.5 % (1/40) of the INH-resistant isolates.

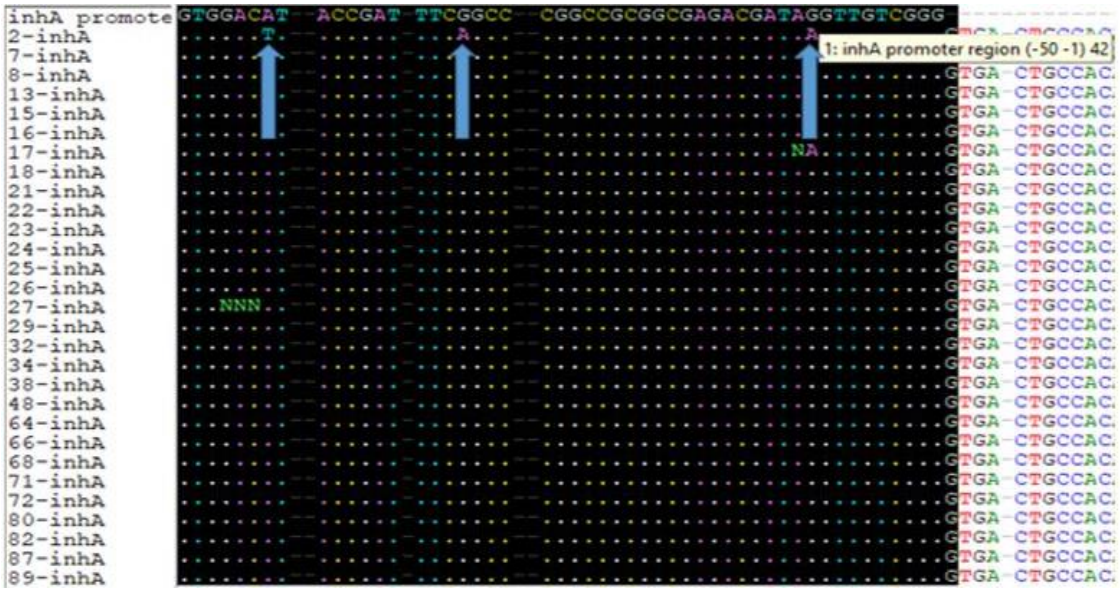


Figure 4.5: Nucleic Acid sequence alignment analyses of the *inhA* locus showing sequence variations between selected isolates and the wild type *inhA* promoter

The nucleotide changes at specific positions in the *katG* loci are presented in Table 4.4.

Table 4.4. Frequency of mutations in *katG* gene region associated with INH resistance detected by sequence alignment.

Location of Mutation	**Nucleotide changes**	**Amino acid changes**	**No. (%) of Isolates N=40**
Codon 315	TCG-ACG	Ser – Thr (S315T)	23 (57.5)
Codon 315	AGC-ATC	Ser - Iso (S315I)	7(17.5)
Codon 329	GAC-GGC	Asp -Gly(D329G)	6 (15)
Codon 307	GGA-GCA	Gly -Ala(G314A)	4 (10)

The most frequent *katG* mutation was a transversion mutation at codon 315 in which serine was substituted for threonine (S315T), Table 4.4

75 percent (n=40) of the MTBC isolates analysed had mutations at codon 315 with the most prevalent mutation being the *katG* S315T, which accounted for 77 percent

(n=30) of the *katG*315 mutations (isolates 7 ,8, 15, 16, 18, 21, 22, 23, 24, 27, 29, 32, 34, 38, 48, 68, 72, 87, 97, 92, 99, 101 and 107). Isolates 72, 68 and 97 only had the *katG* mutation alone while isolate 2, had mutations in the *inhA* promoter and *rpoB* in addition to the *katG* 315 mutation. Isolate17 had *rpoB* and *inhA* mutations. No mutations were observed in isolate 71.

CHAPTER FIVE

DISCUSSION

5.0 OVERVIEW

This chapter discusses the research findings in accordance with the research objectives.

