GENOTYPIC CHARACTERIZATION OF DRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* AMONG THE NEW AND PREVIOUSLY TREATED TUBERCULOSIS CASES, FROM HEALTH FACILITIES ACROSS SEVEN PROVINCES OF ZAMBIA

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Genotypic Characterization of Drug-Resistant *Mycobacterium Tuberculosis* among the New and Previously Treated Tuberculosis Cases, from Health Facilities Across Seven Provinces of Zambia

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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This work is dedicated to my wife Mrs Mumena Florence Masuka Biemba, mother Ms Mumena Elizabeth Mailoni, and family members who supported and encouraged me throughout my postgraduate studies.

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ABBREVIATIONS AND ACRONYMS

BCG	Bacille Calmette-Guérin
DNA	Deoxyribonucleic acid
FLQs	Fluoroquinolones
GLI	Global Laboratory Initiative
***gyr*A**	Gyrase A gene
***gyr*B**	Gyrase B gene
HLS	Hain Life Science
HIV	Human immunodeficiency virus
INH	Isoniazid
INNO-LIPA	Innogenetics line probe assay
MDR/RR-TB	Multidrug-resistant/rifampicin resistant tuberculosis
MDR-TB	Multidrug-resistant tuberculosis
MOH	Ministry of Health
MTB	*Mycobacterium tuberculosis*
MTB-DR	*Mycobacterium tuberculosis* drug-resistance
MTBDR*plus*	*Mycobacterium tuberculosis* drug-resistance first-line assay
MTBDR*sl*	*Mycobacterium tuberculosis* drug-resistance second-line assay
Pre-XDR TB	Pre-extensively drug-resistant tuberculosis

RIF	Rifampicin
RNA	Ribonucleic acid
***rpo*B**	Ribonucleic acid polymerase *β*eta-subunit gene
TDR-TB	Totally drug-resistant tuberculosis
UNITAID	International facility for the purchase of diagnostics and drugs for diagnosis and treatment of HIV/AIDS, malaria and TB
IUATLD	International union against tuberculosis and lung diseases
UNZA	University of Zambia
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis

ABSTRACT

Drug-resistant tuberculosis (DR-TB) is one of the major public health problems globally. The emergence of *Mycobacterium tuberculosis* (MTB) resistant strains is a serious public health challenge to the prevention and control of TB globally. Zambia is ranked among countries with a high-burden of TB, TB/HIV and multidrug-resistant/rifampicin resistant tuberculosis (MDR/RR-TB) in the world. Drug-resistant TB is causing high mortality and morbidity rates in Zambia. Drug-resistant TB is mainly caused by mutations in the target genes of MTB. In this study, sputum samples obtained from the new and previously treated cases of TB were examined for DR-MTB. Sputum specimens were processed using the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method. Mycobacterial DNA was extracted from the processed sputum using the Genolyse technique. Extracted DNA was subjected to multiplex polymerase chain reaction (PCR) amplification and reverse hybridization. Drug-resistance and mutations in MTB were detected using the Hain genotype MTBDR*plus* ver 2.0 and MTBDR*sl* ver 2.0 assays for first- and second-line anti-TB drugs, respectively. A total of 329 positive sputum specimens were analysed for drug-resistant TB. One hundred and two (102) specimens came from the new TB cases and 227 from the previously treated TB cases. Among the new TB cases, 3.9% had rifampicin mono-resistance (RIFr), 12.8% isoniazid mono-resistance (INHr), and 17.7% had MDR. While among the previously treated TB cases 10.1% had RIFr, 6.6% INHr, 33.0% MDR, 1.8% polydrug-resistance, and 0.8% had pre-extensively drug-resistance (pre-XDR). The *rpo*B MUT 3 (Ser531Leu) mutation was the most frequent (8.6%), conferring resistance to RIF, among the new TB cases. The same mutation was the most frequently detected (10.9%), conferring resistance to RIF, among the previously treated TB cases. The *Inh*A MUT 1 (Cys15Thr) mutation was the most frequent (28.6%), conferring resistance to INH, among the new TB cases, while the *kat*G MUT 1 (Ser315Thr 1) mutation was the most frequent (6.7%), conferring resistance to INH, among the previously treated TB cases. The *rpo*B MUT 3 (Ser531Leu) and the *kat*G MUT 1 (Ser315Thr 1) mutations were the most frequent (14.3%), conferring resistance to both RIF and INH, among the new TB cases. The same mutations were the most frequently detected (18.5%), conferring resistance to both RIF and INH, among the previously treated TB cases. The *rpo*B MUT 2A (His526Tyr) and *gyr*A MUT 1 (Ala90Val) mutations were the most frequent (1.7%), conferring resistance to both RIF and fluoroquinolones (FLQs), among the previously treated TB cases. Two MTB isolates had pre-XDR among the previously treated TB cases. The first isolate had a mutation profile; *rpo*B MUT 2A (His526Tyr), *kat*G MUT 1 (Ser315Thr), and *gyr*A MUT 3C (Asp94Gly). The second isolate had the profile; *rpo*B MUT 2B (His526Asp), *kat*G MUT 2 (Ser315Thr 2), and *eis* MUT 1 (Cys14Thr). These two combination mutation profiles had the same frequency of 0.8%. Drug-resistant TB is prevalent in Zambia, especially among the previously treated TB cases. This calls for intensified drug-resistance surveillance. It is recommended to diagnose DR-TB early using the Hain genotype MTBDR ver 2.0 assays. There is also an urgent need to use anti-microbials appropriately to prevent or minimize antimicrobial resistance.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Tuberculosis (TB) is one of the leading causes of death globally (Prasad *et al.,* 2019; Kar *et al.,* 2019). This airborne infectious disease killer causes high mortality and morbidity rates (Oudghiri *et al.,* 2018; Baya *et al.,* 2019; Ukwamendua *et al.,* 2019), it kills one person every 21 seconds (Allue-Guardia *et al.,* 2021; Mumena *et al.,* 2021; Mumena *et al.,* 2022). The current TB mortality is severely impacted by the coronavirus disease-2019, a highly infectious disease caused by a virus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Polack *et al.,* 2020; WHO, 2021). Coronavirus disease-2019 pandemic has reversed years of progress made in the fight against TB. Globally TB targets have gone off-track and many years of progress reversed (WHO, 2021). The burden of TB is driven by the emergence and spreading of drug-resistant *Mycobacterium tuberculosis* (*M. tuberculosis*) strains (Chisompola *et al.,* 2020; Welekidan *et al.,* 2020; Allue-Guardia *et al.,* 2021). TB with the human immunodeficiency virus (HIV) co-infection has led to an increase in different types of drug-resistant TB (Swain *et al.,* 2020). *M. tuberculosis* is the major causative agent for human TB among the species of the *Mycobacterium tuberculosis* complex (MTBC), as it causes 97-99% of TB cases (Ates *et al.,* 2015).

Globally 10.0 million individuals contract TB annually and from that number, 1.6 million individuals die from the disease (Sakamoto *et al.*, 2019). The World Health Organisation (WHO) global TB report of 2022, revealed that in 2021, 10.6 million people fell sick with TB, with 1.6 million deaths reported globally (WHO, 2022). While the 2021 report revealed that in 2020 there were 10.1 million people that got TB globally, with 1, 514, 000 TB deaths reported (WHO, 2021). The 2020 report revealed that in 2019 there were 10.0 million people who got sick of TB globally, with 1,408,000 deaths (WHO, 2020). In 2018 there were 10.0 million people who got sick of TB globally with 1,451,000 deaths (WHO, 2019a). In 2017, 10.0 million people developed TB globally, of which 1.6 million died from the disease (WHO, 2018; Honeyborne *et al.,* 2019). In 2016, 10.4 million cases of TB occurred, with 1.7 million

deaths worldwide (Leung *et al.,* 2018). In 2015, the WHO global TB report showed that the number of patients who had active TB exceeded 20.0 million worldwide, and that there were 9.6 million newly diagnosed cases of TB and 1.5 million of the cases resulted in deaths (Yang and Wu, 2019). Globally TB is more common among men than among women and affects mainly adults in their productive age (Ahmed, 2018).

Africa has a high prevalence of TB, in 2016, 2.6 million cases of TB were reported, with 417, 000 deaths (Ismail *et al.,* 2018). Tuberculosis related morbidity and mortality rates are very high in many African countries (Rachow *et al.,* 2019). Drug-resistant TB is a serious public health problem in sub-Saharan Africa, especially for countries that have a high-burden of both HIV and TB cases (Mekonnen *et al.,* 2015). Drug-resistant TB is posing a huge obstacle for many countries to achieve the set World Health Organization end TB targets (Namburette *et al.,* 2016; WHO, 2018). The top 10 countries in Africa that have the highest cases of multidrug-resistant- or rifampicin resistant- tuberculosis (MDR/RR-TB) are: Nigeria, South-Africa, Democratic Republic of Congo, Mozambique, Ethiopia, Angola, Kenya, Tanzania, Cote d'Ivoire, and Zambia (Ismail *et al.,* 2018).

Zambia is ranked among countries with a high-burden of TB, TB/HIV and MDR/RR-TB in the world (Mulenga *et al.,* 2010; Malama *et al.,* 2013a; WHO, 2019a; WHO, 2021). Tuberculosis is one of the major public health problems in the country, which is causing high mortality and morbidity rates (Kapata *et al.,* 2013; Masenga *et al.,* 2017; Monde *et al.,* 2016; Munthali *et al.,* 2017). The following were TB case notifications to the World Health Organization: 40,726 (2020), 36,866 (2019), 35,922 (2018), 37,203 (2017), 40,153 (2016), 41,588 (2015), 42,716 (2014), 45,793 (2013), 45,269 (2012), 45,594 (2011), 48,616 (2010) (Lungu *et al.,* 2022). Zambia has a high-burden of MDR/RR-TB, which is becoming a growing public health problem in the country (Kasapo *et al.,* 2017; Ismail *et al.,* 2018; WHO, 2021). The current prevalence rate of MDR/RR-TB in Zambia is 2.4% among the new TB cases and 18% among the previously treated TB cases (WHO, 2020).

Nigeria was ranked the third highest TB burdened country in the world and the first in Africa in 2018 (Akinsola *et al.,* 2018). In 2020, a total of 135,784 TB cases were

detected and notified to the World Health Organization, while, in 2018 106,533 cases of TB were notified (WHO, 2019a, WHO, 2021). This country also has high cases of TB/HIV and drug-resistant TB (Onyedum *et al.,* 2017). In 2020, the prevalence rate of MDR/RR-TB among the new TB cases was 6% and among the previously treated TB cases was 32%, while in 2016 the prevalence among the new TB cases was 4.3% and among the previously treated TB cases was 25% (Onyedum *et al.*, 2017; Dayyab *et al.,* 2022).

South-Africa has one of the highest incidence rates of TB in the world, and the highest cases of MDR-TB and extensively drug-resistant tuberculosis (XDR-TB) (Kapwata *et al.,* 2017; Shah *et al.*, 2017; Mcintosh *et al.,* 2018; Jones *et al.,* 2019; Scott *et al.,* 2019). In 2019, a total of 209,545 new and relapse TB cases were detected and notified to the World Health Organization (WHO, 2020). In 2018, a total of 235,652 TB cases were detected and notified (WHO, 2019a). In 2016, 19,073 cases of MDR/RR-TB were identified, a huge increase from 8,072 cases in 2011 (Rensburg *et al.,* 2019). KwaZulu-Natal, Western Cape, and the Eastern Cape provinces have the highest cases of MDR-TB and XDR-TB in South-Africa (Klopper *et al.*, 2013; Maharaj *et al.,* 2016). KwaZulu-Natal has one of the highest cases of XDR-TB in the world (Kapwata *et al.,* 2017).

The Democratic Republic of the Congo (DRC) is among the top 10 highest TB burdened countries in the world, based on the severity of the disease burden (WHO, 2018). In 2017, DRC ranked 9th position in the world and 2nd in Africa with regards to the TB burden (Robert and Many, 2019). In 2015 and 2016, DRC detected and notified 120,508 and 132,515 TB cases respectively (Bisuta-fueza *et al.*, 2019). In 2017, DRC had an estimated TB incidence of 322/100,000 with 60,000 TB deaths (Bulabula *et al.,* 2019). In 2017, 3,400 cases of MDR/RR-TB were detected and notified to the World Health Organization (Robert and Many, 2019). The antimicrobial drug-resistance survey data of 2018 in DRC showed that the prevalence of rifampicin-resistant TB among the new TB patients was 2.2% and 16.7% for the previously treated TB cases (Kayomo *et al.,* 2018).

Mozambique is also one of the top 20 highest TB burdened countries in the world based on absolute number of incident TB cases (WHO, 2019a). Tuberculosis is a serious public health problem and the leading cause of death in Mozambique (Gudo *et al.,* 2010). The mortality and morbidity burden of TB are high (Schacht *et al.,* 2019). In 2017, the incidence rate of TB in Mozambique was 551 per 100,000 population (Valencia *et al.,* 2017; Schacht *et al.,* 2019). Drug-resistant TB is a challenge in Mozambique (Namburete *et al.,* 2016). The prevalence rate of MDR/RR-TB in 2018 was 3.7% among the new TB cases and 20% among the previously treated TB cases (WHO, 2019a).

Drug-resistant TB is a serious public health problem globally (Daoqun *et al.,* 2017). TB control strategies are being hampered by the successful transmission of drug-resistant *M. tuberculosis* strains which are causing MDR and XDR-TB in the human population (Pholwat *et al.,* 2016; Nguyen *et al.,* 2019). The World Health Organization global TB report of 2022, revealed that in 2021, 141, 953 people developed MDR/RR-TB and 25, 038 had pre-extensively drug-resistant (pre-XDR) or extensively drug-resistant tuberculosis (XDR-TB) (WHO, 2022). The global prevalence of MDR/RR-TB in 2021 stood at 3.6% among the new TB cases and 18% among the previously treated TB cases (WHO, 2022). While the 2021 report revealed that 132, 222 cases of MDR/RR-TB and 25, 681 of pre-XDR/XDR-TB were detected in 2020 (WHO, 2021). The global prevalence of MDR/RR-TB in 2020 was 3.5% among the new TB cases and 18% among the previously treated TB cases (WHO, 2021). In 2019, 206, 030 cases of MDR/RR-TB were detected and notified globally (WHO, 2020). The global prevalence of MDR/RR-TB in 2019 was 3.3% among the new TB cases and 17.7% among the previously treated TB cases (WHO, 2020; Santos *et al.,* 2021). In 2018, 186, 772 cases of MDR/RR-TB were detected and notified globally (WHO, 2019a). The global prevalence of MDR/RR-TB was 3.4% and 18% among the new TB cases and previously treated TB cases respectively (WHO, 2019a). In 2017, 160, 684 cases of MDR/RR-TB were detected and notified globally (WHO, 2018). From the total detected and notified only 139,114 MDR-TB/RR-TB (87%) were enrolled on treatment and 21,570 MDR-TB/RR-TB cases were not, giving a 13% gap of not treated MDR-TB/RR-TB cases (WHO, 2018). The global prevalence of MDR/RR-TB was 3.5% among the new TB cases and 18% among the previously treated TB cases, in

2017 (WHO, 2018). In 2017, 8.5% of TB patients diagnosed with MDR-TB progressed to developing XDR-TB (WHO, 2018). Extensively drug-resistant TB is already spreading throughout all the regions of the world with 9.4 million new cases and 1.7 million reported deaths annually (Takawira *et al.,* 2017). In 2016, 153, 119 cases of MDR/RR-TB were detected and notified globally (WHO, 2017). The global prevalence of MDR/RR-TB was 4.1% and 19% among the new TB cases and previously treated TB cases respectively, in 2016 (WHO, 2017). Globally the prevalence of isoniazid mono-resistance among the new TB cases was 7.2 % and 11.6% among the previously treated TB cases in 2018 (WHO, 2019a).

The most effective strategy to halt the rise of drug-resistant TB cases is early detection and effective treatment. Rapid molecular tools enable the early detection of drug-resistant TB (Solo *et al.,* 2020). Line probe assays (LPAs) are molecular diagnostic assays that are used in detecting *M. tuberculosis* and its resistance to anti-TB drugs (Lynn, 2018). The Hain genotype MTBDR*plus* ver 2.0 assay is an LPA that is used for the qualitative identification of *M. tuberculosis* and its resistance to rifampicin (RIF) and isoniazid (INH), the key first-line anti-TB drugs (Ombura *et al.,* 2016). While the Hain genotype MTBDR*sl* ver 2.0 assay is used for the qualitative detection of *M. tuberculosis* and its resistance to second-line anti-TB drugs (Schön *et al.,* 2017). These assays can use either clinical or cultivated specimens to identify *M. tuberculosis* and its resistance (Eddabra and Benhassou, 2018). Line probe assays also detect gene mutations that confer resistance to anti-TB drugs (Charoenpak *et al.,* 2020). Line probe assays are recommended by the World Health Organization for diagnosis, guidance on empirical treatment and for surveillance of drug-resistant TB (Namburete *et al.,* 2016).

1.2 Statement of the problem

Zambia is ranked among countries with a high-burden of TB, HIV-associated TB (HIV/TB), and MDR/RR-TB in the world (WHO, 2021). TB causes high mortality and morbidity rates in Zambia (Lung *et al.,* 2022). With an estimated TB incidence of 333 per 100, 000 population, Zambia is ranked among the top 30 high TB burdened countries globally (WHO, 2021). In 2021, Zambia notified a total of 50, 825 TB cases (WHO, 2022). While in 2019, 59, 000 TB cases were estimated, of which only 36, 866

(63%) where diagnosed, notified, and initiated on treatment (WHO, 2021). This left a gap of 22, 134 (37%) of undiagnosed TB cases (WHO, 2021). Drug-resistant TB is associated with high mortality and morbidity rates (Takawira *et al.,* 2017). Multidrug-resistant/rifampicin resistant -TB is prevalent and a growing public health problem in Zambia, it has reversed successes made in the TB control programme, country-wide (Kapata *et al.*, 2013; Kasapo *et al.,* 2017). It is associated with a low treatment success rate, high mortality, and morbidity rates (WHO, 2019). The inappropriate use of rifampicin and isoniazid has resulted in the emergence of MDR/RR-TB and XDR-TB (Migliori *et al.*, 2010). The proportion of MDR/RR-TB in Zambia has risen from 0.3% among the new TB cases and 8.1% among the previously treated TB cases in 2014 to 2.4% in new TB cases and 18% in previously treated TB cases in 2018 (Solo *et al.,* 2020b). The World Health Organization global TB report of 2019 estimated an incidence of 2,700 MDR/RR-TB cases in Zambia, while in 2016 the report showed that there were 1,500 MDR-TB cases notified in 2015, of which 196 patients were bacteriologically confirmed MDR-TB cases, but only 99 patients were put on treatment (MOH, 2017). The patients that are not initiated on treatment get worse and continue to spread drug-resistant *M. tuberculosis* strains to members in their households and to others in the community (Leung *et al.,* 2013). The World Health Organization projected that in 2021 and beyond, Zambia will have a high-burden of both MDR-TB and rifampicin-resistant TB cases (MOH, 2017).

The primary method used for the diagnosis of TB in Zambia is sputum smear microscopy (Turnbull *et al.,* 2011). This diagnostic method is simple, quick, specific, and inexpensive. However, it has a low sensitivity and lacks specificity for identifying *M. tuberculosis* resistant strains (Tripathi *et al.,* 2014). Mycobacterial culture, phenotypic and genotypic drug-susceptibility testing are not performed routinely in most public health facilities in Zambia, and only three public laboratories are able to do so (MOH, 2017). Lack of laboratory facilities coupled with the lack of molecular diagnostic tests and equipment, has been identified as an obstacle to the success of most TB programmes (Turnbull *et al.*, 2011). Physicians face a challenge when patients are diagnosed with active pulmonary tuberculosis (PTB). They are left with no option, but to start the patient on first-line anti-TB drugs, with the hope that the *M. tuberculosis* strains detected are drug-susceptible strains. However, if drug-resistant

M. tuberculosis strains develop, they are rarely noticed, and this ultimately results in the development of the different forms of drug-resistant TB and the rapid transmission of drug-resistant *M. tuberculosis* strains to other people (Shin *et al.,* 2010; Leung *et al.,* 2013; Manjelievskaia *et al.,* 2016; Ombura *et al.,* 2016).

1.3 Justification of the study

Tuberculosis is a global emergency and a disease of public health importance (Ombura *et al.,* 2016; Gyimah *et al.,* 2019). Drug-resistant TB is a public health crisis globally. Early detection and empirical treatment of drug-resistant TB is currently a challenge for Zambia (MOH, 2017; WHO, 2018). Drug-resistant TB needs to be diagnosed early and treated empirically. If not, one form of drug-resistant TB worsens and develops into another critical type, for example: mono-drug resistant TB can develop into multidrug-resistant TB, which can develop into pre-extensively drug-resistant tuberculosis and eventually into extensively drug-resistant tuberculosis. Extensively drug-resistant tuberculosis can ultimately develop into totally drug-resistant TB, if it is not detected early and treated empirically (Udwadia, 2016; Osman *et al.,* 2019; Pradipta *et al.,* 2019; Riccardi *et al.,* 2019).

This study investigated the prevalence of drug-resistance and mutations associated with resistance in *M. tuberculosis* strains isolated among the new and previously treated TB cases, using molecular diagnostic assays. Molecular diagnostic assays are better than phenotypic methods like microbiological culturing, because they detect *M. tuberculosis* and its resistance to anti-TB drugs within a very short time (Palomino *et al.,* 2005). It is very important to detect drug-resistant TB quickly because patients that are infected with active disease may die or transmit drug-resistant *M. tuberculosis* strains to other people before treatment is initiated (Nachega and Chaisson, 2003). Molecular diagnostic tests help to diagnose TB very quickly, and enable the patient to be initiated on treatment promptly (thus reducing mortality and morbidity rates), as well as help to control the transmission of *M. tuberculosis* (Palomino *et al.,* 2005). The routine monitoring of drug-resistance patterns of *M. tuberculosis* and TB control programmes need to continue inorder to maintain a robust TB surveillance system (Sitienei *et al.,* 2017). This study helped in the early detection and prompt treatment

of patients infected with drug-resistant *M. tuberculosis* strains. Line probe assays are more beneficial to patient care & management as results are produced within 2 hours, unlike culture methods which take 1-2 months (Ssengooba *et al.*, 2018; Kasaro *et al.*, 2020).

1.4 Research questions

i. What is the prevalence of drug-resistant TB among the new and previously treated TB cases, from health facilities across seven provinces of Zambia?
ii. What is the association between drug-resistant TB to age and gender among the new and previously treated TB cases, from health facilities across seven provinces of Zambia?
iii. What mutations are present in drug-resistant *M. tuberculosis* isolates, among the new and previously treated TB cases, from health facilities across seven provinces of Zambia?

1.5 Objectives

1.5.1 General objective

To characterise drug-resistant *M. tuberculosis*, among the new and previously treated tuberculosis cases, from health facilities across seven provinces of Zambia.

1.5.2 Specific objectives

i. To determine the prevalence of drug-resistant TB, among the new and previously treated TB cases, from health facilities across seven provinces of Zambia.
ii. To determine the association between drug-resistant TB to age and gender, among the new and previously treated TB cases, from health facilities across seven provinces of Zambia.
iii. To determine mutations in drug-resistant *M. tuberculosis* isolates, among the new and previously treated TB cases, from health facilities across seven provinces of Zambia.

CHAPTER TWO

LITERATURE REVIEW

2.1 Causative agents of tuberculosis

The *Mycobacterium tuberculosis* (*M. tuberculosis*) is the major causative agent for human TB among the species of the *Mycobacterium tuberculosis* complex (MTBC), as it causes 97-99% of TB cases (Ates *et al.*, 2015). The other MTBC agents include: *M. africanum, M. bovis, M. microti, M. caprae, M. canettii, M. mungi, M. pinnipedii, M. suricattae, M. dassie,* and *M. orygis* (Caulfield and Wengenack, 2016; Damene *et al.*, 2020; Bespiatykh *et al.*, 2021; Kanabalan *et al.*, 2021). All the microbial agents that belong to the MTBC can cause TB in humans or other animals (Zhu *et al.*, 2016; Brhane *et al.*, 2017). Some of the species of the MTBC are adapted to humans (*M. tuberculosis* and *M. africanum*), while others to animals (*M. bovis, M. microti, M. pinnipedii, M. mungi, M. orygis, and M. caprae*), and others are smooth bacilli (*M. canettii)* (Al-Saeedi & Al-Hajoj, 2017; Senghore *et al.*, 2020; Kanabalan *et al.*, 2021; Khoshnood *et al.*, 2021). The *M. tuberculosis* is acid-fast, non-motile, non-sporulating, has a thick mycolic cell wall, weakly gram-positive bacteria, has a slow growth rate in microbiological cultures, is able to replicate in macrophages, and develops resistance to anti-TB drugs (Sakamoto, 2012; Gordon and Parish, 2018).

2.2 Transmission of tuberculosis

Tuberculosis is mainly transmitted through inhalation of air contaminated with *M. tuberculosis*, when an infected person coughs, sneezes, talks or spits, infectious nuclei droplets containing tubercle bacilli are projected into the air and are inhaled by a nearby person (Bañuls *et al.*, 2015). Once the infectious droplets containing *M. tuberculosis* are inhaled they move to the lungs (Nardell, 2016). In the lungs they cause infection and eventually TB (Churchyard *et al.*, 2017). Tuberculosis can also be transmitted by drinking contaminated, unpasteurized milk or by eating infected dairy products (Asebe, 2017). This route of transmission is common with *M. bovis* a member of the MTBC (Torres-gonzalez *et al.*, 2016). *M. bovis* can infect both humans and animals (Lan *et al.*, 2016). In animals *M. bovis* causes bovine TB (BTB), while in

humans it causes zoonotic TB (ZTB), which can either be pulmonary tuberculosis (PTB) or extra-pulmonary tuberculosis (EPTB) (Malama *et al.*, 2013a; Malama *et al.*, 2013b). The most common type of EPTB that *M. bovis* causes is tuberculous lymphadenitis (TBLA), affecting mostly the cervical lymph nodes (Berg *et al.*, 2015).

