Determination of the association of minimum inhibitory concentrations of antituberculosis drugs and resistance mutations of clinical isolates of *Mycobacterium tuberculosis* from Limpopo Province, South Africa

By

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Dissertation

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DECLARATION

I declare that the dissertation '**Determination of the association of minimum inhibitory concentrations of antituberculosis drugs and resistance mutations of clinical isolates of *Mycobacterium tuberculosis* from Limpopo Province, South Africa'** hereby submitted to the University of Limpopo, for the degree of Master of Science in Medical Sciences has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

Signature  Date: 07 October 2024

DEDICATION

This research project is dedicated to my family (Father Andries and Mother Dineo Malope), who always supported me throughout my studies. Your support, love and comfort made this possible.

I thank you.

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ABSTRACT

Background: Tuberculosis (TB) maintains its position as the leading global infectious disease, and South Africa is among the countries most affected by drug-resistant strains. The situation of tuberculosis is exacerbated by the emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), which presents significant challenges to TB control efforts, especially in regions such as Limpopo Province, where high transmission rates and limited resources worsen the problem.

Genetic mutations are the primary drivers of drug resistance, altering the effectiveness of both first-line and second-line antituberculosis drugs. Despite the revolution in TB detection and treatment brought about by molecular diagnostics, it remains necessary to deepen our understanding of how these genetic mutations impact the level of drug resistance. The minimum inhibitory concentration (MIC) of anti-TB drugs, which indicates the lowest concentration needed to inhibit bacterial growth, plays a crucial role in clinical decision making. However, current diagnostic tools often do not account for the correlation between MIC levels and specific resistance mutations, especially for newer drugs like Bedaquiline.

This study aimed to address the gap by examining the relationship between MICs and resistance mutations in *Mycobacterium tuberculosis* clinical isolates from Limpopo province. By utilizing advanced molecular techniques such as whole genome sequencing (WGS) and phenotypic drug susceptibility testing.

Methods: This cross-sectional, quantitative study analyzed 281 clinical isolates from the tuberculosis repository of the Polokwane National Health Laboratory Service (NHLS). The MICs for levofloxacin (LVX), moxifloxacin(MXF), and bedaquiline (BDQ) were determined using the BD BACTEC MGIT 960 system. Whole genome sequencing (WGS) was performed on phenotypically resistant clinical isolates to identify mutations associated with drug resistance. Statistical analyses, including T-tests and logistic regression, were performed to correlate MICs with specific resistance mutations.

Results: A total of 147 drug-resistant tuberculosis clinical isolates met the study inclusion criteria and were tested for MIC. Seventy-seven (52.4%) isolates were from

men in the age group of 26-45 years with a mean age of 39 (IQR 18 to 46) years. A total of 139 (94.6%) were susceptible to LVX while 144 (98.0%) and 128 (87.1%) were susceptible to MXF and BDQ, respectively. Of the eight fluoroquinolone (FLQ)-Not resistant clinical isolates, most were associated with mutations in the *gyr*A gene. The most common mutations were substitutions in Condon 94 of 6 (75.5%), leading to low-level resistance. In bedaquiline-resistant clinical isolates, mutations were identified in the *Rv*0678, *Rv1979c,* and *atp*E genes, with most mutations linked to increased levels of resistance.

Conclusions In conclusion, this study identified key mutations in *Mycobacterium tuberculosis* clinical isolates from Limpopo province that are associated with resistance to LVX, MXF, and BDQ. These findings highlight the need for continuous monitoring of resistance mutations and MIC levels to inform clinical decision-making and optimize treatment regimens for DR-TB (drug resistant tuberculosis). Understanding the genetic drivers of drug resistance is crucial for developing effective diagnostic tools and therapeutic strategies.

Keywords: Tuberculosis; Drug-resistant tuberculosis; Levofloxacin; Moxifloxacin; Bedaquiline; Minimum inhibitory concentration; mutation; critical concentration: Whole genome sequencing.

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LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTION

Publications

1. **Malope P, Lekalakala-Mokaba RM, Said HM, Omar SV, Rukasha I**: Molecular characterization of levofloxacin, moxifloxacin, and bedaquiline in drug-resistant tuberculosis clinical isolates in Limpopo province, South Africa, 2024. To be submitted for publication.
2. **Malope P, Lekalakala-Mokaba RM, Ngcamu D, Omar SV, Rukasha I:** Phenotypic characterization of DR-TB clinical isolates against levofloxacin, moxifloxacin, and bedaquiline in Limpopo province, South Africa, 2024. Submitted for publication in SAMJ.
3. **I Rukasha, STS Pheeha, MR Lekalakala, and P Malope:** Determination of anti-COVID-19 IgG and IgM seroprevalence among pregnant women at Pietersburg Hospital, Limpopo Province, South Africa. Published Online; *S Afr Med J 2024*;114(3): e702. https://doi.org/10.7196/SAMJ.2024.v114i3.702

Conference Contributions

1. **Malope P, Lekalakala-Mokaba RM, Rukasha I:** Drug-resistance profiles of tuberculosis from Limpopo Province, South Africa 2011 -2019. 7th SA TB Conference 2022: 13 -16th September Durban ICC Convention Centre, South Africa (Poster presentation)

2. **Mokgatsana ZQ, Mukavhanyedzi D, Kaapu KG, Malope P, Lekalakala-Mokaba, MR, Rukasha I**: Seroprevalence of anti-SARS-CoV-2 IgM and IgG antibodies in pregnant women, in Pietersburg Hospital, Limpopo Province FIDSSA Conference: 3-5 November 2022 (oral presentation)

3. **Malope P, Kaapu KG, Lekalakala-Mokaba RM, Rukasha I:** Association of HIV infection with the unfavourable drug-resistant pulmonary tuberculosis treatment outcomes, Limpopo, South Africa: FHS Research Day Conference 2023: 18 – 19 September University of Limpopo (oral presentation)

4. **Malope P, Kaapu KG, Milanzi R, Lekalakala-Mokaba RM, Rukasha I:** Association of HIV infection with the of unfavourable drug-resistant pulmonary tuberculosis treatment outcomes, Limpopo, South Africa**:** PathReD: Enriching

Health Through Research 2023 31 August - 03 September 2023 Pathology Research and Development Congress (Poster presentation).

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LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
APM	agar proportion method
AST	antibacterial susceptibility testing
CCs	critical concentrations
DNA	deoxyribonucleic acid
DR-TB	drug-resistant tuberculosis
DST	drug susceptibility testing
ECOFF	epidemiological cut-off
HIV	human immunodeficiency virus
INH	isoniazid
MDR-TB	multi drug-resistant tuberculosis
MGIT	mycobacterium growth indicator tube
MICs	minimum Inhibitory Concentrations
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NHLS	National Health Laboratory Services
NICD	National Institute of Communicable Diseases
RIF	rifampicin
BDQ	bedaquiline
MXF	moxifloxacin
LVX	levofloxacin
RR-TB	rifampicin-resistant tuberculosis
TB	tuberculosis
WHO	World Health Organization
XDR-TB	extensively drug-resistant tuberculosis

DEFINITION OF CONCEPTS

The terms in this study will be used as defined below unless otherwise stated.

Antituberculosis agents: These are a group of drugs used to treat tuberculosis (Khawbung, Nath and Chakraborty, 2021).

Clinical isolates: Refers to an organism isolated from an infected host and propagated in a culture (Pitt, Boampong, Day, *et al.*, 2022).

Drug susceptibility testing: Measurement of the ability of an antibiotic or other antimicrobial agent to inhibit *in vitro* microbial growth (Kowalska-Krochmal and Dudek-Wicher, 2021).

Minimum Inhibitory Concentration: Lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after incubation, under strictly controlled *in vitro* conditions (Chikezie, 2017; Kowalska-Krochmal and Dudek-Wicher, 2021)

Resistance mutation: This is a mutation in a bacterial gene that allows bacteria to become resistant to treatment with a particular antibiotic drug (Jian, Zeng, Xu, *et al.*, 2021)

CHAPTER 1

INTRODUCTION

1.1 General Background

Tuberculosis (TB) continues to be a significant global health issue and ranks among the top 10 causes of death worldwide, surpassing HIV/AIDS in terms of global mortality (WHO, 2020a, 2023). The impact of tuberculosis is particularly felt in low and middle-income countries, with seven nations responsible for 64% of new TB cases. South Africa bears a heavy burden, contributing to 3.6% of global TB infections (WHO, 2020a).

The challenge of drug-resistant TB (DR-TB) adds to the complexity, with multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) presenting significant concerns. Multidrug resistant TB involves resistance to rifampicin (RR-TB) and isoniazid (INH) (WHO, 2018a, 2020a, 2023). Extremely drug resistant TB includes resistance to fluoroquinolones and at least one additional Group A drug, such as LVX, MXF, BDQ, and linezolid (WHO, 2018a, 2020a, 2023). South Africa ranks third globally in treating DR-TB, following Russia and India (WHO, 2022a).

The global increase in DR-TB cases underscores the importance of rapid and accurate drug susceptibility testing (DST). The WHO End TB Strategy, which aims to eliminate TB by 2035, emphasizes the need for comprehensive DST (WHO, 2018a, 2022a). Current phenotypic DST methods, including Lowenstein-Jensen, 7H11 agar media, and liquid-based systems such as MGIT 960, typically classify TB isolates as resistant or susceptible based on a single concentration (Rancoita, Cugnata, Gibertoni Cruz, *et al.*, 2018; Nonghanphithak, Kaewprasert, Chaiyachat, *et al.*, 2020; Campelo, Cardoso de Sousa, Nogueira, *et al.*, 2021; Köser, Georghiou, Schön, *et al.*, 2021). However, these methods oversimplify resistance as binary, disregarding varying levels of drug resistance, which is crucial for treatment decision-making (Gygli, Keller, Ballif, *et al.*, 2019).

Molecular diagnostics has revolutionized the diagnosis of tuberculosis, offering rapid and cost-effective methods to detect drug resistance. However, these tools often do not consider how different genetic mutations impact drug resistance levels in various TB drugs. Consequently, research has turned to exploring the relationship between

drug resistance mutations and the minimum inhibitory concentration (MIC) of drugs, which represents the lowest concentration required to inhibit bacterial growth (Heysell, Pholwat, Mpagama, *et al.*, 2015; Rancoita, Cugnata, Gibertoni Cruz, *et al.*, 2018; Barilar, Battaglia, Borroni, *et al.*, 2024).

Studies indicate a strong correlation between MIC levels and specific mutations in genes encoding drug targets, providing a more comprehensive understanding of resistance mechanisms (Klopper, Warren, Hayes, *et al.*, 2013; Klopper, Heupink, Hill-Cawthorne, *et al.*, 2020; Nonghanphithak, Kaewprasert, Chaiyachat, *et al.*, 2020). Minimum inhibitory concentration data offer valuable information to clinicians, enabling them to adjust drug dosages or treatment regimens to enhance patient outcomes (Ruesen, Riza, Florescu, *et al.*, 2018; Kowalska-Krochmal and Dudek-Wicher, 2021). For example, understanding the MIC for a particular strain allows physicians to better tailor therapies based on the strain's resistance profile. Furthermore, research demonstrates that molecular predictions of drug resistance can be improved by correlating MIC levels with specific genetic mutations, particularly for newer and repurposed drugs such as BDQ and linezolid (Kambli, Ajbani, Sadani, *et al.*, 2015; Banerjee and Patel, 2023).

This study focused on examining the association between the MICs of key second-line anti-TB drugs (LVX, MXF, and BDQ) and the resistance mutations found in clinical isolates from Limpopo Province, South Africa. The study aimed to: i. Determine MICs of second-line anti-TB drugs using the BD BACTEC MGIT 960 phenotypic drug susceptibility testing method. ii. Identify genetic mutations associated with drug resistance by performing whole genome sequencing (WGS) on resistant isolates. iii. Correlate MICs with specific resistance mutations to assess how these mutations influence resistance to LVX, MXF, and BDQ. Integrating both phenotypic and genotypic data. Furthermore, this study seeks to provide a more comprehensive understanding of drug resistance in Limpopo Province, which has a high burden of TB and DR-TB. Correlating MICs with specific mutations could allow for more personalized TB treatment approaches, ultimately reducing treatment failures and limiting the spread of DR-TB.

The findings of this study could contribute to a better understanding of how MIC data can improve TB management. As TB programs increasingly move toward

personalized treatment strategies, the integration of molecular diagnostics with detailed resistance profiling would be critical to inform future guidelines. Additionally, the results could contribute to the development of new diagnostic tools that incorporate MIC testing into routine diagnostics, giving clinicians more comprehensive data for guiding treatment decisions.

1.2 Research Problem

Molecular diagnostics are considered the most promising route to achieve rapid universal drug susceptibility testing for *Mycobacterium tuberculosis* complex (MTBC). However, to date, molecular diagnostics have focused largely on first-line drugs and predict susceptibilities in a binary manner (classifying strains as susceptible or resistant). Furthermore, the mutation sites of TB drug resistance vary between different countries and regions and change over time. Therefore, it is necessary to continuously associate specific mutations that lead to drug resistance with specific MICs for specific drugs, especially second-line drugs. This is necessary to predict resistance levels, which can be utilized to direct the development of novel assays that enhance clinical decision-making and yield fast findings.

Thus, the aim of the study was to determine the association of the MICs of anti-TB drugs (Levofloxacin and Moxifloxacin and Bedaquiline) with resistance mutations of *Mycobacterium tuberculosis* clinical isolates for profiling drug resistance.

1.3 Purpose of the Study

1.3.1 Aim:

To determine the association of MICs of anti-TB drugs with resistance mutations of *Mycobacterium tuberculosis* clinical isolates for profiling drug resistance.

1.3.2 Objectives:

1.3.2.1 Determine the MICs of anti-TB drugs by performing susceptibility testing on *Mycobacterium tuberculosis* clinical isolates using second-line drugs (levofloxacin and moxifloxacin) and a new drug (bedaquiline) using the BD BACTEC MGIT 960 phenotypic drug susceptibility testing based method.

1.3.2.2 Determine genetic mutations of the phenotypic resistant strain for the second line and new drugs by whole genome sequencing (WGS).

1.3.2.3 Determine the association of different genetic resistance mutations with the MIC of *Mycobacterium tuberculosis* clinical isolates for second-line and new anti-TB drugs.

1.4 Research Question:

What is the association of MICs of anti-TB drugs with genetic mutations of DR-TB *Mycobacterium tuberculosis* isolates from Limpopo province?

1.5 Significance of the Study

This study determined the genetic variation of *Mycobacterium tuberculosis* in this region and its drug susceptibility profiles at the MIC level. The resistance level expressed by MICs is important to guide therapeutic decision-making. This could assist healthcare providers developing appropriate treatment regimens to maximize effective treatment outcomes and minimize the risk of drug-resistant tuberculosis development. Furthermore, knowing the circulating strain in the region would help create point-of-care assays to diagnose patients in the hospital.

Health care workers

This study would inform clinical decision-making when treating patients with drug-resistant tuberculosis in Limpopo Province. Healthcare workers can tailor their treatment plans to improve patient outcomes by having knowledge of the circulating strains and drugs they are susceptible to.

1.6 Theoretical framework

The theoretical framework is a diagrammatic outline of the direction of the entire protocol. It serves as a guide for construction, supports the basis of the study done, and provides the structure of the whole dissertation. The framework consists of concepts and theories that are linked in the same field (Grant et al., 2014).

The theoretical framework of this study is based on the theory of biological evolution of Charles Darwin, which states that “all species of an organism arise and develop through the natural selection of small, inherited variations that increase the individual's ability to compete, survive and reproduce”. The new generations harbour a feature that enables their survival in adverse environments; in the current study, the drug-resistant *Mycobacterium tuberculosis* (RR/MDR-TB and XDR-TB) is dominant. The

environment filters out organisms that are poorly fitted, which is called survival of the fittest according to the Darwinism theory of evolution. In the current study, the wild-type strain of *Mycobacterium tuberculosis* is filtered in the presence of a certain concentration (MIC) of an antituberculosis drug and parish. However, resistant associated variant strains survive despite the presence of the antibiotic; this is due to the acquired mutation within the *Mycobacterium tuberculosis genome* against the drugs, which supports the theory of Darwinism of evolution. The main route of development of resistance to *Mycobacterium* is through; i, under drug-resistant dosage treatment or non-compliant treatment, leading to ii, gene mutation within the bacterial genome, which protects the cell against the effects of anti-TB drugs (Figure 2.1). This study aimed to determine the association between the minimum inhibitory concentration of the antituberculosis drug and the resistance mutation in Limpopo province.

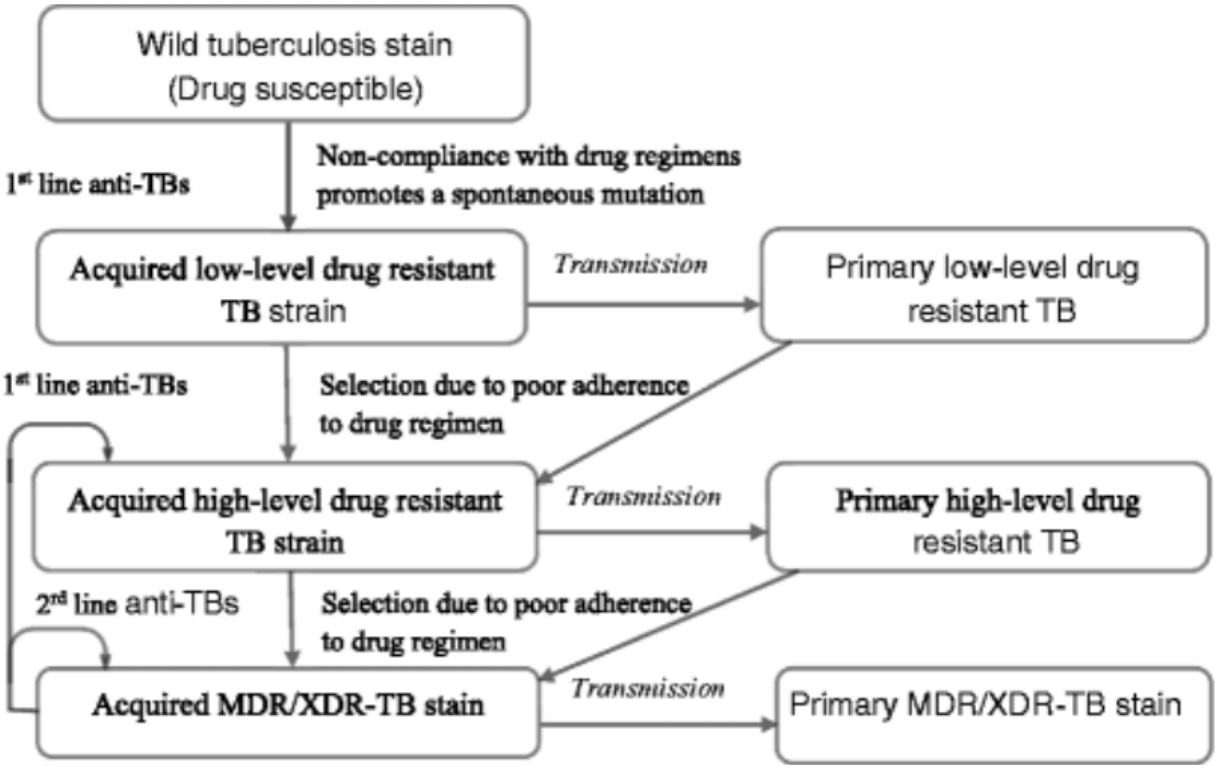

Figure 1.1: Simplified theoretical stepwise development of drug resistance in *Mycobacterium tuberculosis* due to mutations. High-level drug resistance is the result of an accumulation of chromosomal mutations in drug targets, which may be enhanced

by epistatic interactions. The accumulation of mutations in and out of the drug targets, working in concert, may yield or catalyse the evolution of MDR-/XDR-TB strains.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

Tuberculosis remains one of the main causes of the global health problem and ranks alongside the human immune-deficiency virus (HIV) as the leading cause of death worldwide (WHO, 2023). Geographically, the burden of tuberculosis (TB) is highest in Asia and Africa. The African region has approximately a quarter (25%) of the world's cases and death rates relative to the population (WHO, 2020a). South Africa is ranked among the 22 high-risk countries, contributing approximately 80% of the total global burden of TB cases (WHO, 2020a; Chakaya, Khan, Ntoumi, *et al.*, 2021). Historically, TB has been known to humanity since ancient times and was present in the environment for about 150 million years ago (Daniel, 2006; Barberis, Bragazzi, Galluzzo, *et al.*, 2017).

The *Mycobacterium tuberculosis* species belong to the Mycobacteriaceae family, under the order Actinomycetales and Genus Mycobacterium (Koch and Mizrahi, 2018). *Mycobacterium tuberculosis* is a non-encapsulated, non-spore-forming, non-motile, and slow-growing aerobic bacillus (Gordon and Parish, 2018; Koch and Mizrahi, 2018). Microscopically, the bacilli appear as slightly curved rods, with filamentous and branching cords (Chinta, Saini, Glasgow, *et al.*, 2016).

The mycobacterial cell envelope consists of three covalently connected macromolecules, which form peptidoglycans that contain residues of meso-diaminopimelic acid, N-glycosylated muramic acid, arabinogalactan, and mycobacterial acids (Besra and Brennan, 2011; Daffé, 2015). The laboratory strain of *Mycobacterium tuberculosis* H37Rv was completely sequenced in 1998 and found to comprise 4 411 532 base pairs (bp) (Kashyap, Bhullar, More, *et al.*, 2014; Guyeux, Sola and Refrégier, 2019; Wibberg, Price-Carter, Rückert, *et al.*, 2021). Transmission of *Mycobacterium tuberculosis* is by inhalation of droplet nuclei through the mouth or nasal passages of the upper respiratory tract (Farhat, Shapiro, Kieser, *et al.*, 2013; Chinta, Saini, Glasgow, *et al.*, 2016; Churchyard, Kim, Shah, *et al.*, 2017). The clinical manifestations of tuberculosis occur gradually in phases, including latent, primary progressive diseases, and extrapulmonary diseases. In addition, the clinical

manifestations vary depending on age, immune status, and coexisting diseases (Cardona, 2016). People with latent tuberculosis do not have symptoms of the disease, feel no illness, or have no infectious diseases (Bucsan, Rout, Foreman, *et al.*, 2019).

Screening and treatment for the prevalence of *Mycobacterium tuberculosis* are often recommended for high-risk groups, including persons from regions and populations where tuberculosis is endemic (Africans and Asian descendants), people with a high risk of reactivation diseases, including HIV-positive people (Ai, Ruan, Liu, *et al.*, 2016; Ang, Vasconcelos-Santos, Sharma, *et al.*, 2018). Tuberculosis (TB) prophylaxis, also known as tuberculosis preventive therapy (TPT), a course of anti-tuberculosis medication that prevents the development of TB disease. Recommended for people who are at higher risk of developing TB, such as those who: Have been exposed to TB or Have HIV or other conditions that weaken their immune system.

For the diagnosis of *Mycobacterium tuberculosis*, sputum smear microscopy is the most commonly used method for both dead tuberculosis and active tuberculosis, especially in a limited-resource environment (Yuen, Amanullah, Dharmadhikari, *et al.*, 2015; Graham and Sekadde, 2019). The culture of *Mycobacterium tuberculosis* is the current reference method for the diagnosis of tuberculosis or the gold standard method (Heysell, Pholwat, Mpagama, *et al.*, 2015; Ahmad, Mokaddas, Al-Mutairi, *et al.*, 2016).