5.1 MOST PREVALENT MTBC SPECIES

The current study is the first to document the prevalent MTBC species, type and frequency of mutations associated with RIF and INH resistance at the University Teaching Hospitals (UTH) Tuberculosis (TB) Laboratory. The results from this study will augment those from recent related studies on molecular TB drug resistance studies conducted in Zambia which focused on mutations associated with resistance in fluoroquinolone such as PZ (Mitarai *et al*., 2005; Bwalya *et al*., 2018). Results from these earlier studies concluded that screening for PZ resistance in patients with MDR-TB is necessary as approximately half of MDR-TB patients also have PZ resistance.

In this study, 40 *M. tuberculosis* complex isolates were analysed and results revealed that all the 40 isolates were *M. tuberculosis*; no other MTBC species were detected. TB patients in these 3 provinces can therefore be treated with standard first-line TB drugs. These findings are comparable to a study conducted by Bayraktar *et al.* (2011) in Turkey that demonstrated that the most prevalent MTBC species among clinical isolates between 2007 and 2010 was *M. tuberculosis* (94.1 percent) followed by *M. bovis* which accounted for 4.3 percent of the isolates. Most studies have demonstrated that of the species of MTBC, *M. tuberculosis* is the most common cause of tuberculosis in humans worldwide, except in some parts of West Africa where *M. africanum* is widely distributed (Chihota *et al*., 2018). *M. tuberculosis*, historically prevalent in Europe and America could have been introduced in Africa from either Europe or Asia and subsequently spread through

pastoralism, mining and war (Chihota *et al.*, 2018). Non isolation of *M. africanum* in this study partially confirms the distribution of *M. tuberculosis* lineages in Africa as documented by Chihota *et al.* (2018).

Parsons *et al.* (2003) documented that despite the MTBC species being genetically closely related, they differ in certain phenotypes, pathogenicity, host and geographic distribution with *M. tuberculosis* being the most common species in Europe and America, while *M. africanum* is mostly found in African TB patients of African origin. Due to limited diagnostic capacity and because tuberculosis caused by other species in the MTBC is rare, identification of those species is not usually performed in routine mycobacteriological laboratory work (Pinsky and Banaei**,** 2008). However, those species differ in terms of host tropism, phenotype pathogenicity, geographic distribution, and drug resistance (Brosch *et al.*, 2002; Parsons *et al.*, 2003), which may have distinct public health implications and may affect the selection of appropriate treatment regimens (Niemann *et al.*, 2000).

Worldwide, 3.1 percent of tuberculosis cases in humans are attributable to *M. bovis* and that *M. bovis* is isolated in the range 0.4 -10 percent of sputum isolates in African patients (WHO, 1998). Cosivi *et al*. (2013) documented that approximately 85% of the cattle and 82% of the human population of Africa are in areas where bovine TB is either partly controlled or not controlled at all. In developing nations, Zambia included, little is known of the relative frequency with which *M. bovis* causes extrapulmonary TB due to limited laboratory capacity for the culture and typing of tubercle bacilli. A study conducted by Mposhy *et al*. (1983) in Zaire, reported to have isolated *M. bovis* from gastric secretions in two of five patients with pulmonary TB. In the same study, the prevalence of the bovine TB in local cattle was approximately 8% by tuberculin testing and isolation of *M. bovis*. In another study conducted in Egypt, nine out of twenty randomly selected patients with TB peritonitis were infected with *M. bovis*, and the remaining with *M. tuberculosis* (Nafeh *et al*., 1992).

Although *M. bovis* was not detected from the human isolates in this study, a survey conducted by Malama *et al.* (2013) in Zambia between 2008 and 2011 reported a prevalence of 1.0 percent *M. bovis* among the sample population. Another study conducted in the southern province of Zambia by Malama *et al.* (2014) documented

a prevalence of 5.5 percent which falls within the figure estimated for developing countries, by the WHO, (2010). Infection of humans with *M. bovis* poses a diagnostic challenge as most methods currently being used for routine detection of *mycobacteria* in Zambia do not differentiate MTBC species which exposes those infected with *M. bovis* to inappropriate TB treatment (Brosch *et al.*, 2002). Furthermore, in studies conducted in Namwala District in the Southern province of Zambia, Sydney Malama *et al*. (2014) and Girja *et al*. (2013) documented having isolated *M. tuberculosis* from cattle and *M. bovis* from raw milk respectively. The implication for public health is that humans especially children and HIV/AIDS infected individuals could be infected with *M. tuberculosis* and *M. bovis* if they consumed unpasteurized milk from infected cattle due to possible spill back of infection from cattle to humans as previously documented by Srivastava *et al*. (2008) in a study conducted in northern India in which *M. tuberculosis* was isolated in cattle milk.