2.3 Pathogenesis of tuberculosis

The *M. tuberculosis* is transmitted through the air in 98% of the cases, when an individual with TB coughs (Dunn *et al.,* 2016). Once the infectious nuclei droplets are inhaled by an individual, *M. tuberculosis* gets moved to the lungs- specifically in the alveoli where they get phagocytosed by special cells called alveoli macrophages (Sakamoto, 2012; Dunn *et al.,* 2016). In immunocompetent individuals, the immune system clears off the infection, while in immunocompromised individuals, *M. tuberculosis* subverts degradation by alveoli macrophages and replicates inside the macrophages for many weeks. As the *M. tuberculosis* multiply, they are carried into regional lymph nodes by alveoli macrophages and spread haematogenously to other key sites of the body namely; lung apices, meninges, spleen, peritoneum, vertebrae, lymph nodes, genital tract, and urinary tract (Dunn *et al.,* 2016). During this period most individuals infected are asymptomatic, but develop specific immunity (cell-mediated immunity and delayed- type hypersensitivity) to the *M. tuberculosis*, and are said to have latent TB infection (Dunn *et al.,* 2016; Long *et al.,* 2022). However, in immune-compromised individuals TB infection progresses quickly to TB disease, due to weakened immune system (Dunn *et al.,* 2016). *M. tuberculosis* infection can be active or latent (Kebede, 2019). Active TB infection is characterized by the formation of granulomatous structures- bacterial laden, caseous necrotic lesions, and liquefaction (Ravimohan *et al.,* 2018; Kebede, 2019). While latent TB infection is characterized by the formation of calcified and fibrotic lesions (Kebede, 2019).

In summary the establishment of TB infection and the disease TB depends on four key steps: the phagocytosis of the *M. tuberculosis*; multiplication of the tubercle bacilli; containment of the latent phase of TB infection; occurrence of active lung infection and finally development of pulmonary TB (Bañuls *et al.,* 2015). *M. tuberculosis* infects mainly the lungs (causing pulmonary TB) and can disseminate to

other sites and infect other parts of the body for example: the meninges, pleura, bones, joints, urogenital tract, peritoneal cavity, and lymphnodes (causing extra-pulmonary TB) (Lee, 2015).

2.4 Signs and symptoms of tuberculosis

Tuberculosis can be classified into two namely pulmonary TB and extra-pulmonary TB (Lee, 2015). Pulmonary TB has the following classical signs and symptoms: chronic cough, sputum production, fever, loss of appetite, loss of weight, night sweats, and haemoptysis (Zumla *et al.,* 2013). Patients suffering from extra-pulmonary TB initially may have similar signs and symptoms to those with pulmonary TB for example fever, night sweats, and weight loss. However, they also develop signs and symptoms that are specific to the body site affected with extra-pulmonary TB, for example: when the lymph nodes are infected they swell and become inflamed as in TB lymphadenitis; meningitis in central nervous system TB; pericarditis in pericardium TB; pleurisy in TB of the pleura and peritonitis in TB peritonitis (Ahmed, 2018).

2.5 Types of drug-resistant tuberculosis

There are different types of drug-resistant TB (DR-TB), which include: mono drug-resistant tuberculosis, MDR-TB, pre-extensively drug-resistant TB (pre-XDR-TB), extensively drug-resistant TB (XDR-TB), and extremely drug-resistant tuberculosis (XXDR-TB) or total drug-resistant TB (TDR-TB) (Kar *et al.,* 2019; Muzondiwa *et al.,* 2019). Drug-resistant TB is responsible for causing high mortality and morbidity rates globally (Udwadia and Furin, 2019). Drug-resistant TB cases are increasing globally, and are posing a threat to the elimination of TB (Nguyen *et al.,* 2019; Romanowski *et al.,* 2019).

2.5.1 Mono drug-resistant tuberculosis

Mono drug-resistant TB is defined as a type of TB characterized by resistance to one first-line anti-TB drug only (WHO, 2014). Examples of mono drug-resistant TB include: rifampicin resistant TB (RR-TB), and isoniazid resistant TB (INHr-TB). Rifampicin resistant TB is a type of TB that is caused by *M. tuberculosis* strains that

are resistant to rifampicin (WHO, 2019b). Isoniazid resistant TB is a type of TB that is caused by *M. tuberculosis* strains that are resistant to isoniazid (WHO, 2019b), it is more common than rifampicin (RIF) resistance, and is a growing public health issue globally because policy and research directions are only focused on RIF resistance, which is considered as a surrogate marker for MDR-TB (Mumena *et al.,* 2021).

2.5.2 Multidrug-resistant tuberculosis

Multidrug-resistant tuberculosis (MDR-TB) is a type of drug-resistant TB that is caused by *M. tuberculosis* strains that are resistant to both isoniazid (INH) and rifampicin (RIF) (Sloan and Lewis, 2015). Rifampicin and isoniazid are key first-line anti-TB drugs (Seung *et al.,* 2015). The emergence of drug-resistant *M. tuberculosis* strains is currently the major drawback to ending the global TB crisis (Fonseca *et al.,* 2015; Sidiq *et al.,* 2019). Multidrug-resistant tuberculosis is more complex to manage than drug-susceptible TB (DS-TB) (Fikre *et al.,* 2019). The mortality rate is very high in patients infected with MDR-TB than in those infected with DS-TB (Fikre *et al.,* 2019). The major set-backs in the fight against drug-resistant TB have been: late diagnosis and treatment of drug-resistant TB using inappropriate regimens (Marahatta, 2010; Chen *et al.,* 2018). It is a challenge for most developing countries globally to quickly diagnose drug-resistant TB and treat these cases empirically until they are declared cured (Chen *et al.,* 2018). Patients suffering from multidrug-resistant tuberculosis/rifampicin resistance tuberculosis that are not managed well, spread drug-resistant *M. tuberculosis* strains in their communities (Sidiq *et al.,* 2019). Relatives who live in homes with patients infected with drug-resistant *M. tuberculosis* strains are at a very high risk of acquiring these resistant strains (Huynh and Marais, 2019). Nosocomial transmission of drug-resistant *M. tuberculosis* strains to health care workers as well as to HIV-infected patients has been reported in clinical settings (Marahatta, 2010).

2.5.3 Pre extensively drug-resistant tuberculosis

Pre-extensively drug-resistant tuberculosis (Pre-XDR-TB) was defined as resistance to isoniazid and rifampicin, plus any one fluoroquinolone (levofloxacin, gatifloxacin, or moxifloxacin) or any one of the second-line injectable drugs (capreomycin,

kanamycin, or amikacin), but has now been re-defined by the World Health Organization as TB that is resistant to both rifampicin and isoniazid, plus any fluoroquinolone (Mumena *et al.,* 2021; WHO, 2021). Poor case management of MDR-TB leads to a high prevalence of pre-XDR TB, which can eventually become extensively drug-resistant TB (XDR-TB) (Mumena *et al.,* 2021). Detection of Pre-XDR TB among MDR-TB patients helps to prevent treatment failure of MDR-TB and also enables appropriate steps to be undertaken to prevent progression of Pre-XDR to XDR-TB (Mumena *et al.,* 2021; Shibabaw *et al.,* 2020; Kashongwe *et al.,* 2020).

2.5.4 Extensively drug-resistant tuberculosis

Extensively drug-resistant tuberculosis (XDR-TB) was defined as a type of drug-resistant TB that is caused by *M. tuberculosis* strains that are resistant to the first-line drugs, isoniazid and rifampicin, plus any fluoroqinolone, and at least any one of the three second-line injectable drugs (Piubello *et al.,* 2018), but has now been re-defined by the World Health Organization as resistance to rifampicin, plus any fluoroquinolone, plus at least one of the drugs linezolid or bedaquiline (WHO, 2021; Espinosa-pereiro *et al.,* 2022). When a patient has both XDR-TB and HIV infection, XDR-TB progresses faster and become more severe than when the patient is infected with XDR-TB only (Seung *et al.,* 2015).

2.5.5 Totally drug-resistant tuberculosis

Totally drug-resistant tuberculosis (TDR-TB) is a type of drug-resistant TB that is caused by *M. tuberculosis* strains that are resistant to all first-line anti-TB drugs and to all second-line anti-TB drugs (Akbar *et al.,* 2013). Totally drug-resistant tuberculosis has been reported in India, Iran, Italy and South-Africa (Migliori *et al.,* 2012; Akbar *et al.,* 2013). Despite totally drug-resistant tuberculosis only being reported in these four countries, it may also be present in other countries (Parida *et al.,* 2015). However, inadequate molecular diagnostic laboratory facilities, especially in resource limited countries, make it difficult for cases of TDR-TB to be detected (Parida *et al.,* 2015). Poorly managed extensively drug-resistant TB can progress to totally drug-resistant TB (Mumena *et al.,* 2021).

2.6 Treatment of tuberculosis

The bacteria *M. tuberculosis* can be susceptible or resistant to anti-TB drugs (Kar *et al.*, 2019). The treatment of drug-susceptible TB is easier, short, and less expensive, while that for drug-resistant TB is difficult, long and very expensive (WHO, 2019b). In most cases the treatment out-come for drug-resistant TB is poor (Mbuagbaw *et al.*, 2019). According to the World Health Organization TB report of 2017, the global treatment success rate for multidrug-resistant TB stood at 55% and that of XDR-TB at 34% (Li *et al.*, 2019). Empirical treatment of TB is key in its control (Nurwidya *et al.*, 2018).

2.6.1 Classification of anti-tuberculosis drugs

The anti-TB drugs can be classified into five groups (based on their efficacy, safety profiles, drug-class, and experience of use): group [i]. first-line anti-TB drugs (rifampicin, isoniazid, pyrazinamide, and ethambutol); group [ii]. Injectable drugs (capreomycin, amikacin, kanamycin, and streptomycin); group [iii]. Floroquinolones (moxifloxacin, ofloxacin, levofloxacin, and gatifloxacin); group [iv]. Oral bacteriostatic drugs (cycloserine, terizidone, prothionamide, ethionamide, and para-amino salicyclic acid); and group [v]. anti-TB drugs with limited information or unclear efficacy or long-term safety (linezolid, clofazimine, amoxicillin/clavulanate, carbapenems (meropenem and imipenem-cilastatin), high-dose isoniazid, clarithromycin, thioacetazone, delamanid, and bedaquiline) (Palmero *et al.*, 2015; Seung *et al.*, 2015; Sloan and Lewis, 2015). Drugs in the following groups ii, iii, and iv are known as second-line drugs, while those in group v are third-line drugs (Palmero *et al.*, 2015). Patients suffering from pre-extensively drug-resistant TB and extensively drug-resistant TB are treated with drug combinations that include those from group v, because they are usually resistant to most of the other remaining anti-TB drugs (Palmero *et al.*, 2015; Shibabaw *et al.*, 2020).

The second- and third-line anti-TB drugs are expensive, have severe side effects, and require well trained clinicians to administer, as well as equipped laboratory facilities for drug-susceptibility testing (DST) (Kashongwe *et al.*, 2020). These requirements are difficult to achieve in many low- and middle-income countries (Kashongwe *et al.*,

2020). Access to second-line DST is poor in many countries, this causes resistant strains *M. tuberculosis* to spread successfully unrecognized causing different forms of drug-resistant (DR-TB) (Seung *et al.,* 2015).

The drugs pretomanid, bedaquiline, and delamanid were recently approved for use in the management of DR-TB. These drugs have tremendously improved the treatment success rate in patients suffering from MDR/RR-TB (Dookie *et al.,* 2018; Espinosa-pereiro *et al.,* 2022). Additionally, repurposed drugs, such as clofazimine and linezolid have also improved the treatment of MDR/RR-TB as alternative drugs (Pecora *et al.,* 2021; Espinosa-pereiro *et al.,* 2022). Treatment for DR-TB requires a drug regimen that contains a minimum of 4 anti-TB drugs to reduce relapse, improve efficacy and prevent further development of resistance.

2.6.2 Treatment of drug-susceptible tuberculosis

The treatment of susceptible TB requires the use of first-line anti-TB drugs rifampicin, isoniazid, pyrazinamide and ethambutol (Briffotaux *et al.,* 2019). Currently drug-susceptible TB is treated for a period of 6 months, and involves two key treatment phases: the first phase is called the "initial phase", which involves treating the TB patient with four first-line anti-TB drugs for 2 months, while the second phase is called the "continuation phase", which involves treating the TB patient with rifampicin and isoniazid for an additional period of 4 months (Briffotaux *et al.,* 2019). Pyrazinamide has greatly reduced the duration of treating drug-susceptible TB from a period of 9-12 months to 6 months (Shi *et al.,* 2014). The six months treatment is called a "short-course anti-TB therapy" (Ahmed, 2018).

2.6.3 Treatment of drug-resistant tuberculosis

Drug-resistant TB is more difficulty to treat than drug-susceptible TB (WHO, 2019b). The treatment out-come for drug-resistant TB is poor, in most cases (Mbuagbaw *et al.,* 2019). The types of drug-resistant TB, include: monodrug-resistant TB (isoniazid-resistant TB, rifampicin-resistant TB, pyrazinamide- resistant TB, ethambutol-reistant TB), poly drug-resistant TB, MDR-TB, pre-XDR-TB, XDR-TB and TDR-TB (Kar *et al.,* 2019; Muzondiwa *et al.,* 2019; Mumena *et al.,* 2022).

2.6.3.1 Treatment of isoniazid mono-resistant tuberculosis

Isoniazid mono-resistant TB (INHr-TB) is becoming common globally (Garcia *et al.*, 2018). INHr-TB can be treated using the following drugs: rifampicin, ethambutol, pyrazinamide, and levofloxacin for a period of six months (WHO, 2019b). Levofloxacin is the first choice fluoroquinolone used in the treatment of INHr-TB because, it has a good characterized safety profile, and has less known drug interactions (WHO, 2019b). The treatment of INHr-TB can be prolonged beyond six months for patients that have extensive cavitary disease or those who fail to convert to smear-negative or culture- negative after successfully completing treatment (WHO, 2019b). The treatment outcome for INHr-TB can sometimes be poor (Olson *et al.*, 2019).

2.6.3.2 Treatment of multidrug-resistant/rifampicin resistant tuberculosis

Multidrug-resistant/rifampicin resistant tuberculosis (MDR/RR-TB) can be treated for 9-12 months in a standardized shorter treatment regimen, and for 18-20 months in longer treatment regimens. The shorter treatment regimen comprises of all-oral bedaquiline-containing regimen in eligible patients who have not been exposed to second-line anti-TB drugs, and in whom resistance to fluoroquinolones has been excluded (WHO, 2020). The longer treatment regimen for MDR/RR-TB involves the use of three groups of anti-TB drugs namely; group A (moxifloxacin/levofloxacin, linezolid, bedaquiline); group B (clofazimine, cycloserine/terizidone); and group C (pyrazinamide, ethambutol amikacin/streptomycin, delamanid, imipenem-cilastatin, para-aminosalicylic acid, ethionamide/prothionamide, meropenem) (Tiberi *et al.*, 2017; WHO, 2019b; WHO, 2020). Meropenem and imipenem-cilastatin are supposed to be administered with clavulanic acid which is available only in formulations combined with amoxicillin (amoxicillin-clavulanic acid). Amoxicillin-clavulanic acid should not be used without imipenem-cilastin or meropenem (WHO, 2019b). Therefore to treat MDR/RR-TB, the physician needs a combination of at least five effective drugs: three drugs from group A and one drug from group B and C (WHO, 2019b). Multidrug-resistant/rifampicin-TB is a very serious drug-resistant airborne disease (Weyer *et al.*, 2017).

2.6.3.3 Treatment of extensively drug-resistant tuberculosis

Extensively drug-resistant tuberculosis (XDR-TB) can be treated using a regimen comprising of seven effective drugs that is: two core drugs (linezolid, bedaquiline), one companion drug (clofazimine or cycloserine), one other companion drug (meropenem or ertapenem or imipenem-cilastatin, plus amoxicillin-clavulanate or delamanid or para- aminosalicyclic acid), and three supporting drugs (one fluoroquinolone (levofloxacin, or moxifloxacin), one second-line injectable drug (kanamycin, or amikacin, or capreomycin), and (high-dose isoniazid) (Caminero *et al.,* 2017). Using this regimen XDR-TB can be treated for a period of 13-15 months (Caminero *et al.,* 2017). Patients suffering from Pre-extensively TB or XDR-TB have fewer treatment options, and their treatment success rates are very low globally (Kashongwe *et al.,* 2020).

2.7 Mechanisms for drug-resistance to anti-tuberculosis drugs

M. tuberculosis resistance is attributed to a number of factors some of which include: the mycolic acid, lipid layer of the cell wall, presence of *β*-lactamase enzymes, presence of efflux pumps, and the development of mutations in the target genes of *M. tuberculosis* (Eduardo and Palomino, 2011; Mumena *et al.,* 2021). The mycolic acid, lipid layer of *M. tuberculosis* makes the cell wall to become less permeable to a number of anti-TB drugs (Gokulan and Varughese, 2018). The efflux pumps play a role of pumping several antimicrobial agents out of the cells of *M. tuberculosis* (Nasiri *et al.,* 2017; Gokulan and Varughese, 2018). The *β*-lactamase enzyme of *M. tuberculosis* inactivates the *β*-lactam antibiotics thus causes resistance to this class of antibiotics (Gokulan and Varughese, 2018). The development of mutations in the target genes of *M. tuberculosis* is a major mechanism through which resistance to anti-TB drugs occurs (Gygli *et al.,* 2017; Ghajavand *et al.,* 2019).

2.7.1 Drug target genes and mutations conferring resistance to first-line anti-tuberculosis drugs

The first-line anti-TB drugs used in the treatment of TB are; rifampicin, isoniazid, pyrazinamide and ethambutol (Briffotaux *et al.,* 2019). *M. tuberculosis* resistance to

each one of the first-line anti-TB drugs has been detected in TB patients (Dookie *et al.,* 2018). Resistance to first-line anti-TB drugs is mainly caused by mutations in the following genes: *rpo*B gene for resistance to rifampicin; *inh*A, *kas*A, *ahp*C, *kat*G and *ndh* genes for resistance to isoniazid; *embB* gene for resistance to ethambutol; and finally the *pnc*A and *pan*D genes for resistance to pyrazinamide (Damtie *et al.*, 2014).

2.7.1.1 Drug target genes and mutations conferring resistance to isoniazid

Isoniazid (INH) resistance is brought about by mutations in several genes of *M. tuberculosis* such as the *kat*G, *inh*A, *ahp*C, *kas*A, *oxy*R, *fur*A , *fab*G1-*inh*A, and *ndh* genes (Palomino and Martin, 2014; Nasiri *et al.,* 2017; Mumena *et al.,* 2021). However, Current research has shown that resistance to isoniazid can also be caused by an upregulation of efflux pumps or isoniazid inactivators (Hsu *et al.,* 2020). Mutations in the *kat*G, *inh*A and its promoter, and the *oxy*R-*ahp*C intergenic region frequently confer resistance to INH (Havumaki *et al.,* 2017; Hameed *et al.,* 2018; Mumena *et al.,* 2021). While mutations in the following genes *oxy*R, *fur*A, *ndh*, *ahp*C, *kas*A, and *fab*G1-*inh*A infrequently confer resistance to INH (Nasiri *et al.,* 2017; Mumena *et al.,* 2021). Recent studies have also found that mutations in the *dfr*A gene cause resistance to INH (Dookie *et al.,* 2018). Mutations in the *inh*A gene cause resistance to both INH and ethionamide which share the same binding site on the promoter region (Katia *et al.,* 2018). The most frequently identified mutation in the *kat*G gene is the Ser315Thr1, which confer a high-level resistance to INH (Dookie *et al.,* 2018; Nguyen *et al.,* 2019; Dean *et al.,* 2020; Mumena *et al.,* 2021). While that in the *inh*A gene is the C-15T, which confer low-level resistance to INH (Dean *et al.,* 2020; Mumena *et al.,* 2021). The two mutations *kat*G MUT (Ser315Thr1) and *inh*A MUT (C-15T) account for 80% of resistance to INH (Nasir *et al.,* 2017; Torres-Gonzalez *et al.,* 2016). The four most frequently identified mutations in the *inh*A gene that are associated with resistance to INH are Cys15Thr, Thr8Cys, Thr8Ala, and Ala16Gly (Namburete *et al.,* 2016; Mumena *et al.,* 2021). Isoniazid resistance that is associated with mutations in the *kat*G gene occurs before rifampicin resistance (Dookie *et al.,* 2018). Mutations in the *kat*G gene can therefore, serve as a key marker for pre-MDR TB (Manson *et al.,* 2017).

2.7.1.2 Drug target genes and mutations conferring resistance to rifampicin

Mutations within a hypervariable region of the *rpo*B gene, which codes for the *β*-subunit ribonucleic acid polymerase confer resistance to rifampicin (RIF) in 95% of *M. tuberculosis* clinical isolates (Louw *et al.,* 2011; Muthaiah *et al.,* 2017; Hameed *et al.,* 2018; Almutairi *et al.,* 2019; Singh *et al.,* 2019). About 96% of RIF- resistance occurs within the RIF resistance determining region (RRDR) which is also called the "hot-spot region" (HSR), covering codons 507-533 of the *rpo*B gene (Hameed *et al.,* 2018). Mutations at codons 531, 526, and 516 in the *rpo*B gene are the most commonly identified in RIF-resistant *M. tuberculosis* isolates (Hameed *et al.,* 2018). Mutations at codons 529, 526, 518, and 516 confer low-level resistance to RIF, whereas mutations at codons 526-531 show the highest frequency and are associated with a high-level resistance to RIF (Hameed *et al.,* 2018; Nguyen *et al.,* 2019). Mutations at codon 531 are associated with cross-resistance to rifabutin (Hameed *et al.,* 2018). The most frequent mutations associated with resistance to RIF in the rpo*B* gene are Ser531Leu, His526Asp, His526Tyr, and Asp516Val (Namburete *et al.,* 2016; Nguyen *et al.,* 2019). These point mutations involve changes in the positions of amino acids, and can be an insertion, deletion, or missense (Muthaiah *et al.,* 2017; Zaw *et al.,* 2018).

2.7.1.3 Drug target genes and mutations conferring resistance to ethambutol

Mutations in genes that confer resistance to ethambutol (EMB), occur in specific regions known as ethambutol resistance-determining regions (ERDR) or "hot-spot regions" (HSR) (Ramazanzadeh *et al.,* 2016). EMB resistance in *M. tuberculosis* is caused by mutations in the following genes: *emb*A, *emb*B, *emb*C, *emb*R, *Ubi*A, *aft*A and *ini*A genes (Xu *et al.,* 2015; Al-Saeedi & Al-Hajoj, 2017; Dookie *et al.,* 2018; Hameed *et al.,* 2018). The most common mechanism for resistance to EMB is mutation in the *emb*B gene, occurring at codon 306 (Dookie *et al.,* 2018). Mutations at codon 406 and 497 within the *emb*B gene have also been detected (Khosravi *et al.,* 2019). For example the mutations Met306Leu and Met306Val at codon 306 in the *emb*B gene are associated with resistance to EMB (Hameed *et al.,* 2018). Novel mutations *emb*B Gly43Cys, *emb*B Gly554Asn, and *emb*B Ser412Pro in the *emb*B gene also confer resistance to EMB (Al-Saeedi & Al-Hajoj, 2017). Mutations in the *ubi*A gene in-

conjunction with mutations in the *emb*B gene have been found to cause a high-level resistance to EMB (Hameed *et al.,* 2018; Tulyaprawat *et al.,* 2019). Studies have also shown that the high-level resistance to EMB develop via a stepwise acquisition of mutations in the *emb*B, *ubi*A, and *emb*C genes (Lingaraju *et al.,* 2016). From the total of 98% of mutations that occur in the *emb*B CAB locus of the *emb*B gene in resistant *M. tuberculosis* isolates, 70% are found in codon 306, 406, or 497, while 13% of the mutations are found out side of the three regions between codons 296 and 426, and 15% are in the *emb*C-*emb*A intergenic region (Hameed *et al.,* 2018). The embCAB operon comprises of three genes *emb*A, *emb*B, and *emb*C. Therefore, mutations in this operon are associated with resistance to EMB (Khosravi *et al.,* 2019).

2.7.1.4 Drug target genes and mutations conferring resistance to pyrazinamide

Mutations in the *pnc*A, *pan*D, *rps*A, *clp*C1(Rv3596c), and Rv2783c genes of *M. tuberculosis* confer resistance to pyrazinamide (PZA) (Dookie *et al.,* 2018; Katia *et al.,* 2018; Hameed *et al.,* 2018; Nangraj *et al.,* 2021). However, mutations in the *pnc*A and its promoter region are the most frequently identified as they account for 72-99% of resistance to PZA (Dookie *et al.,* 2018; Nangraj *et al.,* 2021). The most frequent mutations in the *pnc*A gene are: Asp49Asn, Tyr64Ser, Trp68Gly, and Phe94Ala (Nangraj *et al.,* 2021). Studies have also shown that PZA resistance is strongly associated with rifampicin resistance (Dookie *et al.,* 2018). This finding confirms that the burden of PZA resistance is in patients who have rifampicin resistance (Dookie *et al.,* 2018).

2.7.2 Drug target genes and mutations conferring resistance to second-line anti tuberculosis drugs

The second-line anti-TB drugs are key in the management of drug-resistant TB, and they include: fluoroquinolones, aminoglycosides, streptomycin, cycloserine, ethionamide, prothionamide, para-amino salicylic acid, and cyclic poly-peptides (Palomino and Martin, 2014; Dookie *et al.,* 2018). Drug-resistance to all anti-TB drugs has been reported in some countries (Oppong *et al.,* 2019). *M. tuberculosis* resistance is mainly caused by mutations in the target genes (Liu *et al.,* 2019). Mutations in the *rps*L, *rrs* and *gid* genes cause resistance to streptomycin, while mutations in the *gyr*A

and *gyr*B genes cause resistance to the fluoroquinolones. Mutations in the *rrs*, *eis* and *tly*A genes cause resistance to the aminoglycosides and cyclic polypeptide antibiotics (Chen *et al.,* 2019a).