The detection of genetic mutations associated with resistance to certain antibiotics using molecular methods is considered an instrument for research and diagnostics (Erkose Genc, Satana, Yildirim, *et al.*, 2018). DNA sequencing-based approaches are considered reference molecular tests for detecting mutations and provide the highest level of information (Morini, Sangiuolo, Caporossi, *et al.*, 2015).

2.2 Classification of *Mycobacterium tuberculosis*

Mycobacterium is a genus of Actinobacteria and a member of the Mycobacteriaceae family (Table 3.1) (Gordon and Parish, 2018). The name *Mycobacterium tuberculosis* is derived from the Greek words 'fungi' (myces) and 'small rod' (bacteria), suggesting that when samples are cultured, mycobacteria can grow on solid surfaces, producing mould-like colonies (Gordon and Parish, 2018).

Table 2 1: Taxonomic classification of *Mycobacterium tuberculosis* (Palomino, Martin, Von Groll, *et al.*, 2008; Gordon and Parish, 2018).

Kingdom	**Bacteria**
Phylum	Actinobacteria
Class	Actinobacteria
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Corynebacteriaceae
Family	Mycobacteriaceae
Genus	*Mycobacterium*
Species	*M. tuberculosis*

2.3 Characteristics and properties of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is a slow growing aerobic bacillus, that is non-encapsulated, non-spore-forming, and non-motile (Gordon and Parish, 2018; Koch and Mizrahi, 2018). The bacilli appear as slightly curved rods microscopically, occasionally branching and filamentous. They form mould-like pellicles in a liquid medium, hence the name Mycobacterium, which is derived from the word "mould", meaning fungus-like bacterium (Chinta, Saini, Glasgow, *et al.*, 2016). *Mycobacterium tuberculosis* is an obligate intracellular pathogen that multiplies within phagocytic cells, especially macrophages and monocytes (Chinta, Saini, Glasgow, *et al.*, 2016; Libbing, McDevitt, Azcueta, *et al.*, 2019). The bacilli are slow-growing pathogenic *Mycobacterium species*, characterised by a 12-to-24-hour division rate and a long culture period characterised by visible growth in solid media, and liquid media which can be seen in 3 to 8 weeks. *Mycobacterium tuberculosis* has an unusual waxy coating on its cell wall (surface), primarily due to the presence of mycolic acid (Gordon and Parish, 2018). This coating renders the cell impervious to Gram staining, and as a result, it cannot appear as Gram-negative or Gram-positive.

2.4 Transmission of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is carried in airborne particles, called droplet nuclei of 1 to 5 micrometres in diameter (Farhat, Shapiro, Kieser, *et al.*, 2013). Infectious droplets

nuclei are generated when persons with pulmonary or laryngeal tuberculosis (TB) cough, sneeze, shout, or sing (Farhat, Shapiro, Kieser, *et al.*, 2013; Churchyard, Kim, Shah, *et al.*, 2017; Jiang, Liu, Ji, *et al.*, 2020; Reichler, Khan, Sterling, *et al.*, 2020). Depending on the environment, these tiny particles can be suspended in the air for several hours and are removed several hours later (Churchyard, Kim, Shah, *et al.*, 2017).

Transmission occurs when a person inhales droplets containing *Mycobacterium tuberculosis,* and the droplets traverse the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli of the lungs (Farhat, Shapiro, Kieser, *et al.*, 2013; Chinta, Saini, Glasgow, *et al.*, 2016; Churchyard, Kim, Shah, *et al.*, 2017). Other modes of transmission are much less common and include cutaneous or mucous inoculation. Inoculation as a method of transmission of *Mycobacterium tuberculosis* has been reported in laboratory personnel (Churchyard, Kim, Shah, *et al.*, 2017). A rare cause of digestive transmission of TB can occur with *M. bovis*, most commonly through cow's milk (Churchyard, Kim, Shah, *et al.*, 2017). Transmission of *Mycobacterium tuberculosis* is more likely to be high in crowded places like public transport, schools, workplaces, prisons, and healthcare facilities (Jiang, Liu, Ji, *et al.*, 2020; Reichler, Khan, Sterling, *et al.*, 2020).

2.5 Clinical Manifestations of Pulmonary and extra-pulmonary TB

The clinical manifestations of tuberculosis (TB) progress in stages that include latent, primary progressive disease, and extrapulmonary disease. The clinical manifestations of TB vary depending on age, immune status, and co-existing diseases (Cardona, 2016). The individuals with latent TB does not have symptoms or feel ill and is not infectious. (Bucsan, Rout, Foreman, *et al.*, 2019). Approximately a third of the world's population is infected with *Mycobacterium tuberculosis* without symptoms (Bucsan, Rout, Foreman, *et al.*, 2019; Tantivitayakul, Juthayothin, Ruangchai, *et al.*, 2020). The *Mycobacterium tuberculosis* organisms are enclosed and inactive. However, viable bacteria can survive in necrotic matter for several years or even their entire life if the immune system is not compromised (Ahmad, 2010). Active TB occurs only in between 5 % and 10 % of people exposed to *Mycobacterium tuberculosis* (Essone, Leboueny, Maloupazoa Siawaya, *et al.*, 2019). Although co-infection with HIV/AIDS is the most significant cause of the progression of active diseases, other factors associated with

transplant-related, diabetes, sepsis, renal failure, older age, malnutrition, smoking, chemotherapy, and immune suppression may trigger the reactivation of secret tuberculosis (Cardona, 2016; Lee, 2016).

The manifestations of TB symptoms include progressive fatigue, malaise, weight loss, low fever accompanied by chills and night sweat (Wanzala, Kiptoo, Githui, *et al.*, 2019; Rameshwari, Jayashree, Anuradha, *et al.*, 2021). The classic wasting TB characteristic is due to lack of appetite, resulting in loss of fat and weakened tissue. In most patients, the cough develops and eventually develops into a productive cough with purulent sputum. The sputum may also be bloody (Haemoptysis) due to the destruction of vessels on the walls of the cavity or the formation of aspergilloma in the old cavity, due to a secondary infection by the mould aspergillus (Rameshwari, Jayashree, Anuradha, *et al.*, 2021).

Extrapulmonary disease occurs in more than 20 - 34 % of immune-competent patients, and with immune suppression, the risk of extrapulmonary tuberculosis increases. Miliary tuberculosis progresses rapidly and is difficult to diagnose due to its systemic and nonspecific signs and symptoms such as fever, weight loss, and muscle weakness (Rameshwari, Jayashree, Anuradha, *et al.*, 2021). The most serious place for extrapulmonary tuberculosis is the central nervous system, where infection can almost cause deadly tuberculosis or space-occupying tuberculomas. Other locations involved in non-pulmonary TB include bones, joints, pleura, lymphatic, genitourinary systems, pericardial tuberculosis, gastrointestinal tuberculosis, and showing symptoms such as difficulty in swallowing (Ravikumar P., 2017; Rameshwari, Jayashree, Anuradha, *et al.*, 2021; Sharma, Mohan and Kohli, 2021).

2.6 Treatment

A 6-month treatment regimen composed of four first-line tuberculosis (TB) medicines, namely isoniazid, rifampicin, ethambutol, and pyrazinamide, is recommended for the treatment of drug-susceptible TB (WHO, 2021).

According to the WHO 2020 consolidated guideline for tuberculosis treatment, in patients with confirmed rifampicin-susceptible and isoniazid-resistant tuberculosis, it is recommended to give patients rifampicin, ethambutol, pyrazinamide, and levofloxacin for a duration of 6 months (WHO, 2020b).

Management of MDR-TB or rifampicin-resistant tuberculosis consists of shorter and longer regimens (WHO, 2020b). A shorter all-oral bedaquiline-containing regimen of 6 - 9 months is recommended in eligible patients with confirmed multidrug- or rifampicin-resistant tuberculosis (MDR/RR-TB). Patients must have not been exposed to treatment with second-line TB medications for more than 1 month, and resistance to fluoroquinolones has to be excluded (WHO, 2020b). In patients on a long-term regimen, the composition of the regimen includes three drugs from Group A and at least one drug from Group B to ensure that treatment begins with at least four effective tuberculosis agents. If bedaquiline is discontinued, at least three drugs are included for the remainder of treatment. In the event that one or two of the Group A agents are utilized, both Group B agents must be included. When agents of Groups A and B alone are not sufficient to complete the regimen, agents from Group C are added.

Table 2.2: Grouping of antituberculosis drugs recommended for use in longer MDR-TB regimens (WHO, 2020b).

Groups and steps	Medicine	Abbreviation
Group A: Include all three medicines	Levofloxacin or Moxifloxacin	Lvx Mfx
	Bedaquiline	Bdq
	Linezolid	Lzd
Group B: Add one or both medicines.	Clofazimine	Cfz
	Cylcoserine or terizidone	Cs Trd
Group C: Add to complete the regimen when medicines from Group a and Group B cannot be used.	Ethambutol	Emb
	Pyrazinamide	Pza
	Imipenem-cilastain or meropenem	Ipm-Cln Mpm
	Amikacin or Streptomycin	Akm S
	Ethionamide or prothionamide	Eth pto
	p-anminosalicyclic acid	Pas

2.7 Diagnosis

2.7.1 Latent Disease

Screening and treatment of latent tuberculosis (TB) infections is often recommended for high-risk groups. This includes people from areas and populations where TB is an epidemic (Africans and Asian descendants), People at high risk of reactivation include HIV-positive patients, those receiving immunosuppressive drug therapy, and those who have recently been in contact with a person infected with TB. (Ai, Ruan, Liu, *et al.*, 2016; Ang, Vasconcelos-Santos, Sharma, *et al.*, 2018). Latent infections can be diagnosed by a Mantoux test, also known as a tuberculin skin test, or by an interferon-gamma release test. (IGRA) (Rameshwari, Jayashree, Anuradha, *et al.*, 2021). Interferon-gamma release assays are more unique and specific to *Mycobacterium tuberculosis* than the tuberculin skin test (TST) (Riazi, Zeligs, Yeager, *et al.*, 2012). Chest radiography is one of the most commonly used instruments for stratifying TB risks and to assess for asymptomatic active disease (Rameshwari, Jayashree, Anuradha, *et al.*, 2021).

2.7.2 Diagnosis of active TB infection

2.7.2.1 Classical microscopy

Sputum smear microscopy is the most common method to diagnose both dead and active tuberculosis (TB), especially in a limited resource environment. (Yuen, Amanullah, Dharmadhikari, *et al.*, 2015; Graham and Sekadde, 2019). Enhanced visualisation of *Mycobacterium tuberculosis* is performed using a light-emitting diode (LED), fluorescent microscopy using auramine–O or auramine-rhodamine dyes (Caulfield and Wengenack, 2016; Vilchèze and Kremer, 2017). Enhanced further visualisation is achieved using acid-fast staining techniques including Ziehl-Neelsen and Kinyoun methods (Vilchèze and Kremer, 2017; Gordon and Parish, 2018). However, once stained, *Mycobacterium tuberculosis* is resistant to acid alcohol decolorization, hence it is called an acid-fast bacillus (Obioma and Wokem, 2018).

2.7.2.2 Culture

The culture method is the gold standard for the diagnosis of *Mycobacterium tuberculosis* in the laboratory (Caulfield and Wengenack, 2016). Traditionally, solid

egg or agar medium has been used to isolate mycobacteria (Heysell, Pholwat, Mpagama, *et al.*, 2015; Ahmad, Mokaddas, Al-Mutairi, *et al.*, 2016). One of the main disadvantages of this test is that it takes a long turnaround period, from 3 to 4 weeks of incubation, before the results are obtained (Pandey, Pant, Rijal, *et al.*, 2017; Reed, Basu, Butzler, *et al.*, 2017; Xia, Zheng, Zhao, *et al.*, 2017). This delay could result in the patient's death and increased transmission of diseases. To shorten turnaround times for DST of *Mycobacterium tuberculosis*, several liquid culture-based systems have been developed (Krüüner, Yates and Drobniewski, 2006; Palomino, Martin, Von Groll, *et al.*, 2008). The introduction of semi-automated and automated liquid-based culture systems has reduced detection times to approximately 10 days. These liquid culture-based system methods include the BACTEC 460 and BACTEC 960 system, the BacT/Alert 3D and MB/Bact and VersaTREK system (Palomino, Martin, Von Groll, *et al.*, 2008). However, the use of liquid-based systems is limited in developing countries due to the requirement of heavy, expensive instrumentation and the absence of appropriately trained human resources (Krüüner, Yates and Drobniewski, 2006; Palomino, Martin, Von Groll, *et al.*, 2008).

2.7.2.3 Genotypic assays for detection and susceptibility testing of *Mycobacterium tuberculosis*

To further shorten the turnaround time for *Mycobacterium tuberculosis* detection tests, several genotypic assays were introduced (Ahmad, Mokaddas, Al-Mutairi, *et al.*, 2016; Meaza, Kebede, Yaregal, *et al.*, 2017). Genotypic methods are based on the detection of mutations in genes known to be associated with anti-TB drugs, allowing the development of in-house molecular and commercial tests (Meaza, Kebede, Yaregal, *et al.*, 2017). Some of the commercially available genotypic methods include line-probe assays such as the INNO-LIPA® Rif TB assay (Innogenetics, Ghent, Belgium), GenoType MTBDRplus (Hain Lifescience Nehren, Germany), and GenoType MTBDRsl (Hain Lifescience Nehren, Germany) (Ahmad, Mokaddas, Al-Mutairi, *et al.*, 2016; Rahman, Sahrin, Afrin, *et al.*, 2016; Schön, Miotto, Köser, *et al.*, 2017). Line probe assays employ a conventional multiplex polymerase chain reaction (PCR) and reverse hybridization to immobilise probes on a membrane strip (Rahman, Sahrin, Afrin, *et al.*, 2016; Schön, Miotto, Köser, *et al.*, 2017). The technology relies on the removal (absence and presence of these bands) of a wild-type band or the emergence of bands that correlate particular mutations that suggest the presence of a resistant

strain while the presence of all bands correlate to the wild type strain. (Rahman, Sahrin, Afrin, *et al.*, 2016).

The INNO-LIPA® Rif TB assay is a line-probe assay that simultaneously detects *Mycobacterium tuberculosis* and DST targeting *rpo*B for available RIF resistance respectively available (Rahman, Sahrin, Afrin, *et al.*, 2016). The GenoType MTBDRplus is a line-probe assay based on the DNA-Strip technology (Meaza, Kebede, Yaregal, *et al.*, 2017). GenoType MTBDRplus allows simultaneous molecular identification of the *Mycobacterium tuberculosis* complex and its resistance to RIF and INH by detecting common mutations in the *ka*tG and *inh*A genes respectively available (Meaza, Kebede, Yaregal, *et al.*, 2017; Kazemian, Kardan-Yamchi, Bahador, *et al.*, 2019). The equivalent line probe assay for the detection of resistance to second-line drugs is performed by the GenoType MTBDRsl assay (Rahman, Sahrin, Afrin, *et al.*, 2016; Tomasicchio, Theron, Pietersen, *et al.*, 2016). The GenoType MTBDRsl assay is used for the detection of resistance against ethambutol (EMB), fluoroquinolones (such as ofloxacin (OFL) and moxifloxacin (MXF), injectable antibiotics (capreomycin, AMI, KAN and viomycin) by targeting the most common mutations in the *emb*B, *gry*A, and *rr*s genes respectively (Dominguez, Boettger, Cirillo, *et al.*, 2016; Tomasicchio, Theron, Pietersen, *et al.*, 2016; Jian, Yang, Yang, *et al.*, 2018). The advantages of MTBDRsl, include the detection of XDR-TB in patients previously diagnosed with MDR-TB and obtaining results within four to five hours (Rahman, Sahrin, Afrin, *et al.*, 2016; Jian, Yang, Yang, *et al.*, 2018). A significant constraint of line probe assay is the restricted number of mutations that can be identified in a single examination. (Degertekin, Hussain, Tan, *et al.*, 2009; Nathavitharana, Cudahy, Schumacher, *et al.*, 2017). Line-probe assays only allow the detection of the most frequent mutations leading to resistance (Kazemian, Kardan-Yamchi, Bahador, *et al.*, 2019). If there are mutations outside the targeted areas, the results of the assay could be wrongly interpreted as susceptible (Dominguez, Boettger, Cirillo, *et al.*, 2016).

2.7.2.4 RT-PCR (Real-time PCR) techniques for susceptibility testing of *Mycobacterium tuberculosis*

Real-time PCR (RT-PCR) involves a correlation between the quantity of PCR products and the amount of fluorescence signals emitted. (Rahman, Sahrin, Afrin, *et al.*, 2016). The early 1990s saw the development of GeneXpert® MTB/RIF (Cepheid) (Rahman,

Sahrin, Afrin, *et al.*, 2016; Singh, Pandey, Mehta, *et al.*, 2016). The GeneXpert® MTB/RIF is a fully automated molecular-based real-time PCR method that allows rapid diagnosis of TB directly from sputum (Ahmad et al., 2016). GeneXpert also tests for RIF by targeting the *rpoB* gene (Meldau, Peter, Theron, *et al.*, 2014; Singh, Pandey, Mehta, *et al.*, 2016). Rifampicin is the key determinant of treatment failure, so detection of rifampicin resistance is proposed as a proxy for MDR-TB diagnosis (Meldau, Peter, Theron, *et al.*, 2014; Singh, Pandey, Mehta, *et al.*, 2016). GeneXpert is easy to perform, easy to interpret, and results can be read within two hours (Massi, Biatko, Handayani, *et al.*, 2017). The GeneXpert MTB/RIF revolutionized the management of *Mycobacterium tuberculosis* infections by providing a faster and more accurate diagnosis. GeneXpert has improved TB programs in 80% of the WHO countries (Massi, Biatko, Handayani, *et al.*, 2017; WHO, 2020a).

2.7.2.5 Limitations of Molecular Tests

In general, the sensitivity of molecular tests for all drugs is lower than the phenotypical DST methods (Miotto, Zhang, Cirillo, *et al.*, 2018). This is due to the fact that all possible genes or mechanisms involved in resistance (some are not yet identified) are not all covered in established molecular tests. Therefore, a variable proportion of resistance species will not be detected (Seifert, Catanzaro, Catanzaro, *et al.*, 2015). Furthermore, it is unclear for all genetic alterations that have been discovered, whether certain mutations are linked to resistance or appear as single nucleotide polymorphisms (Miotto, Zhang, Cirillo, *et al.*, 2018). Another limitation of molecular testing is the identification of hetero-resistant strains, defined as a mixture of resistant and sensitive strains. The detection limit is approximately 10% or more in a mixture of wild-type and mutant DNA (Schön, Miotto, Köser, *et al.*, 2017). If the resistance of the isolate is smaller than this, it cannot be detected by molecular methods, while classical sensitivity tests may give a more sensitive test result in these cases (Cohen, Abeel, Manson McGuire, *et al.*, 2015). One of the major limitations of sequencing is the presence of compensatory genes. Compensatory mutations, silent, or unknown mutations could indicate false resistance (Cohen, Abeel, Manson McGuire, *et al.*, 2015). Thus, as a result, conventional drug susceptibility testing remains critical and relevant.

2.7.3 DNA Sequencing

DNA sequencing-based methods are considered reference molecular testing methods for detecting mutations and provide the highest level of information (Ali, Hasan, McNerney, *et al.*, 2015). The sequencing can be performed both manually and automated. First-generation sequencing generally refers to "Sanger sequencing", while next-generation sequencing refers to any high-throughput method developed after Sanger sequencing, such as whole genome sequencing (Lu and Lu, 2017).

Sanger sequencing, also known as the 'chain termination method', was developed by the English biochemist Frederick Sanger and his colleagues in 1977 (Brownlee, 2015). This method is designed to determine the sequence of nucleotide bases in a piece of DNA (commonly less than 1,000 bp in length) (Prasongkit, Feliciano, Rocha, *et al.*, 2015). Whole genome sequencing is also known as full genome sequencing, a laboratory procedure that determines the order of bases in the genome of an organism in one process. The WGS has revolutionized the biosciences and has been proven to be essential and invaluable to the identification of gene functions and their involvement in diseases (Ali, Hasan, McNerney, *et al.*, 2015).

2.7.4 The use of MICs in drug susceptibility testing of *Mycobacterium tuberculosis*

In the diagnostic laboratory, drug susceptibility testing of *Mycobacterium tuberculosis* strains differs significantly from standard procedures in clinical microbiology, for non-mycobacterial strains (Böttger, 2011). In many other microorganisms, a series of drug dilutions is used to determine the minimum inhibitory concentration required to inhibit bacterial growth in vitro. Current *Mycobacterium tuberculosis* DST protocols categorize clinical isolates as drug-resistant or drug-susceptible based on their ability to proliferate in the presence of a largely single concentration known as the "critical concentration." (Böttger, 2011; Kambli, Ajbani, Sadani, *et al.*, 2015; Hicks, Giffen, Culviner, *et al.*, 2020). The critical concentration is defined as the lowest concentration of an anti-TB agent in vitro, which will inhibit the growth of 99% of phenotypically wild-type strains of the *Mycobacterium tuberculosis* complex (Böttger, 2011; Dominguez, Boettger, Cirillo, *et al.*, 2016; WHO, 2018b).

According to this definition, the critical concentration correlates with the epidemiological "cut-off" concentration and is an epidemiological parameter used to separate "wild type" strains from "nonwild type" strains that can grow in the presence of higher drug concentrations (Böttger, 2011; Ellington, Ekelund, Aarestrup, *et al.*, 2017). There are several concerns with the use of critical concentrations. i) In the diagnostic laboratory, an isolate will always be classified as resistant, regardless of whether high, moderate, or low levels of resistance are present (Böttger, 2011; Kambli, Ajbani, Sadani, *et al.*, 2015). However, empirical data have unequivocally demonstrated that the chromosomal loci responsible for resistance in *Mycobacterium tuberculosis* are not comprised of a singular genetic locus, such as increased expression of an efflux pump or transcriptional regulator induction, but rather result from a compounding of several distinct mutations (Böttger, 2011). These investigations have also demonstrated that resistance in *Mycobacterium tuberculosis* is highly variable and is not necessarily a consistent biological entity (Böttger, 2011; Schön, Miotto, Köser, *et al.*, 2017). In the case of *Mycobacterium tuberculosis*, this heterogeneous resistance encompasses low, moderate and high-level drug resistance phenotypes.

ii) Critical concentrations have little scientific support, especially when it comes to second-line drugs, which can be used at a level too close to the presumed critical concentration (Satta et al., 2018). Instead, of being supported by scientific data, the critical concentrations are determined by expert consensus. (Lange, Alghamdi, Al-Shaer, *et al.*, 2018; Lange, Chesov, Heyckendorf, *et al.*, 2018). Specialist committees use data from antimicrobial susceptibility testing techniques, population simulation tools, pharmacology and pharmacodynamics, clinical trial technologies, and bacterial population dynamics to standardize critical concentrations. Among these committees are the Clinical and Laboratory Standards Institute (CLSI) of the United States of America and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). (Lange, Chesov, Heyckendorf, *et al.*, 2018)

Therefore, the validity of critical concentrations in determining *Mycobacterium tuberculosis* as susceptible or resistant has been questioned by experts (Walzl, McNerney, du Plessis, *et al.*, 2018). To reflect and account for the biological complexity of drug resistance, the straightforward classification of clinical *Mycobacterium tuberculosis* isolates as resistant or susceptible based on

susceptibility testing at "single concentration" may need to be revised and supplemented with quantitative measures of resistance testing (Böttger and Springer, 2008; Böttger, 2011). Even in the 1960s, when present test protocols were devised, limitations in mycobacterial drug susceptibility testing were identified. Canetti in 1969 wrote: “We consider that the best type of sensitivity test is a fully quantitative determination in which the organisms’ capability of growth on medium containing a wide range of drug concentrations is known”, this type of test provides comprehensive information on the resistance level. (Canetti, Froman, Grosset, *et al.*, 1963; Böttger, 2011; Schön, Miotto, Köser, *et al.*, 2017).