While significant amount of work has been done particularly in Namwala district of Zambia and the epidemiology of bovine TB is well documented, effective control and elimination strategies are not being adequately addressed. There still remains a gap in the knowledge of the true extent of the burden of *M. bovis* infections among humans in Zambia especially among the pastoral communities where people and cattle cohabit more closely and raw milk and milk products form the basis of their diet (Girja *et al*., 2013). Because of the animal and public health consequences of *M. bovis*, disease surveillance programs in humans should be considered a priority, especially in areas where risk factors are present. Research is needed to determine when *M. bovis* is of zoonotic importance and what the underlying mechanisms of transmission are Cosivi *et al*. (2013).

5.2 PREVALENCE OF MDR-TB AMONG NEW AND PREVIOUSLY TREATED CASES

The review of the laboratory records and patient information submitted to the laboratory reviewed that thirty-nine out of the forty patients from whose sputum the study isolates were recovered had MDR-TB. Four out of the thirty-nine patients had been previously treated with the standard first-line TB drugs. Resistance in these individuals was therefore likely Acquired or Secondary Resistance, defined as drug

resistance occurring in patients who have a history of previous TB treatment. Thirty-five of the thirty-nine patients did not have any history of previous TB treatment. These individuals likely had Initial Resistance (drug resistance in a newly diagnosed patient who has never received anti-TB drugs that occurs when a patient develops tuberculosis after being infected by another person who has resistant tubercle bacilli) as defined in the National Tuberculosis and Leprosy Program (NTP), (MOH, 1986). The high numbers of MDR-TB cases among the newly diagnosed individuals is indicative of high person to person transmission rate. There is therefore need for sensitisation, early diagnosis and proper case management (appropriate treatment and quarantine of patients with drug resistant TB) should be enhanced so as to break TB transmission. Unfortunately, this study could to determine epidemiological linkages of the isolates.

In the period under review (January 2013 to June 2014), there were a total of 2,419 cases of TB recorded at the UTH TB laboratory of which 2,277 were newly diagnosed cases. The prevalence of MDR-TB among newly and previously treated cases was 1.5 percent and 2.8 percent respectively. These figures compare with the documented 1.8 percent and 2.3 MDR-TB among newly and previously treated cases respectively, which have been reported for Zambia (Mulenga *et al*., 2010).

5.3 *KATG MUTATIONS*

The most prevalent observed mutation S315T, is a significant loss of catalase-peroxidase activity (Gonzalez *et al.,* 1999) that has been reported to result into poor binding of the drug to the enzyme catalase peroxidase which in turn limits drug activation and brings about INH resistance (Yu *et al*., 2003). The results of this study show that mutations at position 315 of the *katG* gene are concerned with INH resistance in 75 percent of the *Mycobacterium tuberculosis* species circulating in Eastern, Lusaka, Western and provinces of Zambia. All the mutations in the *katG* gene were characterised as single nucleotide changes. The high percentage of mutations at position 315 of the *katG* gene (75 percent, n=40) demonstrates the importance of this codon in the development of INH resistance in *Mycobacteria tuberculosis* complex (MTBC). These results on one hand agree with results of a similar study by Bakonyte *et al.* (2003) in Lithuania that documented 83.9 percent

mutation rates at codon 315 of the *katG* gene and those of Caws *et al*., 2006 who documented that 71 percent of INH-resistant *Mycobacterium tuberculosis* isolates from a Hospital in Vietnam carried an S315T mutation in *katG*. On the other hand, the results of this study do differ from those by Haas *et al.* (1997) in a study that documented a 60 percent mutation rate at codon 315 as well as a 60 percent mutation rate documented by Aslan *et al.* (2008) in southern Turkey, 65.4 percent in Australia (Lavender *et al*., 2004), 66.2 percent in Poland (Sajduda *et al*., 2004) and 41.3 percent in Spain.