2.7.2.1 Drug target genes and mutations conferring resistance to streptomycin

Streptomycin (STR) resistance by *M. tuberculosis* is caused by mutations in the *rps*L , *rrs*, and *gid*B genes (Katia *et al.,* 2018; Hameed *et al.,* 2018; Nguyen *et al.,* 2019). The following mutations confer resistance to STR, *rps*L (Lys43Arg, Lys88Gln, Lys88Arg, Cys117Thr), *rrs* (Cys517Thr, Ala514Cys, Ala906Gly, Ala907Cys), *gid*B (Ala183Val, Gly71Arg, Tyr22His, Gly37Arg, Pro75Ser, Gly76Asp, Ile81Thr, Phe100Leu, Val124Gly, Ala134Gly, Ala138Pro, Ser149Arg, Leu152Ser, and Gly157Arg) (Bwalya *et al.,* 2021). Mutations in the *rps*L and *rrs* genes are the major mechanisms that confer resistance to STR in *M. tuberculosis*, they account for 60-70% of resistance to STR (Dookie *et al.*, 2018). Recent studies have revealed that mutations in the *gid*B gene cause low-level resistance and accounts for 33% of resistance to STR in clinical *M. tuberculosis* isolates (Palomino and Martin, 2014; Dookie *et al.,* 2018; Katia *et al.,* 2018). The most frequently identified mutation in the *rps*L gene is the replacement of lysine with arginine at position 43 and 88 (Hameed *et al.*, 2018). While in the *rrs* gene is the mutation Ala80Pro (Hameed *et al.*, 2018). *M. tuberculosis* strains that are resistant to streptomycin confer cross-resistance to amikacin and kanamycin also (Hameed *et al.,* 2018).

2.7.2.2 Drug target genes and mutations conferring resistance to aminoglycosides and cyclic poly-peptide antibiotics

Aminoglycosides and the cyclic polypeptide antibiotics are second-line drugs that are used in the treatment of drug-resistant TB (Dookie *et al.,* 2018). The two key aminoglycosides are kanamycin (KAN) and amikacin (AMK), while capreomycin (CAP) and viomycin (VIO) are key cyclic polypeptide antibiotics (Palomino and Martin, 2014). The three drugs KAN, AMK, and CAP are called second-line injectable drugs (SLIDs) (Dookie *et al.*, 2018). Mutations in the *rrs*, *eis*, *tly*A genes result in resistance to the SLIDs (Palomino and Martin, 2014). Mutations in the *rrs* gene cause resistance to all the three SLIDs, and is the most common molecular mechanism for

resistance to this class of anti-TB drugs (Dookie *et al.,* 2018). Mutations in the *rrs* gene, specifically at positions 1400, 1401, and 1483 base pair (bp) are associated with a high-level resistance to both AMK and KAN in KAN-resistant *M. tuberculosis* strains (Hameed *et al.,* 2018). The mutation Ala1401Gly in the *rrs* gene confer a high-level resistance to AMK and KAN along with cross-resistance to CAP. While the mutation Cys1402Thr or Gly1484Thr is associated with resistance to CAP and a cross-resistance to KAN or VIO (Hameed *et al.,* 2018). Mutations in the *rrs* gene are also associated with resistance to CAP and VIO (Nasiri *et al.,* 2017). Mutations in the *eis* gene confer low-level resistance to KAN (Hameed *et al.,* 2018). Mutations in the *rrs* gene accounts for about 70-80% resistance to CAP and AMK as well as 60% resistance to KAN in resistant *M. tuberculosis* isolates worldwide (Dookie *et al.,* 2018). Mutations in the *eis* gene cause about 80% low-level resistance to KAN but not to AMK (Dookie *et al.,* 2018). While mutations in the *tly*A gene cause about 3% resistance to CAP (Palomino and Martin, 2014; Dookie *et al.,* 2018). Cross-resistance to streptomycin and KAN occur due to mutations in the *whi*B7 gene of *M. tuberculosis* (Zhang and Yew, 2015). Mutations in the *tly*A gene also results in resistance in both CAP and VIO (Palomino and Martin, 2014).

It is worth noting that the most frequent mutations in the *rrs* gene include; Ala1401Gly, Cys1402Thr, and Gly1484Thr (Charoenpak *et al.,* 2020; Zimenkov *et al.,* 2013). While those in the *eis* gene include; Gly37Thr, Cys14Thr, Cys12Thr, Gly10Ala, Cys2Ala (Charoenpak *et al.,* 2020). The *eis* (Cys14Thr) mutation is very specific for resistance to KAN than the *eis* (Gly10Cys) and *eis* (Cys12Thr) mutations (Bablishvili *et al.,* 2017).

2.7.2.3 Drug target gene and mutations conferring resistance to fluoroquinolones

Fluoroquinolones (FLQs) are second-line anti-TB drugs, examples of those used in the treatment of drug-resistant TB include: levofloxacin, ofloxacin, gatifloxacin and moxifloxacin (Jabeen *et al.,* 2015; Tiberi *et al.,* 2017; Mamatha and Shanthi, 2018). Mutations in the quinolone resistance determining region (QRDR) of both *gyr*A and *gyr*B genes of *M. tuberculosis* cause resistance to FLQs (Nasiri *et al.,* 2017; Dookie *et*

al., 2018; Katia *et al.,* 2018; Hameed *et al.,* 2018; Mumena *et al.,* 2021). Mutations in the *gyr*A gene cause a high-level resistance to FLQs, while mutations in the *gyr*B gene cause a low-level resistance to FLQs. However, combined mutations in both the *gyr*A and *gyr*B genes result in a high-level resistance to FLQs (Zhang and Yew, 2015). The most common mutations in the *gyr*A gene are: Gly88Cys, Gly88Ala, Ala90Val, Ser91Pro, Asp94Gly, Asp94Ala, Asp94His, Asp94Asn and Asp94Tyr. While those in the *gyr*B gene are: Glu540Val, and Asn538Asp (Hameed *et al.,* 2018; Mumena *et al.,* 2021). Mutations in the *gyr*B gene are not frequently found among *M. tuberculosis* clinical isolates (Hameed *et al.,* 2018). Mutations in both *gyr*A and *gyr*B genes, such as; Asn538Ile (*gyr*B)-Asp94Ala (*gyr*A) and Ala543Val (*gyr*B)- Asp94Asn/Asp94Gly (gyrA) cause very high-resistance to FLQs (Hameed *et al.,* 2018; Zhang and Yew, 2015). Cross-resistance among the FLQs occurs (Palomino and Martin, 2014). Resistance to ofloxacin causes resistance to other FLQs (Mamatha and Shanthi, 2018). FLQ resistance is one of the most important criterion that is used for defining "extensively drug-resistant TB" (Malik *et al.,* 2012).

The second mechanism through which *M. tuberculosis* develops resistance to FLQs is the use of efflux pumps (Jabeen *et al.,* 2015; Nasiri *et al.,* 2017). These biological pumps remove the FLQ drugs out of the mycobacterial cells (Jabeen *et al.,* 2015).

2.8 Causes of drug-resistant tuberculosis

Drug-resistant tuberculosis is caused by a number of factors, some of which include; delayed diagnosis, interruption of treatment, poor adherence to treatment, poor compliance to treatment, wrong treatment prescription, or previous treatment of TB using inadequate or sub-therapeutic or ineffective anti-TB drugs (Lange *et al.,* 2014; Seung *et al.,* 2015; Seifert *et al.,* 2015; Paudel, 2017; Rumende *et al.,* 2018). Drug-resistant TB can also be caused by direct infection with resistant *M. tuberculosis* strains (Shah *et al.,* 2017). Gene mutations in *M. tuberculosis* are a major mechanism responsible for causing drug-resistant TB (Kanji *et al.,* 2019). These point mutations in chromosomal genes, can be an insertion, deletion or missense (Eddabra & Neffa, 2020). Another key mechanism for drug resistance is the use of efflux pumps, these biological pumps remove anti-TB drugs out of the mycobacterial cells resulting in

resistance to anti-TB drugs (Jabeen *et al.,* 2015; Liu *et al.,* 2019). Resistance to anti-TB drugs in *M. tuberculosis* can also be caused by the *β*-lactamase enzymes which inactivates the *β*-lactam antibiotics (Gokulan and Varughese, 2018). Drug-resistant *M. tuberculosis* strains are also able to resist or tolerate the toxic pharmacological effects of anti-TB drugs (Ombura *et al.*, 2016).

2.9 Risk factors for drug-resistant tuberculosis

Drug-resistant tuberculosis has several risk factors, some of which include; HIV-TB co-infection, failure to respond to first-line anti-TB drugs, relapse TB case after a full treatment course with first-line anti-TB drugs, treatment after default, exposure to a known case of drug-resistant TB (Seung *et al.,* 2015; Stosic *et al.,* 2017). The other risk factors for acquiring drug-resistant TB are; overcrowding and poor ventilation (in hospitals, prisons, dormitories, and market places), immunocompromised state, smoking, indoor and outdoor pollution (Udwadia, 2016; Shah *et al.,* 2018). A household or hospital with patients infected with drug-resistant TB is a risk factor for acquiring drug-resistant TB (Lange *et al.,* 2014).

2.10 Prevention of tuberculosis

Tuberculosis can be prevented through the implementation of infection prevention and control measures in healthcare settings, provision of adequate ventilation (in clinical settings, prisons, homes, and in boarding facilities or dormitories), limiting contact with TB infected patients, intensified or active case finding for TB, vaccination using the Bacillus Calmette-Guerin (BCG) vaccine, provision of isoniazid preventative therapy, provision of highly active anti-retroviral therapy to those who are HIV positive and through pasteurization of milk (Frigati *et al.,* 2011; Hermans *et al.,* 2012; Orme, 2015; Mohajan, 2015; Verkuijl and Middelkoop, 2016; Yotebieng *et al.,* 2016; Silva *et al.,* 2018; Sabasaba *et al.,* 2019; Geremew *et al.,* 2022).

2.10.1 Infection prevention and control

The World Health Organization recommends four levels of infection prevention and control for TB; managerial controls, administrative controls, environmental controls,

and personal respiratory protection (Verkuijl and Middelkoop, 2016). Managerial controls, establishes and oversee infection prevention and control for TB measures, these control measures ensure that tools and management structures are in place to support the implementation of the "3 lines of infection prevention and control for TB defense" (administrative control, environmental control, and personal respiratory protection) (Verkuijl and Middelkoop, 2016).

Administrative controls are policies and work practices that help to reduce the risk of exposure, infection, and disease to healthcare workers, staff, and patients in health care settings by ensuring rapid diagnosis, isolation, and treatment of patients and staff with TB (Chen *et al.,* 2016; Verkuijl and Middelkoop, 2016). Administrative control measures include; promptly identifying individuals with signs and symptoms of TB (screening and triage), separating and/ or isolation of infectious patients, adhering to cough etiquette and respiratory hygiene, minimizing time infectious patients spend in health care facilities, and reducing diagnostic delays by use of rapid diagnostics (Verkuijl and Middelkoop, 2016). Providing a package of prevention and care intervention for healthcare workers is also part of administrative controls and include; HIV counselling and testing, HIV prevention, anti-retroviral therapy, and isoniazid preventive therapy, for HIV-positive healthcare workers (Verkuijl and Middelkoop, 2016). HIV infection with delayed diagnosis of TB and poor infection prevention and control increases the risk of transmission of multidrug-resistance strains of TB (Gandhi *et al.,* 2013; Liang *et al.,* 2010). HIV-positive healthcare workers should be protected from direct exposure to patients with known or suspected TB, redeploying them to areas of lower transmission risk in healthcare facilities (Verkuijl and Middelkoop, 2016). It suffices to say that the main goals of administrative control measures are to ensure; rapid diagnosis, isolation, and treatment of patients and staff with TB (Chen *et al.,* 2016).

Environmental controls are equipment or practices that prevents or reduce the spread of TB by reducing the concentration of infectious respiratory aerosols (infectious respiratory droplet nuclei) in the air (Verkuijl and Middelkoop, 2016). Environmental control measures include ventilation (natural and mechanical), ultraviolet germicidal irradiation, and high efficiency particulate air filtration. Natural ventilation is the most

practical and cost-effective control measure that relies on the movement of air through open windows and doors, ensuring sufficient air changes per hour (Verkuijl and Middelkoop, 2016). Mechanical ventilation is needed in high-risk settings with poor natural ventilation (Chen *et al.,* 2016). Germicidal ultraviolet air disinfection such as the use of ultraviolet germicidal irradiation or germicidal ultraviolet fixtures is recommended as a low-cost complementary system to the natural and mechanical ventilation (Shenoi *et al.,* 2010; Baral and Koirala, 2022). High efficiency particulate air filtration can also be used to clean the air (Verkuijl and Middelkoop, 2016).

Personal respiratory protective gear is the recommended final barrier to protect healthcare workers, patients and the community from inhaling infectious droplet nuclei in settings where the concentration of infectious droplet nuclei cannot be adequately reduced by both administrative and environmental control measures (Chen *et al.,* 2016). Personal respiratory protective gear includes; surgical masks, and particulate respirators (Yates *et al.,* 2016). The respirators protect healthcare workers from acquiring respiratory diseases such as TB, COVID-19, and others (Yates *et al.,* 2016; Polack *et al.,* 2020; WHO, 2021). It is worth noting that respirators are indicated to protect staff and visitors from inhaling infectious droplet nuclei, while surgical masks are indicated for patients-to reduce the spread of infectious droplet nuclei in the air (Yates *et al.,* 2016). For respirators to be effective they need to be fit tested (Dheda *et al.,* 2017).

Tuberculosis infection prevention and control is a vital component of the World Health Organization Stop TB strategy for large reductions in TB incidence, TB mortality, and treatment costs faced by patients with TB, and contribute to strengthening of healthcare systems (Baral and Koirala, 2022). Tuberculosis infection prevention and control implementation contributes to reducing the transmission of *M. tuberculosis* resistant strains to healthcare workers, HIV patients, and the general public (Verkuijl and Middelkoop, 2016; Westhuizen *et al.,* 2019; Cegielski *et al.,* 2021). However, the neglect of infection prevention and control for TB remains an important gap in the provision of high-quality care in high TB burden countries (Westhuizen *et al.,* 2019; Westhuizen *et al.,* 2022). Gaps in implementation of infection prevention and control for TB in health care facilities predisposes healthcare workers to nosocomial

transmission of *M. tuberculosis* resistant strains (Apriani *et al.,* 2022). Healthcare workers with limited knowledge on infection prevention and control for TB also contribute to an increased risk of transmission of *M. tuberculosis* resistant strains not only to themselves but also other patients, and the general public (Apriani *et al.,* 2022). Healthcare workers in frontline services, especially triage staff, must be competent in infection prevention for TB (Curran *et al.,* 2006).

2.10.2 Active case finding for tuberculosis

One of the cornerstones of the World Health Organization Stop TB strategy is increased detection of TB using intensified or active case finding (Hermans *et al.,* 2012). Active case finding for TB is defined as a systematic screening for TB disease outside health care facilities (Nagaraja *et al.,* 2021). Active case finding involves actively searching for undiagnosed active TB disease in a defined population, usually in high-risk groups, high-prevalence congregate communities, or contact of TB patients (Deya *et al.,* 2021; Nagaraja *et al.,* 2021). A patient with infectious TB, who has not yet been initiated on treatment will transmit the tubercle bacilli to 10-15 people per year (Deya *et al.,* 2021). To reduce this transmission, active case finding strategies are required (Deya *et al.,* 2021). Active case finding is one of the key strategies that prevents and controls TB by reducing diagnostic delays and the period of infectiousness and transmission of *M. tuberculosis*, as well as ensuring prompt treatment of TB patients (Bigogo *et al.,* 2018; Deluca *et al.,* 2019; Deya *et al.,* 2021).

2.10.3 Vaccination

The Bacillus Calmette-Guérin (BCG) vaccine is currently the only available and licensed vaccine used for the prevention of TB (Orme, 2015). The World Health Organization recommends universal vaccination using the BCG vaccine to prevent TB. A single dose of BCG vaccine needs to be administered to infants, at birth or as soon as possible after birth in settings where TB is highly endemic or in settings where there is a high-risk of exposure to TB (Cernuschi *et al.,* 2018). There is a proven benefit of BCG vaccination in children than in adults (Roy *et al.,* 2019). The Bacillus Calmette-Guérin vaccine protects against TB meningitis and miliary TB in children, this vaccine has a protective efficacy of 60-80% (Sweeney *et al.,* 2019). Studies have

shown that BCG vaccine can offer protection against TB for up to ten years (Cernuschi *et al.,* 2018).

2.10.4 Isoniazid preventive therapy

Isoniazid preventive therapy is an effective therapy for preventing TB (Frigati *et al.,* 2011). It is one of the 3 I's recommended by the World Health Organization in the prevention of TB in people living with HIV (Yotebieng *et al.,* 2016). The 3 I's are; Isoniazid preventive therapy, intensified TB case finding and infection control (Satiavan *et al.,* 2018; Sabasaba *et al.,* 2019; Tiruneh *et al.,* 2019). Isoniazid preventive therapy is effective in preventing TB in both HIV-negative and HIV-positive individuals (Tiruneh *et al.,* 2019). It also cures latent TB infection and ultimately reduces the risk of TB reactivation and disease in the future (Rhines *et al.,* 2018). Isoniazid preventive therapy is provided as a daily dose for 6 months to prevent TB in people living with HIV and in children that are contacts to bacteriologically confirmed TB positive cases (Abossie & Yohanes, 2017; Little *et al.,* 2018; Kagujje *et al.,* 2019; Sabasaba *et al.,* 2019). Isoniazid preventive therapy reduces mortality and prevents morbidity due to TB in people living with HIV (Briggs *et al.,* 2015; Juszkiewicz *et al.,* 2019; Kagujje *et al.,* 2019).

2.10.5 Provision of highly active anti-retroviral therapy

Human immunodeficiency virus infection is the strongest risk factor for the development of TB, in those who have latent TB infection or new *M. tuberculosis* infection (Hermans *et al.,* 2012). People living with HIV are 21-37 times more likely to develop TB than HIV uninfected persons (Yen *et al.,* 2018; Owiti *et al.,* 2019; Geremew *et al.,* 2022; Tiruneh *et al.,* 2022). HIV down-regulates the immune system directly by destroying the host CD4+ T- cells and increases the risk of TB infection (Yen *et al.,* 2018; Geremew *et al.,* 2022). PLWHIV develop a variety of different opportunistic infections in their life span, especially when their CD4+ T- cell count is less than 250 cells/mm^3 (Shenoy *et al.,* 2017; Beshaw *et al.,* 2021). Tuberculosis is the most common opportunistic infection that causes high mortality and morbidity rates in people living with HIV (Manosuthi *et al.,* 2016; Yen *et al.,* 2018; Tiruneh *et al.,* 2022). Highly active anti-retroviral therapy improves the survival of people living with

HIV, it reconstitutes the immune function and enhances the immune response to *M. tuberculosis,* thus averting TB incidence (Geremew *et al.,* 2022). Early initiation of highly active anti-retroviral therapy is a key component to prevent and control the HIV-associated TB syndemic (Suthar *et al.,* 2012). Indeed, highly active anti-retroviral therapy has a potent TB preventive effect in people living with HIV, even in those with advanced immunodeficiency (Harris *et al.,* 2018). The most recent World Health Organization recommendation on highly active anti-retroviral therapy, stipulates that all people living with HIV should be initiated on highly active anti-retroviral therapy regardless of clinical stage or CD4+ T- cell count, this recommendation provide a consideration TB preventive benefit in high HIV prevalence settings (Harris *et al.,* 2018). Isoniazid preventive therapy combined with highly active anti-retroviral therapy among people living with HIV reduces the risk of TB and mortality compared with highly active anti-retroviral therapy alone (Harris *et al.,* 2018; Geremew *et al.,* 2022).

2.10.6 Pasteurization

M. bovis causes bovine TB in animals, and zoonotic TB in humans, which can either be pulmonary or extra-pulmonary TB (Malama *et al.,* 2013a; Malama *et al.,* 2013b; Gormley and Corner, 2018; Vayr *et al.,* 2018; Kemal *et al.,* 2019). Infected animals shed *M. bovis* through respiratory infectious aerosols, saliva, feces, urine, milk, and discharging lesions (Kuria, 2019). Therefore, *M. bovis* can be transmitted from animals to humans by inhalation of infectious aerosols, or by consumption of unpasteurized milk (Chauhan, 2019). The incidence of pulmonary TB caused by *M. bovis* is higher in occupationally exposed individuals such as livestock keepers, dairy workers, and slaughterhouse workers (Asebe, 2017; Chauhan, 2019). Zoonotic TB affects humans and can be prevented by: pasteurizing milk before drinking it; avioding direct contact with secretions or excretions of infected animals such as cattle; and adhering to hygiene standards strictly when handling domestic and wild animals (Silva *et al.,* 2018; Vayr *et al.,* 2018; Chauhan *et al.,* 2019).

2.11 Tackling the global drug-resistant tuberculosis crisis

The emergence of MDR-TB and XDR-TB is an obstacle to effective control of TB (Shah *et al.,* 2018). Drug-resistant TB is a serious public health problem globally (Daoqun *et al.,* 2017). In order to improve TB control, it is important to track the spread of *M. tuberculosis,* identify index cases, and detect outbreak cases (Lacoma *et al.,* 2017). Monitoring and proper management of patients infected with drug-resistant *M. tuberculosis* strains is key to effective control of drug-resistant TB globally (Sitienei *et al.,* 2017). The World Health Organization's proposed five key priority actions to tackle the global drug-resistant TB crisis are: provision of high quality management of drug-susceptible TB; expand the rapid testing and diagnosis of drug-resistant TB cases; provide quick access to effective treatment regimens and proper care of patients infected with drug-resistant TB; practicing of effective infection prevention and control; and increase political will and financing of TB programmes globally (Pai and Memish, 2016; Rendon *et al.,* 2017).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was conducted at the National Reference, Chest Diseases Laboratory, in Lusaka, Zambia from March to September, 2020. The Chest Diseases Laboratory is a National Reference mandated to perform confirmatory phenotypic and genotypic tests for *M. tuberculosis* resistance to first-and second-line anti-TB drugs. Therefore, different health facilities across the ten provinces of Zambia submit sputum specimens for further testing. Sputum specimens for the study came from health facilities in seven provinces of Zambia; Northern, Lusaka, Southern, Eastern, Central, Muchinga, and Western provinces.

From the Northern Province, 9 health facilities submitted sputum specimens; Location Urban Clinic, Luwingu District Hospital, Chilubi Mainland District Hospital, Chitimukulu Rural Health Centre, Senga Hill District Hospital, Kaputa District Hospital, Mpulungu Urban Clinic, Kasama General Hospital, and Mbala General Hospital. From Lusaka Province, 10 health facilities submitted sputum specimens; University Teaching Hospital, Chawama Clinic, Kafue General Hospital, Kamwala Clinic, Mwandi Mission Hospital, Mother Theresa Clinic, Mutendele Mission Hospital, Chaisa Health Centre, Yeta Hospital, and Coptic Hospital. From Southern Province, 10 health facilities submitted sputum specimens; Monze Mission Hospital, Nansenga Mission Hospital, Nangoma Mission Hospital, Zambia Sugar Hospital, Mazabuka General Hospital, Shampande Urban Clinic, Maramba Clinic, Magoye Rural Health Centre, Siavonga General Hospital, and Kabuyu Rural Health Centre (figure 3.1).

From Eastern Province, 12 health facilities submitted sputum specimens; Chipata Central Hospital, Saint Francis Mission Hospital, Lumezi Mission Hospital, Kamoto Mission Hospital, Petauke District Hospital, Chadiza Urban Clinic, Nyanje Mission Hospital, Petauke Urban Clinic, Kapata Urban Clinic, Katete Urban Clinic, Mnukwa Rural Health Centre, and Lundazi District Hospital. From Central Province, 5 health

facilities submitted sputum specimens; Kabwe General Hospital, Kabwe Urban Clinic, Kabwe Central Hospital, Itezhi-tezhi Hospital, and Kapiri- Mposhi Urban Clinic. From Muchinga Province, 2 health facilities submitted sputum specimens; Chilonga Mission Hospital and Nakonde Urban Clinic. From Western Province, 3 health facilities submitted sputum specimens; Limulunga General Hospital, Lewanika General Hospital, and Lwampa Mission Hospital (figure 3.1).

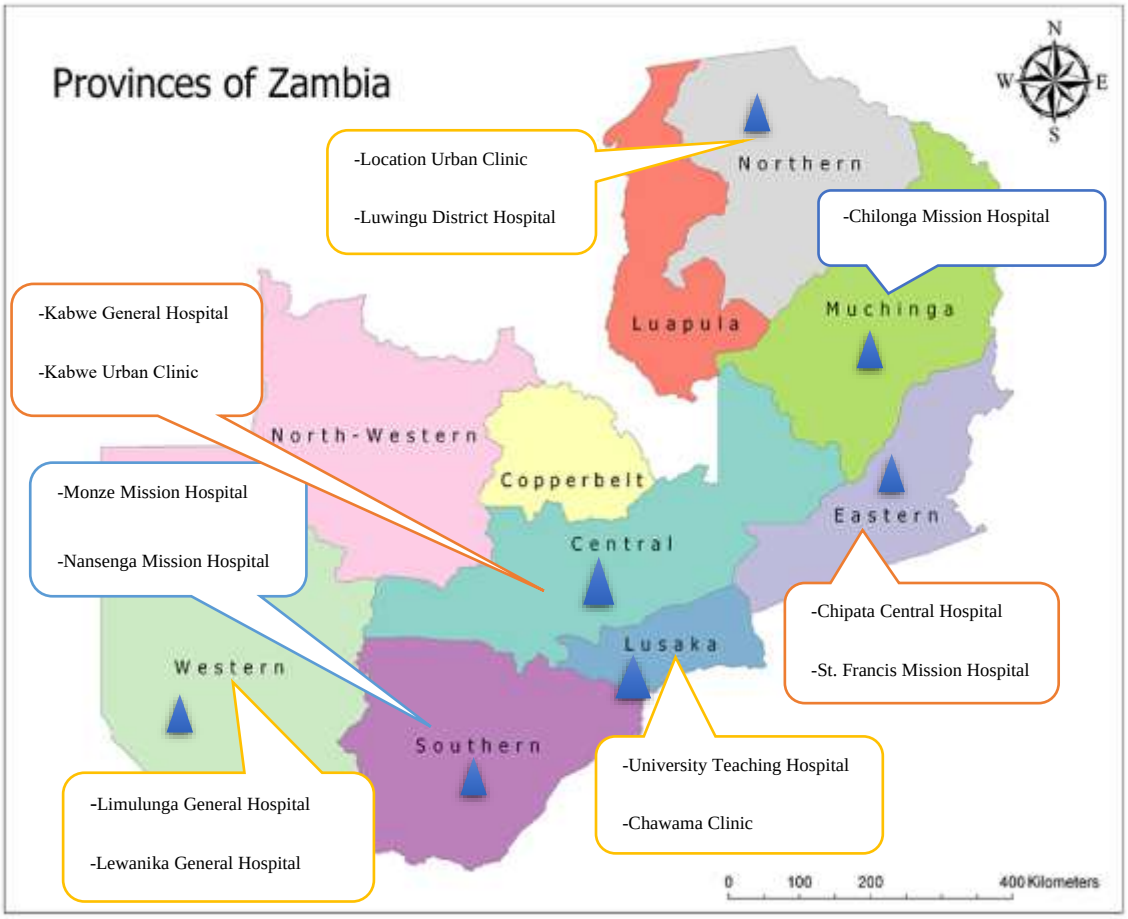

Figure 3.1: Map of Zambia highlighting seven provinces and some of the health facilities that submitted sputum specimens to the Chest Diseases Laboratory

3.2 Study design

This was a cross-sectional study carried out on the new and previously treated TB cases, from health facilities across seven provinces of Zambia.