As a result, the use of MICs is long overdue and should be done in a way that minimizes the expense and time needed to obtain MIC results. (Böttger, 2011). Standardized procedures for quantitative drug susceptibility testing of first-line and second-line drugs are crucial since they are a need for future research to determine how resistance heterogeneity affects treatment outcomes. i.e., linking the data from quantitative resistance testing with clinical outcomes (Böttger, 2011).

A variety of methods have been used in MIC testing (quantitative measurement of resistance) of *Mycobacterium tuberculosis* which includes i) antimicrobial gradient method which encompasses the Epsilometer test (Etest), ii) agar-based MIC which includes Löwenstein-Jensen medium (L-J medium) or 7H10, iii) macro-dilution MIC based tests which have the MGIT 460 and 960 systems. iv) micro-dilution MIC-based methods that have Alamar blue plates and Sensititre MYCOTB plate (TREK Diagnostic Systems) (Jian, Yang, Yang, *et al.*, 2018; Rancoita, Cugnata, Gibertoni Cruz, *et al.*, 2018). However, in this study, the BD BACTEC MGIT 960 method was used to determine the MIC.

2.7.5 Correlating MIC levels of drugs with specific genetic mutations in *Mycobacterium tuberculosis*

Not all genetic mutations in *Mycobacterium tuberculosis* have been shown to influence phenotypic resistance to drugs equally and several published research indicates that MIC values closely correspond to the position and character of the encoded amino-acid substitution (Portelli, Phelan, Ascher, *et al.*, 2018). Thus, molecular testing significantly reduces the turnaround time to obtain results, but the MIC associated with certain types of mutation may be necessary because not all mutations are associated

with resistance. (Ahmad, Mokaddas, Al-Mutairi, *et al.*, 2016). Linking particular mutations together prior to the successful application of these technologies, drug resistance must be associated with certain minimum inhibitory concentrations (MICs) for a particular class of drugs. (Kambli, Ajbani, Sadani, *et al.*, 2015). This information is especially helpful for injectable anti-TB medications, rifamycins, INH, and fluoroquinolones. This is exemplified by the definitions of MDR-TB and XDR-TB which are based on these compounds (Dominguez, Boettger, Cirillo, *et al.*, 2016). The varied minimum inhibitory concentrations (MIC) found in drug-resistant *Mycobacterium tuberculosis* could have significant therapeutic consequences. (Fonseca, Knight and McHugh, 2015).

According to research, mutations in the RRDR region of the *rpo*B gene have been found in more than 95% of RIF-resistant isolates for rifampicin (Hameed, Fang, Liu, et al., 2022; Sinha, Srivastava, Tripathi, et al., 2020). However, some *rpo*B mutations do not correlate with resistance to RIF or rifabutin (RFB). (Sinha, Srivastava, Tripathi, *et al.*, 2020; Yadav, Singh, Sharma, *et al.*, 2021). Mutations in codons 531, 526, and 513 in the RRDR are typically linked to elevated RIF resistance levels. Alternatively, low levels of resistance to RIF are associated with amino acid substitutions caused by particular mutations at codons 511, 514, 515, 516, 518, 522, and 533. (Sinha, Srivastava, Tripathi, *et al.*, 2020).

Furthermore, rpoB mutations that provide a high level of resistance to RIF (MICs >32ug/ml) in *Mycobacterium tuberculosis* have been shown to be associated with cross-resistance to RFB (MICs >4) (Hamze, Ismail, Rahmo, *et al.*, 2015). However, clinical isolates with MICs greater than 8 ug/ml, indicating lower levels of RIF resistance, were still found to be phenotypically susceptible to RFB at a critical concentration of 0.5 ug/ml. (Georghiou, Penn-Nicholson, de Vos, *et al.*, 2021). Mutations in S315T and S315G in the *kat*G gene have been linked to high-level resistance to isoniazid and ethionamide in *Mycobacterium tuberculosis*, while mutations in the *Inh*A region, including G-17T, T-8A, and C-15T, among others, are linked to lower levels of resistance (Georghiou, Penn-Nicholson, de Vos, *et al.*, 2021).

Concerning fluoroquinolones, MGIT testing has demonstrated a direct relationship between the different *gyr*A mutations found and the degrees of phenotypic resistance to ofloxacin (OFLX) and MXF. (Kambli, Ajbani, Sadani, *et al.*, 2015). Although codons

88 and 94 were associated with higher levels of fluoroquinolone resistance, mutations involving codons 89, 90, and 91 were associated with lower levels of resistance (Avalos, Catanzaro, Catanzaro, *et al.*, 2015; Kambli, Ajbani, Sadani, *et al.*, 2015). Thus, these findings imply that while ASP94 Aspn/Tyr/Gly mutations may require the use of an alternate drug, clinical *Mycobacterium tuberculosis* isolates with *gyr*A mutations in Ala90Val and Ser91Pro may be able to be treated with greater doses of fluoroquinolones. (Kambli, Ajbani, Sadani, *et al.*, 2015). Consequently, a publication clarified how genotypic data can be interpreted in relation to TB regimens and detailed the clinical implications of mutations found by molecular techniques. (Qiao, Ren, Guo, *et al.*, 2021).

Recent research has explored the relationship between the MICs of antituberculosis drugs specifically LVX, MXF, and BDQ; and the emergence of drug resistance in *Mycobacterium tuberculosis*. A study conducted in southwest China examined the susceptibility of MDR-TB isolates to these antibiotics, emphasizing the critical role of MIC assessment in identifying resistance patterns (Zheng, He, Jiao, *et al.*, 2021). A study from mid-East of China showed that 37% of fluoroquinolone resistance events were in MDR-TB isolate, and in Shanghai it was reported that 25.1% of MDR-TB strains exhibited mutations in the gyrA gene, a marker for FQ resistance with MICs above the known critical concentration (Dreyer, Mandal, Dev, *et al.*, 2022). Where else for BDQ, mutations in the Rv0678 gene, which causes the overexpression of efflux pump MmpS5-MmpL5, were found to be the driver of resistance in the drug and to result in cross-resistance between the two drugs (Clofazimine) (Zheng, He, Jiao, *et al.*, 2021; Omar, Ismail, Ndjeka, *et al.*, 2022).

Another study evaluated a broth microdilution assay for routine MIC determination of 14 antituberculosis drugs, including LVX, MXF, and BDQ, based on the EUCAST reference protocol. This study demonstrated high agreement between phenotypic drug susceptibility testing and genotypic results derived from whole-genome sequencing, underscoring the potential of MIC testing in guiding treatment regimens (Mansjö, Espinosa-gongora, Samanci, *et al.*, 2024).

Despite these advancements, comprehensive evaluations of MIC distributions and their role in resistance development remain limited. Variability in laboratory methodologies, differing definitions of resistance thresholds, and inconsistent clinical

responses highlight the need for harmonized MIC-based resistance studies. Thus, in this study, we determined the association of genetic polymorphism and resistance at the MIC level, for second and new drugs to link to the corresponding mutations obtained from genetic mutations. The MICs will be determined using the BD BACTEC MGIT 960 method and relevant genes will be sequenced using whole genome sequencing (WGS).

CHAPTER 3

METHODOLOGY

3.1 Research Design

The study had three major components, namely the determination of MICs using the BD BACTEC MGIT 960 phenotypic drug susceptibility testing-based method, the WGS on phenotypic resistant strains of *Mycobacterium tuberculosis* and linking resistance mutations to MICs. This study was cross-sectional, quantitative, and prospective in design.

Cross-sectional- is the study that an investigator measures the outcome and the exposures in the study participants at the same time (Setia, 2016). This study was a cross-sectional study to determine the MIC trends and mutations of the most potent drugs that are used to treat tuberculosis in Limpopo province.

Quantitative: Quantitative research is a systematic investigation of phenomena by gathering quantifiable data and performing statistical, mathematical, or computational techniques (Toye, Williamson, Williams, *et al.*, 2016). The study was considered quantitative because the data collected were statistically analyzed to access the MIC of the drugs and the susceptibility patterns of *Mycobacterium tuberculosis* clinical isolates.

Prospective: is an epidemiologic study in which groups of individuals are selected based on factors that are to be examined for possible effects on the same outcomes (Suharta and Suarjana, 2018). Clinical isolates from tuberculosis patients were collected to determine the trends of MIC using the MGIT 960 phenotypic drug susceptibility testing method.

3.2 Study area

The study area was Limpopo Province which covers 123,910 km^2 with an estimated population of 5,982, 584 million (Stats-SA, 2019). The province is situated in northern South Africa bordering Mozambique, Zimbabwe, and Botswana. Limpopo Province shares international borders with districts and provinces of three countries:

Limpopo Province is divided into five district municipalities: Capricorn, Mopani, Sekhukhune, Vhembe, and Waterberg. The population of Limpopo province contributes 10,2 % of South Africa's population of 5, 982,584 million, according to STATS SA (Stats-SA, 2019). This makes Limpopo the fifth most populated province in the country (Stats-SA, 2019)**.**

3.3 Study population

The study aimed at clinical isolates cultured positive for the *Mycobacterium tuberculosis* complex that was resistant to drugs from patients admitted in wards and outpatients in all regional hospitals and clinics in Limpopo province whose samples were sent to national health laboratory service (NHLS) at Pietersburg Hospital.

3.4 Sample size

$$N = \frac{Z_{1-a/2}^2 \times p(1-p)\, D}{d^2}$$

Sample size formula by (Suresh and Chandrashekara, 2012).

$Z_{1-\alpha/2}$ = standard normal deviation at the level of significance which was 1.96.

D = design effect which was 1.

d = absolute error or precision that was 0.05.

P = proportion or prevalence, which was 2.1%

N= the sample size

$$\text{Sample Size} = \frac{Z_{1-a/2}^2 \times p(1-p)\, D}{d^2} = \frac{(1.96)^2 \times (0.021) \times (1-0.021) \times 1}{(0.05)^2} = 255$$

Added 10% sample attrition =255+26 =281

In this study, a minimum of 281 clinical isolates were collected.

3.5 Sampling method

Simple stratified random sampling is a method of sampling that involves the division of a population into smaller subgroups called strata. Samples were selected from

strata, so each member of the subset had the same opportunity to be chosen as part of the sampling process (Acharya, Prakash, Saxena, *et al.*, 2013).

Simple stratified random sampling was applied, allowing clinical isolates of patients with known profile strata (RR-TB, MDR-TB, Pre-XDR-TB, and XDR-TB) of drug-resistant tuberculosis to be collected. The number of clinical isolates to be collected in each stratum was determined based on the prevalence of the drug-resistant tuberculosis profile. From each selected DR-TB stratum, each clinical isolate had an equal chance of being collected.

3.6 Sample collection

In this study, clinical isolates with a known profile of drug-resistant tuberculosis stored in the NHLS TB repository were used. The degree of drug resistance included rifampicin-resistant tuberculosis, multidrug-resistant tuberculosis, pre-extensive tuberculosis, and extensive-resistant tuberculosis. The selected samples were sub-cultured to ensure viability and for purity check:

Sub-Culturing

In a clean decontaminated BSC II (Biosafety Cabinet level II), all DR-TB isolates were sub-cultured following the standard procedures of the BACTEC MGIT 960 system (Becton Dickinson, Sparks, Md). Briefly, the BACTEC MGIT- Medium contains 7ml of Middlebrook 7H9 broth base and a fluorescent sensor that detects the concentration of oxygen embedded at the bottom of medium. An aliquot of 0.8 ml of BACTEC 960 supplement OADC (oleic acid, albumin, dextrose, and catalase) from (Becton Dickinson, Sparks, Md) and 0.5ml suspension containing DR-TB isolates was added in a MGIT culture tube and were incubated in the BACTEC MGIT 960 system until a positive signal was flagged. The fluorescent due to bacterial growth in the MGIT tube is continuously monitored. The luminous chemical exhibits sensitivity to the existence of dissolved oxygen inside the broth. The small amount of fluorescent is first visible, but as mycobacteria develop and respire, they absorb oxygen and release the compound's fluorescent light. The algorithm in the system software determines the proportional growth ratio.

The tubes were examined prior to further analysis, where any growth or contamination would render the outcome invalid when inoculated on blood agar. Samples of 0.1 ml

of concentrated specimen were inoculated on blood agar. Blood agar plates were examined for contamination on day 1, 2 and 3 for the appearance of any visible colonies. All plates were sealed with CO2-permeable tape, placed in plastic bags, and incubated at 37°C in 5 to 10% CO2

3.7 Inclusion criteria

In the current study, clinical isolates of all patients tested for tuberculosis were included. The study included drug-resistant tuberculosis clinical isolates, primarily RR-TB, MDR-TB, Pre-XDR-TB, and XDR-TB.

Of the total of 288 DR-TB clinical isolates collected, 141 (49%) were excluded from the study (90 were duplicates, 7 were contaminants, and 44 had no growth after subculture. A total of 147 of the clinical isolates met the inclusion criteria of the study for the MICs testing, of which 96 (65.3%) were RR-TB, 42 (28.6%) were MDR-TB, 7 (4.8%) were pre-XDR-TB, and 2 (1.4%) were XDR-TB **(Figure 3.1)**. Of the 147 clinical isolates tested for MICs of LVX, MXF, and BDQ, only 24 (16.3%) isolates were sent for WGS.

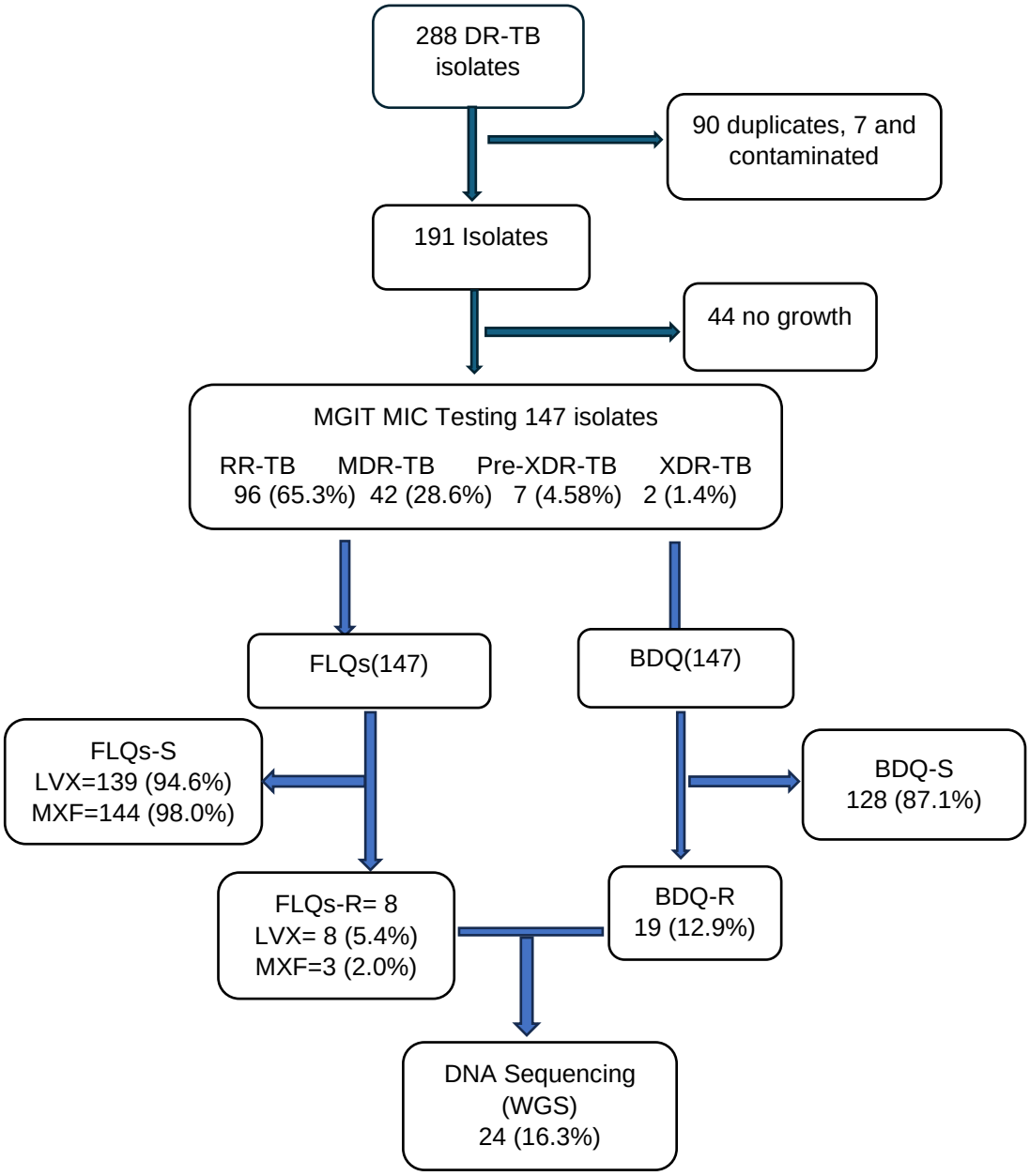

Figure 3.1: Flow diagram of the number of DR-TB isolates screened, eligible, and included for MICs testing and whole genome sequencing. MXF-S (moxifloxacin sensitive), MXF-R (moxifloxacin resistant), LVX-S (levofloxacin sensitive), LVX-R (levofloxacin resistant), BDQ-S (bedaquiline sensitive), BDQ-R (bedaquiline resistant).

3.8 Exclusion criteria

In this study, clinical isolates from patients with susceptible TB (not resistant to any antituberculosis drug) were excluded from the study. Duplicates and isolates that did

not grow after sub-culturing or growing contaminants even after a series of decontamination were removed from the study.

3.9 Data collection

This was a quantitative study (drug susceptibility testing) assay; data was collected using laboratory methods to determine the MICs of antituberculosis drugs used in Limpopo Province. A BD BACTEC MGIT 960 DST method was used to determine the MIC see below and in(Appendix 1). Furthermore, DNA extraction, real-time PCR and WGS (Appendix 1) were applied to selected *Mycobacterium tuberculosis* clinical isolates for profiling drug resistance-associated mutation of RR-TB, MDR-TB, Pre-XDR-TB, and XDR-TB strains.

Preparation of the drugs concentrations and MIC testing

MIC testing and drug preparation MXF, LVX and BDQ MIC testing was performed using the MGIT 960 system following the standard protocol for DST of first-line drugs (Becton Dickinson, USA). The LVX powder was obtained from Sigma Aldrich/Meck USA), Moxifloxacin from Becton Dickinson, USA and BDQ was obtained from J&J, USA.

A 672 µg/ml stock solution of LVX was prepared by dissolving 33.6mg of LVX powder in 50ml of sterile water and 0.1N of NaOH (37.5 sterile water and 12.5 0.1N of NaOH) and stored in small aliquots at -20°C until further use. “Prepare 0.1 N NaOH by dissolving 4g of NaOH in 100ml of distilled water. Do 1:10 dilution in distilled water to achieve a 0.1N NaOH”. On the day of testing, the working solution was prepared from the thawed stock solution. Thereafter, the test concentrations were made by two-fold serial dilutions ranging between 0.125 and 8.0 µg/ml (working solutions). All the dilutions were made in sterile water. Any leftovers of working solutions were discarded.

To prepare the stock solution of MXF a 489µg MXF vial from BD was dissolved in 1.5 ml of dimethyl sulfoxide (DMSO), making a stock solution of 332 µg/ml. Thereafter, the test concentrations were made by two-fold serial dilutions ranging between 0.06 and 4.0 µg/ml (working solutions).

To prepare the stock solution of BDQ a 20.23mg of BDQ (fumarate salts) powder was dissolved in 25ml is DMSO to make a working solution of 672ug/ml and stored in small

aliquots at -20°C until further use. Thereafter, the test concentrations were made by two-fold serial dilutions ranging between 0.016 and 2.0 µg/ml (working solutions). M. tuberculosis H37Rv (ATCC 27294) was included for each batch as a control at all sites.

The MIC of the drugs were determined using the BACTEC™ MGIT™ 960 DST method. The tests were prepared by adding 0.8ml of OADC(growth supplement) in a BACTEC MGIT tubes - Medium contains 7ml of Middlebrook 7H9 broth. Then 0.1ml of the drugs according to the prepared concentration were added in the MGIT containing OADC and 0.5ml of the TB suspension was added and the tubes were incubated in the BACTEC MGIT 960 instrument. The EpiCentre TBExist software (Becton Dickinson, USA) was used for interpretation of MIC for this method, and the incubation period extended from the recommended 4 to 28 days, adjusted for slow growing drug-resistant isolates. The MGIT MIC was defined as the lowest concentration of drug-containing tube reported having a growth Unit <100 of the Mycobacterium tuberculosis. The MICs were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. All resistant isolates were sent for DNA extraction, PCR and WGS analysis.

DNA Extraction-(NucliSENS EasyMAG)

For the study of whole genome sequencing, care was taken to confirm purity of each isolate. DNA was extracted using the Nuclisens easyMAG DNA extraction system (BioMérieux, Marcyl'Étoile, France) from a concentrated 1.2 ml volume of positive culture MGIT medium. The samples were heat killed by placing it in an 80°C oven for 20 minutes. Then the samples were loaded into an aerosol free centrifuge for 5 minutes. Then 0.6ml of the supernatant was discarded using a micro-pipette making sure that the pallet was not disturbed. The remaining samples (pallet 0.6ml) were disrupted using a FastPrep-24 Homogenizer for 45 seconds. Afterwards the tubes were centrifuged for 1 minute and 0.5ml of the supernatant was transferred directly into the easyMAG disposable cartridge and loaded on the easyMAG instrument for extraction. After extraction the DNA (50µl) was transferred into a clean 1.5ml tube. Then the DNA extracts were quantified using the Qubit 4 Fluorometer (Life Technologies, Carlsbad, California, United States).

WGS(whole Genome Sequencing)

For WGS, the quantified DNA extracts were sent to the NICD (Sequencing core) for sequencing. The DNA libraries were prepared using the Illumina Nextera DNA Flex Library Preparation kit (Illumina, San Diego, California, United States). Libraries were sequenced on the Illumina NextSeq 550 sequencing system using the NextSeq 500/550 High Output Kit v2.5 (300 Cycles). Sequencing analysis was completed using CLC Genomics Workbench v11 (Qiagen, Venlo, the Netherlands) to detect any variants (single nucleotide polymorphism, substitution, insertions, and deletions) against the Mycobacterium © tuberculosis reference genome (NC000962.3). The lineages were allocated using the SNP bar coding as described by Coll et al.17.

3.9.1 Data analysis

The results were loaded in Excel and analysed using Statistical Packages for the Social Sciences (SPSS) version 28. Categorical variables were expressed as absolute numbers or percentages. Demographic data were expressed in bar graphs and pie charts to show the pictorial presentation of the data. The results were analysed by keeping a laboratory log sheet to record all the MIC results. A Chi-square comparison was used to compare the relation of the different DR-TB profiles with the associated demographics. Descriptive statistics were used to determine the mean MIC for each drug. The mean difference was considered significant with a P value of <0.05. The T-test was used to compare the susceptibility of FLQ (levofloxacin and moxifloxacin), and logistic regression was used to determine the association between different genetic polymorphisms and resistance at the MIC level.