A study of MDR isolates in central China found a very high percentage (100 percent) of *katG* mutations at codon 315 (Hu *et al*., 2010). Differences in the prevalence of mutations in different countries could be explained by geographically based prevalence and mutation frequency differences associated with different sub lineages of *M. tuberculosis* within the East Asian lineage. Another explanation is that there may be a higher selective pressure for *katG*315 mutations in MDR isolates (Iwamoto *et al.*, 2008). Although S315T is the most common mutation worldwide, the exact functional effect of the mutation remains unclear. It is however hypothesised that the threonine residue may block access to the active site and reduce the INH affinity of the enzyme, or may alter redox potentials and local hydrogen bonds (Bertrand *et al*., 2004).

The second most prevalent mutation concerned with INH resistance in this study was Ser315I. These findings do not compare with findings of most other studies that have reported that the second most prevalent mutations in the *KatG* gene is the mutation occurring at codon 465 (Caws *et al*., 2006; Sajduda *et al*., 2006; Lavender *et al*., 2005). The difference could be explained by the fact that mutations differ based on geographical location.

Of all the *katG* mutations, the S315T mutation has been the most widely studied mutation worldwide and has been used in the GenoType MTBDRplus (Shu-Ting *et al*., 2015) programme. In this study, of the 40 INH-resistant isolates, 30 had the *katG*315 mutation with 23, (N=30) demonstrating the *katG* Ser315Thr mutation. Only two had mutations in the *inhA* promoter (at positions -10, -33 and -44). The findings of this study are not consistent with the results of Baker *et al*. (2005), who found 63 and 22 percent of the *katG* Ser315Thr mutation and mutation at -15 in the

inhA promoter of INH-resistant isolates respectively. This could be attributed to the fact that the number of isolates analysed in this study was relatively smaller. Furthermore, most studies suggest that mutations in various loci and those that contribute to drug resistance display geographical variations (Morgan *et al*., 2005; and Ling *et al*., 2008).

Overall, the findings confirmed the previous observation that the Ser315Thr mutation is found most often, occurring in approximately 40 percent of all isoniazid-resistant isolates (Aslan *et al.,* 2008; Haas *et al.,* 1997).

5.4 *RPOB* GENE MUTATIONS

The results of this study demonstrate that the most prevalent *rpoB* mutations are transition mutations at positions 450 in which serine was substituted for leucine (S450L) and 445 in which histidine was substituted for tyrosine (H445T). These accounted for 48.7 percent and 20.5 percent (n=39) of rifampicin resistance in the analysed MTB isolates respectively. These results compare with findings of Ramaswamy *et al.* (2004), who documented S450L and H445T as the most frequent mutations but do not compare with Aslan *et al.* (2008) who reported that mutations at codon 531 are the most common mutations (41 - 60 percent) responsible for rifampicin resistance in most *Mycobacterium tuberculosis* species. The next most frequent mutations affecting codons in this gene region were at codon 516 (12.8 percent) which also compare with the study by Aslan *et al.* (2008) which reported 7.3 percent to 16 percent mutation rate at codon 516. This study also detected mutations at codon 524 and mutations not previously reported were found at codons 535 in which glutamine was substituted for glutamic acid (CAA-GAA, Q535E) and 545 in which leusine was substituted for arginine (CTG-CCG, L545R). This result demonstrates the varying geographical distribution of the *rpoB* mutations in distinct regions of different countries (Morgan *et al*., 2005, Ling *et al*., 2008).

In one phenotypically RIF resistant isolate (2.6 percent) no nucleotide changes were detected in the *rpoB* gene. Resistance could therefore, be due to other mutations occurring in the gene region not screened in study, probably *Kas*A and/or *ahpC*.

5.5 MUTATION IN THE *INHA* REGULATORY REGION

In this study, two out of all isolates analysed for RIF and INH resistance had nucleotide changes in the *inhA* regulator region. Isolate 17 had nucleotide changes at position -10 while isolate 2 had nucleotide changes at position -10 as well as on positions -33 and -44 accounting for 5 percent resistance rate. This differs from results of several studies that have demonstrated that *inhA* regulator region mutations appeared with low frequency ranging between 10-25.8 percent in INH resistant isolates with the most common being -15CT (Cardoso *et al*., 2004). The difference in the frequency could be attributed to the comparatively smaller number of samples analysed in this study as well as varying geographical distribution (Morgan *et al*., 2005, Ling *et al*., 2008).