3.3 Study population

The study population was the new and previously treated TB case, from health facilities across seven provinces of Zambia.

3.3.1 Inclusion criteria

i. Any newly diagnosed TB patient (regardless of age diagnosed with drug-sensitive or smear-positive pulmonary TB at least 1+ on the IUATLD/WHO scale on smear microscopy) who has never been treated for TB or has taken anti-TB drugs for less than 1 month, with a productive cough able to submit at least 3-5 millilters of sputum specimen.
ii. Any previously treated TB patient who has received 1 month or more of anti-TB drugs in the past.

The World Health Organization defines a new TB case as a newly registered episode of TB in a patient who has never been treated for TB or has taken anti-TB medicines for less than 1 month. While previously treated TB cases refers to patients who have received 1 month or more of anti-TB medicines in the past. Previously treated cases may have been treated with a first-line regimen for drug-susceptible TB or a second-line regimen for drug-resistant TB forms (WHO, 2019b; Tahseen *et al.*, 2020). Based on the most recent course of treatment the previously treated TB cases can be classified as; relapse TB patients, treatment after failure patients, treatment after loss to follow-up patients, and treatment after default patients (Cohen *et al.*, 2018).

3.3.2 Exclusion criteria

i. Patients with tuberculosis who were not able to produce sputum were excluded from the study.
ii. Patients who had no episode of tuberculosis or had no previous history of being treated for tuberculosis were excluded from the study.

3.4 Sample size determination

The sample size was calculated using the Cochran's formular (Kasiulevičius *et al.*, 2006). The confidence level at 95%, margin of error 5% and the Z-score of 1.96. The prevalence rate of MDR-TB/RR-TB in Zambia among the new TB cases was 7.1% (Masenga *et al.*, 2017). The sample size was calculated using the Cochran's formular for prevalence studies shown below:

$$n = \frac{\mathrm{Z}^2 \times \mathrm{P}\,(1-\mathrm{P})}{\mathrm{e}^2}$$

Where:

$n =$ sample size

Z =1.96, at 95% confidence level

P = prevalence rate of MDR/RR-TB among the new TB cases was 7.1% (0.071)

e = margin of error is 5% (0.05)

$$\therefore n = \frac{1.96^2 \times 0.071\,(1-0.071)}{0.05^2} = 102$$

The same Cochran's formular above was used to calculate the sample size for the previously treated TB cases. The prevalence rate of MDR-TB/RR-TB in Zambia, among the previously treated TB cases was 18% (P = 0.18) in 2019 (WHO, 2019a; WHO, 2020). Therefore, plugging in the figures into the formular we have the following equation below:

$$\therefore n = \frac{1.96^2 \times 0.18\,(1-0.18)}{0.05^2} = 227$$

The calculated sample size for the new and previously treated TB cases was 102 and 227 positive sputum specimens, respectively. Therefore, the final total sample size for this study was 329 (102 +227) TB positive sputum specimens.

3.5 Sampling technique

This study used a stratified random sampling technique. In this technique, stratification was used to group the TB positive sputum specimens into two groups (the first group had specimens coming from the "new TB cases", while the second group from the "previously treated TB cases"). Then a simple random sampling technique was applied to pick sputum specimens by a random process using a random number table (table of random numbers, appendix IV), from each group. From the first group, 102 TB positive sputum specimens were randomly picked over a period of seven (7) months (from that total 5 sputum specimens came from the Northern province, 7 from Southern province, 22 from Lusaka province, 63 from Eastern province, 2 from Central province, 2 from Muchinga province, and 1 from Western province), while from the second group, 227 TB positive sputum specimens were randomly picked over the same period of time (from that total 9 came from Northern province, 84 from Lusaka province, 26 from Southern province, 83 from Eastern province, 17 from Central province, 5 from Muchinga province, and 3 from Western province). Therefore, the total sputum specimens collected for the study over a period of seven months were 329 sputum specimens.

3.6 Laboratory procedures

3.6.1 Biosafety measures

Specimen processing for line probe assay was performed in a laboratory equipped with class II biosafety cabinets, strictly following biosafety precautions and infection prevention principles. Appropriate personal protective equipment (PPE) was used such as: gloves, fluid-resistant laboratory coats, gowns, aprons, head caps, N95 masks, protective goggles and rubber boots. Laboratory equipment were decontaminated before and after use, using 70% isopropyl alcohol. While the work surfaces were disinfected daily using a disinfectant, 0.1% sodium hypochlorite (NaOCl). The

biohazardous waste generated in the laboratory were segregated and treated, then taken to the incinerator for inceration by a trained biosafety officer. Biosafety precautions and infection prevention principles were adhered to strictly, from the point of specimen reception through to processing, analysis and disposal of biohazardous waste (Chen *et al.,* 2016; Yates *et al.,* 2016; Polack *et al.,* 2020; WHO, 2021).

3.6.2 Specimen processing

Sputum specimen processing involved three techniques; digestion, decontamination, and concentration processes. These processes were carried out in a level III laboratory, equipped with class III biosafety cabinets. The digestion and decontamination processes were done using the mucolytic agent N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) to liquefy the sputum specimens, so as to release the tubercle bacilli and expose the normal flora for decontamination. In brief 0.25g of NALC (N-acetyl-L- cysteine) was added in clean falcon tubes, then 50 millilitres of a mixture of 4% NaOH (sodium hydroxide) and 2.9 % $C_6H_5O_7$ (citrate) was added to the NALC. A physical examination of the sputum specimens was carried out and the volume of each sputum specimen in the falcon tube was recorded. An equal volume of the NALC-NaOH-citrate mixture was added to an equal volume of sputum, and then allowed to stand for 15 minutes for digestion and decontamination of the sputum. One (1) millilitre of phosphate buffer solution [pH 6.8] was added to each specimen mixture to neutralize the NaOH, dilute the homogenate and reduce its viscosity and specific gravity. The specimen mixtures were then centrifuged at 3,000 revolutions per minute (rpm) for 20 minutes to concentrate the acid-fast bacilli. After discarding the supernatant in a disinfectant (5% phenol), the sediment was re-suspended in 3 millilitres phosphate buffer solution and used for fluorescent microscopy and line probe assay (Satapathy *et al.,* 2014; Allen *et al.,* 2016; Jha *et al.,* 2019).

3.6.3 Fluorescent staining technique and microscopy

Sputum smears were prepared from the processed sputum specimens. Clean frosted-end microscope glass slides were labelled with a specific identification number appearing on the specimen container and requisition form for each TB patient. Then a small portion of sputum was picked using a sterilized wire loop and smeared on the

microscope glass slide, to make a regular oval smear covering an area of 3 cm x 2 cm (Alfred *et al.,* 2014). The sputum smear slides were then placed on a slide rack, to allow them to air-dry at room temperature, away from direct sunlight. Once dried the sputum smear slides were fixed by passing them over a flame 2-3 times (Alfred *et al.,* 2014). The slides were then stained using the fluorescent staining technique for fluorescence microscopic examination. The first step involved flooding the dried and well-fixed smears with auramine-O-phenol stain (a fluorochrome primary stain) for 20 minutes, then rinsing thoroughly with distilled water (GLI, 2013). The decolouriser 0.5% acid- alcohol was poured on the smears and left for 3 minutes. Thereafter, each slide was gently rinsed using distilled water, and a counterstain 0.5% potassium permanganate was flooded on each slide for 1 minute, then washed off, and the slides allowed to air-dry (GLI, 2013).

The stained sputum smears were examined under a fluorescent microscope, starting with the objective lens of 200 times magnification for screening of the slides, then with the objective lens of 400 times magnification for confirmation and quantification of the slides (Imaz *et al.,* 2018). Each of the slides was examined for at least 5 minutes to accurately identify a positive or negative sputum smear, this is a technical recommendation by the International Union Against Tuberculosis and Lung Diseases (IUATLD) (Marais *et al.,* 2008). The sputum smear results were reported according to the World Health Organization and the IUATLD recommended reporting scale, which stipulates that: zero acid-fast bacilli (AFB) seen per 40 fields is a negative result; 1-19 AFB seen per 40 fields is a positive scanty result; 20-199 AFB seen per 40 fields is a 1+ positive result; 5-50 AFB seen field is a 2+ positive result; and more than 50 AFB seen per field is a 3+ positive result (Imaz *et al.,* 2018). The acid-fast bacilli appear as bright yellow fluorescent rods against a dark background created by the counterstain potassium permanganate when examined under a fluorescent microscope (Marais *et al.,* 2008). Only those sputum specimens that were confirmed positive for *M. tuberculosis* complex by microscopy were subjected to molecular testing for resistance to the first-and second-line anti-TB drugs.

3.6.4 Molecular detection of drug-resistant *Mycobacterium tuberculosis* strains

The extraction of DNA from *M. tuberculosis*-positive sputum specimens was done using the Genolyse technique according to the manufacturer's instructions (Hain Life Science, Germany). Detection of drug-resistance genes in DNA samples was achieved by using two molecular line probe assays; Hain genotype MTBDR*plus* ver 2.0 and MTBDR*sl* ver 2.0 assays, that utilise a polymerase chain reaction (PCR)-based assay and a hybridization assay performed on an automated GT Blot 48 device. Both assays were performed strictly according to the manufacturer's instructions (Hain Life Science, Germany). The Hain genotype MTBDR*plus* ver 2.0 assay was used for detecting MTBC resistance against first-line ant-TB drugs, while the genotype MTBDR*sl* ver 2.0 assay was used for detecting MTBC resistance to second-line anti-TB drugs (Dunn *et al.,* 2016). Line probe assays uses three key steps: DNA extraction, DNA amplification, and reverse hybridization (Nurwidya *et al.,* 2018).

3.6.4.1 Genotype MTBDR*plus* ver 2.0 molecular assay

3.6.4.1.1 Extraction of mycobacterial pure genomic DNA

The extraction of *M. tuberculosis* DNA from positive sputum specimens was done using the Genolyse procedure (Hain Life Science, Germany), where 500 microliters (µL) of decontaminated sputum specimens were dispensed in 1.0 ml screw capped cryo-vial then centrifuged for 15 minutes at 10,000 revolutions per minute (rpm) in a centrifuge with an aerosol tight rotor. After centrifugation the supernatant was discard. One hundred (100) µL of lysis buffer (A-LYSIS) was added to the sediment and re-suspended. The mixture was then incubated on a heat block for 5 minutes at a temperature of 95°C. After incubation, 100 µL of neutralization buffer (A-NB) was added to the mixture then vortexed for 5 minutes. Thereafter, the solution was centrifuged for 5 minutes at full speed in a centrifuge. The final produced solution was a purified *M. tuberculosis* DNA solution. This extracted mycobacterial genomic DNA was subjected to multiplex polymerase chain reaction (PCR) amplification, then to reverse hybridization (Ombura *et al.,* 2016; Addo *et al.,* 2017; Ogari *et al.,* 2019).

3.6.4.1.2 Pre-polymerase chain reaction process

The amplification process had two steps namely: the pre-polymerase chain reaction (pre-PCR) and polymerase chain reaction (PCR) processes. The pre-PCR process was done in a laboratory equipped with class I biosafety cabinets. The pre-PCR step involved preparing the master mix solution by mixing 10µL of amplification-A (AM-A) with 35µL of amplification-B (AM-B) in new PCR tubes. Therefore, the final total volume of the master mix solution was 45µL in each PCR tube. The number of PCR tubes prepared, containing the master mix solution was dependent on the number of specimens being analysed with controls included in each test.

3.6.4.1.3 Polymerase chain reaction process

The polymerase chain reaction (PCR) process was carried out in a laboratory, different from the one where the pre-PCR process was done to prevent cross contamination of the specimens. Each PCR tube was labelled with the sample identification number, 5µL of the extracted *M. tuberculosis* DNA was dispensed in each PCR tube containing 45µL of the master mix solution (except to the negative control), bringing the final volume to 50µL. The master mix solution comprises of; PCR buffer, 4 deoxynucleoside triphosphates (dNTPs) [(deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanine triphosphate (dGTP), and deoxythymine triphosphate (dTTP)], forward primer, reverse primer, Taq DNA polymerase, template DNA, magnesium chloride ($MgCl_2$), and PCR molecular biology grade sterile water. The mixture was then loaded in a programmed real time PCR thermocycler for amplification of the drug-resistant determining region of the target gene, using incorporated biotinylated primers. The PCR process in the thermocycler took place at the following set conditions: initial denaturation at 95°C for 15 minutes, 1 cycle; denaturation at 95°C for 30 seconds, 20 cycles; annealing at 65°C for 2 minutes, 20 cycles; denaturation at 95°C for 25 seconds, 30 cycles; annealing at 50°C for 40 seconds, 30 cycles; extension at 70°C for 40 seconds, 30 cycles; and final extension at 70°C for 8 minutes, 1 cycle. The amplified *M. tuberculosis* DNA samples were then subjected to reverse hybridization (Ombura *et al.*, 2016; Addo *et al.*, 2017; Kadri, 2021).

3.6.4.1.4 Hybridization of the amplified mycobacterial DNA

The third major process in genotyping using the Hain genotype MTBDR*plus* ver 2.0 assay was reverse hybridization. Reverse hybridization is a process that involve the binding of DNA amplicons to specific oligonucleotide probes that are embeded onto a nitrocellulose membrane strip (Bang *et al.,* 2011).

The process of reverse hybridization was carried out as follows: 20 microlitres (µL) of denaturation solution (DEN) was dispensed in each well of the tray. Then 20 µL of the amplified DNA sample was added to the DEN solution in each of the wells used, pipetted up and down to mix, then incubated at room temerature for 5 minutes. After incubation, 1 ml of pre-warmed hybridization (HYB) buffer was added carefully to each of the wells and the tray was then shaken gently until the solution had a homogenous colour. The line probe assay strips were labeled with a specific sample identification number (appearing on the specimen container and the requisition form) then placed in each well of the tray following a sequence. The tray was then placed on the shaking platform of the twincubator, and incubated for 30 minutes at 45°C. When the incubation period was over the HYB buffer was aspirated completely from each well and 1 ml of the stringent wash solution (STR) was added to each strip in the well and incubated for 15 minutes at 45°C on a shaking platform of the twincubator. When the incubation period was over, the STR solution was removed completely from each well. Each strip was then washed once using 1 ml of the rinse solution (RIN) for 1 minute in a twincubator. When rinsing was over, the RIN solution was poured out completely, then 1 ml of diluted conjugate (CON) added to each strip in the well, and the tray incubated for 30 minutes in a twincubator. When the incubation was over, the diluted CON solution was aspirated and each strip washed twice for 1 minute with 1 ml of RIN solution and once for 1 minute with 1 ml of distilled water on a shaking twincubator platform. When the washing process was over, all the solution was poured out completely from each well of the tray. Then 1ml of diluted substrate (SUB) added to each strip in the well of the tray and incubated for 20 minutes. When the incubation period was over, the strips were gently rinsed twice using 1ml of distilled water for 1 minute. Distilled water in each well was not removed, so as to assist with the picking and transferring of the strips (using forceps) to an absorbent paper for drying. Once

the strips dry, evaluation and interpretation is required (HLS, 2015a; Ombura *et al.,* 2016; Addo *et al.,* 2017).

3.6.4.1.5 GenoScanning of the hybridized nitrocellulose membrane strips

GenoScanning is the final stage that involves analysis and interpretation of molecular data generated on nitrocellulose membrane strips (DNA technology strips) after the process of hybridization. GenoScanning was done using a GenoScan system analyser (Hain Life Science, Germany), that is designed to rapidly analyse and accurately interpret all hybridization patterns generated on membrane strips, using its innovative software reader. The GenoScan analyzed and interpreted *M. tuberculosis* resistance and mutations associated with resistance, to first-and second-line anti-TB drugs (Addo *et al.,* 2017).

3.6.4.2 Genotype MTBDR*sl* ver 2.0 molecular assay

The Hain genotype MTBDR*sl* ver 2.0 assay was performed and interpretated in the same manner as for the Hain genotype MTBDR*plus* ver 2.0 assay, the only difference is that the Hain genotype MTBDR*sl* ver 2.0 assay tests for resistance to second-line anti-TB drugs, while the Hain genotype MTBDR*plus* ver 2.0 assay tests for resistance to first-line anti-TB drugs (HLS, 2015b).

3.6.5 Quality control

Quality control procedures were carried out strictly in line with the manufacturer's instructions (Hain Life Science, Germany). The following controls were used in the study procedure: an extraction positive control (*M. tuberculosis* strain H37 RV), an extraction negative control (molecular grade water) and the PCR master mix negative control. The extraction positive control used was sensitive to all the standard anti-TB drugs. This positive control specimen clearly showed a valid positive test result. The extraction negative control and PCR master mix negative control specimens clearly showed a valid negative test result by showing only the presence of the conjugate control band and the amplification control band on the line probe assay strips for both first- and second-line probe assays. The negative control checked for any possibility

of contamination. This negative control sample played a critical role in checking whether quality standards were adhered to, from the point of specimen reception to preparation, through to processing and analysis (HLS, 2015a; HLS, 2015b).

The Hain genotype MTBDR*plus* ver 2.0 test strips contain five in-built control zones: A conjugate control zone (checks the binding of the conjugate onto the test strip and ensure that the correct chromogenic reaction occurs), an amplification control zone (checks whether a successful amplification reaction has occurred) and finally the three locus control zones (*rpoB, katG, and inhA* – which helps to quality check the sensitivity of the reaction for each of the gene loci tested). The extraction positive control was used, which is susceptive to all the standard anti-TB drugs. The positive control specimen showed valid positive test signals by clearly showing the presence of the following bands on the first-line probe assay- GenoType MTBDR*plus*: conjugate control (CC), amplification control (AC), tub (MTB), *rpo*B wild type probe 1, *rpo*B wild type probe 2, *rpo*B wild type probe 3, *rpo*B wild type probe 4, *rpo*B wild type probe 5, *rpo*B wild type probe 6, *rpo*B wild type probe 7, *rpo*B wild type probe 8, *kat*G wild type, *inh*A wild type probe 1 and *inh*A wild type probe 2. With the absence of the following bands: *rpo*B MUT 1, *rpo*B MUT 2A, *rpo*B MUT 2B, *rpo*B MUT 3, *kat*G MUT 1, *kat*G MUT 2, *inh*A MUT 1, *inh*A MUT 2, *inh*A MUT 3A and *inh* A MUT 3B (HLS, 2015a).

The Hain genotype MTBDR*sl* ver 2.0 test strips contain six in-built control zones: a conjugate control zone (checks the binding of the conjugate onto the test strip and ensure that the correct chromogenic reaction occurs), an amplification control zone (checks whether a successful amplification reaction has occurred), and finally the four locus control zones (*gyrA, gyrB, rrs, and eis* – which helps to quality check the sensitivity of the reaction for each of the gene loci tested). The extraction positive control specimen also showed valid positive signals on the second-line probe assay strip- GenoType MTBDR*sl* by clearly showing the presence of the following bands: conjugate control (CC), amplification control (AC), tub (MTB), *gyr*A wild type probe 1, *gyr*A wild type probe 2, *gyr*A wild type probe 3, *gyr*B wild type, *rrs* wild type probe 1, *rrs* wild type probe 2, *eis* wild type probe 1, *eis* wild type probe 2, and *eis* wild type probe 3. With the absence of the following bands: *gyr*A MUT 1, *gyr*A MUT 2, *gyr*A

MUT 3A, *gyr*MUT 3B, *gyr*A MUT 3C, *gyr*A MUT 3D, *gyr*B MUT 1, *gyr*B MUT 2, *rrs* MUT 1, *rrs* MUT 2 and *eis* MUT 1. All the above three types of controls used in this study passed the quality control test (HLS 2015b).

3.7 Statistical analysis

Data was first entered in Microsoft excel spread sheets then cleaned up and analysed using STATA version 13.0 statistical software (StataCorp, Lakeway Drive, College Station, Texas, USA). Pearson's Chi-square test was used to find the association between the different types of drug-resistant TB to age and gender. A *p*-value of less than 0.05 was considered to be statistically significant at 95% confidence interval. Frequencies and percentages were used to determine the level of drug-resistant TB among the new and previously treated TB cases.

3.8 Ethics approval

Ethics approval was sought from the University of Zambia, School of Health Sciences, Research and Ethics Committee (UNZA-HSREC) (Ethics approval letter ID number: 20203101001) (Appendix I), while the final clearance and approval to conduct the study was obtained from the National Health Research Authority (NHRA) (Appendix II). Permission to conduct the study was obtained from the Ministry of Health, Headquarters, as well the Lusaka Provincial Health Office and the Chest Diseases Laboratory in Lusaka, Zambia (Appendix III). The study did not involve any direct contact with the TB patients. Confidentiality, intergrity, professionalism, and good clinical laboratory practice (GCLP) were maintained throughout the study period. Confidentiality was maintained by using study-specific numbers to identify the samples, and not names for the patients.

CHAPTER FOUR

RESULTS

4.1 Demographic characteristics of the study participants

In this study, a total of 329 sputum specimens were analysed, which translates to 329 TB cases analysed for drug-resistant TB. The cases were categorized into two groups: the first group comprised 102(31%) new TB cases, while the second group comprised 227(69%) previously treated TB cases. Among the new TB cases, 80 (78.4%) were males and 22 (21.6%) females, with an age range of 1-90 years and a median age of 33 (Table 4.1). In the previously treated TB cases, there were 166 (73.1%) males and 61 (26.9%) females, with an age range of 4-78 years (median age 34 years) (Table 4.1).

The new TB cases were on the standard first-line TB treatment which comprised; 2 months of intensive phase (treatment using rifampicin, isoniazid, pyrazinamide, and ethambutol), and 4 months of continuation phase (treatment using rifampicin and isoniazid) (Briffotaux *et al.,* 2019). While the previously treated TB cases were on a treatment regimen which comprised; 4 months of intensive phase (treatment using levofloxacin, ethionamide, kanamycin, ethambutol, pyrazinamide, high-dose isoniazid, and clofazimine), and 5 months of continuation phase (treatment using levofloxacin, clofazimine, pyrazinamide, and ethambutol) (WHO, 2016; Mase and Chorba, 2019; Jang and Chung, 2020). The previously treated TB cases comprised; treatment failure cases, lost to follow-up cases, relapse cases and defaulter cases. The classification of TB patients into the new and previously treated TB cases is important for surveillance of drug-resistance and treatment. The type of treatment for the new and previously treated TB cases differs, thus a great need for categorization of these cases (Cohen *et al.,* 2018).

Table 4.1: Demographic data of the new and previously treated TB cases

New TB Cases (N=102)			Previously Treated TB Cases (N=227)		
Variables	**n**	**%**	**Variables**	**n**	**%**
Gender			**Gender**		
Females	22	21.6	Females	60	26.4
Males	80	78.4	Males	167	73.6
Age (years)			**Age (years)**		
1-19	5	4.9	4-20	8	3.5
20-39	65	63.7	21-37	128	56.4
40-59	26	25.5	38-54	70	30.8
60-79	5	4.9	55-71	17	7.5
80-99	1	1.0	72-88	4	1.8

Abbreviations: N, total number of cases; **n,** number of cases; **TB,** tuberculosis; **%,** percentage; **percentage = (n/N) x 100%.**

4.2 Prevalence of drug-resistant tuberculosis among the new and previously treated tuberculosis cases

The prevalence of the different forms of drug-resistant TB among the new TB cases was as follows: 3.9% (4/102) had rifampicin mono-resistance, 12.8% (13/102) isoniazid mono-resistance, and 17.7% (18/102) had both rifampicin and isoniazid resistance, which is multidrug-resistance, and finally 65.7% (67/102) were susceptible to all first-and second-line anti-TB drugs (Table 4.2). Drug-resistant TB was more prevalent in male cases than female cases, and the age range most affected with drug-resistance was 20-39 years among the new TB cases (Tables 4.2 and 4.3).

The prevalence of the different forms of drug-resistant TB among the previously treated TB cases was as follows: 10.1% (23/227) had rifampicin mono-resistance, 6.6% (15/227) isniazid mono-resistance, 33.0% (75/227) had both rifampicin and isoniazid resistance, which multidrug-resistance, 1.8% (4/227) had poly drug-resistance, 0.9% (2/227) had pre-extensively drug-resistant TB, and finally 47.6% (108/227) of the case were susceptible to all first-and second-line anti-TB drugs (Tables 4.2). The prevalence of drug-resistant TB was more common in males than in females. The age range most affected with drug-resistant TB was 21-37 years among the previously treated TB cases (Table 4.4).