3.10 Reliability and validity

Reliability refers to the replicability of the processes and the results (Heale and Twycross, 2015; Souza, Alexandre and Guirardello, 2017). Reliability was addressed by perfoming all assays in duplicate. The discrepancy results were repeated. All methods were carried out according to the manufacturer's instructions.

Validity refers to the degree of accuracy of measurements which are accurate (Heale and Twycross, 2015; Souza, Alexandre and Guirardello, 2017). In this study controls H37Rv strain (ATCC 27294) strain of *Mycobacterium tuberculosis*) were run concurrently with the test and calibrated equipment to ensure the accuracy of the results.

3.12 Bias

Bias is a form of systematic error that can affect scientific investigations and distort the measurement process (Jamieson, 2020). In this study, sampling bias was avoided by using simple stratified random sampling in which clinical isolates had the same chance of being selected in the group, and methodological bias was avoided by using validated data collection or laboratory methods. Statistical bias was avoided by calculating the sample size and using appropriate statistical tests.

3.13 Ethical considerations

3.13.1 Permission to conduct the study

Permission to conduct the study was obtained from the Turfloop Research Ethics Committee (TREC) for ethical clearance (Appendix 3) and the Academic Affairs and Research Management System (AARMS) of the National Health Laboratory Service (NHLS) / National Institute of Communicable Diseases (NICD) (Appendix 3) to use clinical isolates and facilities.

3.13.2 Anonymity and Confidentiality

Anonymity refers to keeping the participant's identities unknown in all documents resulting from research, hence the use of research ID (Wallace, 1999). For this study, no personal information from the ~~real~~ patients was written on the reports obtained. The clinical isolates that were obtained were identified using a unique identifier instead of using the patient's identity.

Confidentiality means that the results of the participants must only be known by the individual who conducts the research and the participants (Beltran-Aroca, Girela-Lopez, Collazo-Chao, *et al.*, 2016). According to the South African National Health Act, Section 14 Act no. 61, 2003; all information concerning users or participants involving information relating to their health status and treatment should stay confidential. To ensure confidentiality, clinical and laboratory information and the health status of clinical isolates were not linked to their real names and, therefore cannot be revealed.

3.13.3 Privacy

Researchers signed confidentiality forms to ensure discretion in using the information about the results (Appendix 2).

3.13.4 Harm

Refers to any pain or discomfort participants may be exposed to during the study, particularly during sample collection. In this study, this was not applicable since the clinical isolates were collected from the NHLS-TB stored samples (repository).

3.14 Financial support

The financial support for the project was obtained from NHLS Academic Affairs and Research Management division.

3.15 Publications

The results of the study were presented at conferences and would be published in scientific journals.

CHAPTER 4

RESULTS

Results, Interpretation of Findings.

4. Introduction

This chapter outlines the results of the 147 clinical isolates derived from the methodologies utilized in this study. It begins with a brief overview of the data set described and analyzed, as illustrated in Figure 4.1.

The chapter then investigates the demographics of the study population, detailing key characteristics such as age, gender, and the district's municipalities of the population from which the clinical isolates originated. This demographic analysis sets the stage for a deeper examination of the frequency of the different DR-TB profiles observed in the study clinical isolates and highlights significant patterns. A Chi-square comparison was used to compare the relation of the different DR-TB profiles with the associated demographics. Furthermore, the chapter shows the phenotypic and genotypic susceptibility patterns of the clinical isolates of DR-TB.

The key component of this chapter is the association of MICs with resistant mutations of the DR-TB clinical isolates. This analysis scrutinizes the MICs of resistant phenotypes and links them with resistant mutations, highlighting the nature of mutations at a particular drug concentration. Additionally, it shows the distribution of the lineages and the associated drug-resistant profiles.

This chapter provides a clear understanding of the study's findings by methodically presenting the data and emphasizing important statistical analyses, setting the stage for the discussion and implications of the study to follow.

The figure below highlights the overview of the results sections interpreted and discussed in this study chronologically (Figure 4.1).

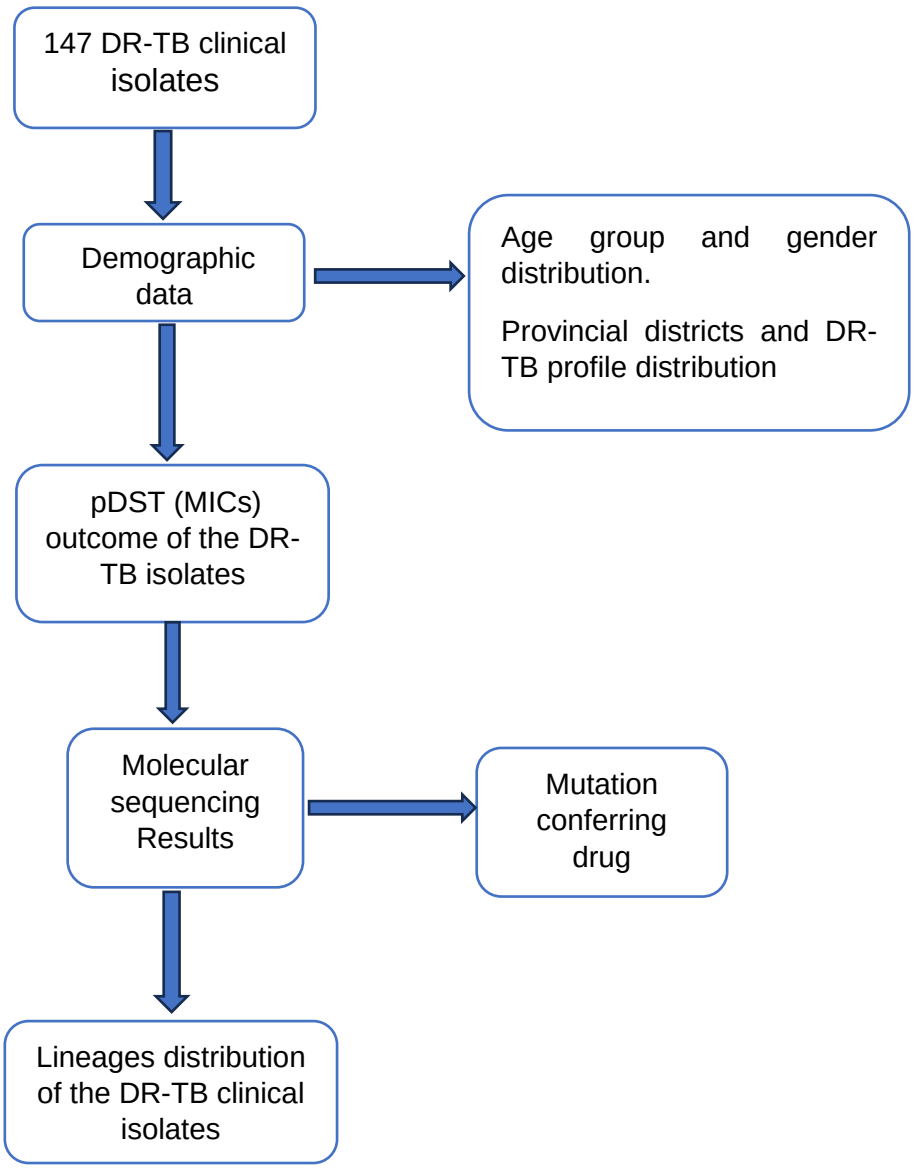

Figure 4.1: Summary of the Overview of Chapter 4

4.1 Demographic results

4.1.1 Age

A total of 147 clinical isolates of DR-TB were included in the study, of which the majority were within the age range of 31 to 45 years (41.5%), followed by 16 to 30 years (30.6%) and 46 to 60 years (19.7%) with less than 15 years (1.4%), being the lowest age range recorded (Figure 4.2). The mean age in these patients was found to be 39 years with an interquartile range of 28 to 46 years. No significant association

was found between age group and drug-resistant TB profiles (P = 0.510), suggesting that the distribution of resistance was independent of age in these samples (Table 4.1).

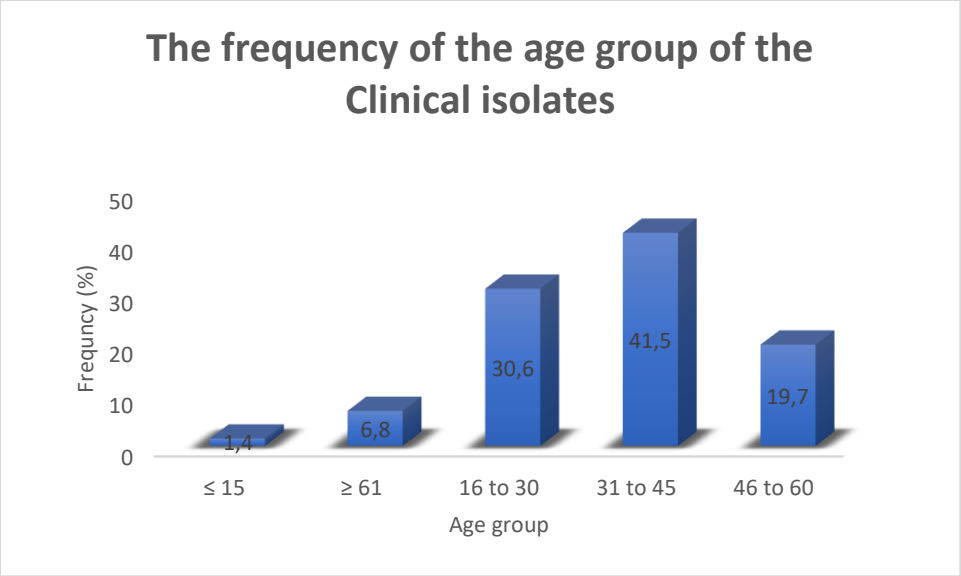

Figure 4.2 Distribution of the age group within clinical isolates.

4.1.2 Gender

The analysis has shown that the majority of clinical isolates were from males 52.4% (77) followed by females with 47.0% (69) and one (0.7%) isolate uncategorized in the cohort of this study (Figure 4.3). The male group had a high number of DR-TB, with RR-TB 51 (66.2%) being the predominant profile, followed by 22 (28.6%) MDR-TB. The female group had a high number of pre-XDR-TB with four (5.8%) clinical isolates compared to the male group with three (3.9%) (Table 4.1). The gender was not significantly associated with drug-resistant TB profiles (P-value 0.873), indicating that the distribution of resistance in these samples was not influenced by gender.

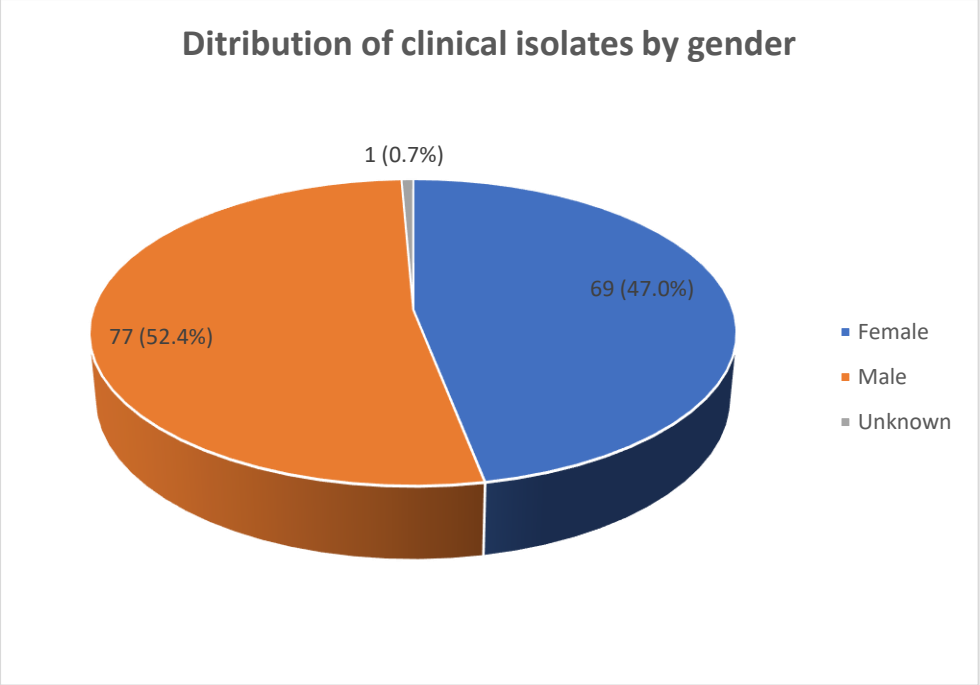

Figure 4.3: The figure illustrates the distribution of clinical isolates by gender.

4.1.3 Distribution by Provincial Districts.

Of the total of 147 clinical isolates analyzed in the study, the provincial district analysis shows that the majority 33.4% (49) of the clinical isolates were from the Capricorn district followed by the Vhembe district with 21.1% (31), while Mopani and Waterberg were both found in 16.3 % (24) of the clinical isolates, with Sekhukhune being the least with 12.9 % (19). However, there was no significant association between districts and drug-resistant TB profiles with a P value of 0.718, indicating that the distribution of resistance of the samples was independent of the geographical location (Figure 4.4).

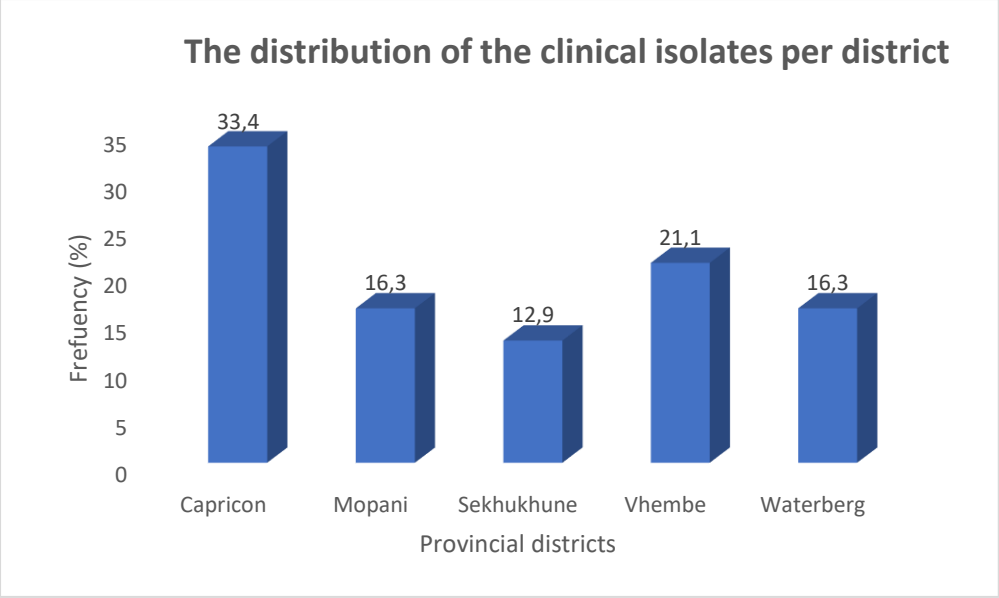

Figure 4.4: The figure illustrates the distribution of clinical isolates by provincial districts.

4.1.4 The distribution of the DR-TB profile by demographic data.

The analysis has shown that of the 147 clinical isolates included in the study, 96 (65.3%) were RR-TB, followed by MDR-TB, pre-XDR, and XDR-TB with 42 (28.6%), seven (4.8%), and one (1.4%) isolate, respectively. Regression analysis has shown that demographic characteristics were not significantly associated with the DR-TB profile with P value > 0.05 indicating that the distribution of resistance of the samples was independent of demographic characteristics (Table 4.1).

	RR-TB N (%)	MDR-TB N (%)	PreXDR-TB N (%)	XDR-TB N (%)	P-value	Total N (%)
Total isolates per category	96 (65.3)	42 (28.6)	7 (4.8)	2 (1.4)		147 (100)
Gender						
Male	51 (66.2)	22 (28.6)	3 (3.9)	1 (1.3)		7 7(52.4)
Female	44 (63.8)	20 (29.0)	4 (5.8)	1 (1.4)	0.873	69 (46.9)
Unknown	1 (100)	0 (0)	0 (0)	0 (0)		1 (0.8)
Age group						
≤ 15	1 (50.0)	1 (50.0)	0 (0)	0 (0)		2 (1.4)
16 – 25	16 (66.7)	5 (20.8)	2 (8.3)	1 (4.2)		24 (16.3)
26 – 35	24(55.8)	17(39.5)	1(2.3)	1(2.3)		43 (29.3)
36 – 45	28 (71.8)	8 (20.5)	3 (7.7)	0 (0)		39 (26.5)
46 – 55	17 (81.0)	4 (19.0)	0 (0)	0 (0)	0.510	21(14.3)
56 – 65	9 (69.2)	4 (30.8)	0 (0)	0 (0)		13 (8.8)
≥ 66	1 (20.0)	3 (60.0)	1 (20.0)	0 (0)		5 (3.4)
Location (Districts)						
Vhembe	21 (67.7)	8 (25.8)	2 (6.5)	0 (0)		31 (21.1)
Capricorn	32 (65.3)	14 (28.6)	1 (2.0)	2 (4.1)		49 (33.4)
Mopani	14 (58.3)	8 (33.3)	2 (8.3)	0 (0)	0.718	24 (16.3)
Waterberg	15 (62.5)	7 (29.2)	2 (8.3)	0 (0)		24 (16.3)
Sekhukhune	14 (73.7)	5 (26.3)	0 (0)	0 (0)		19 (12.9)

Table 4.1: Demographic distribution of the DR-TB profile data.

4.2 Phenotypic drug susceptibility outcomes of drug-resistant tuberculosis isolates.

Of the 147 isolates tested for MIC, a total of 139 (94.6%) were susceptible to LVX while 144 (98.0%) and 128 (87.1%) were susceptible to MXF and BDQ, respectively. Most DR-TB isolates 19 (12.9%) were resistant to BDQ, followed by eight (5.4%) exhibiting resistance to LVX, while only three (2.0%) isolates were resistant to MXF (Table 2). Overall, MXF exhibited the most potent activity against probable FLQ-susceptible *Mycobacterium tuberculosis* isolates, demonstrating a MIC_{50} of 0.06 µg/ml and a MIC_{90} of 0.125 µg/ml, which were lower than those of LVX (MIC50 of 0.25 µg/ml and MIC90 of 0.5 µg/ml) (Table 4.2).

Drug	MIC range µg/ml	Strain	Total number of Isolates 147 (100%)	Number of isolates in the MIC									Breakpoint (µg/ml)	Total number of resistant isolates No (%)	No (%) of resistant isolate
				0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8			
LVX	0.12 - 8	RR	96	0	0	0	14	68	14	0	0	0	0	1	8 (5.4)	0
		MDR	42	0	0	0	3	32	5	2	0	0	0			0
		Pre-XDR	7	0	0	0	0	1	0	0	3	2	1			6(85.7)
		XDR	2	0	0	0	0	0	0	0	1	0	1			2(100)
MXF	0.06 - 4	RR	96	0	0	63	31	1	1	0	0	0	0	0.5	3 (2.0)	0
		MDR	42	0	0	18	22	2	0	0	0	0	0			0
		Pre-XDR	7	0	0	1	0	0	2	2	1	1	0			2(28.6)
		XDR	2	0	0	0	0	0	1	0	1	0	0			1(50.0)
BDQ	0.015 - 2	RR	96	0	0	0	3	21	41	17	14	0	0	1	19 (12.9)	14(15.2)
		MDR	42	0	0	0	1	10	20	8	2	0	0			2(4.9)
		Pre-XDR	7	0	0	0	0	0	1	5	1	0	0			1(28.6)
		XDR	2	0	0	0	0	0	0	0	2	0	0			2(100)

Table 4.2: Minimum inhibitory concentrations and susceptibility breakpoints for each antibiotic.

Figure 4.5 illustrates the susceptibility trends for levofloxacin. Most 68.7% of the drug-resistant tuberculosis clinical isolates were susceptible at a concentration of 0.25 ug/ml (MIC_{50}). This is followed by 12.9% of isolates with a borderline concentration of 0.5ug/ml **(Figure 4.5).**

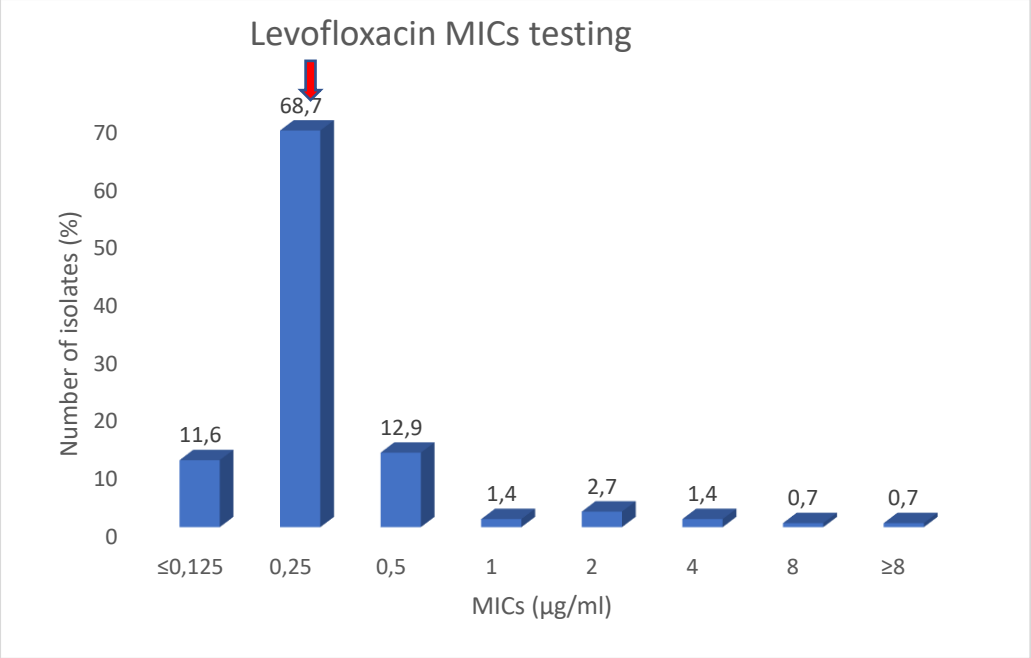

Figure 4.5: Figure illustrates the susceptibility trends of levofloxacin at different drug concentrations. The arrow indicates the MIC_{50}.

Moxifloxacin has been shown to have a higher susceptibility rate at a concentration of ≤0.06 ug/ml with 55.8% clinical isolates sensitive, followed by 0.125 ug/ml with 36.1% and a lower rate of resistance of borderline of 2% at a concentration of 0.5ug/ml **(Figure 4.6)**.