5.6 RIFAMPICIN MONO RESISTANCE (RMR)

In 2010, rifampicin mono resistant (RMR) TB accounted for 0.3 percent, 0.3 percent, and 0.1 percent of primary TB cases, and 1.9 percent, 0 percent, and 0.2 percent of secondary TB cases in Germany, United Kingdom, and Poland, respectively (EU/EEA report, 2010). A prospective study carried out in France, documented that RMR TB was a rare event. The overall observed proportion of 0.12 percent is similar to those reported in other countries with similar epidemiological profile of TB such as Western Europe (Meyssonnier *et al.*, 2014). This study reports rifampicin mono resistance in 2.6 percent (n= 39) of all the rifampicin resistant *Mycobacterium tuberculosis* isolates analysed. Previously, Mulenga *et al*. (2010) documented RMR frequency of 1.3 percent among new cases, and 3.2 percent among previously treated cases while a review of national MDR-TB data by Kapata *et al*. (2013) documented 4.8 percent RMR. The discrepancy could be due to the difference in the time period during which the cases were analysed. It has been reported that resistance against RIF usually occurs together with other drugs, especially isoniazid, and for this reason rifampicin resistance is considered a surrogate marker for MDR-TB (Caws *et al*., 2006). Molecular techniques such as the GeneXpert MTB/RIF assay could therefore be used for screening MDR-TB cases in Eastern, Lusaka and Western provinces of Zambia.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.0 OVERVIEW

This chapter presents the conclusion of the study and some recommendations.

6.1 CONCLUSION

This study presents important findings on the type of species as well as frequency and different kinds of mutations occurring at various target loci concerned with rifampicin and isoniazid resistance in clinical isolates resistant to RIF and INH in Eastern, Lusaka and Western Provinces of Zambia.

1. M*ycobacterium tuberculosis* was the only species in all drug resistant isolates collected at the University Teaching Hospitals Tuberculosis Laboratory between January 2013 and June 2014. Therefore, TB patients in Lusaka, Western and Eastern Provinces of Zambia can be treated using the standard first-line treatment regimen which includes pyrazinamide that is known to be ineffective in *M. bovis* infection.
2. The most common *katG* mutation in INH resistant isolates was S315T. Mutation at codon 315 of *katG* gene fragment is responsible for INH resistance in 77 percent of *M. tuberculosis* isolates at the University Teaching Hospital while the most common *rpoB* mutation in Rif resistant isolates was Ser450Leu, which accounted for 48.7 percent of resistance to rifampicin.

   The results of this study suggest that mutations differ depending on region and therefore determination of frequency of mutations associated with drug resistance in isolates from various regions is important for improving rapid and specific molecular diagnostic techniques.
3. The most common encountered combinations of mutations responsible for MDR-TB in Eastern, Lusaka and Western provinces of Zambia at the *katG* loci was S315T while at the *rpoB* loci it was S450L.

The findings of this study are consistent with the results of other studies such as that of Haas *et al*. (1997) and Aslan *et al*. (2008) who found that 60 percent of INH-resistant isolates had the *katG* Ser315Thr mutation and Ramaswamy *et al*. (2004) who documented S450L as the most prevalent mutation among RIF resistant isolates. Molecular diagnostic tests based on detecting these predominant mutations could therefore, be useful for the rapid detection of multi drug resistant tuberculosis in Eastern, Lusaka, Western parts of Zambia.

6.2 RECOMMENDATIONS

It is recommended that:

1. More studies should be conducted in the other regions of Zambia to determine the prevalent MTBC species especially in regions where humans and cattle inhabit more closely to better inform treatment and management of tuberculosis cases in Zambia.

2. Further studies with a larger sample size should be carried out country wide to determine frequency of INH and RIF mutations.

3. The distribution of mutations responsible for resistance to RIF and INH should be mapped to better inform development and use of molecular diagnostic techniques.

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