Table 4.2: Prevalence of drug-resistance among the new and previously treated TB cases

Type of drug-resistance	New TB cases (N=102) n (%)	Previously tretaed TB cases (N=227) n (%)
Susceptible	67 (65.7%)	108 (47.6%)
Mono-drug resistance		
RIF mono-resistance	4 (3.9%)	23 (10.1%)
INH mono-resistance	13 (12.8%)	15 (6.6%)
Multi-drug resistance		
RIF+INH	18 (17.7%)	75 (33.0%)
Poly-drug resistance		
RIF+FLQs	0 (0%)	4 (1.8%)
Pre-extensively drug resistance		
RIF+INH+FLQs	0 (0%)	1 (0.4%)
RIF+INH+KAN	0 (0%)	1 (0.4%)

Abbreviations: N, total number of cases; **n,** number of cases ; **%,** percentage; **RIF,** rifampicin; **INH,** isoniazid; **FLQs,** fluoroquinolones; **KAN,** kanamycin; **TB,** tuberculosis; **Prevalence = (n/N) x 100%.**

4.3 Association of drug-resistant tuberculosis to age and gender among the new tuberculosis cases

When the different types of drug-resistant TB (rifampicin mono-resistance, isoniazid mono-resistance, and multidrug-resistance), among the new TB cases were tested for association to age using the Pearson's Chi-square test, all the *p*- values were above the 0.05 level of significancy at 95% confidence interval (Table 4.3).

Similarly when the different types of drug-resistant TB (rifampicin mono-resistance, isoniazid mono-resistance, and multidrug-resistance), among the new TB cases were tested for association to gender using the Pearson's Chi-square test, all the *p*- values were above the 0.05 level of significancy at 95% confidence interval (Table 4.3).

Table 4.3: Association of drug-resistant tuberculosis to demographic variables among the new TB cases

Demographic variables	Monodrug-resistance (N=17)		Multidrug-resistance (N=18)
	RIFr (N=4) n (%)	INHr (N=13) n (%)	RIFr+INHr (N=18) n (%)
Gender			
Females	1 (25%)	2 (15.4%)	2 (11.1%)
Males	3 (75%)	11 (84.6%)	16 (88.9%)
Pearson's Chi-square (X^2)- value	**1.4165**	**2.0583**	**1.8915**
P-value	**0.493**	**0.357**	**0.388**
Age (years)			
1-19	0 (0%)	0 (0%)	1 (5.6%)
20-39	2 (50%)	11 (84.6%)	14 (77.8%)
40-59	1 (25%)	2 (15.4%)	3 (16.7%)
60-79	1 (25%)	0 (0%)	0 (0%)
80-99	0 (0%)	0 (0%)	0 (0%)
Pearson's Chi-square (X^2)- value	**6.0982**	**6.9422**	**5.5664**
P-value	**0.636**	**0.543**	**0.696**

Abbreviations: N, total number of resistant cases; **n,** number of resistant cases; **%,** percentage; **RIFr,** rifampicin-resistance; **INHr,** isoniazid-resistance; **TB,** tuberculosis; Percentage = (n/N) X 100%; *P-value* < 0.05 statistically significant; *P-value* > 0.05 not statistically significant.

4.4 Association of drug-resistant tuberculosis to age and gender among the previously treated tuberculosis cases

When the different types of drug-resistant TB, among the previously treated TB cases were tested for association to age using the Pearson's Chi-square test, all the *p*- values were above the 0.05 level of significancy at 95% confidence interval (Table 4.4).

Similarly when the different types of drug-resistant TB, among the previously treated TB cases were tested for association to gender using the Pearson's Chi-square test, all the *p*- values were above the 0.05 level of significancy at 95% confidence interval (Table 4.4).

Table 4.4: Association of drug-resistant tubercuosis to demographic variables among the previously treated TB cases

Variables	Monodrug-resistance (N=38)		MDR (N=75)	PDR (N=4)	Pre-XDR (N=1)	Pre-XDR (N=1)
	RIFr (N=23) n (%)	INHr (N=15) n (%)	RIFr+INHr n (%)	RIFr+FLQr n (%)	RIFr+INHr+FLQr n (%)	RIFr+INHr+KANr n (%)
Gender						
Females	6 (26.1%)	5 (33.3%)	23 (30.7%)	1 (25%)	1 (100%)	0 (0%)
Males	17 (73.9%)	10 (66.7%)	52 (69.3%)	3 (75%)	0 (0%)	1 (100%)
Pearson's Chi-square (X^2) value	**4.3554**	**5.0371**	**1.0331**	**0.0043**	**2.7956**	**0.3609**
P- value	**0.499**	**0.284**	**0.309**	**0.948**	**0.095**	**0.548**
Age (years)						
4-20	1 (4.3%)	0 (0%)	2 (2.7%)	0 (0%)	0 (0%)	0 (0%)
21-37	18 (78.3%)	9 (60%)	45 (60%)	1 (25%)	1 (100%)	0 (0%)
38-54	4 (17.4%)	4 (26.7%)	22 (29.3%)	2 (50%)	0 (0%)	0 (0%)
55-71	0 (0%)	2 (13.3%)	6 (8.0%)	1 (25%)	0 (0%)	1 (100%)
72-88	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pearson's Chi-square (X^2) value	**26.0315**	**18.5882**	**2.5878**	**3.0787**	**0.7769**	**12.4076**
P- value	**0.165**	**0.291**	**0.629**	**0.545**	**0.942**	**0.125**

Abbreviations: N, total number of resistant cases**; n,** number of resistant cases**; %,** percentage**; MDR,** multidrug-resistance**; PDR,** polydrug-resistance**; Pre-XDR,** pre-extensively drug resistance**; RIFr,** rifampicin-resistance**; INHr,** isoniazid-resistance**; FLQr,** fluoroquinolone-resistance**; KANr,** kanamycin-resistance**;** Percentage = (n/N) x 100%; *P-value* < 0.05 statistically significant; *P-value* > 0.05 not statistically significant.

4.5 Frequency of mutations conferring drug-resistance in *Mycobacterium tuberculosis* resistant isolates detected among the new tuberculosis cases

Mutations associated with drug-resistance were detected in the *rpo*B, *kat*G, and *inh*A genes of *M. tuberculosis* resistant isolates identified among the new TB cases. Overall, 34.3% (35/102) of the *M. tuberculosis* resistant isolates had mutations detected among the new TB cases. From a total of 35 resistant *M. tuberculosis* isolates identified, 4 isolates had resistance to rifampicin (RIF) among the new TB cases and the most frequent mutation conferring resistance to RIF was the *rpo*B MUT 3 (Ser531Leu), with a frequency of 8.6% (3/35). The second frequently detected mutation conferring resistance to RIF was the *rpo*B MUT 1(Asp516Val), with a frequency of 2.9%(1/35) (Table 4.5).

From a total of 35 resistant *M. tuberculosis* isolates identified among the new TB cases, 13 isolates had resistance to isoniazid (INH), and the most frequent mutation conferring resistance to INH was the *Inh*A MUT 1 (Cys15Thr), with a frequency of 28.6% (10/35). The second frequently detected mutation was the *kat*G MUT 1 (Ser315Thr 1), with a frequency of 8.6% (3/35) (Table 4.5).

From a total of 35 resistant *M. tuberculosis* isolates identified among the new TB cases, 18 isolates had multidrug-resistance (resistance to both rifampicin and isoniazid), and the most frequent mutation conferring resistance to both rifampicin and isoniazid was a combination of *rpo*B MUT 3 (Ser531Leu) and *kat*G MUT 1 (Ser315Thr 1), with a frequency of 14.3% (5/35). The second frequently detected mutation was a combination of *rpo*B WT 3 (Asp516Val) and *kat* MUT 1 (Ser 315Thr1), with a frequency of 5.7% (2/35) (Table 4.5).

4.6 Frequency of mutations conferring drug-resistance in *Mycobacterium tuberculosis* resistant isolates detected among the previously treated tuberculosis cases

Mutations associated with drug-resistance were detected in the *rpo*B, *kat*G, *inh*A, *gyr*A, and *eis* genes of *M. tuberculosis* resistant isolates identified among the new TB cases. Overall, 52.4% (119/227) of the *M. tuberculosis* resistant isolates had mutations detected among the previously treated TB cases. From a total of 119 resistant *M. tuberculosis* isolates identified among the previously treated TB cases, 23 isolates had resistance to rifampicin (RIF), and the most frequent mutation conferring resistance to RIF was the *rpo*B MUT 3 (Ser 531Leu), with a frequency of 10.9% (13/119) (Table 4.6).

From a total of 119 resistant *M. tuberculosis* isolates identified among the previously treated TB cases, 15 isolates had resistance to isoniazid (INH), and the most fequent mutation conferring resistance to INH was the *kat*G MUT (Ser315Thr1), with a frequency of 6.7% (8/119). The second frequently detected mutation was the *Inh*A MUT 1 (Cys15Thr), with a frequency of 4.2% (5/119) (Table 4.6).

From a total of 119 resistant *M. tuberculosis* isolates identified among the previously treated TB cases, 75 isolates had mutidrug-resistance (resistance to both rifampicin and isoniazid), and the most frequent mutation conferring resistance to both rifampicin (RIF) and isoniazid (INH) was a combination of *rpo*B MUT 3 (Ser 531Leu) and *kat*G MUT 1 (Ser 315Thr1), with a frequency of 18.5% (22/119). The second frequently detected mutation was a combination of the *rpo*B MUT 2B (His526Asp) and *kat*G MUT 1 (Ser 315Thr 1), with a frequency of 16.8% (20/119). The third frequently detected mutation was a combination *rpo*B MUT 3 (Ser531Leu) with *Inh*A MUT 1 (Cys15Thr), with a frequency of 1.7% (2/119) (Table 4.6).

From a total of 119 resistant *M. tuberculosis* isolates identified among the previously treated TB cases, 4 isolates had resistance to both RIF and fluoroquinolones (FLQs), and the most frequent mutation conferring resistance to both RIF and FLQs was a combination of the *rpo*B MUT 2A (His526Tyr) and *gyr*A MUT 1 (Ala90Val), with a frequency of 1.7% (2/119) (Table 4.6).

From a total of 119 resistant *M. tuberculosis* isolates identified among the previously treated TB cases, 2 isolates had pre-extensively drug-resistance (pre-XDR). The first isolate had resistance to both rifampicin and isoniazid plus fluoroquinolones, while the second isolate had resistance to both rifampicin and isoniazid plus kanamycin. The first mutation responsible for conferring resistance to both rifampicin and isoniazid plus fluoroquinolones was a combination of; *rpo*B MUT 2A (His526Tyr), *kat*G MUT 1 (Ser315Thr 1), and *gyr* A MUT 3C (Asp94Gly). This combination mutation had a frequency of 0.8% (1/119). The second mutation responsible for conferring resistance to both rifampicin and isoniazid plus kanamycin was a combination of; *rpo*B MUT 2B (His526Asp), *kat*G MUT 2 (Ser315Thr 2), and *eis* MUT (Cys14Thr). This combination mutation had a frequency of 0.8% (1/119) (Table 4.6).

Table 4.5: Frequency of mutations detected in *Mycobacterium tuberculosis* resistant isolates among the new TB cases

Genes implicated in drug-resistance	Hybridization pattern (s) (n=35)	Mutations detected	No. of MTB isolates	Frequency (%) (n=35)
	Mono drug resistance (n=17)			
*rpo*B	**Rifampicin (n=4)**			
	*rpo*B MUT 1	Asp516Val	1	1/35 (2.9%)
	*rpo*B MUT 3/Δ *rpo*B WT 8	Ser531Leu	3	3/35 (8.6%)
*Inh*A *or kat*G	**Isoniazid (n=13)**			
	*Inh*A MUT 1/Δ *Inh*A WT 1	Cys15Thr	10	10/35 (28.6%)
	*kat*G MUT 1	Ser315Thr 1	3	3/35 (8.6%)
	Multidrug-resistance (n=18)			
*rpo*B + *kat*G *or* *Inh*A	Δ *rpo*B WT 8Δ *kat*G WT	Ser531Leu C-315	1	1/35 (2.9%)
	*rpo*B MUT 3/Δ *rpo*B WT 8 *kat*G MUT 1/Δ *kat*G WT	Ser531Leu Ser315Thr 1	5	5/35 (14.3%)
	Δ *rpo*B WT 2 *Inh*A MUT 1	Glu510His Cys15Thr	1	1/35 (2.9%)
	*rpo*B MUT 3 *Inh*A MUT 1	Ser531Leu Cys15Thr	1	1/35 (2.9%)
	Δ *rpo*B WT 2 *Inh*A MUT 1/Δ *Inh*A WT 1	Glu510His Cys15Thr	1	1/35 (2.9%)
	*rpo*B MUT 2B/Δ *rpo*B WT 7 *kat*G MUT 1/Δ *kat*G WT	His526Asp Ser315Thr 1	1	1/35 (2.9%)
	Δ *rpo*B WT 3/Δ *rpo*B WT 4 *kat*G MUT 1/Δ *kat*G WT	Asp516Val Ser315Thr 1	2	2/35 (5.7%)
	Δ *rpo*B WT 1 Δ *rpo*B WT 2 Δ *rpo*B WT 8 *Inh*A MUT 1/Δ *Inh*A WT 1	C 505-509 Glu510His Ser531Leu Cys15Thr	1	1/35 (2.9%)
	Δ *rpo*B WT 8 *kat*G MUT 2	Ser531Leu Ser315Thr 2	1	1/35 (2.9%)
	*rpo*B MUT 2B *rpo*B MUT 3 *kat*G MUT 1	His526Asp Ser531Leu Ser315Thr 1	1	1/35 (2.9%)
	*rpo*B MUT 2B *kat*G MUT 1	His526Asp Ser315Thr 1	1	1/35 (2.9%)
	*rpo*B MUT 2A /Δ *rpo*B WT 7 *kat*G MUT 1/Δ *kat*G WT	His526Tyr Ser315Thr 1	1	1/35 (2.9%)
	*rpo*B MUT 3/ Δ *rpo*B WT 8 *Inh*A MUT 1/Δ *Inh*A WT 1	Ser531Leu Cys15Thr	1	1/35 (2.9%)

Abbreviations: Δ, deletion; **WT,** wild type; **MUT,** mutation; **ΔWT/MUT,** deletion of the wild-type probe and presence of mutation; **C,** codon; **TB,** tuberculosis; **MTB,** *Mycobacterium tuberculosis*; **Ala,** Alanine; **Asp,** Aspartic acid; **Cys,** Cysteine; **Glu,** Glutamic acid; **Gly,** Glycine; **His,** Histidine; **Leu,** Leucine; **Ser,** Serine; **Thr,** Threonine; **Tyr,** Tyrosine; **Val,** Valine.

Table 4.6: Frequency of mutations detected in *Mycobacterium tuberculosis* resistant isolates among the previously treated TB cases

Genes implicated in drug-resistance	Hybridization pattern (s) (n=119)	Mutations detected	No. of MTB isolates	Frequency (%) (n=119)
*rpo*B	**Mono drug resistance (n=38)**			
	Rifampicin (n=23)			
	*rpo*B MUT 3/ Δ *rpo*B WT 8	Ser531Leu	13	13/119 (10.9%)
*Inh*A *and/or kat*G	**Isoniazid (n=15)**			
	*Inh*A MUT 1/Δ *Inh*A WT 1	Cys15Thr	5	5/119 (4.2%)
	*kat*G MUT 1/ Δ *kat*G WT	Ser315Thr 1	8	8/119 (6.7%)
	*kat*G MUT1	Ser315Thr 1	1	1/119 (0.8%)
	*Inh*A MUT 1/Δ *Inh*A WT 1	Cys15Thr		
*rpo*B + *kat*G *&/or Inh*A	**Multidrug-resistance (n=75)**			
	*rpo*B MUT 3/Δ *rpo*B WT 8	Ser531Leu	22	22/119 (18.5%)
	*kat*G MUT 1/Δ *kat*G WT	Ser315Thr 1		
	*rpo*B MUT 2B/Δ *rpo*B WT 7	His526Asp	20	20/119 (16.8%)
	*kat*G MUT1/ Δ *kat*G WT	Ser315Thr 1		
	*rpo*B MUT 1	Asp516Val	1	1/119 (0.8%)
	*kat*G MUT1/ Δ *kat*G WT	Ser315Thr 1		
	*rpo*B MUT 2B	His526Asp	1	1/119 (0.8%)
	*rpo*B MUT 3	Ser531Leu		
	*kat*G MUT1	Ser315Thr 1		
	*Inh*A MUT 1	Cys15Thr		
	*rpo*B MUT 2A	His526Tyr	1	1/119 (0.8%)
	*kat*G MUT1/ Δ *kat*G WT	Ser315Thr 1		
	*rpo*B MUT 1/Δ *rpo*B WT 3/ Δ *rpo*B WT 4	Asp516Val	1	1/119 (0.8%)
	*kat*G MUT 2/ Δ *kat*G WT	Ser315Thr 2		
	*rpo*B MUT 3/Δ *rpo*B WT 8	Ser531Leu	2	2/119 (1.7%)
	*Inh*A MUT 1/Δ *Inh*A WT 1	Cys15Thr		
	*rpo*B MUT 2B	His526Asp	1	1/119 (0.8%)
	*Inh*A MUT 1	Cys15Thr		
*rpo*B + *gyr*A	**Poly drug- resistance (n=4)**			
	*rpo*B MUT 2 A	His526Tyr	1	1/119 (0.8%)
	Δ *gyr*A WT 2	C 89-93		
	Δ *gyr*A WT 3	C 92-96		
	*rpo*B MUT 2 A/ Δ *rpo*B WT 7	His526Tyr	2	2/119 (1.7%)
	*gyr*A MUT 1/ Δ *gyr*A WT 2	Ala90Val		
	*rpo*B MUT 2 A/ Δ *rpo*B WT 7	His526Tyr	1	1/119 (0.8%)
	Δ *gyr*A WT 2	C 89-93		
*rpo*B + *kat*G + *gyr*A	**Pre- extensively drug-resistance (n=2)**			
	*rpo*B MUT 2A/Δ *rpo*B WT 7	His526Tyr	1	1/119 (0.8%)
	*kat*G MUT 1 /Δ *kat*G WT	Ser315Thr 1		
	*gyr*A MUT 3C/Δ *gyr* A WT 3	Asp94Gly		
*rpo*B + *kat*G + *eis*	*rpo*B MUT 2B/Δ *rpo*B WT 7	His526Asp	1	1/119 (0.8%)
	*kat*G MUT 2/Δ *katG* WT	Ser315Thr 2		
	eis MUT 1	Cys14Thr		

Abbreviations: Δ, deletion; **WT,** wild type; **MUT,** mutation; **ΔWT/MUT,** deletion of the wild-type probe and presence of mutation; **C,** codon; **TB,** tuberculosis; **MTB,** *Mycobacterium tuberculosis*; **Ala,** Alanine; **Asp,** Aspartic acid; **Cys,** Cysteine; **Glu,** Glutamic acid; **Gly,** Glycine; **His,** Histidine; **Leu,** Leucine; **Ser,** Serine; **Thr,** Threonine; **Tyr,** Tyrosine; **Val,** Valine.

CHAPTER FIVE

DISCUSSION

5.1 Introduction

The aim of this study was to characterise drug-resistant *M. tuberculosis* in positive sputum specimens among the new and previously treated TB cases. This study highlights the growing threat posed by the emergence of resistant *M. tuberculosis* strains responsible for causing the different types of drug-resistant TB in Zambia. The majority of patients identified with drug-resistant TB had a history of being previously treated for TB and interrupted first-line anti-TB therapy. These factors are predictors of the emergence of drug-resistant TB (Parida *et al.,* 2015; Asgedom *et al.,* 2018; Elduma *et al.,* 2019). In this study, drug-resistant TB was found to be high among the previously treated TB patients than among the new TB patients. Drug-resistant TB is high among adult males than females, because of social stigma, cultural habits and poor health seeking behaviour among males (Adejumo *et al.,* 2018).

5.2 Prevalence of drug-resistant tuberculosis

5.2.1 Prevalence of drug-resistant tuberculosis among the new tuberculosis cases

5.2.1.1 Prevalence of rifampicin mono-resistant tuberculosis

The prevalence of rifampicin resistant (RR)-TB in this study was 3.9%, among the new TB cases. In Zambia rifampicin resistance is mainly acquired via primary transmission, among the new TB cases (Masenga *et al.,* 2017; Su *et al.,* 2021). A similar study done in Zimbabwe found the prevalence of RR-TB to be 4.0% among the new TB cases; this result was attributed to a history of travel to a high TB burdened country (Timire *et al.,* 2019). A study in Ethiopia reported the prevalence of RR-TB at 7.6%, among the new TB cases. This study found that RR-TB, among the new TB cases was mainly due to active person-to-person transmission or the existence of undiagnosed new RR-TB cases in the communities (Arega *et al.,* 2019). A study done in Nigeria reported a high prevalence of RR-TB at 22.5%, among the new TB cases.

This high prevalence was mainly attributed to symptomatic contact with drug-resistant TB, among the new TB cases (Adejumo *et al.,* 2018).

5.2.1.2 Prevalence of isoniazid mono-resistance tuberculosis

The prevalence of isoniazid mono-resistance (INHr)-TB in our study was 12.8%, among the new TB cases. Isoniazid mono-resistance is the most common type of drug-resistant TB in the world (Monde *et al.,* 2023), it is increasing among the new TB cases in Zambia due to primary transmission of *M. tuberculosis* strains carrying INHr, and also due to poor absorption of isoniazid, resulting from the sub-therapeutic drug concentrations (Solo *et al.,* 2021). The findings of this study are consistent with what others found, for instance; studies in South-Korea and Pakistan reported 11% and 9.8%, respectively, for INHr-TB, among the new TB cases (Jhun and Koh, 2019; Tahseen *et al.,* 2020). A low prevalence of INHr, among the new TB cases was reported in Tanzania (7.6%) (Hoza *et al.,* 2015), and Ethiopia (9.5%) (Seyoum *et al.,* 2014). Isoniazid resistance is more common than rifampicin resistance (Monde *et al.,* 2023), and is a growing public health problem globally because; research and policy directions are only focused on rifampicin resistance as a surrogate marker for multidrug-resistant TB (Sulis *et al.,* 2020). Tahseen and colleagues argue as to why rifampicin resistance is regarded a surrogate marker for multidrug-resistant TB, when in fact it is INHr that increases the risk of developing multidrug-resistant TB, and is the most common type of drug-resistance globally (Jhun and Koh, 2019; Tahseen *et al.,* 2020; Monde *et al.,* 2023). The growing problem of INHr is being ignored globally by policy makers and research direction (Sulis *et al.,* 2020).

5.2.1.3 Prevalence of multidrug-resistant tuberculosis

The prevalence of multidrug-resistant tuberculosis (MDR-TB) in this study was high, 17.7% among the new TB cases. This high prevalence among the newly diagnosed TB patients is of concern, and requires an urgent intervention to improve the quality of TB control to interrupt the transmission of multidrug-resistant TB. This high prevalence was mainly attributed to; direct contact of new TB cases with known MDR-TB cases, and living in crowded slums with poor ventilation. Improved laboratory diagnostic network has also led to a number of drug-resistant TB cases to be identified, including

MDR-TB (Kapata *et al.,* 2013). Multidrug-resistant-TB is a growing public health threat in Zambia (Kasapo *et al.,* 2017; MOH, 2017). The findings of this study are similar with what other studies reported; India reported a prevalence of MDR-TB of 11.4% among the new TB cases. This study reported that nosocomial transmission of MDR-TB among the new TB cases in clinical settings was triggered by compromised infection control measures (Singh *et al.,* 2020). A study done in Ethiopia reported a prevalence of MDR-TB of 11.6%, among the new TB cases. This study concluded that the increased prevalence of MDR-TB, among the new TB cases was an indicator for on-going primary transmission of MDR-TB (Welekidan *et al.,* 2020).

Multidrug-resistant TB is one of the most serious public health problems in the world (Diandé *et al.,* 2019; Olson *et al.,* 2019). Its prevalence is increasing in every part of the world today, in both the new and previously treated TB cases (Muthaiah *et al.,* 2017).

5.2.2 Prevalence of drug-resistant tuberculosis among the previously treated tuberculosis cases

5.2.2.1 Prevalence of rifampicin mono-resistant tuberculosis

The prevalence of rifampicin resistant (RR)-TB in this study was 10.1%, among the previously treated TB cases. Being previously treated for TB is a key predictor for the acquisition of the different types of drug-resistant TB. The majority of the previously treated TB cases in this study were treatment failures, relapse cases, treatment defaulters, or lost to follow-up, and were co-infected with HIV (Kapata *et al.,* 2013; Masenga *et al.,* 2017; Cohen *et al.,* 2018). Rifampicin resistant TB is significantly associated with HIV positivity, among the previously treated TB cases (Salaam-Dreyer *et al.,* 2021), and Zambia has a high prevalence of HIV infection (Mutembo *et al.,* 2019). HIV is associated with the emergence of rifampicin resistant TB through an increased risk of acquisition of resistance during TB treatment. This is solely attributed to altered pharmacokinetics, associated with drug malabsorption (Mulenga *et al.,* 2010; Salaam-Dreyer *et al.,* 2021). Impaired drug absorption is one of the reasons for the ineffectiveness of TB treatment, particularly with drugs such as rifampicin and ethambutol (Monde *et al.,* 2023). The roll-out of GeneXpert MTB/RIF analysers to

most health facilities in Zambia has improved the detection of RR-TB cases (Kasaro *et al.,* 2020; Monde *et al.,* 2021). On the other hand delayed diagnosis of RR-TB cases, increases the risk for RR spreading in the community (Su *et al.,* 2021).

The findings of this study were similar with what other studies reported; A study done in Zambia reported a prevalence for RR-TB at 7.1%, among the previously treated TB cases (Masenga *et al.,* 2017). A study done in Zimbabwe found the prevalence of RR-TB to be 14.2%, among the previously treated TB cases (Timire *et al.,* 2019). This result was attributed to risk factors significantly associated with RR-TB namely; previous history of TB treatment, HIV infection, and history of travel to a high TB burdened country (Timire *et al.,* 2019). The association between HIV infection and RR-TB was attributed to acquired drug-resistance arising from "preferential adherence" to anti-retroviral drugs at the expense of anti-TB drugs, among TB/HIV co-infected patients (Timire *et al.,* 2019). A study in South-Africa found the prevalence of RR-TB to be 8.8%, among the previously treated TB cases (Coovadia *et al.,* 2013; Diriba *et al.,* 2019). This result was attributed to HIV associated malabsorption of anti-TB drugs, previous mismanagement of TB, poor adherence, drug-to-drug interactions (among the HIV/TB co-infected) and increased rates of extra-pulmonary tuberculosis (Coovadia *et al.,* 2013). In Burkina Faso, the prevalence of RR-TB was found at 14.5%, among the previously treated TB cases; in this study, previous treatment for TB was found to be significantly associated with rifampicin resistance (Diandé *et al.,* 2019). Nigeria reported a 7.3% prevalence of RR-TB, among the previously treated TB cases (Ukwamedua *et al.,* 2019).