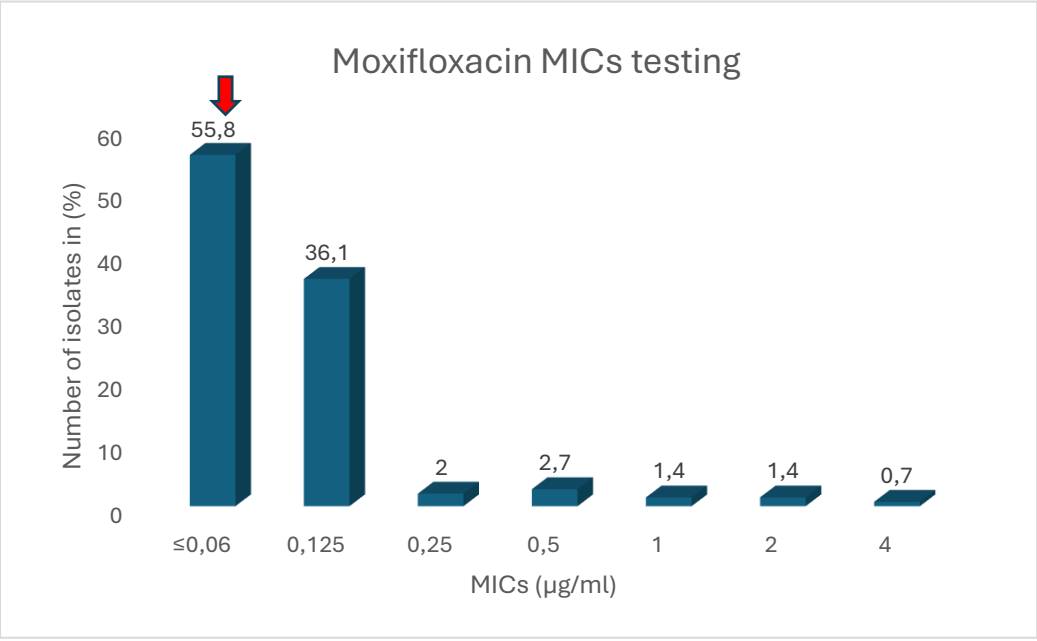

Figure 4.6: Figure illustrates the susceptibility trends of moxifloxacin at different drug concentrations. The arrow indicates the MIC_{50}.

Bedaquiline has shown a higher susceptibility rate at a concentration of 0.5 ug/ml with 42.2% of clinical isolates being sensitive, followed by 0.25 ug/ml with a sensitivity rate of 21.1%. Furthermore, 20.4% of the clinical isolates were sensitive at the borderline concentration of 1 ug/ml, and 12.9% of the clinical isolates were considered resistant (**Figure 4.7).**

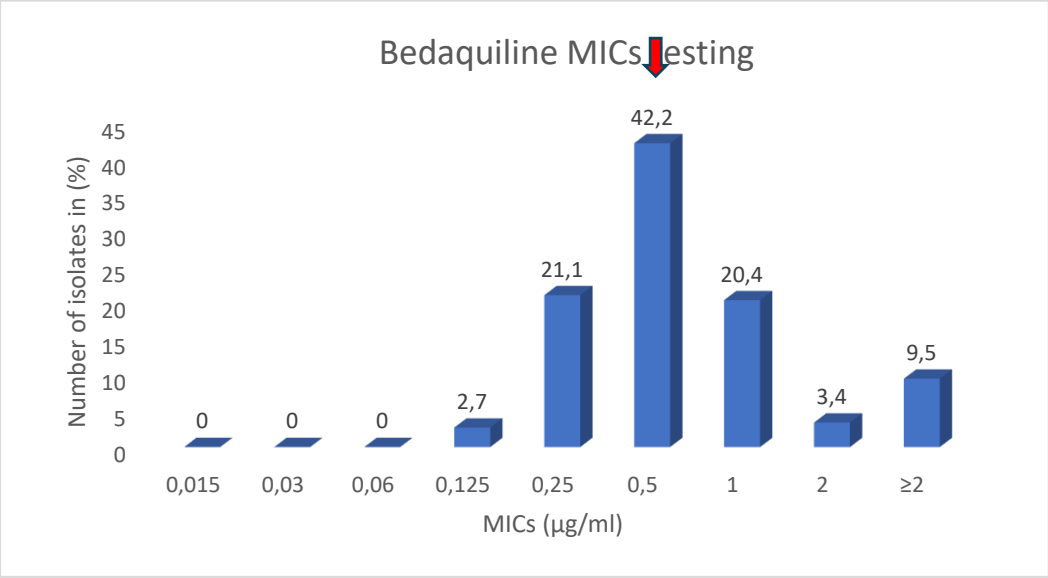

Figure 4.7: Susceptibility trends of bedaquiline at different drug concentrations. The arrow indicates the MIC_{50}.

4.3 Molecular sequencing results

Of the 147 clinical isolates tested for MICs of LVX, MXF, and BDQ only 24 clinical isolates were sent for whole genome sequencing. These clinical isolates were selected for WGS because they were resistant to current drugs tested at the MIC level, as shown in Figure 4.8. Of the 24 clinical isolates, seven (29.2%) were resistant to LVX, three (1.25%) were resistant to MXF and 19 (79.2%) were resistant to BDQ.

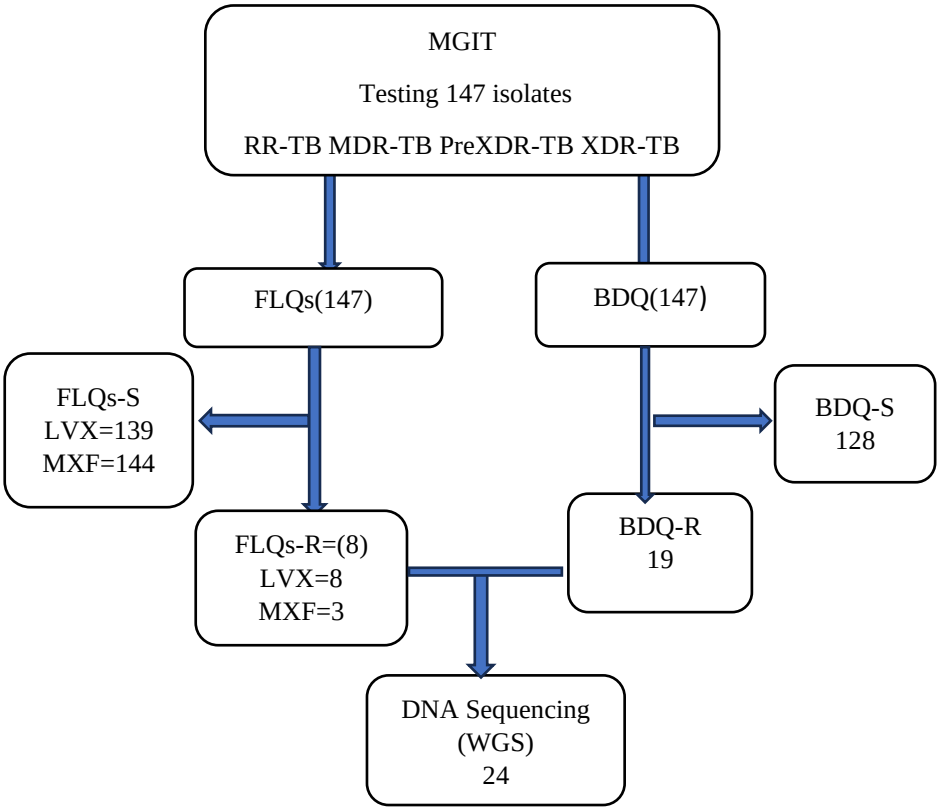

Figure 4.8: The figure illustrates the path through which clinical isolates were subjected to be illegible for DNA sequencing.

4.3.1 Mutation conferring drug resistance

4.3.1.1 Fluoroquinolone sequencing results

Of the FLQ-resistant isolates, eight (100%) are resistant to LVX, three (37.5%) resistant to MXF, and three (37.5%) cross-resistant to LVX and MXF. Mutation was observed within the *gyr*A, *gyrB,* and *Rv*2752c genes. The most common mutation in the *gyr*A gene was the substitution in Condon 94 six (75.5%), resulting in amino acid change for Asp to three (37.5%) Gly, one (12.5%) Ala, and one (12.5%) Asn. The subsequent common substitution occurred at codon 90 with the substitution of Ala to Val four (50%).

Only one substitution was identified in the *gyr*B gene associated with resistance one (12.5%) Arg446His. Another substitution was identified within the *Rv*2752c gene Gly161Ser three (37.5%) and Pro123Leu one (12.5%) (Table 4.3).

FLQs MIC	pDST	Gene	No of Isolates n (%)	Nucleotide change	Amino acid change	WGS Final Confidence grading
8	R	*gyr*A	1 (4.2)	C269T	Ala90Val	R
			1 (4.2)	A281C	Asp94Ala	R
			1 (4.2)	G280A	Asp94Asn	R
		*Rv*2752c	1 (4.2)	G368A	Pro123Leu	S
4	R	*gyr*A	2 (8.3)	A281G	Asp94Gly	R
		*Rv*2752c	1 (4.2)	C481T	Gly161Ser	S
2	R	*gyr*A	1 (4.2)	A281G	Asp94Gly	R
			3 (12.5)	C269T	Ala90Val	R
1	S	*gyr*B	1 (4.2)	G1337A	Arg446His	R
≤ 0.5	S	*gyr*A	1 (4.2)	A281G	Asp94Gly	R
			1 (4.2)	G739A	Gly247Ser	R
		*Rv*2752c	2 (8.3)	C481T	Gly161Ser	S

Table 4.3: Distribution of mutations encoding FLQ resistance with the associated MIC. pDST (phenotypic drug susceptibility testing).

The different types of mutations in the *gyr* genes were significantly associated with different levels of resistance to FLQ drugs with a value of P value =0.022 for LVX and p=0.41 for MXF. Substitutions of Ala90Val and Asp94Gly were associated with a low level of resistance to LVX and MXF, although the Asp94Gly mutation is known to be associated with high levels of resistance to LVX and MXF (Table 4.4).

Mutation (n=9)	LVX			MXF		
	MIC ≤ 4 (n=6/ 66.7%)	MIC > 4 (n=33/ 3%)	p-Value	MIC ≤ 2 (n=7/ 77.7%)	MIC > 2 (n=2/ 22.2%)	p-Value
Ala90Val	3	1	0.022	3	1	0.041
Asp94Gly	3	0		3	0	
Asp94Asn	1	0		0	1	
Asp94Ala	0	1		0	1	
Arg446His	1	0		1	0	

Table 4.4: Association of *gyr* mutations with different FLQs resistance levels.

4.3.1.2 Bedaquiline sequencing results

Among the 19 confirmed clinical isolates phenotypically resistant to BDQ, the mutation was observed in 16 (84.2%) isolates within the *Rv*0678, *Rv*1979c, and *atp*E genes. The most common mutations 15 (79%) were found in the *Rv*0678 gene with substitution within codon Arg82Leu two (12.5%), Arg30Trp one (6.3%), Arg89Trp one (6.3%), Ser151Pro one (6.3%), Arg96Trp one (6.3%) and Ala102Val one (6.3%). Insertion was also identified, resulting in the amino acid change of Lle67fs two (12.5%), Tyr92fs one (6.3%), Glu49fs two (12.5%), Leu142fs one (6.3%) and Lle108fs one (6.3%). Deletion within Arg156fs one (6.3%), Lle67fs two (12.5%), and Lle67fs two (12.5%) was associated with intermediate resistance to BDQ. The second mutation was in the identified *Rv*1979c gene, with substitution of the amino acid change Arg409Gln one (6.3%) and Glu38Asp two (12.5%) but was not associated with phenotypic resistance. Lastly, a substitution mutation within the *atp*E gene was found in one (6.3%) clinical isolate Table 4.5.

BDQ MIC	pDST	Gene	No of Isolates n (%)	Nucleotide change	Amino acid change	WGS Final Confidence grading
≥ 2	R	*Rv*0678	1 (4.2)	A88T	Arg30Trp	R
			1 (4.2)	T451C	Ser151Pro	R
			2 (8.3)	G245T	Arg82Leu	R
			1 (4.2)	C265T	Arg89Trp	R
			1 (4.2)	C286T	Arg96Trp	R
			1 (4.2)	424dupC	Leu142fs	R
			1 (4.2)	144dupC	Glu49fs	R
			2 (8.3)	198dupG	Lle67fs	R
			1 (4.2)	275dup	Try92fs	R
			1 (4.2)	321dup	Lle108fs	R
			1 (4.2)	46delG	Arg15fs	R
			2 (8.3)	198delG	Lle67fs	R
		*atp*E	1 (4.2)	C198G	Lle66Met	R
2	R	*Rv*0678	1 (4.2)	C305T	Ala102Val	R
			1 (4.2)	144dupC	Glu49fs	R
1	S	*Rv*1979c	1 (4.2)	C114G	Glu38Asp	R
			1 (4.2)	C1226T	Arg409Gln	R

Table 4.5: Distribution of mutations encoding bedaquiline resistance with the associated MIC. pDST (phenotypic drug susceptibility testing).

The different types of mutations in the *Rv*0678 genes were not significantly associated with different levels of resistance to bedaquiline drugs (P value > 0.05). Frameshifts Arg151fs, Glu49fs, lle67fs, Tyr92fs, ll108fs, Glu49fs and the substitution of Arg82leu, Arp89Trp, Arg96Trp, Ser151Pro and Ala102Val were associated with an increased level of resistance of bedaquiline (Table 4.6).

Gene	Mutation (n=16)	BDQ		P-Value
		MIC ≤ 2 (n=2/ 12.5%)	MIC >2 (n=14/ 87.5%)	
*Rv*0678	Arg151fs	0	1	0.374
	Glu49fs	0	1	
	Arg82leu	0	0	
	lle67fs	0	4	
	Tyr92fs	0	1	
	ll108fs	0	1	
	Leu142	0	1	
	Ala102Val	1	0	
	Arp89Trp	0	1	
	Arg96Trp	0	1	
	Ser151Pro	0	1	
	Glu49fs	1	1	
*atp*E	lle66Met	0	1	

Table 4.6: Association of *Rv*0678 and *atp*E mutations with different levels of resistance to bedaquiline.

4.3.2: Distribution of lineages of drug-resistant clinical isolates.

Whole genome sequencing confirmed heterogeneous TB epidemiology among our clinical isolates with the four major *M. tuberculosis* complex lineages: Lineage 1; East African Indian, Lineage 2; East Asian (including Beijing), Lineage 3; Indo-Oceanic and Lineage 4; Euro-American (Haarlem, H37Rv-like, LAM and X-type). Of the 24 sequenced clinical isolates, the most represented lineages were Euro-American (15/

62,5%) and East Asian (Beijing) (7/ 29,2%) followed by the East African Indian (1/ 4,2%) and Indo-Oceanic (1/ 4,2%) lineage, respectively.

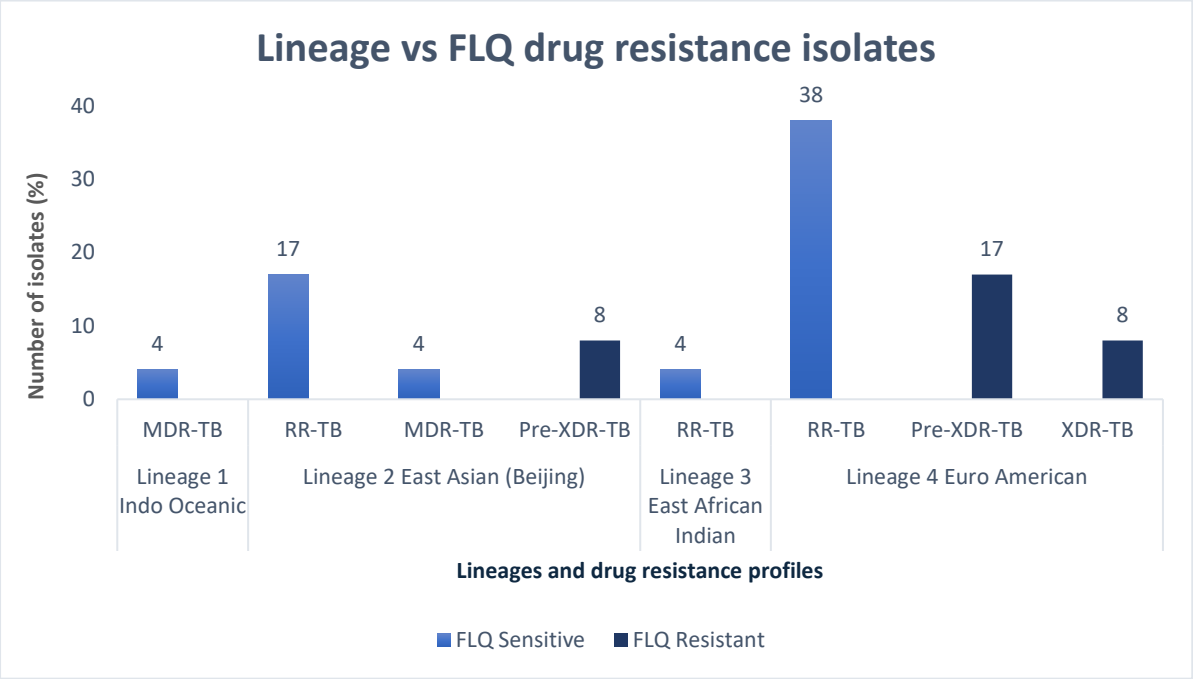

Figure 4.9: Distribution of lineages of fluoroquinolone-resistant clinical isolates.

Of the eight FLQs resistant clinical isolates, six (75%) were of the Euro-American (L4), where four (66.7%) were pre-XDR-TB and two (33.3%) were XDR-TB isolates. Lastly, two (25%) were of the East Asian (Beijing) (L2) and pre-XDR-TB clinical isolates.

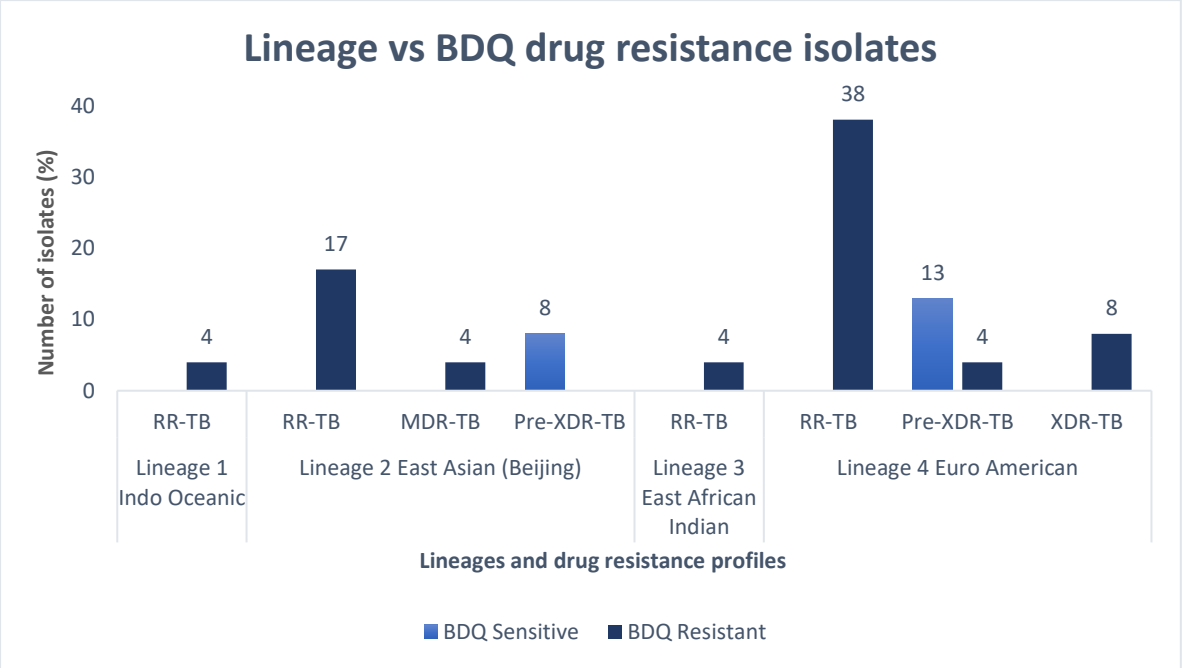

Figure 4.10: Distribution of lineages of bedaquiline-resistant clinical isolates.

Of the 19 bedaquiline-resistant clinical isolates, the majority 12 (63%), were of the Euro-American (L4) lineage, nine (75%) were RR-TB followed by two (16.7%) XDR-TB, and one (8.3%) pre-XDR-TB isolates. The second most common lineage was East Asia (Beijing) (L2) with five (26.3%) clinical isolates, while RR-TB and MDR-TB were 4 (80.0%) and one (20.0%). Finally, Indo-Oceanic (L1) with one (5.2%) clinical isolate of the RR-TB profile and East African one (5.2%) clinical isolate of the RR-TB profile (Figure 4.10).

Summary of Findings

In summary, the study identified key resistance mutations associated with levofloxacin, moxifloxacin, and bedaquiline resistance in *Mycobacterium tuberculosis* clinical isolates from Limpopo Province. Mutations in *gyr*A were linked to fluoroquinolone resistance, while *Rv*0678, *Rv*1979c, and *atp*E mutations contributed to varying levels of bedaquiline resistance. These findings emphasize the importance of monitoring genetic mutations and MIC levels to guide personalised treatment regimens for drug-resistant tuberculosis.

CHAPTER 5

Discussion

With the global rise in drug-resistant tuberculosis, access to rapid and comprehensive drug susceptibility testing (DST) is a major component toward the end-TB strategy in 2035. Most of the DST methods currently used in laboratories, give categorically as resistant or susceptible. The phenotypic DST relies on critical concentration, neglecting the biological complexity of TB, which involves low, moderate, and high-level resistance. Furthermore, the genotypic assays detect only prominent mechanisms of resistance, hotspot genomic regions, or frequent mutations largely on first-line drugs and predict susceptibilities in a binary manner (classifying strains as either susceptible or resistant), and for second-line drugs, it's non-exhaustive because not all resistance genotypes are known, particularly for the new and repurposed drugs. Furthermore, the mutation sites of TB drug resistance vary between different countries, and regions and change over time. Thus, specific mutations conferring drug resistance need to be continually associated with specific MICs for given drug classes,

particularly for second-line drugs. The level of resistance, reflected by MIC, is important in guiding therapeutic decision-making for clinicians treating patients, to determine whether to increase the dosage or change the regimen.

Moreover, phenotypic drug resistance allows for a holistic approach to DST, allowing for the recognition of non-genetic causes of resistance, as well as novel and compensatory genetic causes of drug resistance. Using phenotypic MIC and genotypic WGS methods in a province with a low incidence of TB is crucial to determining drug resistance and monitoring phenotypic and genotypic susceptibility changes for anti-TB drugs, particularly new and repurposed drugs. Thus, we conducted this study to investigate the susceptibility patterns of LVX, MXF, and BDQ for clinical isolates of DR-TB at the MIC level and associated the resistant clinical isolates with the selected resistant mutation from the five districts of Limpopo Province. To our knowledge, this was the first study conducted such research in the Limpopo province.

The analysis showed that most of the clinical isolates of DR-TB (41.5%) of our study population were from patients between 31 and 45 years of age, with a mean age of 39 years. The dominance of the age group 31- 45 was similar to studies conducted by Ismail *et al* in South Africa, by Peter *et al* in Tanzania, and globally by the WHO (Ismail, N.A., Mvusi, Nanoo, *et al.*, 2018; Torokaa, Kileo, Urio, *et al.*, 2023; WHO, 2023). The age group has been listed as the economically active age group, as many individuals in this group are in the prime of their careers, contributing significantly to the economy (Png, Yoong, Phan, *et al.*, 2016; Seloma, Makgatho and Maimela, 2023). Furthermore, the age group is often exposed to various environments and interactions, which could increase their risk of TB infections, especially in crowded areas, which can facilitate the transmission of TB (Seifert, Aung, Besler, *et al.*, 2021; Humayun, Chirenda, Ye, *et al.*, 2022; Seloma, Makgatho and Maimela, 2023).

More than half (52%) of the clinical isolates were from male patients while female patients represented 47%. Higher male numbers are an indicator that men are at increased risk of developing DR-TB compared to females, which was confirmed by prior equivocal evidence (Humayun, Chirenda, Ye, *et al.*, 2022; Seloma, Makgatho and Maimela, 2023; Noman, Islam, Aktar, *et al.*, 2024). Men often exhibit higher rates of exposure to risk factors such as tobacco smoking, or occupational hazards from excessive alcohol consumption, and the prevalence of comorbidities like HIV (Behera,

Pannu and Behera, 2022). Furthermore, socioeconomic disparities may cause men to be less likely to seek timely medical care, leading to delayed diagnosis and treatment, which can contribute to the development of drug-resistant tuberculosis (Marçôa, 2018; Humayun, Chirenda, Ye, *et al.*, 2022; Noman, Islam, Aktar, *et al.*, 2024). This reluctance is influenced by several factors, including societal expectations about masculinity, lower health literacy, and a tendency to downplay symptoms (Schlichthorst, Sanci, Pirkis, *et al.*, 2016; Beia, Kielmann and Diaconu, 2021).

Geographically, most of the clinical isolates were from the Capricorn district and the Vhembe district with 33.4% and 21.1%, respectively, followed by Mopani and Waterberg with both 6.3%. The higher number of cases of DR-TB in the Capricorn district is possibly due to Capricorn being the economic hub of Limpopo Province and the capital city of Polokwane. Furthermore, the district is comprised of a diverse population which includes the student population, inner city with peri-urban, low-density areas on the outskirts, and township areas. The high incidence of DR-TB might be due to the immigration of people from other districts seeking employment, further education, and better health services (SAHO, 2011; Fofana, Moultrie, Scott, *et al.*, 2023). Furthermore, the only TB reference laboratory in the province is within the district, which further justifies the high number of DR-TB cases detected.

Phenotypic drug susceptibility testing

In this study, we observed that an average of 95.7% of the DR-TB clinical isolates were susceptible to fluoroquinolones with LVX accounting for 94.6% and MXF accounting for 96.7%. Most of the clinical isolates were susceptible at the concentration of 0.25µg/ml (68.0%/100) for LVX and at 0.06µg/ml (55.8%/82) for MXF followed by 0.5 µg/ml (12.9%/19) and 0.125µg/ml (36.1%/53) respectively.

Furthermore, the study reports an average of 3.4% of the FLQs resistance rate of which LVX constituted 4.8% and MXF 2.0%. These resistance results are low compared to those of the study by Zheng *et al*., in the Southwest of China, which reported 50% resistance to LVX and 38.6% to MXF among MDR-TB strains and 76% resistance to LVX and 73% resistance to MXF resistance in southern China (Hameed, Tan, Islam, *et al.*, 2019; Zheng, He, Jiao, *et al.*, 2021). These high numbers of FLQ resistance in China are thought to be due to the misuse of FLQs in the treatment of

undiagnosed bacterial infections (Zhang, Lu, Wang, *et al.*, 2014; Hameed, Tan, Islam, *et al.*, 2019; Che, Lu, Zhu, *et al.*, 2024). This outcome indicates that the use of FLQs in Limpopo Province is still acceptable, with less than 5% reporting resistance to FLQs (WHO, 2019; Singh, Kumari, Gupta, *et al.*, 2021). Keeping resistance rates low is crucial for the success of TB treatment programs, as it ensures that these powerful antibiotics can continue to be used effectively against multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB).

Bedaquiline in the current study, on the other hand, had a susceptibility rate of 87.1%. Most of these susceptible isolates were susceptible at a concentration of 0.5µg/ml (42.2%/62) of the drug followed by 0.25 µg/ml with (21.1/31%). These susceptibility rates were comparatively lower than those found in a study by Kaniga *et al*, where they reported 97.9% in a study over five years in countries that included India, Lithuania, Pakistan, the Philippines, South Africa, South Korea, Taiwan, Thailand, Turkey, Vietnam, and the United States (Kaniga, Hasan, Jou, *et al.*, 2022).

However, on the other hand, the current study found a BDQ resistance level of 12.9% in Limpopo province. These resistance levels were comparatively lower than those reported in Cape Town, where more than 50% of DR-TB isolates developed resistance (Derendinger, Dippenaar, De Vos, *et al.*, 2023). The variation in resistance may be due to the Western Cape study focusing only on DR-TB patients who were culture positive after 4 months of a BDQ-containing regimen (Derendinger, Dippenaar, De Vos, *et al.*, 2023), while in the current study, we focus on all clinical isolates of RR-TB. Furthermore, Cape Town has a higher number of RR/MDR-TB cases than Limpopo, which may have high use and exposure to the drug (Ismail, N.A., Mvusi, Nanoo, *et al.*, 2018). Furthermore, the resistance level was comparatively higher than the BDQ DST results found in a study conducted in multicounty surveillance that included South Africa, which reported resistance of only 2.1% (Kaniga, Hasan, Jou, *et al.*, 2022). Furthermore, this value was comparatively higher than that reported in Southwest China, which reported only 5% BDQ resistance (Zheng, He, Jiao, *et al.*, 2021). Country-wise, France reported 1.0% (Veziris, Bernard, Guglielmetti, *et al.*, 2017a), Russia 1.3% (Peretokina, Krylova, Antonova, *et al.*, 2020) and China 2.2 – 3.9 of BDQ resistance (Pang, Zong, Huo, *et al.*, 2017; Yang, Park, Jang, *et al.*, 2017). The low

sensitivity of BDQ DST reported in this study may be due to differences in epidemic strains, medication history, and the local genotype of epidemic strains.

The growing resistance to BDQ observed in this study may be a caution against the blanket use of the BDQ-containing regimen in all RR-TB, as is currently the treatment guideline in South Africa without knowing the susceptibility outcome (WHO, 2022c). The indiscriminate use of BDQ on all RR-TB strains may explain the increase in the number of strains resistant to BDQ in Limpopo province.

One of the main concerns in this study is the findings of borderline susceptibility (clinical breakpoint of the drug) at a drug concentration of 0.5 µg/ml for FLQs (12.9% for LVX and 2.7% for MXF) and 1 µg/ml for BDQ (20.4%). These isolates must be monitored, as they are on the verge of becoming resistant the antituberculosis drugs. In patients with systemic drug concentrations below ideal levels, these isolates with borderline susceptibility may be especially vulnerable to insufficient bacterial killing resulting in relapse or worse case drug resistance development (Mpagama, Houpt, Stroup, *et al.*, 2013). Knowing the percentage of isolates that are borderline susceptible is crucial and should be monitored because this has an impact on treatment options.

The WHO has approved the use of the BPaLM regimen (bedaquiline, pretomanid, linezolid, and moxifloxacin) as part of treatment for MDR/pre-XDR-TB (WHO, 2022b). However, in South Africa, LVX is the primary FLQ of choice over MXF (National Department of Health, 2023). Despite the higher in vitro susceptibility, MXF has been associated with cardiotoxicity (low QT interval), making LVX the preferred FLQ in South Africa, although it has been associated with musculoskeletal disorders in pediatric patients (Maitre, Petitjean, Chauffour, *et al.*, 2017; Täubel, Prasad, Rosano, *et al.*, 2020; Cohen and Moorhouse, 2023; Vanino, Granozzi, Akkerman, *et al.*, 2023). In a study by Sidamo *et al*., who compared the treatment outcomes of LVX and MXF-based regimens, the LVX-based regimen group had a better overall treatment success rate than the MXF-based group (Sidamo, Shibeshi, Yimer, *et al.*, 2021). However, another study revealed that these drugs have similar efficacy in the treatment of RR-TB patients (Chopra, Bansal and Bansal, 2020). The current study showed findings comparable to those of Guan *et al,* in which MXF was found to be more effective than LVX (Guan and Liu, 2020). As a result, the current study recommends the use of MXF

for the treatment of RR/MDR-TB patients based on the resistance rates reported. A study by Maitre *et al*. and Radtke *et al.* also recommended the use of MXF for the treatment of MDR-TB based on the susceptibility outcome (Maitre, Petitjean, Chauffour, *et al.*, 2017; Radtke, Hesseling, Winckler, *et al.*, 2022).

Molecular characterization

The phenotypic drug susceptibility testing highlighted that there were 8 (5.4%) clinical isolates that were resistant to FLQs after MICs testing of which 75.0% (6) were pre-XDR and 25.0% XDR-TB. This resistance was associated with an increase in MIC, ranging from 0.5 – 8µg/ml of the drugs. The isolates were resistant to FLQs due to mutations in *gyr*A and *gyrB.* The analysis showed that a total of 87.5% of the clinical isolates resistant to FLQs were shown to harbour mutation within the *gyr*A gene in the quinolone resistance determining region (QRDR). Interestingly, these results align with the findings of Kabir *et al.* and Nehru *et al.,* reporting that the frequency of *gyr*A mutations is higher than that of *gyr*B and other resistant associated genes (Kabir, Tahir, Mukhtar, *et al.*, 2020; Nehru, Jose Vandakunnel, Brammacharry, *et al.*, 2024). Most of the mutations were associated with codon 94 being the predominant mutation site with the substitution of Asp94-Gly, and Asp followed by codon 90 with the substitution of Ala90Val. This result aligns with the study by Gardee *et al.* in South Africa, where they reported that the majority of mutations, 56.5% were observed at codon 94 of the *gyr*A gene (Gardee, Dreyer, Koornhof, *et al.*, 2017).

Similarly to our findings, a study by Kabir *et al.* in Pakistan, Aubry *et al.* in, and Sayadi *et al.* in Iran revealed that mutations mainly alter codons 94 (replacing aspartic acid with glycine, D94G) and 90 (replacing alanine with valine, A90V) (Aubry, Veziris, Cambau, *et al.*, 2006; Kabir, Tahir, Mukhtar, *et al.*, 2020; Sayadi, Zare, Jamedar, *et al.*, 2020). This reported occurrence of mutation within codon 94 in this study is comparably higher to the one reported in southwest China and Russia reporting 75% and 83% of the occurrence respectively (Mokrousov, Otten, Manicheva, *et al.*, 2008; Zheng, He, Jiao, *et al.*, 2021). Our findings further showed that one of the FLQ-resistant clinical isolates was associated with a mutation of the *gyr*B gene with the substitution of Arg446His.

Additionally, most of the mutations were associated with the substitution of alanine (Ala) and asparagine (Arg), these are extracurricular amino acids that help the brain

operate, flush out toxins, and help synthesize blood cells. Any mutation-related functional alterations in these non-essential amino acids may make it more difficult to produce proteins necessary for cell growth, maintenance, and repair mechanisms (Aaina, Venkatesh, Usharani, *et al.*, 2021; Nehru, Jose Vandakunnel, Brammacharry, *et al.*, 2024).

However, in terms of bedaquiline (BDQ), our study identified that the 19 phenotypically confirmed resistant clinical isolates had mutations in the known resistance-associated genes of BDQ (*Rv*0678, *Rv*1979c, and *atp*E). Although resistance to BDQ was initially reported to be due to target-based mutations in the *atp*E gene encoding subunit C of the ATP synthase complex (Ismail, Ismail, Omar, *et al.*, 2019; Degiacomi, Sammartino, Sinigiani, *et al.*, 2020; Chesov, Chesov, Maurer, *et al.*, 2022; Omar, Ismail, Ndjeka, *et al.*, 2022). Non-target-based mutations in the *Rv*0678 (*mmp*R) gene encoding the MmpS5-MmpL5 efflux pump repressor were found to predominating in our study, present in 79 % (15) of the clinical isolates resistant to BDQ. A study conducted in South Africa reported that *Rv*0678 is the most common cause of resistance to BDQ, which was similar to our findings (Omar, Ismail, Ndjeka, *et al.*, 2022). Most of the associated variants resistant (RAV) to BDQ were RR-TB strains with 73.7% (14) compared to the other DR-TB profiles. The high number of RR-TB strains resistant to BDQ noted in the study was alarming, which calls for the need for more studies to investigate the resistance patterns of BDQ, particularly in RR-TB strains, to salvage this increasing resistance and maintain the efficacy of the new drug.

Some studies showed that mutations within *Rv*0678 could be present prior to BDQ treatment both *in vitro* and *in vivo* (Veziris, Bernard, Guglielmetti, *et al.*, 2017b; Villellas, Coeck, Meehan, *et al.*, 2017; Chawla, Martinez, Kumar, *et al.*, 2018). However, they could not exclude the fact that some of the patients were originally infected with strains already resistant to bedaquiline or clofazimine, of which several factors argue against the possibility (Villellas, Coeck, Meehan, *et al.*, 2017; Degiacomi, Sammartino, Sinigiani, *et al.*, 2020). The indiscriminate use of BDQ in all RR-TB strains or naturally acquired *Rv*0678 mutant strains may explain the increase in the number of BDQ-resistant strains in Limpopo province. The drug was not highly used for the treatment of all patients with RR-TB during the sampling period (2021 to 2022) of the study.

Furthermore, in the study, we identified a clinical isolate that had a mutation within the *atp*E gene (Ile66Met). This mutation is known to be associated with high-level bedaquiline resistance (Karmakar, Rodrigues, Holt, *et al.*, 2019). Although mutation in this gene is less common compared to *Rv*0678 since BDQ now forms part of the standard regimen for the treatment of DR-TB, continued surveillance of drug resistance to BDQ is necessary. Lastly, in this study, we found isolates that harbored a mutation within the *Rv1*979c gene and were associated with low-level resistance to BDQ with a MIC of 1 µg/ml. Variants in *Rv*1979c have previously been reported in less than 1% of *M. tuberculosis* isolates resistant to BDQ without prior exposure to the drug (Ismail, Omar, Joseph, *et al.*, 2018; Saeed, Shakoor, Razzak, *et al.*, 2022).These findings were similar to those reported in a study by *et al.* reporting that a single nucleotide polymorphism (SNP) was seen in the *Rv*1979c gene and was associated with intermediate resistance to BDQ (Saeed, Shakoor, Razzak, *et al.*, 2022).

Linking minimum inhibitory concentrations to whole genome sequence-predicted drug resistance

The different types of mutations in the *gyr* genes in this study were significantly associated with different levels of resistance to FLQ drugs with a $p < 0.01$. Substitutions of Ala90Val, Asp94Asn, and Arg446His were predominately associated with a low level of resistance to LVX and MXF with MICs ranging from 2 – 4 µg/ml in this study. These findings were similar to other studies, demonstrating that clinical isolates with the Ala90Val substitution mutation convey low FLQ resistance (Kambli, Ajbani, Sadani, *et al.*, 2016; Couvin, David, Zozio, *et al.*, 2019; WHO, 2019; Uddin, Ather, Nasrin, *et al.*, 2021). Interestingly, these findings suggest that while low-level resistance is common, these mutations may still permit some therapeutic benefit from fluoroquinolones if appropriately dosed.

However, we observed a discordance with the findings of this study, the substitution mutation of Asp94Gly was predominately associated with low levels of resistance to LVX and MXF; however, previous studies have associated the mutation with high-level resistance to fluoroquinolones (Willby, Sikes, Malik, *et al.*, 2015; Huo, Ma, Li, *et al.*, 2020; Momen, Achraf, Abdelmajid, *et al.*, 2021; Hu, Chi, Feng, *et al.*, 2023). This discordance may be due to the geographical variation of the clinical isolates analyzed in the study. Furthermore, another substitution of Asp94Ala was associated with a high

level of FLQs resistance in this study, with MICs ranging above 4 µg/ml and 2 µg/ml for LVX and MXF, respectively. Similar results were seen in a study by Kabir *et al* in Pakistan (Kabir, Tahir, Mukhtar, *et al.*, 2020).

In terms of bedaquiline (BDQ), on the other hand, mutations within the *Rv*0678, *Rv*1979c, and *atp*E genes were not significantly associated with different levels of resistance to the drug with a p = 0.374 in this study. Most (79%) of the clinical isolates had a mutation with the *Rv*0678 gene, with a MIC greater than two (MIC > 2 µg/ml). This concentration is known to confer a high level of bedaquiline resistance (Van Anh Nguyen, Anthony, Bañuls, *et al.*, 2018; Mirzayev, Viney, Linh, *et al.*, 2021; Saeed, Shakoor, Razzak, *et al.*, 2022). The majority of the RAVs harbored these frameshifts Arg151fs, Glu49fs, lle67fs, Tyr92fs, ll108fs, Glu49fs and the substitution of Arg82leu, Arp89Trp, Arg96Trp, Ser151Pro and Ala102Val. These findings were similar to those found in a study conducted by Saeed *et al* (Saeed, Shakoor, Razzak, *et al.*, 2022).

Furthermore, in this study, a RAV with a mutation in *atp*E was detected, with a MIC of greater than 2 µg/ml. This mutation is known to be associated with high-level bedaquiline resistance (Karmakar, Rodrigues, Holt, *et al.*, 2019). Lastly, in this study, we found isolates that harbored a mutation within the *Rv*1979c gene with a MIC equal to 1 µg/ml, which was associated with resistance to low-level BDQ. This finding indicates a genotypic and phenotypic discordance, as the clinical isolates had a sensitive pDST and resistant associated mutation. Findings of this magnitude necessitate the need to integrate genotypic and phenotypic DST in the diagnosis of DR-TB for clinicians to make informed decisions when designing the patient's treatment regimen. Furthermore, variants in *Rv*1979c have previously been reported in ≤ 1% of *M. tuberculosis* isolates without prior exposure to the drug (Ismail, Omar, Joseph, *et al.*, 2018; Saeed, Shakoor, Razzak, *et al.*, 2022). This finding again cautions against the indiscriminate use of TB drugs, as this can further perpetuate the dilemma of drug-resistant TB.

Limitations

Exclusion of Susceptible Isolates: The study excluded drug-susceptible TB isolates, focusing only on drug-resistant strains. Although this allowed for a detailed examination of resistant mutations and MICs, it limited the ability to compare these results with susceptible strains. A comparative analysis could have provided a more complete picture of the resistance mechanisms by differences between resistant and susceptible isolates.

Lack of Clinical Correlation: While the study identified specific mutations linked to drug resistance, it did not include clinical data such as patient outcomes, treatment responses, or other relevant factors that could provide a more holistic view of how these mutations affect TB treatment. Incorporating clinical outcomes would have enhanced the translational relevance of the findings, linking genetic resistance patterns with real-world patient care.

CHAPTER 6

CONCLUSION

In conclusion, our study found that the phenotypic DST in Limpopo Province has shown that more than 10% of RR/MDR-TB isolates have BDQ resistance and up to 20% possess borderline resistance, while up to 5% and 12% FLQs (MXF and LVX) resistance and borderline resistance respectively. Most of these resistant clinical isolates were from male patients within the age group of 31 to 45 years of age and residing in the Capricorn district. The resistance pattern would not have been shown holistically by using current genotypic methods. The findings of this study show that it is important to continuously monitor the MIC of individual drugs to prevent the gradual loss of drug activity that can consequently compromise the clinical utility of available anti-TB drugs. Thus, highlighting the periodic use of phenotypic DST in developing countries is of paramount importance.

Furthermore, whole genome sequencing has shown that the driving force of FLQs resistance in Limpopo Province was mutations within the *gyr,* particularly in codon 94 and codon 90 which were mainly associated with low resistance. *Gyr* genes were significantly associated with different levels of resistance to FLQ drugs with a p-value of less than 0.05. For BDQ resistance, the driving force was the mutation within the *Rv*0678 gene that was associated with high-level resistance to BDQ in this study, and some resistance was associated with the mutation of the *atp*E genes that were notably associated with high-level resistance.

This research highlights the critical role of MIC testing and genetic analysis in the fight against DR-TB. The study demonstrates that while the majority of isolates remain susceptible to second-line and new drugs, the presence of resistance-associated mutations in some isolates requires careful consideration in treatment planning.

FUTURE RESEARCH

More research is needed to correlate the MICs of drug-resistant tuberculosis clinical isolates with the resistant mutation to understand their susceptibility patterns, particularly to the currently used drugs. Research is needed to look at the MICs of the currently used drugs over a longer period to get a better broader picture of the trends of MICs over time. Furthermore, more studies are needed to develop a phenotypic DST method (assay) that determines the MICs of the current drugs that are used to treat the different phenotypes of TB in one single test (run), to alleviate the tremendous, tedious, and laborious currently used phenotypic DST method.

Future research on unknown drug resistance mechanisms rather than the already known genotypic and phenotypic resistance-associated factors need to be investigated. Some studies suggest that proteomic approaches coupled with bioinformatics could be useful for the characterization of novel proteins that could be related to drug resistance, especially when no related mutations could explain it. Factors such as the role of efflux pumps, inadequate limits of detection of sequencing technologies, and random errors associated with resistance need to be holistically studied to limit or end the situation of tuberculosis which is aggravated by the emergence of drug-resistant tuberculosis strains. Lastly, more research is needed to develop protocols to simplify the current WGS analysis and to make the technique adaptable in routine laboratories, as it is relevant in the fight against the diagnosis of drug-resistant TB.

RECOMMENDATIONS

Comprehensive diagnostic protocols must be implemented to incorporate both phenotypic and genotypic testing in routine clinical practice to ensure accurate identification of drug-resistant TB strains. The use of minimum inhibitory concentration (MIC) testing alongside whole genome sequencing (WGS) can offer a more comprehensive understanding of resistance profiles, enabling more informed treatment decisions. Investment in the development and deployment of rapid point-of-care diagnostic tools that can swiftly identify drug-resistance mutations is crucial. These tools should be designed to be cost-effective and accessible in resource-limited settings, thus enabling timely treatment adjustments.

National and regional surveillance programs should be established or enhanced to monitor the prevalence of drug-resistant TB. This includes tracking resistance patterns, conducting epidemiological studies to identify trends, and correlating these with clinical outcomes to better inform public health strategies. Educational initiatives aimed at healthcare providers and the general public should be launched to raise awareness about TB and drug resistance. These campaigns should emphasize the importance of early diagnosis, adherence to treatment regimens, and the need for routine follow-up care.

Encouragement should be given for the adoption of personalized treatment strategies based on individual resistance profiles. By correlating MIC values with specific genetic mutations, clinicians can optimize drug selection and dosages, reducing the risk of treatment failure and improving patient outcomes. Support for research initiatives aimed at discovering new anti-TB drugs, especially those that target previously unexploited mechanisms of action, is crucial. Exploring combination therapies that include both existing and new agents can enhance treatment efficacy against drug-resistant TB.

Collaboration among healthcare providers, researchers, and public health officials should be fostered to share data on drug resistance and treatment outcomes. This collaborative approach can facilitate the development of evidence-based guidelines and enhance TB control efforts. Given the high co-infection rates between TB and HIV, it is important to integrate TB control programs with HIV treatment and prevention

programs. This integrated approach can help improve the overall management of both diseases and address the unique challenges faced by co-infected individuals.

Advocacy for increased funding and resource allocation for TB research and control programs is essential. Government agencies, non-governmental organizations, and international health bodies should prioritize TB eradication efforts and invest in healthcare infrastructure to combat drug resistance. Training for healthcare professionals on the interpretation of MIC results and genetic testing for drug resistance should be provided. This training will equip clinicians with the necessary skills to make informed treatment decisions based on resistance profiles.

Conducting longitudinal studies to evaluate the effectiveness of personalized treatment strategies and the impact of specific mutations on treatment outcomes is crucial. This research can provide valuable data to refine clinical practices and improve management protocols. Enhancing healthcare systems to ensure consistent access to quality TB care, including diagnostics and treatment, is crucial. Strengthening supply chains for essential medications, laboratory resources, and training can improve the overall effectiveness of TB control efforts.

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APPENDICES

Appendix 1: Methods

1. Preparation of the drugs concentrations

MIC testing and drug preparation MXF, LVX and BDQ MIC testing was performed using the MGIT 960 system following the standard protocol for DST of first-line drugs (Becton Dickinson, USA). The LVX powder was obtained from Sigma Aldrich/Meck USA), Moxifloxacin from Becton Dickinson, USA and BDQ was obtained from J&J, USA.