Other studies found a high prevalence for RR-TB, among the previously treated TB cases for instance: A cross-sectional study done in Ethiopia found the prevalence of RR-TB at 17.1%, among the previously treated TB cases (Mulu *et al*., 2017). Another study in Ethiopia reported the prevalence of RR at 27.4%, among the previously treated TB cases (Arega *et al.,* 2019). A study done in Lagos, Nigeria reported the prevalence of RR at 26.7% among the previously treated TB cases (Adejumo *et al.,* 2018). These studies found that the high prevalence of RR-TB, among the previously treated TB cases was significantly associated with; poor adherence, history of previous treatment for TB, and improved laboratory diagnostic networks (Mulu *et al.,* 2017;

Adejumo *et al.,* 2018; Arega *et al.,* 2019). A study done in Iran revealed that none of the *M. tuberculosis* isolates had resistance to rifampicin. This was due to a regular supply of anti-TB drugs, availability of advanced laboratory diagnostic networks, effective treatment of TB patients, and follow-up of TB patients (Jaleta *et al.*, 2017).

Rifampicn resistant TB is associated with a previous history of TB therapy. Therefore, improvement in adherence to treatment halts the emergence or re-emergence of *M. tuberculosis* and RR-TB cases (Araya *et al.,* 2020). Identification of rifampicin resistance has improved due to the wide availability of new rapid molecular technology (Dlamini *et al.,* 2019).

5.2.2.2 Prevalence of isoniazid mono-resistant tuberculosis

The prevalence of isoniazid mono-resistance (INHr)-TB in this study was 6.6%, among the previously treated TB cases. This result is attributed to the factor that most laboratories in Zambia don't have the capacity to detect INH resistance, because the most commonly used GeneXpert MTB/RIF analysers detects only RIF resistance (Monde *et al.,* 2023). Also, the Hain Genotype MTBDR line probe assays, which can detect resistance to both INH and rifampicin, are only available at three TB reference laboratories in Zambia. Therefore, this contributes to the increase in the prevalence rate of INH mono-resistant TB cases, as the majority of the cases are misdiagnosed and subsequently mismanaged (Monde *et al.,* 2023).

The findings of this study were similar with what other studies reported; A study in Zambia reported a prevalence of INHr-TB at 9.8% (Monde *et al.*, 2023), while that in India reported a prevalence of 7.8%, among the previously treated TB cases, respectively (Shivekar *et al.,* 2020). These studies revealed that isoniazid resistance was increasing due to; lack of diagnostic tools to identify INHr-TB, research direction and policy makers are ignoring INHr-TB, misdiagnosis and mismanagement of INHr-TB (Sulis *et al.*, 2020; Shivekar et al., 2020; Monde *et al.,* 2023).

Isoniazid mono-resistance is the most common type of drug-resistant TB in the world (Monde *et al.,* 2023). Novel technologies such as GeneXpert MTB/RIF analysers, which were endorsed in 2008 by the World Health Organisation, do not include INH-

resistance testing on their platforms (Sulis *et al.,* 2020). Many patients with INHr-TB are missed by current diagnostic algorithms which only test for rifampicin resistance (Dean *et al.,* 2020). The development of isoniazid resistant TB is usually the first step in the evolution of multidrug-resistant TB or even more complicated types of drug-resistant TB (Dlamini *et al.*, 2019). Isoniazid-resistant TB is posing a challenge to global efforts for ending the TB epidemic by 2030 (Dean *et al.,* 2019), because it is associated with higher treatment failure and relapse rates, as well as progression to multidrug-resistant TB. Therefore, early detection of INH resistant TB with initiation of effective therapy can significantly increase treatment outcome in INH-resistant populations and reduce progression to multidrug-resistant TB (Huo *et al.,* 2019; Monde *et al.,* 2023).

5.2.2.3 Prevalence of multidrug-resistant tuberculosis

The prevalence of multidrug-resistant (MDR)-TB in this study was high, 33.0% among the previously treated TB cases. This high prevalence is attributed to; poor adherence, irresponsive monitoring of patient progress, inadequate supply/stockout of anti-TB drugs, delayed diagnosis, HIV/TB co-infection, history of previous treatment for TB (Chisompola *et al.,* 2020; Monde *et al.,* 2023). Patients who have a history of previous treatment for TB (treatment failure, defaulter, or relapse cases) are at a greater risk of developing MDR-TB (Welekidan *et al.,* 2020). The prevalence of MDR-TB is 20% higher in HIV-positive individuals than HIV-negative ones (Singh *et al.,* 2020). Multidrug resistant-TB is a growing public health problem in Zambia, especially among the previously treated TB case who are HIV positive (Kasapo *et al.,* 2017; MOH, 2017; Singh *et al.,* 2020).

The finding of this study was consistent with what other studies reported; A study done in Zambia reported a prevalence of MDR-TB at 25.5%, among the previously treated TB cases (Monde *et al.,* 2023), India reported the prevalence of 36.4% (Singh *et al.,* 2020), Ethiopia reported the prevalence of 32.7% (Welekidan *et al.,* 2020), while China reported the prevalence of MDR-TB at 30.4%, among the previously treated TB cases (Liang *et al.,* 2012). Monde and colleagues revealed in their study that the high prevalence of MDR-TB, among the previously treated TB cases in Zambia was

attributed to; poor adherence arising from a high pill burden, adverse effects of anti-TB drugs, long duration of treatment, impaired drug absorption (especially for rifampicin, isoniazid, and ethambutol), drug-to-drug interactions in TB/HIV co-infected patients, poor quality directly observed treatment short course (DOTS) program (Monde *et al.*, 2023). Singh and colleagues revealed that HIV co-infection was the strongest risk factor for the development of MDR-TB, because individuals who are TB/HIV co-infected are immunosuppressed, thus susceptible to acquiring MDR-TB (Singh *et al.*, 2020; Welekidan *et al.*, 2020). Welekidan and colleagues revealed that the high prevalence of MDR-TB cases were attributed to; poor adherence, poor compliance, history of previous treatment for TB, lack of supervision of treatment, lack of monitoring of patient progress, improper treatment regimens, inadequate/irregular supply of anti-TB drugs, late diagnosis, mismanagement of susceptible TB, and poor-quality DOTS program (Welekidan *et al.*, 2020). While Liang and colleagues in their study found that inappropriate treatment of susceptible TB, was the most common cause of MDR-TB (Liang *et al.*, 2012).

Multidrug-resistant TB is not caused by a single factor, but by several factors, including: poor adherence and non-compliance to TB treatment, history of previous treatment (treatment failure, relapse, defaulter, or lost to follow-up case), irregular supply and stock-out of anti-TB drugs, inappropriate TB therapy, delayed diagnosis, initiation of ineffective therapy, and spontaneous chromosomal mutations (Seyoum *et al.*, 2014; Okethwangu *et al.*, 2019; Prasad *et al*, 2019; Iacobino *et al.*, 2020). Also failure to implement effective TB prevention and control measures, facilitates the emergence of the different forms of DR-TB including MDR-TB (Calver *et al.*, 2010; Qian *et al.*, 2015; Vashistha *et al.*, 2018). Multidrug-resistant TB must be managed effectively to reduce mortality, morbidity, and the eventual transmission of *M. tuberculosis* resistant strains (Pires *et al.*, 2014).

5.2.2.4 Prevalence of poly drug-resistant tuberculosis

In this study, poly drug-resistant TB (PDR-TB) was detected, which is defined as resistance to more than one anti-TB drug (other than both isoniazid and rifampicin) (Lobie *et al.*, 2020; Pan *et al.*, 2023). The PDR-TB in this study was resistance to

rifampicin and fluoroquinolone (levofloxacin), with a prevalence of 1.8%, among the previously treated TB cases. This prevalence, despite being low, revealed the existence of resistance to both first- and second-line anti-TB drugs, among the previous treated TB cases. The previously treated TB cases (treatment failures, relapse cases, defaulters, or return after lost to follow-up) have the highest risk for acquiring any type of drug-resistant TB, including PDR-TB (Adam *et al.,* 2016). The majority of the previously treated TB cases in Zambia also have HIV infection (Mutembo *et al.*, 2019). HIV causes rifampicin resistance via alteration in pharmaco-kinetics of rifampicin resulting in malabsorption of the drug (Mulenga *et al.*, 2010; Salam-Dreyer, *et al.,* 2021; Brode *et al.,* 2022). Fluoroquinolones (FLQs) are among the most widely prescribed antibiotics globally, extensively used for the treatment of several bacterial infectious diseases because they have a broad-spectrum activity (Kabir *et al.,* 2020). Fluoroquinolones are over-prescribed and easily accessible as over-the counter medications in many resource-limited countries, Zambia inclusive (Sayadi *et al.,* 2020). This situation has led to the emergence of fluoroquinolone resistance in *M. tuberculosis* (Kabir *et al.,* 2020).

The findings of this study were consistent with what other studies reported; Canada reported a prevalence of 0.2% for PDR-TB, among the previously treated TB cases (Gallant *et al.,* 2017), Sudan reported 6.4% (Adam *et al.,* 2016), and Bangladesh reported 8.6% (Kundu *et al.,* 2020). These studies highlighted that *M. tuberculosis* resistance to fluoroquinolones was attributed to; irrational use of fluoroquinolones in the treatment of several bacterial infections, incomplete adherence to TB treatment, and failure in previous TB treatment (Adam *et al.,* 2016; Kundu *et al.,* 2020).

5.2.2.5 Prevalence of pre-extensively drug-resistant tuberculosis

Pre-extensively drug-resistant tuberculosis (pre-XDR TB) was detected, defined as TB that is resistant to both rifampicin and isoniazid, plus any fluoroquinolone (ofloxacin, levofloxacin, gatifloxacin, or moxifloxacin) or second-line injectable drugs (kanamycin, amikacin, or capreomycin) but not both (Monde *et al.,* 2021). The pre-XDR TB detected was resistance to rifampicin and isoniazid plus fluoroquinolones/kanamycin, with a prevalence of 0.8%, among the previously treated

TB cases. This prevalence, despite being low, revealed the existence of resistance to both first- and second-line anti-TB drugs. This calls for an urgent need to employ antimicrobial stewardship principles in clinical settings. Extensive use of fluoroquinolones in the treatment of other bacterial infections, and the ease of access of these drugs over-the counter without prescriptions have led to the emergence of resistance by *M. tuberculosis*, to these drugs (Kabir *et al.*, 2020; Sayadi *et al.,* 2020).

Studies in other countries reported a high prevalence of pre-XDR, among the previously treated TB cases: Nigeria (17%), Morocco (22.2%), Zimbabwe (27%), Nepal (28%), Bangladesh (16.2%), Vietnam (17.9%), China (34%), Pakistan (38.7%), and India (56%) (Sagonda *et al.,* 2014; Tasnim *et al.,* 2018; Shibabaw *et al.,* 2020; Welekidan *et al.,* 2020). The high prevalence of pre-XDR in these countries was attributed to a widespread use of fluoroquinolones without prescriptions, self-medication using fluoroquinolones, most of the study participants being TB treatment failures and chronic TB cases (Kundu *et al.,* 2020; Welekidan *et al.,* 2020). The resistance to second-line injectable drugs (kanamycin, amikacin, and capreomycin) by *M. tuberculosis* is attributed to treatment defaulting by TB patients, due to the severe side effects which these drugs have, namely; ototoxicity (hearing impairment) and nephrotoxicity (kidney damage) (Isabel *et al.,* 2019). The high prevalence of pre-XDR TB also indicates poor management of MDR-TB cases. Poorly managed MDR-TB cases progress to pre-XDR TB, which eventually get to XDR-TB (Kashongwe *et al.,* 2020; Shibabaw *et al.,* 2020).

5.3 Association of drug-resistant tuberculosis to age and gender among the new and previously treated tuberculosis cases

In this study, no association was found between drug-resistant TB to age and gender among the new and previously treated TB cases. Age and gender were not statistically associated with drug-resistance, which is in agreement with what other studies reported; Sudan (Ali *et al.,* 2019), Nigeria (Uzoewulu *et al.,* 2014), Ethiopia (Brhane *et al.,* 2017), and Egypt (Hosny *et al.,* 2020), Burkina Faso (Diande *et al.,* 2019), Georgia, Russia, and Estonian (Arega *et al.,* 2019).

Studies in the Tigray Region, Northern Ethiopia and Russia found an association between age and multidrug-resistant tuberculosis (MDR-TB) (Welekidan *et al.,* 2020; Bykov *et al.,* 2022), such a result was not found in this study. Another study in Ethiopia found that the history of previous treatment for TB (failures, defaulters, and relapses) and cigarette smoking were associated with MDR-TB (Welekidan *et al.,* 2020). Cigarette smoking was associated with MDR-TB, because cigarette smoking directly causes ciliary dysfunction; this diminishes the immunity of individuals, which makes them prone to primary MDR-TB (Welekidan *et al.,* 2020). Studies have shown that the key predicting/risk factors associated with the development of drug-resistant TB include; history of previous treatment for TB, poor adherence to TB treatment, inadequate supply and stock-out of anti-TB drugs, direct contact with drug-resistant-TB patients, HIV/TB co-infection, a high bacillary load, age, gender, diabetes mellitus, occupation, history of isoniazid prophylaxis therapy, history of imprisonment, and unemployment (Seyoum *et al.,* 2014; Brhane *et al.,* 2017; Dlamini *et al.,* 2019; Okethwangu *et al.,* 2019; Hosny *et al.,* 2020; Iacobino *et al.,* 2020; Singh *et al.,* 2020).

5.4 Drug-resistance associated mutations in *Mycobacterium tuberculosis* resistant isolated among the new tuberculosis cases

5.4.1 Frequency of mutations conferring resistance to rifampicin

In this study the *rpo*B MUT 3 (Ser531Leu) mutation was the most frequently detected (8.6%), conferring resistance to rifampicin, in *M. tuberculosis* resistant strains, among the new TB cases. Rifampicin resistance is mainly acquired through primary transmission, among the new TB cases (Su *et al.,* 2021). The mutation Ser531Leu belongs to a potentially highly transmissible *M. tuberculosis* strain, no wonder it is easily transmitted via primary transmission, among the new TB cases (Vidyaraj *et al.,* 2017). Studies indicate that mutations at codons 531, 526, and 516 of the *rpo*B gene coding for the RNA polymerase β-subunit make up the majority of rifampicin-resistance mutations reported globally (Spinato *et al.,* 2016; Reta *et al.,* 2020).

Other Similar studies reported high results; in Brazil (75%) (Feliciano *et al.,* 2015), Ethiopia (74.2% and 77.1%) (Meaza *et al.,* 2017; Reta *et al.,* 2020), Canada reported 47.8% (Spinato *et al.,* 2016), India reported 46.9% and 19.5% in two separate studies

(Desikan *et al.,* 2016; Shenoy *et al.,* 2018), and Uganda reported 40% (Kigozi *et al.,* 2018). All these results are in agreement with the high transmissibility of the *M. tuberculosis* strain, carrying the Ser531Leu mutation. Based on these results it suffices to say, mutation frequencies vary from one geo-location to another due to differences in the epidemiology of TB, differences in geographic transmission environments for *M. tuberculosis*, as well as differences in treatment combinations used in the management of cases (Kigozi *et al.,* 2018; Reta *et al.,* 2020; Tahseen *et al.,* 2020; Monde *et al.,* 2023).

5.4.2 Frequency of mutations conferring resistance to isoniazid

The *inh*A MUT1 (Cys15Thr) mutation was the most frequently detected (28.6%), in *M. tuberculosis* resistant isolates, among the new TB cases. This was so because the *M. tuberculosis* resistant strains carrying the Cys15Thr mutation are very virulent and highly transmissible. The most frequently detected mutation in the *inh*A gene, that confer low-resistance to isoniazid is the *inh*A Cys15Thr (Jian *et al.,* 2018). A study in California reported a similar frequency (23.0%), for the Cys15Thr mutation (Reta *et al.,* 2020). Other similar studies reported very high frequencies for the *inh*A MUT 1 (Cys15Thr) mutation; Ethiopia (77.5%) (Reta *et al.,* 2020), South-Africa (70.1%) (Click *et al.,* 2020)**,** and India (85.9%) (Prasad *et al.,* 2019). These results from other countries are not surprising because, countries like Ethiopia, South-Africa, and India have a huge-burden of TB and drug-resistant TB, and so the transmissibility rate of the *M. tuberculosis* resistant strains carrying the Cys15Thr is very high (Jian *et al.,* 2018).

5.4.3 Frequency of mutations conferring resistance to both rifampicin and isoniazid

The *rpo*B MUT 3 (Ser531Leu) and the *kat*G MUT1 (Ser315Thr 1) mutations were the most frequent (14.3%), conferring resistance to both rifampicin and isoniazid, in *M. tuberculosis* resistant isolates, among the new TB cases. This was so because the mutation *rpo*B Ser531Leu is carried by virulent and highly transmissible *M. tuberculosis* resistant strains (Vidyaraj *et al.,* 2017). A high frequency of the *kat*G Ser315Thr 1 mutation is associated with countries with a high prevalence of TB, such as Zambia (Solo *et al.,* 2020a). The *kat*G Ser315Thr 1 mutation occur more frequently

in *M. tuberculosis* resistant strains that are responsible for causing multidrug-resistant TB, because it provides isoniazid resistance with the retention of virulence. This in turn allows the Ser315Thr 1 mutated strains to persist in the intracellular environment and become resistant to additional anti-TB drugs (Spinato *et al.*, 2016). The result for this study was lower than what was reported in; Thailand (36.4%), as frequencies for mutations conferring resistance to both rifampicin and isoniazid (Charoenpak *et al.*, 2020). This result is not a surprise at all, because Thailand has a huge-burden of TB and drug-resistant TB, and so the transmissibility rate of the *M. tuberculosis* resistant strains carrying the mutations *rpo*B (Ser531Leu) and *kat*G (Ser315Thr1) is very high (Tseng *et al.*, 2013; Vidyaraji *et al.*, 2017; Prasad *et al.*, 2019).

5.5 Drug-resistance associated mutations in *Mycobacterium tuberculosis* resistant isolates among the previously tuberculosis cases

5.5.1 Frequency of mutations conferring resistance to rifampicin

The *rpo*B MUT 3 (Ser531Leu) mutation was the most frequently detected (10.9%), conferring resistance to rifampicin (RIF), in *M. tuberculosis* resistant strains, among the previously treated TB cases. This result is attributed to the fact the mutation *rpo*B (Ser531Leu) is carried by *M. tuberculosis* resistant strains that are virulent and highly transmissible (Vidyaraji *et al.*, 2017). Studies in other countries reported high frequencies for the *rpo*B (Ser531Leu) mutation; Ethiopia reported 77.1% (Reta *et al.*, 2020), and Uganda reported 40% (Kigozi *et al.*, 2018). These results clearly confirm that the mutation *rpo*B (Ser531Leu) is carried by *M. tuberculosis* resistant strains that are very virulent and highly transmissible (Vidyaraji *et al.*, 2017).

5.5.2 Frequency of mutations conferring resistance to isoniazid

The *kat*G MUT 1 (Ser315Thr 1) mutation was the most frequently (6.7%) encountered mutation conferring resistance to isoniazid, in *M. tuberculosis* resistant strains, among the previously treated TB cases. The mutation *kat*G (Ser315Thr 1) is carried by *M. tuberculosis* mutant strains that are very virulent, highly transmissible, and persistent in intracellular environments, thus become resistant to other anti-TB drugs (Spinato *et al.*, 2016). Studies have shown that *kat*G Ser315Thr 1 mutation is associated with

countries that have a high prevalence of TB, Zambia inclusive (Solo *et al.,* 2020a). Studies in other countries reported high frequencies for the *kat*G (Ser315Thr 1) mutation; Taiwan (50.4%), Myanmar (57.3%) and India (57.8%) (Tseng *et al.,* 2013; Prasad *et al.,* 2019). These results clearly confirm that the mutation *katG* (Ser315Thr 1) is carried by *M. tuberculosis* mutant strains that are very virulent, highly transmissible, and persistent. The results also show that these countries have a high prevalence of TB, because a high frequency of the *kat*G Ser315Thr 1 mutation is associated with countries that have a high prevalence of TB (Solo *et al.,* 2020a).

5.5.3 Frequency of mutations conferring resistance to both rifampicin and isoniazid

The *rpo*B MUT 3 (Ser531Leu) and the *kat*G MUT1 (Ser315Thr 1) mutations were the most frequent (18.5%), conferring resistance to both rifampicin and isoniazid, in *M. tuberculosis* resistant isolates, among the previously treated TB cases. This result was attributed to the fact the mutations *rpo*B (Ser531Leu) and *kat*G (Ser315Thr 1), are carried by very virulent, highly transmissible, and persistent *M. tuberculosis* mutant strains (Vidyaraji *et al.*, 2017; Reta *et al.,* 2020), that are circulating in countries that have a huge-burden of TB, of which Zambia is inclusive (Solo *et al.,* 2020a). A study in Thailand reported the frequency of (36.4%), for the mutations *rpo*B (Ser531) and *kat*G (Ser315Thr1) (Charoenpak *et al.,* 2020). This result confirms that these mutations are carried by resistant *M. tuberculosis* strains that have a competitive fitness and higher transmissibility (Dookie *et al.,* 2018). A high frequency of mutations in the *rpo*B, *kat*G, and *inh*A genes of resistant *M. tuberculosis* strains is facilitated by bacterial genetics (Loiseau *et al.,* 2023).

Studies have shown that *M. tuberculosis* resistant strains that harbour resistance mutations have the following key characteristics; have higher transmission fitness compared to drug-susceptible strains, tolerate the physiological effects of antimicrobials, have a long infectious period, and acquire compensatory mutations that enhance their competitive fitness (Dookie *et al.,* 2018; Loiseau *et al.,* 2023).

5.5.4 Frequency of mutations conferring resistance to both rifampicin and fluoroquinolones

This study also detected other mutations such as the *rpo*B MUT 2A (His526Tyr) and *gyr*A MUT 1 (Ala90Val) in *M. tuberculosis* resistant isolates, with a frequency of 1.7%, that confer resistance to both rifampicin and fluoroquinolones (FLQs), among the previously treated TB cases. The study participants were taking the fluoroquinolone, levofloxacin. Mutations in the 81-base pair (bp) region (codons 507–533) of the *rpo*B gene harbour over 95% of rifampicin resistance in *M. tuberculosis* isolates and high-level rifampicin resistance is associated with point mutations in 531, 526 and 516 codons of the *rpo*B gene (Agonafir *et al.,* 2023). Studies have shown that most mutations associated with fluoroquinolone resistance occur in the *gyr*A gene on codon 90-94 of *M. tuberculosis* resistant strains (Welekidan *et al.,* 2020). The Ala90Val mutation is associated with high-level resistance to FLQs (Kabir *et al.,* 2020). The Ala90Val mutation confers resistance to levofloxacin, but a higher generation fluoroquinolone (moxifloxacin) can still be used at a higher treatment dose. However, if the Asp94Gly mutation is present, both levofloxacin and moxifloxacin become ineffective. The *M. tuberculosis* resistant strains that harbour the Ser91Pro and Asp94Ala mutation are susceptible to moxifloxacin at higher doses, but resistant to levofloxacin. However, the Asp94Asn/Asp94Tyr mutation confers resistance to both moxifloxacin and levofloxacin (Kabir *et al.,* 2020).

A study in Ethiopia reported a high frequency (50%), for the *rpo*B (His526Tyr) and *gyr*A (Ala90Val) mutations (Welekidan *et al.,* 2020). This result confirms that the *rpo*B (His526Tyr) and *gyr*A (Ala90Val) mutations are carried by resistant *M. tuberculosis* strains that have a competitive fitness and high transmissibility (Dookie *et al.,* 2018; Charoenpak *et al.,* 2020).

5.5.5 Frequency of mutations conferring resistance to both rifampicin and isoniazid plus fluoroquinolones or kanamycin

In this study mutations associated with resistance in the *rpo*B, *kat*G, *gyr*A, and *eis* genes, were detected. These mutations were responsible for causing pre-extensively drug-resistant TB (pre-XDR TB). The first isolate had resistance to both rifampicin

and isoniazid plus fluoroquinolones, while the second isolate had resistance to both rifampicin and isoniazid plus kanamycin. The first *M. tuberculosis* isolate had the combination mutation: *rpo*B MUT 2A (His526Tyr), *kat*G MUT 1 (Ser315Thr), and *gyr*A MUT 3C (Asp94Gly). While the second had *rpo*B MUT 2B (His526Asp), *kat*G MUT 2 (Ser315Thr 2), and *eis* MUT 1 (Cys14Thr). Each of the combination mutation had a frequency of 0.8%. The occurrence of these mutations is a confirmation that resistant *M. tuberculosis* strains that have a competitive fitness and high transmissibility rate are circulating (Dookie *et al.,* 2018; Charoenpak *et al.,* 2020). Studies elsewhere, reported high frequencies for these mutations; Morocco (47.6%) (Oudghiri *et al.,* 2018), India (69.5%) (Rufai *et al.,* 2020), and Pakistan (98.1%) (Kabir *et al.,* 2020). These results are not surprising, because in these countries highly transmissible resistant *M. tuberculosis* strains harbouring mutations in the *rpo*B, *kat*G, *gyr*A, and *eis* genes are circulating (Oudghiri *et al.,* 2018; Kabir *et al.,* 2020; Rufai *et al.,* 2020).

The extensive use of fluoroquinolone and/or injectable aminoglycoside antibiotics in the treatment of other bacterial diseases other than TB, has contributed to the evolution of resistance in *M. tuberculosis* strains, associated with mutations in the *gyr*A, *gyr*B, *rrs,* and *eis* gene (Avalos *et al.,* 2015; Oudghiri *et al.,* 2018; Kabir *et al.,* 2020). Variations in mutation frequencies occur due to differences in the; treatment combinations used in the management of drug-resistant TB, epidemiology of TB, and geographic transmission environments for resistant *M. tuberculosis* strains (Araya *et al.,* 2020; Kabir *et al.,* 2020).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Drug-resistant tuberculosis is very prevalent in Zambia, especially multidrug-resistant tuberculosis among the previously treated TB cases. The high prevalence of drug-resistant TB among this category indicates failures in TB management and control. The high prevalence of isoniazid drug-resistant TB, among the newly diagnosed TB cases indicates on-going primary transmission, which suggests the need for enhanced TB control program to interrupt primary transmission.

The identification of drug-target genes with their associated mutations offers therapeutic benefits to patient care and management. The most common mutations identified in this study were; *rpo*B (Ser531Leu, His526Asp, Asp516Val, His526Tyr, and Glu510His) conferring resistance to rifampicin, *kat*G (Ser315Thr 1, and Ser315Thr 2) conferring high-level resistance to isoniazid, *inh*A (Cys15Thr) conferring low-level resistance to isoniazid, *gyr*A (Ala90Val) conferring resistance to fluoroquinolones, and *eis* (Cys14Thr) conferring low-level resistance to kanamycin, and each of these mutations had a varying frequency. The *inh*A (Cys15Thr) mutation was the most frequently detected in *M. tuberculosis* resistant isolates, among the new TB cases. While the *rpo*B (Ser531Leu) mutation was the most frequently identified in resistant isolates, among the previously treated TB cases.

Mutation frequencies vary from one geographical region to another, mainly because of the differences in treatment combinations used in the management of drug-resistant TB. The other reasons are differences in the epidemiology of TB and geographic transmission environments for *M. tuberculosis* resistant strains.

6.2 Recommendations

i. Diagnose drug-resistant TB early using molecular diagnostic assays such as the Hain GenoType MTBDR*plus* ver 2.0 and MTBDR*sl* ver 2.0, line probe assays.

ii. Manage the previously treated TB cases effectively, because drug-resistant TB is very prevalent in this category of TB cases.

iii. Use antimicrobials judiciously, carefully and rationally to prevent or minimize the occurrence of antimicrobial resistance (AMR).

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APPENDICES

Appendix I: Ethics approval letter

UNIVERSITY OF ZAMBIA HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

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Lusaka, 15101
Zambia
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FWA no: 00026270

Protocol ID: 20203101001

31st January 2020

Mr. David Kajoba Mumena
Jomo Kenyata University of Agriculture and Technology
School of Biomedical Sciences
Department of Medical Microbiology
Kenya

Dear Mr. Mumena,

Re: Approval Letter of Protocol ID Number 20203101001

I write to inform you that your study entitled ***'Genotypic Characterization of Drug-resistant Mycobacterium tuberculosis in Positive Sputum Specimens in Lusaka, Zambia'***, submitted by you as the principal investigator to our research ethics committee has been reviewed under the expedited review process and has been **approved**. A **waiver of consent** is also granted for you to use archival tuberculosis specimens at the Chest Diseases Laboratory in Lusaka.

Note that the study approval duration is for one year after which you may renew the protocol. Your renewal date is 30th January 2021.

The Research Ethics Committee expects to be informed about the progress of the project, any adverse events occurring during the study, any revision of the protocol and participant information sheet/informed consent form and ask to be provided a copy of your final report.

You are advised to obtain final study clearance and approval to conduct research in Zambia from the National Health Research Authority (NHRA) before commencing the research project.

Yours faithfully,

Sody M. Munsaka, BSc., MSc., PhD
CHAIRPERSON

Appendix II: Authorization letter from the National Health Research Ethics Board

NATIONAL HEALTH RESEARCH AUTHORITY

Paediatric Centre of Excellence, University Teaching Hospital, P.O. Box 30075, LUSAKA

Tell: +260211 250309 | Email: znhrasec@gmail.com | www.nhra.org.zm

Ref No:..... **Date: 5th March, 2020**

The Principal Investigator
Mr. David Kajoba Mumena
Jomo Kenyata University of Agriculture and Technology
School of Biomedical Sciences
Department of Medical Microbiology
KENYA.

Dear Mr. Mumena,

Re: Request for Authority to Conduct Research

The National Health Research Ethics Board (NHREB) is in receipt of your request for authority to conduct research titled **"Genotypic characterization of drug-resistant Mycobacterium tuberculosis in positive sputum specimens among the new and previously treated tuberculosis cases at the National Reference Chest Diseases Laboratory, in Lusaka Zambia."**
I wish to inform you that following submission of your request to the Board, its review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. **A Material Transfer Agreement is obtained and cleared by the National Health Research Ethics Board should there be any need for samples to be sent outside the country for analysis.**
2. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
3. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
4. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
5. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents

Yours sincerely,

Prof. Patrick Musonda
Chairperson
National Health Research Ethics Board

All correspondences should be addressed to the Director/CEO National Health Research Authority

Appendix III: Authorization letter from the Ministry of Health

All Correspondence should be addressed to the
Permanent Secretary
Telephone: +260 211 253040/5
Fax: +260 211 253344

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH

In reply please quote
MH/101/23/10

NDEKE HOUSE
P. O. BOX 30205
LUSAKA

19 March, 2020

Mumena David Kajoba
University of Zambia
School of Public Health
Box 50110
LUSAKA

RE: PERMISSION TO CONDUCT RESEARCH STUDY

The Ministry of Health is in receipt of your request to conduct Research Study titled **"Genotypic Characterization of Drug-resistant Mycobacterium tuberculosis in Positive Sputum Specimens among the New and Previously Treated Tuberculosis cases, at the National Reference Chest Diseases Laboratory in Lusaka, Zambia".**

I wish to inform you that permission to conduct Research has been granted and information obtained will be used only for the intended purpose as stipulated in the request.

By copy of this letter, Lusaka District Health Director is hereby informed.

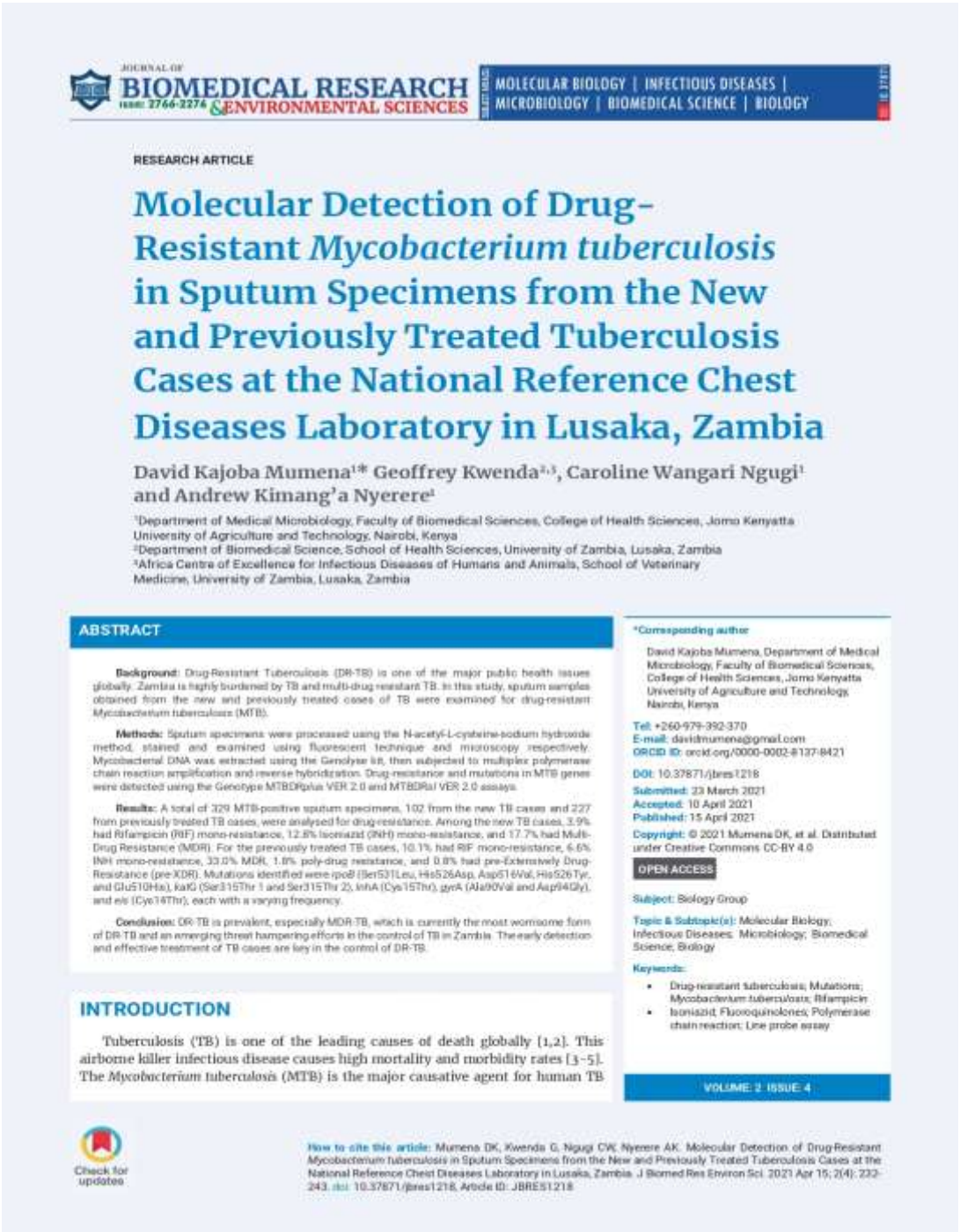
Dr. Kennedy Malama
Permanent Secretary- (TS)
MINISTRY OF HEALTH

cc: PHD- Lusaka
cc: DHD- Lusaka

Appendix IV: Table of random numbers

Row Number	Column Number									
	01–05	06–10	11–15	16–20	21–25	26–30	31–35	36–40	41–45	46–50
01	89,392	23,212	74,483	36,590	25,956	36,544	68,518	40,805	09,980	00467
02	61,458	17,639	96,252	95,649	73,727	33,912	72,896	66,218	52,341	97,141
03	11,452	74,197	81,962	48,443	90,360	26,480	73,231	37,740	26,628	44,690
04	27,575	04,429	31,308	02,241	01,698	19,191	18,948	78,871	36,030	23,980
05	36,829	59,109	88,976	46,845	28,329	47,460	88,944	08,264	00843	84,592
06	81,902	93,458	42,161	26,099	09419	89,073	82,849	09,160	61,845	40,906
07	59,761	55,212	33,360	68,751	86,737	79,743	85,262	31,887	37,879	17,525
08	46,827	25,906	64,708	20,307	78,423	15,910	86,548	08,763	47,050	18,513
09	24,040	66,449	32,353	83,668	13,874	86,741	81,312	54,185	78,824	00718
10	98,144	96,372	50,277	15,571	82,261	66,628	31,457	00377	63,423	55,141
11	14,228	17,930	30,118	00438	49,666	65,189	62,869	31,304	17,117	71,489
12	55,366	51,057	90,065	14,791	62,426	02,957	85,518	28,822	30,588	32,798
13	96,101	30,646	35,526	90,389	73,634	79,304	96,635	06,626	94,683	16,696
14	38,152	55,474	30,153	26,525	83,647	31,988	82,182	98,377	33,802	80,471
15	85,007	18,416	24,661	95,581	45,868	15,662	28,906	36,392	07,617	50,248
16	85,544	15,890	80,011	18,160	34,468	84,106	40,603	01,315	74,664	20,553
17	10,446	20,699	98,370	17,684	16,932	80,449	92,654	02,084	19,985	59,321
18	67,237	45,509	17,638	65,115	29,757	80,705	82,686	48,565	72,612	61,760
19	23,026	89,817	05,403	82,209	30,573	47,501	00135	33,955	50,250	72,592
20	67,411	58,542	18,678	46,491	13,219	84,084	27,783	34,508	55,158	78,742
21	48,663	91,245	85,828	14,346	09,172	30,168	90,229	04,734	59,193	22,178
22	54,164	58,492	22,421	74,103	47,070	25,306	76,468	26,384	58,151	06,646
23	32,639	32,363	05,597	24,200	13,363	38,005	94,342	28,728	35,806	06,912
24	29,334	27,001	87,637	87,308	58,731	00256	45,834	15,398	46,557	41,135
25	02,488	33,062	28,834	07,351	19,731	92,420	60,952	61,280	50,001	67,658

Appendix V: Research article publication



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RESEARCH ARTICLE

Molecular Detection of Drug-Resistant *Mycobacterium tuberculosis* in Sputum Specimens from the New and Previously Treated Tuberculosis Cases at the National Reference Chest Diseases Laboratory in Lusaka, Zambia

David Kajoba Mumena[1]* Geoffrey Kwenda[2,3], Caroline Wangari Ngugi[1] and Andrew Kimang'a Nyerere[1]

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[2]Department of Biomedical Science, School of Health Sciences, University of Zambia, Lusaka, Zambia
[3]Africa Centre of Excellence for Infectious Diseases of Humans and Animals, School of Veterinary Medicine, University of Zambia, Lusaka, Zambia

ABSTRACT

Background: Drug-Resistant Tuberculosis (DR-TB) is one of the major public health issues globally. Zambia is highly burdened by TB and multi-drug resistant TB. In this study, sputum samples obtained from the new and previously treated cases of TB were examined for drug-resistant *Mycobacterium tuberculosis* (MTB).

Methods: Sputum specimens were processed using the N-acetyl-L-cysteine-sodium hydroxide method, stained and examined using fluorescent technique and microscopy respectively. Mycobacterial DNA was extracted using the Genolyse kit, then subjected to multiplex polymerase chain reaction amplification and reverse hybridization. Drug-resistance and mutations in MTB genes were detected using the Genotype MTBDRplus VER 2.0 and MTBDRsl VER 2.0 assays.

Results: A total of 329 MTB-positive sputum specimens, 102 from the new TB cases and 227 from previously treated TB cases, were analysed for drug-resistance. Among the new TB cases, 3.9% had Rifampicin (RIF) mono-resistance, 12.8% Isoniazid (INH) mono-resistance, and 17.7% had Multi-Drug Resistance (MDR). For the previously treated TB cases, 10.1% had RIF mono-resistance, 6.6% INH mono-resistance, 33.0% MDR, 1.8% poly-drug resistance, and 0.8% had pre-Extensively Drug-Resistance (pre-XDR). Mutations identified were *rpoB* (Ser531Leu, His526Asp, Asp516Val, His526Tyr, and Glu510His), *katG* (Ser315Thr 1 and Ser315Thr 2), *inhA* (Cys15Thr), *gyrA* (Ala90Val and Asp94Gly), and *eis* (Cys14Thr), each with a varying frequency.

Conclusion: DR-TB is prevalent, especially MDR-TB, which is currently the most worrisome form of DR-TB and an emerging threat hampering efforts in the control of TB in Zambia. The early detection and effective treatment of TB cases are key in the control of DR-TB.

INTRODUCTION

Tuberculosis (TB) is one of the leading causes of death globally [1,2]. This airborne killer infectious disease causes high mortality and morbidity rates [3-5]. The *Mycobacterium tuberculosis* (MTB) is the major causative agent for human TB

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- Isoniazid; Fluoroquinolones; Polymerase chain reaction; Line probe assay

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among the *Mycobacterium tuberculosis* Complex (MTBC), as it causes 97-99% of TB cases [6]. Globally 10 million individuals contract TB and from this number, 1.6 million individuals die from the disease [7]. The World Health Organization (WHO) global TB report of 2020 revealed that in 2019 there were 10 million people who got sick of TB, with 1,408,000 deaths globally [8].

Drug-resistant TB is a serious public health problem globally [9]. TB prevention, control, eradication and elimination strategies are being hampered by the successful transmission of drug-resistant MTB strains which are causing Multidrug-Resistant (MDR) and Extensively Drug-Resistant (XDR)-TB in the human population [10,11]. In 2020 the global prevalence of MDR/RR (Rifampicin-Resistance) was 3.3% among the new TB cases and 17.7% among the previously treated TB cases [8]. In 2019 was 3.4% and 18% among the new and previously treated TB cases respectively [12]. In 2017, 8.5% of TB patients diagnosed with MDR-TB progressed to developing XDR-TB [12]. XDR-TB is rapidly spreading throughout the world with 9.4 million new cases and 1.7 million reported deaths annually [13]. Globally in 2019 the prevalence of isoniazid mono-resistance among the new TB cases was 7.2% and 11.6% among the previously treated TB cases [8]. Drug-resistant TB is a serious public health threat in sub-Saharan Africa, especially for countries that have a high burden of both HIV and TB cases [14]. Drug-resistant TB is posing a huge obstacle for many countries to achieve the set WHO end TB targets [12,15].

Zambia is ranked among the top ten countries with a high burden of TB in the world [12,16,17]. TB is a major public health problem in the country, and is causing high mortality and morbidity rates [18-21]. Zambia is also among the top 10 countries in Africa with the highest cases of MDR/RR-TB [22]. MDR-TB is indeed a growing public health problem in the country [23]. In 2020, the MDR-TB prevalence rate in Zambia was 2.4% among the new and 18% previously treated TB cases [8]. While in 2019 was 2.8% and 18% among the new and previously treated TB cases respectively [12]. It has been projected that in 2021, Zambia will have a high burden of MDR and rifampicin-resistant TB cases [24].

Drug-resistant TB types include mono drug-resistant (resistance to any single anti-TB drug), poly drug-resistant (resistance to more than one anti-TB drug, other than both Rifampicin (RIF) and Isoniazid (INH) [25], MDR (resistance to at least INH and RIF), pre-XDR (resistance to INH, RIF, plus any one Fluoroquinolone (FLQ) (levofloxacin, ofloxacin, moxifloxacin or gatifloxacin) or any one of the Second-Line Injectable Drugs (SLIDs) (Kanamycin, Amikacin or Capreomycin), XDR (resistance to INH, RIF, plus any FLQ, plus any SLIDs) [26-30]. Poorly managed XDR-TB cases can progress to Totally Drug-Resistant TB (TDR-TB). TDR-TB is a type of DR-TB that is resistant to all first- and second-line anti-TB drugs. TDR-TB has been reported in India, Iran, Italy and South-Africa [31,32].

MTB develops resistance to anti-TB drugs due to a number of factors some of which include poor adherence, poor regimen selection, inadequate supply, and stock-out of anti-TB drugs [30,33]. However, research has shown that MTB resistance is mainly caused by mutations in the target genes and chromosomal replication errors [34,35].

Mutations in the *rpoB* gene, specifically in an 81 base pair region called the Rifampicin-Resistance Determining Region (RRDR) confer resistance to RIF [36,37]. The four most frequent mutations that confer resistance to RIF are Ser531Leu, His526Tyr, His526Asp, and Asp516Val [25]. Mutations in the *katG* and/or *inhA* gene cause resistance to INH frequently [36]. However, mutations in the following genes *ahpC*, *oxyR*, *kasA*, *furA*, *fabG* 1 and *ndh* infrequently cause resistance to INH [37]. The most frequent mutation in the *katG* gene that confers resistance to INH is the Ser315Thr mutation [37]. The four most frequent mutations in the *inhA* gene that confer resistance to INH are Cys15Thr, Ala16Gly, Thr8Cys and Thr8Ala [25]. Mutations occurring in the *katG* cause high-level resistance to INH, while those in the *inhA* cause low-level resistance [38].

Mutations occurring in the Quinolone Resistance Determining Region (QRDR) of both the *gyrA* and *gyrB* genes of resistant MTB strains cause resistance to the FLQs [39,40]. The most frequent mutations in the *gyrA* gene that confer resistance to the FLQs are Gly88Ala, Gly88Cys, Ala90Val, Ser91Pro, Asp94Ala, Asp94Asp, Asp94Tyr, Asp94Gly, and Asp94His. While those in the *gyrB* gene are Asp538Asp, Glu540Val [25]. It is worth noting that mutations in the *gyrA* gene confer a high-level resistance to FLQs, while those in the *gyrB* gene confer low-level resistance [41]. The frequency of mutations in the *gyrA* gene differ from one geographical location to another [41]. These differences are attributed to differences in treatment combinations containing FLQs used, and differences in geographic transmission environments for MTB [42]. Mutations in the *rrs*, *eis* and *tlyA* genes cause resistance to the aminoglycosides and cyclic polypeptide antibiotics [40]. The most frequent mutations in the *rrs* gene are Ala1401Gly, Cys1402Thr and Gly1484Thr [25,39]. While those in the *eis* gene are Gly37Thr, Cys14Thr, Cys12Thr, Gly10Ala, Cys2Ala [25]. The *eis* (Cys14Thr) mutation is very specific for resistance to kanamycin than the *eis* (Gly10Cys) and *eis* (Cys12Thr) mutations [43].

The most effective strategy to halt the rise of DR-TB cases is early detection and effective treatment. Rapid molecular tools enable the early detection of DR-TB [44]. Line Probe Assays (LPAs) are molecular diagnostic assays that are used in detecting MTB and its resistance to anti-TB drugs [45]. The Genotype MTBDR*plus* assay is an LPA that is used for the qualitative identification of MTB and its resistance to RIF and INH, the key first-line anti-TB drugs [46]. While the Genotype MTBDR*sl* assay is used for the qualitative detection of MTB and its resistance to second-line anti-TB drugs [47]. These assays can use either clinical or cultivated specimens to identify MTB and its resistance [48]. LPAs also

detect gene mutations in MTB that confer resistance to anti-TB drugs [25]. LPAs are recommended by the World Health Organization for diagnosis, guidance on empirical treatment and for surveillance of drug-resistant TB [15]. This study aimed to detect drug-resistant MTB in sputum specimens obtained from the new and previously treated TB cases.

MATERIALS AND METHODS

Study design and setting

This was a prospective laboratory-based, cross-sectional study that was carried out on TB positive sputum specimens from the new and previously treated TB cases. The study was conducted at the Chest Diseases Laboratory (CDL), in Lusaka, Zambia, from March 2020 to September 2020. CDL is a National Reference TB laboratory that is located at the National Institute for Scientific and Industrial Research (NISIR). CDL has the mandate to perform diagnosis and surveillance for both drug-susceptible and drug-resistant TB. The other key responsibilities for CDL are to conduct trainings in External Quality Assurance (EQA), biosafety, and diagnosis of TB. CDL performs EQA in TB with all provincial laboratories in the country as well as with the Supra-National TB Reference Laboratory in Kampala, Uganda. EQA is performed through blinded rechecking, proficiency testing, and on-site supervision. CDL is accredited by the Southern African Development Community Accreditation Services (SADCAS) of South-Africa.

Specimen processing

Sputum specimens were decontaminated and digested in a class II Biological Safety Cabinet (BSC II) using the N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH) method. Briefly, 0.25g of NALC (N-acetyl-L- cysteine) was added in clean falcon tubes, then 50 ml of 4% NaOH (Sodium Hydroxide) and 2.9 % $C_6H_8O_7$ (Citrate) mixture was added to the NALC. A physical examination of the sputum specimens was carried out and the volume of each sputum specimen in the falcon tube was recorded. An equal volume of the NALC-NaOH-citrate mixture was added to an equal volume of sputum, then allowed to stand for 15 minutes for digestion and decontamination of the sputum. 1 ml of phosphate buffer solution [pH 6.8] was added to each specimen mixture to neutralize the NaOH, dilute the homogenate and reduce its viscosity and specific gravity. The specimen mixtures were then centrifuged at 3,000 rpm for 20 minutes to concentrate the acid-fast bacilli. After discarding the supernatant into a disinfectant (5% phenol), the sediment was re-suspended in 3 ml phosphate buffer solution and used for fluorescent microscopy and line probe assay. Only confirmed MTB-positive sputum specimens were used for the detection of MTB resistance to the first-and second-line anti-TB drugs.

Detection of drug-resistant *Mycobacterium tuberculosis* complex

DNA extraction from MTB-positive sputum specimens was done using the GenoLyse kit (Hain Life Science, Germany) according to the manufacturer's instructions. Detection of drug-resistance genes in the DNA samples was achieved by two-line probe assays, Genotype MTBDR*plus* and MTBDR*sl* assays (Hain Lifescience, Germany), that utilise a Polymerase Chain Reaction (PCR)-based assay and a hybridization assay performed on an automated GT Blot 48 device (Hain Lifescience, Germany). Both assays were performed strictly according to the manufacturer's instructions. The Genotype MTBDR*plus* assay was used for detecting resistance against first-line ant-TB drugs, while the MTBDR*sl* assay was used for detecting resistance to second-line anti-TB drugs.

Statistical analysis

Data analysis was performed using STATA version 13.0 statistical software (StataCorp, Lakeway Drive, College Station, Texas, USA). Pearson's Chi-square test was used to find the association between the different types of drug-resistant TB to age and gender. A p-value of less than 0.05 was considered to be statistically significant at 95% confidence interval. Frequencies and percentages were used to determine the level of drug-resistant TB among the new and previously treated TB cases.

Ethics consideration

This was a laboratory-based study, with no direct contact with patients. Permission to conduct the study was obtained from the Zambia, Ministry of Health, as well the Lusaka Provincial Health Office and the Chest Diseases Laboratory in Lusaka, Zambia. Confidentiality was maintained using study-specific numbers to identify the samples. Integrity, professionalism, and Good Clinical Laboratory Practice (GCLP) standards were maintained throughout the study period. Ethics approval was sought from the University of Zambia, School of Health Sciences, Research and Ethics Committee (UNZA-HSREC) (Ethics Approval Number: 20203101001), while the final clearance and approval to conduct the study was obtained from the National Health Research Authority (NHRA).

RESULTS

Demographic characteristics of the study participants

In this study, a total of 329 sputum specimens were analysed, which translates to 329 cases for drug-resistance TB. The cases were categorized into two groups: the first group comprised 102 (31%) new TB cases, while the second group comprised 227 (69%) previously treated TB cases. Among the new TB cases, 80 (78.4%) were males and 22 (21.6%) females, with an age range of 1-90 years and a median age of 33 (Table 1).

In the previously treated TB cases, there were 166 (73.1%) males and 61 (26.9%) females, with an age range of

Table 1: Distribution of the new and previously treated TB cases by demographic characteristics.