A 672 µg/ml stock solution of LVX was prepared by dissolving 33.6mg of LVX powder in 50ml of sterile water and 0.1N of NaOH (37.5 sterile water and 12.5 0.1N of NaOH) and stored in small aliquots at -20°C until further use. “Prepare 0.1 N NaOH by dissolving 4g of NaOH in 100ml of distilled water. Do 1:10 dilution in distilled water to achieve a 0.1N NaOH”. On the day of testing, the working solution was prepared from the thawed stock solution. Thereafter, the test concentrations were made by two-fold serial dilutions ranging between 0.125 and 8.0 µg/ml (working solutions). All the dilutions were made in sterile water. Any leftovers of working solutions were discarded.

To prepare the stock solution of MXF a 489µg MXF vial from BD was dissolved in 1.5 ml of dimethyl sulfoxide (DMSO), making a stock solution of 332 µg/ml. Thereafter, the test concentrations were made by two-fold serial dilutions ranging between 0.06 and 4.0 µg/ml (working solutions).

To prepare the stock solution of BDQ a 20.23mg of BDQ (fumarate salts) powder was dissolved in 25ml is DMSO to make a working solution of 672ug/ml and stored in small aliquots at -20°C until further use. Thereafter, the test concentrations were made by two-fold serial dilutions ranging between 0.016 and 2.0 µg/ml (working solutions). *M. tuberculosis* H37Rv (ATCC 27294) was included for each batch as a control at all sites. The MIC was determined to be the lowest drug concentration that inhibited a strain.

Table 3.5 Schematic presentation of the drug concentrations

MXF 4	LVX 8	BDQ 2
MXF 2	LVX 4	BDQ 1
MXF 1	LVX 2	BDQ 0.5
MXF 0.5	LVX 1	BDQ 0.25
MXF 0.25	LVX 0.5	BDQ 0.125
MXF 0.125	LVX 0.25	BDQ 0.06
MXF 0.06	LVX 0.125	BDQ 0.03
		BDQ 0.016

Abbreviations: BDQ-Bedaquline, LVX-Levofloxacin, MXF-Moxifloxacin,

2. DNA Extraction-(NucliSENS EasyMAG)

For the study of whole genome sequencing, care was taken to confirm purity of each isolate. In this investigation, just one isolate specific to a patient was included. DNA was extracted using the Nuclisens easyMAG DNA extraction system (BioMérieux, Marcyl'Étoile, France) from a concentrated 1.2 ml volume of positive culture MGIT medium. The DNA extracts were quantified using the Qubit 4 Fluorometer (Life Technologies, Carlsbad, California, United States).

3. WGS(whole Genome Sequencing)

DNA libraries were prepared using the Illumina Nextera DNA Flex Library Preparation kit (Illumina, San Diego, California, United States). Libraries were sequenced on the Illumina NextSeq 550 sequencing system using the NextSeq 500/550 High Output Kit v2.5 (300 Cycles). Sequencing analysis was completed using CLC Genomics Workbench v11 (Qiagen, Venlo, the Netherlands) to detect any variants (single nucleotide polymorphism, substitution, insertions, and deletions) against the *Mycobacterium © tuberculosis* reference genome (NC000962.3). The lineages were allocated using the SNP bar coding as described by Coll *et al*.17.

Appendix 2: Researcher confidentiality form

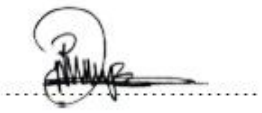
RESEARCH PROJECT CONFIDENTIALITY FORM AT THE UNIVERSITY OF LIMPOPO

Turfloop Campus

RESEARCHER CONFIDENTIALITY FORM

Statement by Researcher:

I, Malope Pleasure, hereby declare that the patient information in this study will not be shared with non-members of the research project and will solely be used for the research project.



Signature

Appendix 3: Approvals

1. School Research Ethics Committee

University of Limpopo
Faculty of Health Sciences
School of Medicine

To: **Prof SM Risenga**
Director: School of Medicine

From: **Prof CJ Sutton**
Chairperson: School Senior Degrees Committee

Date: **21st July 2022**

Subject: **Submission of Master of Science in Medical Sciences**

A research proposal of Mr P Malope for Master of Science in Medical Sciences is hereby submitted for ratification by Faculty Higher Degrees Committee. The proposal was approved by the School Senior Degrees Committee on 22nd June 2022.

Title: "Determination of the association between the minimum inhibitory concentrations of anti-tuberculosis drugs and resistance mutations of *Mycobacterium tuberculosis* clinical isolates from Limpopo Province, South Africa"

Kind regards,

Prof CJ Sutton
Chairperson: School Research Ethics Committee.

Finding solutions for Africa

2. Turfloop Research Ethics Committee

University of Limpopo
Department of Research Administration and Development
Private Bag X1106, Sovenga, 0727, South Africa
Tel: (015) 268 3935, Fax: (015) 268 2306, Email: anastasia.ngobe@ul.ac.za

TURFLOOP RESEARCH ETHICS COMMITTEE

ETHICS CLEARANCE CERTIFICATE

MEETING: 04 December 2023

PROJECT NUMBER: TREC/564/2022: PG - Amended

PROJECT:

Title:	Determination of the association between the minimum inhibitory concentrations of anti-tuberculosis drugs and resistance mutations of Mycobacterium tuberculosis clinical isolates from Limpopo Province, South Africa.
Researcher:	P Malope
Supervisor:	Dr I Rukasha
Co-supervisor/s:	Dr SV Omar Prof RM Lekalakala
School:	Medicine
Degree:	Master of Science in Medical Sciences

PROF D MAPOSA
CHAIRPERSON: TURFLOOP RESEARCH ETHICS COMMITTEE

The Turfloop Research Ethics Committee (TREC) is registered with the National Health Research Ethics Council, Registration Number: **REC-0310111-031**

Note:

i) This Ethics Clearance Certificate will be valid for one (1) year, as from the abovementioned date. Application for annual renewal (or annual review) need to be received by TREC one month before lapse of this period.

ii) Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee, together with the Application for Amendment form.

iii) PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.

3. Academic Affairs and Research Management System

NATIONAL HEALTH LABORATORY SERVICE

Academic Affairs and Research
1 Modderfontein Road, Sandringham, 2031
Tel: +27 (0)11 555 0367/0406
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02 May 2024

Applicant: Pleasure Malope
Institution: University of Limpopo
E-mail Address: pleasuremalope86@gmail.com
Cell: 079 725 0007
Cc: Ivy Rukasha Molebogang Lekalakala

Project Title: DETERMINATION OF THE ASSOCIATION BETWEEN THE MINIMUM INHIBITORY CONCENTRATIONS OF ANTI-TUBERCULOSIS DRUGS AND RESISTANCE MUTATIONS OF MYCOBACTERIUM TUBERCULOSIS CLINICAL ISOLATES FROM LIMPOPO PROVINCE, SOUTH AFRICA

Reference Number: PR2345512

Research Application Type(s):
1. Permission to use NHLS Facilities
2. Request for Samples

RE: APPROVAL LETTER: REQUEST TO ACCESS NHLS RESOURCES FOR RESEARCH PURPOSES

This letter serves to advise that the application requesting permission to conduct the above-mentioned research using the listed NHLS resources has been reviewed and **"Approved"**. Please note that the approval is granted on the condition that you comply with the NHLS Research Material and Data Access Policy and requirements stated below.

1. All material and data requested shall be used as per the research protocol submitted to the NHLS and as approved by the relevant Health Research Ethics Committee (HREC) in South Africa.
2. Access to the NHLS material and/or data shall be limited to the minimum required for successful completion of the approved study and shall be made available ***without patient names and other patient identifiers (including, but not limited to, national identity numbers, hospital/clinic file numbers, addresses and telephone numbers)***.
3. Confidentiality shall be maintained at the participant and institutional level and there shall be no disclosure of personal information or confidential information.
4. Data and/or material shall not be shared with other parties unless approved by the NHLS
5. The material and/or data obtained from the NHLS shall be anonymised and not, for any reason, be used to track or recruit patients as no pre-approval/consent is obtained from patients.
6. Processes shall be discussed with the relevant NHLS departments (i.e. Corporate Data Warehouse (CDW), NHLS Laboratory Management, Operations Office, etc.) and agreed upon.
7. Any amendments to the study requirements, including the use of the material and/or data for purposes not initially disclosed to the NHLS) shall be cleared by an approved HREC and submitted to the NHLS for approval via the AARMS system – https://aarms.nhls.ac.za.
8. The NHLS shall be acknowledged as a source of material and/or data in any output, such as abstracts and journal articles, emanating from the project.
9. A final report of the research study and any published output resulting from this study shall be submitted to the NHLS via AARMS

Please note that this letter constitutes approval by the NHLS Academic Affairs and Research Office. The NHLS entities tasked with providing the material and/data may have additional requirements for access. Use of facility and sample related queries should be directed to Mr Harris Tefo the Business Manager on email: harris.tefo@nhls.ac.za.

Dr Babatyi Malope-Kgokong
National Manager: Academic Affairs and Research

Appendix 4: Raw results

Table 4.1 : Demographic and geographical information of the patients' samples included in the study

RESEARCH ID	Year	TB Profile	Gender	Age	Age group	LOCATION	District	TTD	TTR	BDQ MIC	LEVO MIC	MOXI MIC	BDQ (S OR R)	LEVO (S OR R)	MOXI (S OR R)
OA6724	2022	MDR-TB	Female	42	36 to 45	Seshego Clinic	Capricorn	7;5	12;2	0,5	0,25	0,06	S	S	S
OA3402	2022	MDR-TB	Female	24	16 to 25	Ratshatsha Clinic	Capricorn	11;20	7;18	0,5	0,125	0,06	S	S	S
OA0233	2021	MDR-TB	Female	30	26 to 35	Buite Street Clinic	Capricorn	12;15	9;10	0,125	0,25	0,06	S	S	S
OU1906	2021	MDR-TB	Female	64	56 to 65	Zebediela Hospital	Capricorn	10;3	9;19	0,5	0,5	0,125	S	S	S
NK1264	2021	MDR-TB	Female	70	66 to 75	Botlokwa Hospital	Capricorn	14;17	11;8	0,25	0,25	0,06	S	S	S
NK9898	2021	MDR-TB	Female	27	26 to 35	Botlokwa Hospital	Capricorn	13;14	9;12	0,5	0,25	0,06	S	S	S
OA8436	2021	MDR-TB	Female	30	26 to 35	Seshego hospital	Capricorn	16;7	8;18	0,5	0,25	0,06	S	S	S
OI0392	2022	PreXDR-TB	Female	39	36 to 45	Lebowakgomo Hospital	Capricorn	8;14	8;10	1	0,25	0,06	S	S	S
OF7650	2021	RR-TB	Female	34	26 to 35	Helene Franz Hospital	Capricorn	11;10	9;5	2	0,5	0,06	R	S	S
OA6311	2021	RR-TB	Female	60	56 to 65	Seshego Hospital	Capricorn	8;21	10;19	2	0,25	0,06	R	S	S
OA5874	2021	RR-TB	Female	60	56 to 65	Seshego Hospital	Capricorn	17;12	13;7	0,25	0,125	0,06	S	S	S
NB7384	2021	RR-TB	Female	26	26 to 35	Helene Franz Hospital	Capricorn	8;19	12;3	1	0,5	0,125	S	S	S
OF9117	2021	RR-TB	Female	26	26 to 35	Helene Franz Hospital	Capricorn	7;13	13;1	0,5	0,25	0,06	S	S	S
OA9294	2022	RR-TB	Female	38	36 to 45	Perskebult Clinic	Capricorn	10;8	9;19	0,5	0,25	0,125	S	S	S
OA0182	2021	RR-TB	Female	17	16 to 25	Seshego Hospital	Capricorn	9;21	7;21	0,125	0,25	0,06	S	S	S
OA2914	2021	RR-TB	Female	54	46 to 55	Seshego Hospital	Capricorn	15;5	9;13	2	0,125	0,06	R	S	S
OA6558	2021	RR-TB	Female	38	36 to 45	Pietersburg Hospital	Capricorn	7;19	7;16	0,25	0,125	0,06	S	S	S
OA7920	2021	RR-TB	Female	38	36 to 45	Seshego Hospital	Capricorn	4;12	7;16	1	0,25	0,06	S	S	S
NK6364	2021	RR-TB	Female	45	36 to 45	Matoks Clinic	Capricorn	6;18	9;2	0,5	0,25	0,06	S	S	S
NK5548	2021	RR-TB	Female	46	46 to 55	Botlokwa Hospital	Capricorn	9;5	9;16	2	0,25	0,125	R	S	S

OA7902	2021	RR-TB	Female	47	46 to 55	Seshego Hospital	Capricorn	11;7	11;7	2	0,25	0,125	R	S	S
NM6383	2021	RR-TB	Female	23	16 to 25	Mankweng Hospital	Capricorn	9;22	10;18	0,5	0,25	0,06	S	S	S
OA6335	2021	RR-TB	Female	22	16 to 25	Seshego Hospital	Capricorn	8;8	10;8	0,5	0,125	0,06	S	S	S
OA5512	2021	RR-TB	Female	38	36 to 45	Seshego Clinic	Capricorn	13;15	8;14	0,25	0,25	0,06	S	S	S
OA0197	2021	RR-TB	Female	55	46 to 55	Seshego Hospital	Capricorn	12;1	11;18	1	0,25	0,125	S	S	S
PI4323	2022	XDR-TB	Female	26	26 to 35	Matikwane Hospital	Capricorn	8;18	11;4	2	2	0,5	R	R	S
OA1784	2021	MDR-TB	Male	46	46 to 55	Seshego Clinic	Capricorn	13;5	16;9	0,5	1	0,125	S	S	S
OF9642	2021	MDR-TB	Male	36	36 to 45	Helene Franz Hospital	Capricorn	11;2	10;3	1	0,25	0,125	S	S	S
OF0506	2021	MDR-TB	Male	34	26 to 35	Helene Franz Hospital	Capricorn	9;11	10;20	1	0,25	0,06	S	S	S
OA0531	2022	MDR-TB	Male	39	36 to 45	WF knobel Hospital	Capricorn	6;18	9;0	2	0,125	0,06	R	S	S
OA5207	2021	MDR-TB	Male	67	66 to 75	Seshego Hospital	Capricorn	13;1	9;23	0,5	0,5	0,125	S	S	S
OA8170	2022	MDR-TB	Male	35	26 to 35	Buite Street Clinic	Capricorn	12;9	9;19	1	0,25	0,125	S	S	S
NK1601	2021	MDR-TB	Male	56	56 to 65	Botlokwa Hospital	Capricorn	14,23	9;4	0,25	0,25	0,125	S	S	S
OA3940	2021	RR-TB	Male	23	16 to 25	Seshego Hospital	Capricorn	12;0	9;1	1	0,25	0,06	S	S	S
OA5203	2021	RR-TB	Male	36	36 to 45	Mashashane Clinic	Capricorn	15;20	11;1	0,5	0,25	0,06	S	S	S
OA8987	2021	RR-TB	Male	52	46 to 55	Seshego Hospital	Capricorn	9;8	8;2	1	0,25	0,06	S	S	S
OA2476	2021	RR-TB	Male	34	26 to 35	Seshigo Hospital	Capricorn	10;6	8;6	0,25	0,25	0,06	S	S	S
OA6893	2021	RR-TB	Male	36	36 to 45	Buite Street Clinic	Capricorn	9;11	7;18	0,25	0,25	0,06	S	S	S
NM7309	2022	RR-TB	Male	43	36 to 45	Mankgweng Hospital	Capricorn	12;15	9;5	0,25	0,25	0,06	S	S	S
NM0112	2021	RR-TB	Male	69	66 to 75	Mankweng Hospital	Capricorn	15;2	16;18	0,25	0,25	0,06	S	S	S
OI3882	2021	RR-TB	Male	19	16 to 25	Lebowakgomo Hospital	Capricorn	8;21	11;7	0,5	0,25	0,125	S	S	S
OA7614	2021	RR-TB	Male	20	16 to 25	Seshego Hospital	Capricorn	9;22	8;17	0,25	0,25	0,06	S	S	S

OF0231	2021	RR-TB	Male	42	36 to 45	Helene Franz Hospital	Capricorn	7;19	8;3	0,5	0,25	0,06	S	S	S
OA9857	2021	RR-TB	Male	37	36 to 45	Seshego Hospital	Capricorn	11;14	11;18	0,5	0,125	0,06	S	S	S
OF9704	2021	RR-TB	Male	43	36 to 45	Helene Franz Hospital	Capricorn	8;21	8;5	0,25	0,25	0,125	S	S	S
OA0943	2021	RR-TB	Male	57	56 to 65	WF knobel Hospital	Capricorn	6;8	8;19	0,5	0,25	0,06	S	S	S
OA7253	2021	RR-TB	Male	61	56 to 65	Seshego Hospital	Capricorn	22;1	13;22	0,5	0,25	0,06	S	S	S
OF8819	2022	RR-TB	Male	25	16 to 25	Helene Franz Hospital	Capricorn	8;17	11;2	1	0,25	0,06	S	S	S
OF4644	2022	XDR-TB	Male	22	16 to 25	Helene Franz Hospital	Capricorn	8;8	8;3	2	8	2	R	R	R
NE9362	2021	MDR-TB	Female	21	16 to 25	Kgapane Hospital	Mopani	12;1	10;2	0,25	0,25	0,06	S	S	S
NF3037	2021	MDR-TB	Female	19	16 to 25	Letaba Hospital	Mopani	16;4	13;19	0,5	0,25	0,06	S	S	S
ND2141	2021	MDR-TB	Female	41	36 to 45	Nkhensani Hospital	Mopani	9;19	10;21	0,25	0,25	0,06	S	S	S
NJ5547	2021	MDR-TB	Female	59	56 to 65	Maphutha L Malatje Hospital	Mopani	11;13	11;0	0,5	0,25	0,125	S	S	S
NJ8550	2021	RR-TB	Female	41	36 to 45	Maphutha L Malatje Hospital	Mopani	15;9	11;14	0,25	0,25	0,06	S	S	S
NF6030	2021	RR-TB	Female	45	36 to 45	Letaba Hospital	Mopani	9;11	12;19	1	0,25	0,125	S	S	S
NA9661	2021	RR-TB	Female	17	16 to 25	CN Phatudi Hospital	Mopani	7;0	11;1	0,5	0,5	0,06	S	S	S
NH8254	2021	RR-TB	Female	34	26 to 35	Malamulele Hospital	Mopani	14;11	9;3	1	0,25	0,06	S	S	S
ND1305	2021	MDR-TB	Male	44	36 to 45	Nkhensani Hospital	Mopani	14;11	10;18	1	0,25	0,125	S	S	S
ND9922	2021	MDR-TB	Male	52	46 to 55	Nkhensani Hospital	Mopani	14;4	12;18	0,5	0,5	0,06	S	S	S
NQ7275	2021	MDR-TB	Male	55	46 to 55	Mothupa Clinic	Mopani	14;14	13;18	0,5	0,125	0,25	S	S	S
ND0989	2022	MDR-TB	Male	31	26 to 35	Nkhensani Hospital	Mopani	14;5	13;17	0,25	0,25	0,06	S	S	S
NN7508	2022	PreXDR-TB	Male	45	36 to 45	Sekororo Hospital	Mopani	9;2	9;2	1	8	4	S	R	R
ND2027	2021	PreXDR-TB	Male	21	16 to 25	Nkhensani Hospital	Mopani	6;23	18;22	1	2	0,5	S	R	S
ND4235	2021	RR-TB	Male	43	36 to 45	Nkhensani Hospital	Mopani	12;5	9;20	0,5	0,25	0,125	S	S	S

NA1080	2021	RR-TB	Male	48	46 to 55	CN Phatudi Hospital	Mopani	7;17	7;21	2	0,25	0,125	R	S	S
NF6031	2021	RR-TB	Male	29	26 to 35	Letaba Hospital	Mopani	13;6	8;23	0,5	0,25	0,06	S	S	S
NA2620	2021	RR-TB	Male	43	36 to 45	CN Phatudi Hospital	Mopani	8;12	9;19	2	0,25	0,06	R	S	S
NA3010	2021	RR-TB	Male	38	36 to 45	CN Phatudi Hospital	Mopani	11;5	8;11	0,125	0,125	0,06	S	S	S
NA9628	2021	RR-TB	Male	43	36 to 45	CN Phatudi Hospital	Mopani	8;21	10;4	0,5	0,25	0,125	S	S	S
NF7321	2021	RR-TB	Male	36	36 to 45	Letaba Hospital	Mopani	7;5	9;13	0,25	0,5	0,25	S	S	S
NF5136	2021	RR-TB	Male	36	36 to 45	Letaba Hospital	Mopani	9;21	9;20	2	0,25	0,125	R	S	S
NF1022	2021	RR-TB	Male	20	16 to 25	Letaba Hospital	Mopani	12;3	8;12	0,5	0,25	0,125	S	S	S
NH0985	2021	RR-TB	Male	40	36 to 45	Malamulele Hospital	Mopani	12;7	12;1	0,5	0,25	0,125	S	S	S
OB7493	2021	MDR-TB	Female	22	16 to 25	Dilokong Hospital	Sekhukhune	14;6	9;19	1	0,25	0,125	S	S	S
OE8274	2021	MDR-TB	Female	41	36 to 45	Groblersdal Hospital	Sekhukhune	14;6	9;19	0,5	0,25	0,125	S	S	S
OH5570	2021	RR-TB	Female	29	26 to 35	Philadelphia Hospital	Sekhukhune	14;1	9;14	0,5	0,125	0,06	S	S	S
OH1809	2021	RR-TB	Female	37	36 to 45	Philadelphia Hospital	Sekhukhune	17;1	17;20	0,25	0,5	0,125	S	S	S
OG1496	2021	RR-TB	Female	34	26 to 35	Jane Furse Hospital	Sekhukhune	9;14	10;20	0,5	0,25	0,125	S	S	S
OH8984	2021	RR-TB	Female	44	36 to 45	Philadelphia Hospital	Sekhukhune	9;1	10;18	0,5	0,5	0,125	S	S	S
OB3443	2021	RR-TB	Female	35	26 to 35	Dilokong Hospital	Sekhukhune	19;15	9;22	0,25	0,25	0,06	S	S	S
OH7561	2021	RR-TB	Female	45	36 to 45	Philadelphia Hospital	Sekhukhune	13;6	11;17	0,5	0,5	0,06	S	S	S
OH1646	2021	RR-TB	Female	46	46 to 55	Philadelphia Hospital	Sekhukhune	17;0	9;3	0,25	0,25	0,06	S	S	S
OH9454	2021	RR-TB	Female	24	16 to 25	Valschfontein clinic	Sekhukhune	10;4	9+;15	0,5	0,5	0,125	S	S	S
OB2821	2021	MDR-TB	Male	28	26 to 35	Dilokong Hospital	Sekhukhune	10;5	16;6	1	0,25	0,06	S	S	S
OH7132	2021	MDR-TB	Male	32	26 to 35	Valschfontein clinic	Sekhukhune	14;3	10,2	0,5	0,25	0,125	S	S	S
OB0354	2021	MDR-TB	Male	30	26 to 35	Dilokong Hospital	Sekhukhune	12;0	8;15	0,25	0,25	0,06	S	S	S