New TB Cases (N = 102)			Previously Treated TB Cases (N = 227)		
Variables	Number (n)	Percentage (%)	Variables	Number (n)	Percentage (%)
Gender			**Gender**		
Females	22	21.6	**Females**	60	26.4
Males	80	78.4	**Males**	167	73.6
Age (years)			**Age (years)**		
1 - 19	5	4.9	**4 - 20**	8	3.5
20 - 39	65	63.7	**21 - 37**	128	56.4
40 - 59	26	25.5	**38 - 54**	70	30.8
60 - 79	5	4.9	**55 - 71**	17	7.5
80 - 99	1	1.0	**72 - 88**	4	1.8

Abbreviations: N: Total Number of Cases; n: Number of Cases; TB: Tuberculosis; Percentage = (n/N) x 100%.

4 -78 years (median age 34 years) (Table 1). The previously treated TB cases comprised treatment failure cases, lost to follow-up cases, relapse cases and defaulter cases. The classification of TB patients into the new and previously treated TB cases is important for surveillance of drug-resistance and treatment. The type of treatment for the new and previously treated TB cases differs, thus a great need for categorization of these cases.

Resistance profiles for first-and second-line anti-TB drugs

The prevalence of the different forms of drug-resistant TB among the new TB cases was as follows: 3.9% had rifampicin mono-resistance, 12.8% isoniazid mono-resistance, and 17.7% had both rifampicin and isoniazid resistance (Table 2). Drug-resistant TB was more prevalent in male cases than female cases, and the age range most affected with drug-resistance was 20-39 years among the new TB cases (Tables 2 & 3).

Table 2: Prevalence of drug-resistance among the new and previously treated TB cases.

Type of drug-resistance	New TB cases (N = 102) No. of cases (n) (%)	Previously treated TB cases (N = 227) No. of cases (n) (%)
Susceptible	67 (65.7%)	108 (47.6%)
Monodrug- resistance		
RIF mono-resistance	4 (3.9%)	23 (10.1%)
INH mono-resistance	13 (12.8%)	15 (6.6%)
Multidrug- resistance		
RIF + INH	18 (17.7%)	75 (33.0%)
Polydrug- resistance		
RIF + FLQs	0 (0%)	4 (1.8%)
Pre-extensively drug resistance		
RIF + INH + FLQs	0 (0%)	1 (0.4%)
RIF + INH + KAN	0 (0%)	1 (0.4%)

Abbreviations: N: Total number of cases; No: Number; RIF: Rifampicin; INH: Isoniazid; FLQs: Fluoroquinolones; KAN: Kanamycin; TB: Tuberculosis; Prevalence = (n/N) x 100%.

Table 3: Association between the different types of drug-resistant TB to demographic variables among the new TB cases.

Demographic variables	Mono-drug-resistance (N = 17)		Multidrug-resistance (N = 18)
	RIF (N = 4) n (%)	INH (N = 13) n (%)	RIF + INH n (%)
Gender			
Females	1 (25%)	2 (15.4%)	2 (11.1%)
Males	3 (75%)	11 (84.6%)	16 (88.9%)
Pearson's Chi-square (X^2)- value	1.4165	2.0583	1.8915
p-value	0.493	0.357	0.388
Age (years)			
1-19	0 (0%)	0 (0%)	1 (5.6%)
20-39	2 (50%)	11 (84.6%)	14 (77.8%)
40-59	1 (25%)	2 (15.4%)	3 (16.7%)
60-79	1 (25%)	0 (0%)	0 (0%)
80-99	0 (0%)	0 (0%)	0 (0%)
Pearson's Chi-square (X^2)- value	6.0982	6.9422	5.5664
p-value	0.636	0.543	0.696

Abbreviations: N: Total number of resistant cases; n: Number of resistant cases; RIF: Rifampicin; INH: Isoniazid; TB: Tuberculosis; Percentage = (n/N) X 100%; $p < 0.05$ statistically significant; $p > 0.05$ not statistically significant.

The prevalence of the different forms of drug-resistant TB among the previously treated TB cases was as follows: 10.1% had rifampicin mono-resistance, 6.6% isoniazid mono-resistance, 33.0% had both rifampicin and isoniazid resistance, 1.8% had poly-drug resistance, and finally, 0.8% had pre-extensively drug-resistant TB (Tables 2 & 4). The prevalence of drug-resistant TB was more common in males than in females. The age range most affected with drug resistance TB was 21-37 years among the previously treated TB cases (Table 4).

Association between drug-resistant TB types to age and gender among the TB cases

No association was found between the different types

Table 4: Association between the different types of drug-resistant TB to demographic variables among the previously treated TB cases.

Demographic variables	Monodrug-resistance (N = 38)		MDR (N = 75)	Poly-drug resistance (N = 4)	Pre-XDR (N = 1)	Pre-XDR (N = 1)
	RIF (N = 23) n (%)	INH (N = 15) n (%)	RIF + INH n (%)	RIF + FLQs n (%)	RIF + INH + FLQs n (%)	RIF + INH + KAN n (%)
Gender						
Females	6 (26.1%)	5 (33.3%)	23 (30.7%)	1 (25%)	1 (100%)	0 (0%)
Males	17 (73.9%)	10 (66.7%)	52 (69.3%)	3 (75%)	0 (0%)	1 (100%)
Pearson's Chi-square (X^2) value	4.3554	5.0371	1.0331	0.0043	2.7956	0.3609
p- value	0.499	0.284	0.309	0.948	0.095	0.548
Age (years)						
4 - 20	1 (4.3%)	0 (0%)	2 (2.7%)	0 (0%)	0 (0%)	0 (0%)
21 - 37	18 (78.3%)	9 (60%)	45 (60%)	1 (25%)	1 (100%)	0 (0%)
38 - 54	4 (17.4%)	4 (26.7%)	22 (29.3%)	2 (50%)	0 (0%)	0 (0%)
55 - 71	0 (0%)	2 (13.3%)	6 (8.0%)	1 (25%)	0 (0%)	1 (100%)
72 - 88	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pearson's Chi-square (X^2) value	26.0315	18.5882	2.5878	3.0787	0.7769	12.4076
p- value	0.165	0.291	0.629	0.545	0.942	0.125

Abbreviations: N: Total number of resistant cases; n: number of resistant cases; MDR: Multidrug-Resistance; Pre-XDR: pre-extensively drug resistance; RIF: Rifampicin; INH: Isoniazid; FLQs: Fluoroquinolones; KAN: Kanamycin; Percentage = (n/N) x 100%; $p < 0.05$ statistically significant; *p*-value > 0.05 not statistically significant.

of drug-resistant TB to age and gender among the new or previously treated TB cases. All the p-values were above the significant level of 0.05 at 95% confidence interval (Tables 3 and 4).

Frequency of mutations conferring drug-resistance in M. tuberculosis resistant strains

Mutations were detected in the *rpoB*, *katG*, *InhA*, *gyrA*, and *eis* genes of MTB resistant strains. Overall, 34.3% of the cases had mutations detected among the new TB cases and 52.4% among the previously treated TB cases.

Mutations conferring resistance to Rifampicin

From a total of 4 RIF mono-resistant TB cases detected among the new TB cases, the most frequent mutation conferring resistance to RIF was the *rpoB* MUT 3 (Ser531Leu), with a frequency of 75% (Table 5). Among the previously treated TB cases, a total of 23 RIF mono-resistant cases were detected, and the most frequent mutation was the *rpoB* MUT 3 (Ser531Leu), which had a frequency of 56.5% (Table 6).

Mutations conferring resistance to isoniazid

From a total of 13 INH mono-resistant TB cases detected among the new TB cases, the most frequent mutation conferring resistance to INH was the *InhA* MUT 1 (Cys15Thr), with a frequency of 76.9% (Table 5), while a total of 15 INH mono-resistance cases were detected among the previously treated TB cases, with the most frequent mutation being *katG* MUT 1 (Ser315Thr1), 53.3% (Table 6). MTB resistance to INH was detected in the *inhA* and/or *katG* gene(s).

Mutations conferring resistance to both rifampicin and isoniazid

From a total of 18 multi-drug resistance cases detected among the new TB cases, the most frequent mutation conferring resistance to both RIF and INH was a combination of *rpoB* MUT 3 (Ser531Leu) and *katG* MUT 1(Ser315Thr 1), with a frequency of 27.8% (Table 5). From a total of 75 multi-drug resistant cases detected among the previously treated TB cases, the most frequently detected mutation was a combination of *rpoB* MUT 3 (Ser531Leu) and *katG* MUT 1 (Ser315Thr 1), with a frequency of 29.3% (Table 6).

Mutations conferring resistance to both rifampicin and fluoroquinolones

From a total of 4 poly-drug resistant TB cases detected among the previously treated TB cases, the most frequent mutation conferring resistance to both RIF and fluoroquinolones was a combination of the *rpoB* MUT 2A (His526Tyr) and *gyrA* MUT 1(Ala90Val), with a frequency of 50% (Table 6).

Mutations conferring resistance to both rifampicin and isoniazid plus fluoroquinolones or kanamycin

Two pre-extensively drug-resistant cases were detected among the previously treated TB cases. One of the cases had a mutation profile of *rpoB* MUT 2B (His526Asp), *katG* MUT 2 (Ser315Thr 2), and *eis* MUT 1 (Cys14Thr). While the other case had a mutation profile of *rpoB* MUT 2A (His526Tyr), *katG* MUT 1 (Ser315Thr), and *gyrA* MUT 3C (Asp94Gly). Each of these profiles had a frequency of 50% (Table 6).

Table 5: Frequency of mutations detected in *M. tuberculosis* resistant isolates among the new TB cases.

Genes implicated in drug-resistance	Genotypic profile of drug-resistance	Mutations detected	No. of MTB isolates	Frequency (%)
	Mono drug resistance (n = 17)			
	Rifampicin (n = 4)			
rpoB	rpoB MUT 1	Asp516Val	1	1/4 (25%)
	***rpoB* MUT 3/Δ *rpoB* WT 8**	**Ser531Leu**	**3**	**3/4 (75%)**
	Isoniazid (n = 13)			
inhA* or *katG	***inhA* MUT 1/Δ *inhA* WT 1**	**Cys15Thr**	**10**	**10/13 (76.9%)**
	katG MUT 1	Ser315Thr 1	3	3/13 (23.1%)
	Multidrug-resistance (n = 18)			
rpoB* + *katG* or *inhA	Δ rpoB WT 8 Δ katG WT	Ser531Leu C-315	1	1/18 (5.6%)
	***rpoB* MUT 3/Δ *rpoB* WT 8 *katG* MUT 1/Δ *katG* WT**	**Ser531Leu Ser315Thr 1**	**5**	**5/18 (27.8%)**
	Δ *rpoB* WT 2 *inhA* MUT 1	Glu510His Cys15Thr	1	1/18 (5.6%)
	rpoB MUT 3 *inhA* MUT 1	Ser531Leu Cys15Thr	1	1/18 (5.6%)
	Δ *rpoB* WT 2 *inhA* MUT 1/Δ *inhA* WT 1	Glu510His Cys15Thr	1	1/18 (5.6%)
	rpoB MUT 2B/Δ *rpoB* WT 7 *katG* MUT 1/Δ *katG* WT	His526Asp Ser315Thr 1	1	1/18 (5.6%)
	Δ rpoB WT 3/Δ *rpoB* WT 4 *katG* MUT 1/Δ *katG* WT	Asp516Val Ser315Thr 1	2	2/18 (11.1%)
	Δ *rpoB* WT 1 Δ *rpoB* WT 2 Δ *rpoB* WT 8 *inhA* MUT 1/Δ *inhA* WT 1	C 505-509 Glu510His Ser531Leu Cys15Thr	1	1/18 (5.6%)
	Δ *rpoB* WT 8 *katG* MUT 2	Ser531Leu Ser315Thr 2	1	1/18 (5.6%)
	rpoB MUT 2B *rpoB* MUT 3 *katG* MUT 1	His526Asp Ser531Leu Ser315Thr 1	1	1/18 (5.6%)
	rpoB MUT 2B *katG* MUT 1	His526Asp Ser315Thr 1	1	1/18 (5.6%)
	rpoB MUT 2A /Δ *rpoB* WT 7 *katG* MUT 1/Δ *katG* WT	His526Tyr Ser315Thr 1	1	1/18 (5.6%)
	rpoB MUT 3/Δ *rpoB* WT 8 *inhA* MUT 1/Δ *inhA* WT 1	Ser531Leu Cys15Thr	1	1/18 (5.6%)

Abbreviations: Δ: deletion; WT: Wild Type; MUT: Mutation; ΔWT/MUT: deletion of the wild-type probe and presence of mutation; C: Codon; TB: Tuberculosis; MTB: *Mycobacterium tuberculosis*; Ala: Alanine; Asp: Aspartic acid; Cys: Cysteine; Glu: Glutamic acid; Gly: Glycine; His: Histidine; Leu: Leucine; Ser: Serine; Thr: Threonine; Tyr: Tyrosine; Val: Valine. Mutations with the highest frequency are bold in black.

DISCUSSION

This study highlights the growing threat posed by the emergence of resistant MTB strains responsible for causing the different types of drug-resistant TB in Zambia. The majority of patients identified with drug-resistant TB had a history of being treated for TB previously and interrupted first-line anti-TB therapy. These factors are predictors of the emergence of drug-resistant TB [49-51]. In this study, drug-resistant-TB was found to be high among the previously treated TB patients than among the new TB patients. Drug-resistant TB was high among adult males than females, because of social stigma, cultural habits and poor health-seeking behaviour among males [52].

The prevalence of Rifampicin Resistant (RR)-TB in our study was 3.9% among the new TB cases and 10.1% among the previously treated TB cases. These findings are consistent with the findings reported elsewhere. A previous study conducted in Zambia reported a prevalence for RR at 5.9% among the previously treated TB cases [20]. A similar study conducted in Zimbabwe found the prevalence of RR to be 4.0% among the new TB cases and 14.2% among the previously treated TB cases [53], while a South-African found the burden of RR to be 8.8% among the previously treated TB cases [54]. In Burkina Faso, the prevalence of RR was found at 2.0% and 14.5% among the new and previously treated TB cases, respectively [55]. RR-TB is associated with a history of being treated for TB previously. Therefore, improvement in adherence to treatment halts the emergence or re-emergence of MTB and RR-TB cases [56]. The differences in the reported prevalence for RR-TB from other countries can

Table 6: Frequency of mutations detected in M. tuberculosis resistant isolates among the previously treated TB cases.

Genes implicated in drug-resistance	Genotypic profile of drug-resistance	Mutations detected	No. of MTB isolates	Frequency (%)
	Mono drug resistance (n = 38)			
rpoB	**Rifampicin (n = 23)**			
	***rpoB* MUT 3/Δ *rpoB* WT 8**	**Ser531Leu**	**13**	**13/23 (56.5%)**
InhA and/or *katG*	**Isoniazid (n = 15)**			
	InhA MUT 1/Δ *InhA* WT 1	Cys15Thr	5	5/15 (33.3%)
	***katG* MUT 1/Δ *katG* WT**	**Ser315Thr 1**	**8**	**8/15 (53.3%)**
	katG MUT 1 *InhA* MUT 1/Δ *InhA* WT 1	Ser315Thr 1 Cys15Thr	1	1/15(6.7%)
	Multidrug-resistance (n = 75)			
rpoB + *katG* &/or *InhA*	***rpoB* MUT 3/Δ *rpoB* WT 8 *katG* MUT 1/Δ *katG* WT**	**Ser531Leu Ser315Thr 1**	**22**	**22/75 (29.3%)**
	rpoB MUT 2B/Δ *rpoB* WT 7 *katG* MUT1/Δ *katG* WT	His526Asp Ser315Thr 1	20	20/75 (26.7%)
	rpoB MUT 1 *katG* MUT1/Δ *katG* WT	Asp516Val Ser315Thr 1	1	1/75 (1.3%)
	rpoB MUT 2B *rpoB* MUT 3 *katG* MUT1 *InhA* MUT 1	His526Asp Ser531Leu Ser315Thr 1 Cys15Thr	1	1/75 (1.3%)
	rpoB MUT 2A *katG* MUT1/Δ *katG* WT	His526Tyr Ser315Thr 1	1	1/75 (1.3%)
	rpoB MUT 1 Δ *rpoB* WT 3/Δ *rpoB* WT 4 *katG* MUT 2/Δ *katG* WT	Asp516Val Ser315Thr 2	1	1/75 (1.3%)
	rpoB MUT 3/Δ *rpoB* WT 8 *InhA* MUT 1/Δ *InhA* WT 1	Ser531Leu Cys15Thr	2	2/75 (2.7%)
	rpoB MUT 2B *InhA* MUT 1	His526Asp Cys15Thr	1	1/75 (1.3%)
	Poly drug- resistance (n = 4)			
rpoB + *gyrA*	*rpoB* MUT 2 A Δ *gyrA* WT 2 Δ *gyrA* WT 3	His526Tyr C 89-93 C 92-96	1	1/4 (25)
	***rpoB* MUT 2 A/Δ *rpoB* WT 7 *gyrA* MUT 1/Δ *gyrA* WT 2**	**His526Tyr Ala90Val**	**2**	**2/4 (50%)**
	rpoB MUT 2 A/Δ *rpoB* WT 7 Δ *gyrA* WT 2	His526Tyr C 89-93	1	1/4 (25)
	Pre- extensively drug-resistance (n=2)			
rpoB + *katG* + *gyrA*	*rpoB* MUT 2A/Δ *rpoB* WT 7 *katG* MUT 1 /Δ *katG* WT *gyrA* MUT 3C/Δ *gyr A* WT 3	His526Tyr Ser315Thr 1 Asp94Gly	1	1/2 (50%)
rpoB + *katG* + *eis*	*rpoB* MUT 2B/Δ *rpoB* WT 7 *katG* MUT 2/Δ *katG* WT *eis* MUT 1	His526Asp Ser315Thr 2 Cys14Thr	1	1/2 (50%)

Abbreviations: Δ deletion; WT: Wild Type; MUT: Mutation; ΔWT/MUT: deletion of the wild-type probe and presence of mutation; C: Codon; TB: Tuberculosis; MTB: *Mycobacterium Tuberculosis*; Ala: Alanine; Asp: Aspartic acid; Cys: Cysteine; Glu: Glutamic acid; Gly: Glycine; His: Histidine; Leu: Leucine; Ser: Serine; Thr: Threonine; Tyr: Tyrosine; Val: Valine. Mutations with the highest frequency are bold in black.

be attributed to differences in the sample size used, study settings, study design, the method employed, differences in socio-economic factors, differences in case management of drug-resistant TB, and many other factors.

The prevalence of Isoniazid Mono-Resistance (INHr)-TB in our study was 12.8% and 6.6% among the new and previously treated TB cases, respectively. These findings are consistent with other regions of the world. A low prevalence of INHr among the new TB cases was reported in Tanzania (7.6%) [57], and Ethiopia (9.5%) [58]. Similar studies in South-Korea and Pakistan reported 11% and 9.8%, respectively, for INHr-TB among new TB cases [59,60]. INHr is more common than RR and is a growing public health problem globally because research and policy directions are only focused on RR as a marker for MDR-TB. However, this growing problem of INHr is being ignored globally [61].

MDR-TB is one of the most serious public health problems in the world [55,62]. Its prevalence is increasing in every part of the world today, for both new and previously treated TB cases [63]. In the current study, the prevalence of MDR-TB was high, 17.7% among the new TB cases and 33.0% among the previously treated TB cases. These

findings were consistent with those reported in India in which the prevalence of MDR-TB was found to be 11.4% and 36.4% among the new and the previously treated TB cases, respectively [64]. Another study conducted in Ethiopia reported 11.6% and 32.7% MDR prevalence among the new and previously treated cases, respectively [65], while that in China reported the prevalence of MDR-TB at 30.4% among the previously treated cases [66]. MDR-TB is a growing public health threat in Zambia, and the WHO has projected that by 2021 the country will have a high burden of the disease [23,24]. DR-TB is not caused by a single factor, but by several factors which come into play and some of these include: poor adherence to TB treatment, irregular supply and stock-out of anti-TB drugs, inappropriate TB therapy, delayed diagnosis and initiation of ineffective therapy as well as spontaneous chromosomal mutations [1,30,33,58]. Liang and colleagues in their study found that inappropriate treatment was the most common cause of MDR-TB [66]. Failure to implement effective TB prevention and control measures also facilitates the emergence of the different forms of DR-TB including MDR-TB [67-69]. The key factors above including treatment failure, TB relapse, treatment defaulting, MDR contacts, loss to follow-up cases, and misdiagnosis could be contributing to the reasons for the observed rise in the number of MDR-TB cases in Zambia. MDR-TB must be managed effectively to reduce mortality, morbidity, and the eventual transmission of MTB resistant strains [70].

In this study, the prevalence of poly drug-resistant TB was found to be low (1.8%) among the previously treated TB cases. This finding was similar to studies conducted in Ethiopia (2.3%) and Canada (0.4%) [71,72]. However, studies elsewhere reported a high prevalence of poly drug-resistance among the previously treated: India reported 12.0% prevalence [64], Bangladesh 8.6% [72] and Sudan 6.4% [72]. Poor case management of mono-drug resistant TB results in poly drug-resistant TB.

The prevalence of pre-XDR TB in our study was low (0.8%) among the previously treated TB cases. This result is similar to that reported in Ethiopia, 5.7% [73]. Studies elsewhere reported a high prevalence of pre-XDR: India (56%), China (34%), Nepal (28%), Zimbabwe (27%, Nigeria (17%) and Bangladesh (16.2%) [73-75]. The high prevalence of pre-XDR TB can indicate poor management of MDR-TB cases. Poorly managed MDR-TB cases progress to pre-XDR TB, which eventually get to XDR-TB.

In this study, no association was found between the different types of Drug-Resistance (DR) to age and gender among the new and previously treated TB cases. This finding was similar to other countries such as Sudan [76], Nigeria [77], Ethiopia [78], and Egypt [79]. Age and gender were not associated with drug-resistance because these variables are not significant risk factors. Studies have shown that the key risk factors associated with the development of drug-resistant TB include: history of previous treatment for TB, poor adherence to TB treatment, inadequate supply and stock-out of anti-TB drugs, poor management of TB, and contact with DR-TB patients [30,33,58,78-79].

Drug-resistant MTB strains use various complex mechanisms to inactive or resist anti-TB drugs. Among these are point mutations in chromosomal genes, which can be an insertion, deletion or missense [80-82]. Molecular assays detect mutations that confer resistance to anti-TB drugs. In this study the *rpo*B MUT 3 (Ser531Leu) mutation was the most frequently detected (75%), conferring resistance to rifampicin, among the new TB cases. This result is similar to that reported in Brazil (75%) [83], and in Ethiopia (74.2% and 77.1%) [84,85]. In contrast, other studies elsewhere reported a low frequency of the mutation, with Canada reporting 47.8% [86], India reporting 46.9% and 19.5% in two separate studies [82,87], and Uganda reporting 40% [37]. Based on these results it suffices to say, mutation frequencies vary from one geographical location to another due to differences in the epidemiology of TB, differences in geographic transmission environments for MTB, as well as differences in treatment combinations used in the management of cases.

The *kat*G MUT 1 (Ser315Thr 1) mutation was the most frequently (53.3%) encountered mutation conferring resistance to INH among the previously treated TB cases in this current study. This finding is consistent with studies in Taiwan (50.4%), Myanmar (57.3%) and India (57.8%) [1,88]. Studies have shown that 50-95% of MTB resistant strains have mutations in the *kat*G gene [44]. A high frequency of the *kat*G Ser315Thr 1 mutation is associated with countries with a high prevalence of TB, such as Zambia [44]. The *Inh*A MUT1 (Cys15Thr) mutation was also frequently (76.9%) detected among the new TB cases in this current study and this finding is consistent with findings from similar studies in other countries such as Ethiopia (77.5%) [85], South-Africa (70.1%) [89], and India (85.9%) [1]. It has been shown that the Ser315Thr 1 mutation is the most frequent in the *kat*G gene that confers resistance to INH, while the Cys15Thr mutation is the most frequent in the *Inh*A gene that confer resistance to INH [90].

Mutations conferring resistance to both RIF and INH were detected in our study. These mutations are responsible for causing MDR-TB. The *rpo*B MUT 3 (Ser531Leu) and the *kat*G MUT1 (Ser315Thr 1) mutations were the most frequent (27.8%) conferring resistance to both RIF and INH, among the new TB cases. This same mutation was the most frequent among the previously treated TB cases. These findings are similar to results obtained in Thailand (36.4%) [25].

This study also detected other mutations such as the *rpo*B MUT 2A (His526Tyr) and *gyr*A MUT 1 (Ala90Val) with a frequency of 50%, that confer resistance to both RIF and FLQs among the previously treated TB cases and was consistent

with findings reported in Ethiopia where the mutational frequency was found to be 50% [65]. The Ala90Val mutation is associated with high-level resistance to FLQs [41]. Studies have shown that most mutations associated with FLQ resistance occur in the *gyrA* gene on codon 90–94 of MTB resistant strains [65]. This is in agreement with our findings. Misuse of FLQ antibiotics has contributed to resistance in MTB strains, associated with mutations in the *gyrA* gene [41,42].

In this study other multiple mutations detected were associated with resistance in the *rpoB*, *katG*, *gyrA* and *eis* genes. These combined mutation profiles were responsible for causing pre-XDR TB and each had a frequency of 50%. One of these combination mutations had a profile: *rpoB* MUT 2B (His526Asp), *katG* MUT 2 (Ser315Thr 2), and *eis* MUT 1 (Cys14Thr). The other had *rpoB* MUT 2A (His526Tyr), *katG* MUT 1 (Ser315Thr), and *gyrA* MUT 3C (Asp94Gly). Unlike the high frequency of these mutations in this study, studies elsewhere, such as Morocco and India, reported low mutational frequency in these genes [3,91]. Mismanagement of DR-TB cases result in resistance associated with multiple mutations in MTB genes.

CONCLUSION

Drug-resistant TB is prevalent in Zambia, especially MDR-TB, is hampering efforts in the control of TB. DR-TB is mainly caused by mutations in the target genes of resistant MTB strains. Mutations identified in this study were the *rpoB* (Ser531Leu, His526Asp, Asp516Val, His526Tyr, and Glu510His), *InhA* (Cys15Thr), *gyrA* (Ala90Val and Asp94Gly), *katG* (Ser315Thr 1 and Ser315Thr 2), and *eis* (Cys14Thr), each with a varying frequency. Zambia needs to scale-up on the number of laboratory sites performing genotypic drug-susceptibility testing, effectively treat DR-TB, especially MDR-TB, and implement effective TB control strategies to combat DR-TB.

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