OG0133	2021	RR-TB	Male	55	46 to 55	Tswaing Clinic	Sekhukhune	12;3	14;9	0,5	0,25	0,125	S	S	S
OM6733	2021	RR-TB	Male	52	46 to 55	Maboela Clinic	Sekhukhune	7;16	9;0	2	0,25	0,125	R	S	S
OH2016	2021	RR-TB	Male	21	16 to 25	Elandsdoorn Clinic	Sekhukhune	8;19	10;5	1	0,25	0,125	S	S	S
OQ2968	2021	RR-TB	Male	60	56 to 65	St Rita's Gate Way Clinic	Sekhukhune	19;13	10;4	1	0,25	0,125	S	S	S
OG2760	2021	RR-TB	Male	56	56 to 65	Jane Furse Hospital	Sekhukhune	13;7	9;2	0,5	0,25	0,06	S	S	S
OB6459	2021	RR-TB	Male	46	46 to 55	Burgersfort Clinic	Sekhukhune	8;22	12;6	0,25	0,125	0,06	S	S	S
NP9924	2021	MDR-TB	Male	34	26 to 35	Tshilidzini Hospital	Vhembe	12;9	7;18	0,25	0,25	0,125	S	S	S
NP5844	2021	MDR-TB	Female	26	26 to 35	Tshilidzini Hospital	Vhembe	12;12	5;15	-	0,25	0,06	-	S	S
NP6532	2021	MDR-TB	Female	27	26 to 35	Tshilidzini Hospital	Vhembe	16;20	11;14	0,25	0,5	0,06	S	S	S
NI1182	2022	PreXDR-TB	Female	45	36 to 45	Musina Hospital	Vhembe	5;8	8;0	1	2	1	S	R	R
NP7151	2021	PreXDR-TB	Female	25	16 to 25	Tshilidzini Hospital	Vhembe	7;19	10;1	2	2	0,5	R	R	S
NI7127	2021	RR-TB	Female	33	26 to 35	Musina Hospital	Vhembe	6;5	7;17	0,5	0,25	0,06	S	S	S
NB6068	2022	RR-TB	Female	28	26 to 35	Donald Fraser Hospital	Vhembe	13;3	10;11	2	0,25	0,06	R	S	S
NG2733	2021	RR-TB	Female	55	46 to 55	Louis Trichardt Hospital	Vhembe	12;22	13;20	2	0,25	0,06	R	S	S
NC2989	2022	RR-TB	Female	24	16 to 25	Elim Hospital	Vhembe	7;22	8;5	0,5	0,5	0,06	S	S	S
NP3039	2021	RR-TB	Female	24	16 to 25	Tshilidzini Hospital	Vhembe	10;5	9;19	0,5	0,25	0,06	S	S	S
NB2689	2021	RR-TB	Female	52	46 to 55	Donald Fraser Hospital	Vhembe	9;0	12;19	0,5	0,25	0,06	S	S	S
NG9230	2021	RR-TB	Female	29	26 to 35	Louis Trichardt Hospital	Vhembe	7;19	9;11	0,25	0,25	0,125	S	S	S
NP2236	2021	RR-TB	Female	27	26 to 35	Tshilidzini Hospital	Vhembe	10;3	8;4	0,25	0,25	0,06	S	S	S
NG1057	2021	RR-TB	Female	12	6 to 15	Louis Trichardt Hospital	Vhembe	8;13	9;9	0,25	0,25	0,06	S	S	S
NP2177	2021	RR-TB	Female	29	26 to 35	Tshilidzini Hospital	Vhembe	13;12	13;20	1	0,125	0,06	S	S	S
NG0689	2021	RR-TB	Female	22	16 to 25	Louis Trichardt Hospital	Vhembe	10;21	8;22	0,25	0,25	0,06	S	S	S

NI4968	2021	RR-TB	Female	26	26 to 35	Musina Hospital	Vhembe	8;16	8;16	1	0,5	0,125	S	S	S
NE7192	2021	MDR-TB	Male	33	26 to 35	Bellevue Clinic	Vhembe	16;6	9;7	0,5	0,25	0,125	S	S	S
NPM1948	2022	MDR-TB	Male	45	36 to 45	Tshilidzini Hospital	Vhembe	15;16	15;16	1	0,5	0,125	S	S	S
NI3365	2021	MDR-TB	Male	66	66 to 75	Musina Hospital	Vhembe	12;20	11;13	0,5	0,25	0,125	S	S	S
NB3306	2022	MDR-TB	Male	28	26 to 35	Donald Fraser Hospital	Vhembe	5;2	11;1	0,5	0,25	0,125	S	S	S
NP8807	2022	MDR-TB	Male	34	26 to 35	Tshilidzini Hospital	Vhembe	15,3	10;5	0,5	0,25	0,125	S	S	S
NP6357	2021	RR-TB	Male	24	16 to 25	Tshilidzini Hospital	Vhembe	9;17	12;13	0,5	0,125	0,5	S	S	S
NB8677	2021	RR-TB	Male	54	46 to 55	Donald Fraser Hospital	Vhembe	9;19	9;7	2	0,25	0,06	R	S	S
NB3145	2021	RR-TB	Male	34	26 to 35	Donald Fraser Hospital	Vhembe	7;10	9;20	2	0,5	0,125	R	S	S
NB5645	2022	RR-TB	Male	35	26 to 35	Donald Fraser Hospital	Vhembe	7;11	10;14	1	0,5	0,06	S	S	S
NCM4291	2022	RR-TB	Male	38	36 to 45	Elim Hospital	Vhembe	11;11	9;19	0,5	0,5	0,125	S	S	S
NH7874	2021	RR-TB	Male	43	36 to 45	Malamulele Hospital	Vhembe	15;9	12;	0,5	0,25	0,125	S	S	S
NC1256	2021	RR-TB	Male	45	36 to 45	Olifantshoek Clinic	Vhembe	10;9	10;3	0,5	0,25	0,125	S	S	S
NP2758	2021	RR-TB	Male	48	46 to 55	Tshilidzini Hospital	Vhembe	17;1	11;22	1	0,125	0,06	S	S	S
NP4314	2021	RR-TB	Male	53	46 to 55	Tshilidzini Hospital	Vhembe	8;5	10;3	0,5	0,25	0,06	S	S	S
OD2199	2021	MDR-TB	Female	18	16 to 25	George Masebe Hospital	Waterberg	9;8	10;18	0,5	0,25	0,125	S	S	S
OS2657	2021	MDR-TB	Female	34	26 to 35	Limpopo MDR Unit	Waterberg	12;17	10;5	0,5	0,25	0,125	S	S	S
OM3805	2021	MDR-TB	Female	54	46 to 55	Mokopane Hospital	Waterberg	9;7	8;3	0,25	0,25	0,125	S	S	S
OV3032	2021	MDR-TB	Female	14	6 to 15	Witpoort Hospital	Waterberg	13;22	9;19	0,5	0,25	0,125	S	S	S
OS8448	2021	MDR-TB	Female	25	26 to 35	Bela Bela Hospital	Waterberg	14;20	13;19	2	1	0,25	R	S	S
OS4813	2022	PreXDR-TB	Female	31	26 to 35	Limpopo MDR Unit	Waterberg	7;15	10;22	1	4	2	S	R	R
ON3142	2021	RR-TB	Female	34	26 to 35	Phagameng Hospital	Waterberg	8;16	7;20	0,5	0,125	0,06	S	S	S

ON7457	2021	RR-TB	Female	34	26 to 35	FH Odendaal Hospital	Waterberg	12;23	8;2	1	0,25	0,06	S	S	S
OS5480	2021	RR-TB	Female	34	26 to 35	Limpopo MDR Unit	Waterberg	7;8	9;20	2	0,25	0,06	R	S	S
OM5968	2021	RR-TB	Female	30	26 to 35	Mahwelereng Clinic	Waterberg	6;12	10;7	0,5	0,125	0,06	S	S	S
OC7070	2021	MDR-TB	Male	61	56 to 65	Ellisras Hospital	Waterberg	16;0	10;7	0,25	0,25	0,06	S	S	S
ON9119	2021	MDR-TB	Male	44	36 to 45	Phagameng Hospital	Waterberg	8;5	13;19	1	0,25	0,125	S	S	S
OC0008	2022	PreXDR-TB	Male	68	66 to 75	Ellisras Hospital	Waterberg	7;19	8;4	0,5	4	1	S	R	R
OM9658	2021	RR-TB	Male	44	36 to 45	Mokopane Hospital	Waterberg	8;19	11;12	0,5	0,25	0,125	S	S	S
OS5116	2021	RR-TB	Male	34	26 to 35	Limpopo MDR Unit	Waterberg	14;6	10;3	0,5	0,125	0,06	S	S	S
OC4328	2021	RR-TB	Male	30	26 to 35	Ellisras Hospital	Waterberg	8;6	10;3	1	0,25	0,06	S	S	S
OS3412	2021	RR-TB	Male	56	56 to 65	Limpopo MDR Unit	Waterberg	5;19	10;14	0,5	0,25	0,06	S	S	S
ON9358	2022	RR-TB	Male	51	46 to 55	Phagameng Hospital	Waterberg	11;14	9;19	0,25	0,25	0,06	S	S	S
ON2526	2021	RR-TB	Male	64	56 to 65	Vaalwater Clinic	Waterberg	10;2	6;18	0,125	0,5	0,125	S	S	S
OS2737	2022	RR-TB	Male	32	26 to 35	Bela Bela Hospital	Waterberg	12;6	11;19	0,25	0,25	0,06	S	S	S
OM5241	2021	RR-TB	Male	53	46 to 55	Mokopane Hospital	Waterberg	8;9	9;2	0,5	0,25	0,06	S	S	S
OD9568	2021	RR-TB	Male	19	16 to 25	George Masebe Hospital	Waterberg	8;20	8;20	1	0,25	0,06	S	S	S
OM7128	2021	RR-TB	Male	63	56 to 65	Mahwelereng Clinic	Waterberg	12;15	8;16	0,5	0,25	0,06	S	S	S
OA2441	2022	RR-TB	Male	37	36 to 45	Mahwelereng Clinic	Waterberg	7;14	7;17	0,5	0,25	0,125	S	S	S

Table 4.2 Whole Genome Results

Research ID	Gene	Coverage	Frequency	Average quality	Nucleotide	Aminoacid	Average coverage	variant	drug_class	FINAL_CONFIDENCE_GRADING
NA1080_PM	gyrB	98	100	32,84	C281T	Pro94Leu	102,8	gyrB_Pro94Leu	Fluoroquinolone	Not associated with R
NA1080_PM	gyrA	84	100	33,81	G61C	Glu21Gln	102,8	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
NA1080_PM	gyrA	96	100	33,21	G284C	Ser95Thr	102,8	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
NA1080_PM	gyrA	95	97,89	33,44	G2003A	Gly668Asp	102,8	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
NA1080_PM	mmpL5	100	100	32,44	T2842C	Ile948Val	102,8	mmpL5_Ile948Val	Bedaquiline	Not assoc w R - Interim
NA1080_PM	Rv0678	102	60,78	33,13	A88T	Arg30Trp	102,8	Rv0678_Arg30Trp	Bedaquiline	Associated with R-Interim-WHO ER
NA1080_PM	Rv0678	145	10,34	30,53	465delG	Arg156fs	102,8	Rv0678_Arg156fs	Bedaquiline	Associated with R-Interim-WHO ER
NA2620_PM	gyrA	102	100	32,39	G61C	Glu21Gln	113,83	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
NA2620_PM	gyrA	84	100	33,36	G284C	Ser95Thr	113,83	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
NA2620_PM	gyrA	119	100	33,23	G2003A	Gly668Asp	113,83	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
NA2620_PM	mmpL5	108	100	33,3	T2842C	Ile948Val	113,83	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
NB3145_PM	gyrA	89	97,75	32,71	G61C	Glu21Gln	102,88	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
NB3145_PM	mmpL5	73	98,63	32,94	T2842C	Ile948Val	102,88	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
NB6068_PM	gyrA	97	100	33,38	G61C	Glu21Gln	116,5	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
NB6068_PM	gyrA	98	98,98	33,13	G284C	Ser95Thr	116,5	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
NB6068_PM	gyrA	111	99,1	33,44	G2003A	Gly668Asp	116,5	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
NB6068_PM	mmpL5	95	100	32,82	T2842C	Ile948Val	116,5	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
NB6068_PM	Rv0678	88	30,68	34	144dup	Glu49fs	116,5	Rv0678_Glu49fs	Bedaquiline	Associated with R-Interim-WHO ER
NB6068_PM	Rv0678	111	73,87	33,37	G245T	Arg82Leu	116,5	Rv0678_Arg82Leu	Bedaquiline	CTB-R
NB8677_PM	gyrA	96	98,96	32,91	G61C	Glu21Gln	112,26	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R

NB8677_PM	gyrA	93	100	32,73	G284C	Ser95Thr	112,26	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
NB8677_PM	gyrA	91	100	33,25	G2003A	Gly668Asp	112,26	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
NB8677_PM	Rv0678	107	57,94	33,03	G245T	Arg82Leu	112,26	Rv0678_Arg82Leu	Bedaquiline	CTB-R
ND2027_PM	gyrA	105	100	32,02	G61C	Glu21Gln	112,6	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
ND2027_PM	gyrA	107	100	33,2	C269T	Ala90Val	112,6	gyrA_Ala90Val	Fluoroquinolone	Associated with R
ND2027_PM	gyrA	113	100	33,2	G284C	Ser95Thr	112,6	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
ND2027_PM	gyrA	106	100	33,42	G2003A	Gly668Asp	112,6	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
ND2027_PM	mmpL5	82	100	32,8	T2842C	Ile948Val	112,6	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
NF5136_PM	gyrA	122	100	32,3	G61C	Glu21Gln	112,49	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
NF5136_PM	gyrA	119	99,16	33,61	G284C	Ser95Thr	112,49	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
NF5136_PM	gyrA	105	100	33,77	G2003A	Gly668Asp	112,49	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
NF5136_PM	mmpL5	93	100	33,42	T2842C	Ile948Val	112,49	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
NF5136_PM	atpE	99	97,98	32,99	C198G	Ile66Met	112,49	atpE_Ile66Met	Bedaquiline	Associated with R - Interim
NG2733_PM	gyrA	95	100	32,95	G61C	Glu21Gln	110,72	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
NG2733_PM	gyrA	84	100	33,74	A281G	Asp94Gly	112,2	gyrA_Asp94Gly	Fluoroquinolone	Associated with R
NG2733_PM	gyrA	81	98,77	33,62	G2003A	Gly668Asp	110,72	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
NG2733_PM	mmpL5	69	100	32,61	T2842C	Ile948Val	110,72	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
NG2733_PM	mmpL5	80	100	32,4	G2381A	Thr794Ile	110,72	mmpL5_Thr794Ile	Bedaquiline	Not associated with R - Interim
NG2733_PM	mmpL5	88	100	30,93	C2299T	Asp767Asn	110,72	mmpL5_Asp767Asn	Bedaquiline	Not associated with R
NI1182_PM	gyrA	138	100	33,23	G61C	Glu21Gln	112,2	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
NI1182_PM	gyrA	107	100	32,9	A281G	Asp94Gly	112,2	gyrA_Asp94Gly	Fluoroquinolone	Associated with R
NI1182_PM	gyrA	107	99,07	32,45	G284C	Ser95Thr	112,2	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R

NI1182_PM	gyrA	102	100	33,12	G2003A	Gly668Asp	112,2	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
NI1182_PM	mmpL5	102	100	32,73	T2842C	Ile948Val	112,2	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
NI1182_PM	mmpL5	83	97,59	33,31	G2381A	Thr794Ile	112,2	mmpL5_Thr794Ile	Bedaquiline	Not associated with R - Interim
NI1182_PM	Rv1979c	133	99,25	32,74	C1226T	Arg409Gln	112,2	Rv1979c_Arg409Gln	Bedaquiline	Associated with R
NK5548_PM	gyrA	123	100	33,37	G61C	Glu21Gln	118,04	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
NK5548_PM	gyrA	134	99,25	33,71	G284C	Ser95Thr	118,04	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
NK5548_PM	gyrA	74	100	33,46	G2003A	Gly668Asp	118,04	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
NK5548_PM	mmpL5	94	100	31,83	T2842C	Ile948Val	118,04	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
NK5548_PM	Rv0678	101	42,57	32,6	C265T	Arg89Trp	118,04	Rv0678_Arg89Trp	Bedaquiline	Associated with R-Interim-WHO ER
NK5548_PM	Rv0678	104	21,15	33	C286T	Arg96Trp	118,04	Rv0678_Arg96Trp	Bedaquiline	Associated with R-Interim-WHO ER
NN7508_PM	gyrA	108	100	33,37	G61C	Glu21Gln	118,31	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
NN7508_PM	gyrA	106	100	32,85	C269T	Ala90Val	118,31	gyrA_Ala90Val	Fluoroquinolone	Associated with R
NN7508_PM	gyrA	96	100	33,69	A281C	Asp94Ala	118,31	gyrA_Asp94Ala	Fluoroquinolone	Associated with R
NN7508_PM	gyrA	95	100	33,6	G284C	Ser95Thr	118,31	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
NN7508_PM	gyrA	98	100	32,86	G739A	Gly247Ser	118,31	gyrA_Gly247Ser	Fluoroquinolone	Not associated with R
NN7508_PM	gyrA	105	97,14	33,25	G2003A	Gly668Asp	118,31	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
NN7508_PM	mmpL5	92	98,91	32,92	T2842C	Ile948Val	118,31	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
NN7508_PM	Rv1979c	111	99,1	33,29	C114G	Glu38Asp	118,31	Rv1979c_Glu38Asp	Bedaquiline	Uncertain significance
NN7508_PM	Rv2752c	126	100	32,9	G368A	Pro123Leu	118,31	Rv2752c_Pro123Leu	Fluoroquinolone	Not associated with R
NP7151_PM	gyrA	121	100	33,69	G61C	Glu21Gln	136,2	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
NP7151_PM	gyrA	144	97,92	32,94	C269T	Ala90Val	136,2	gyrA_Ala90Val	Fluoroquinolone	Associated with R
NP7151_PM	gyrA	150	100	33,75	G284C	Ser95Thr	136,2	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R

NP7151_PM	gyrA	116	100	33,66	G2003A	Gly668Asp	136,2	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
NP7151_PM	mmpL5	105	100	32,7	T2842C	Ile948Val	136,2	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
NP7151_PM	Rv0678	107	72,9	33,41	198dup	Ile67fs	136,2	Rv0678_Ile67fs	Bedaquiline	Associated with R-Interim-WHO ER
NP7151_PM	Rv0678	122	13,11	32,62	275dup	Tyr92fs	136,2	Rv0678_Tyr92fs	Bedaquiline	Associated with R-Interim-WHO ER
OA0531_PM	gyrA	101	99,01	33,02	G61C	Glu21Gln	105,13	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
OA0531_PM	gyrA	84	100	33,29	G284C	Ser95Thr	105,13	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OA0531_PM	gyrA	104	100	33,5	G2003A	Gly668Asp	105,13	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
OA0531_PM	mmpL5	79	97,47	32,36	T2842C	Ile948Val	105,13	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OA0531_PM	mmpL5	83	100	32,19	G2381A	Thr794Ile	105,13	mmpL5_Thr794Ile	Bedaquiline	Not associated with R - Interim
OA0531_PM	mmpL5	71	100	30,9	C2299T	Asp767Asn	105,13	mmpL5_Asp767Asn	Bedaquiline	Not associated with R
OA0531_PM	Rv2752c	121	100	33,32	C481T	Gly161Ser	105,13	Rv2752c_Gly161Ser	Fluoroquinolone	Uncertain significance
OA2914_PM	gyrA	80	100	32,7	G61C	Glu21Gln	100,59	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
OA2914_PM	gyrA	89	100	33,82	G284C	Ser95Thr	100,59	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OA2914_PM	gyrA	79	100	33,8	G2003A	Gly668Asp	100,59	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
OA2914_PM	mmpL5	60	100	33,5	T2842C	Ile948Val	100,59	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OA2914_PM	mmpL5	74	100	32,3	G2381A	Thr794Ile	100,59	mmpL5_Thr794Ile	Bedaquiline	Not associated with R - Interim
OA2914_PM	mmpL5	68	100	30,74	C2299T	Asp767Asn	100,59	mmpL5_Asp767Asn	Bedaquiline	Not associated with R
OA2914_PM	Rv0678	77	98,7	31,32	C305T	Ala102Val	100,59	Rv0678_Ala102Val	Bedaquiline	CTB-R
OA2914_PM	Rv2752c	110	100	33,78	C481T	Gly161Ser	100,59	Rv2752c_Gly161Ser	Fluoroquinolone	Uncertain significance
OA2914_PM	Rv2752c	110	100	33,78	C481T	Gly161Ser	100,59	Rv2752c_Gly161Ser	Fluoroquinolone	Not associated with R
OA6311_PM	gyrA	114	100	33,19	G284C	Ser95Thr	118,88	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OA6311_PM	gyrA	118	100	33,27	G2003A	Gly668Asp	118,88	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R

OA6311_PM	mmpL5	97	100	32,99	T2842C	Ile948Val	118,88	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OA6311_PM	mmpL5	101	99,01	31,32	G2381A	Thr794Ile	118,88	mmpL5_Thr794Ile	Bedaquiline	Not associated with R - Interim
OA7902_PM	gyrA	104	100	33,33	C269T	Ala90Val	118,31	gyrA_Ala90Val	Fluoroquinolone	Associated with R
OA7902_PM	gyrA	109	100	33,43	G284C	Ser95Thr	116,51	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OA7902_PM	gyrA	93	100	33,18	G2003A	Gly668Asp	116,51	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
OA7902_PM	mmpL5	90	100	33,22	T2842C	Ile948Val	116,51	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OA7902_PM	mmpL5	80	100	32,22	G2381A	Thr794Ile	116,51	mmpL5_Thr794Ile	Bedaquiline	Not associated with R - Interim
OA7902_PM	mmpL5	91	100	30,79	C2299T	Asp767Asn	116,51	mmpL5_Asp767Asn	Bedaquiline	Not associated with R
OA7902_PM	Rv0678	107	28,04	33,47	321dup	Ile108fs	116,51	Rv0678_Ile108fs	Bedaquiline	Associated with R-Interim-WHO ER
OA7902_PM	Rv0678	105	78,1	33,34	424dup	Leu142fs	116,51	Rv0678_Leu142fs	Bedaquiline	Associated with R-Interim-WHO ER
OC0008_PM	gyrA	98	100	33,76	G61C	Glu21Gln	110,8	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
OC0008_PM	gyrA	104	100	33,35	A281G	Asp94Gly	110,8	gyrA_Asp94Gly	Fluoroquinolone	Associated with R
OC0008_PM	gyrA	103	100	33,4	G284C	Ser95Thr	110,8	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OC0008_PM	gyrA	87	100	33,75	G2003A	Gly668Asp	110,8	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
OC0008_PM	mmpL5	86	98,84	32,94	T2842C	Ile948Val	110,8	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OC0008_PM	mmpL5	88	100	32,61	G2381A	Thr794Ile	110,8	mmpL5_Thr794Ile	Bedaquiline	Not associated with R - Interim
OC0008_PM	mmpL5	78	100	29,69	C2299T	Asp767Asn	110,8	mmpL5_Asp767Asn	Bedaquiline	Not associated with R
OC0008_PM	Rv2752c	48	100	32,17	1171delC	Arg391fs	110,8	Rv2752c_Arg391fs	Fluoroquinolone	Uncertain significance
OC0008_PM	Rv2752c	96	95,83	33,91	C481T	Gly161Ser	110,8	Rv2752c_Gly161Ser	Fluoroquinolone	Uncertain significance
OC0008_PM	Rv2752c	96	95,83	33,91	C481T	Gly161Ser	110,8	Rv2752c_Gly161Ser	Fluoroquinolone	Not associated with R
OF4644_PM	gyrA	101	99,01	33,32	G61C	Glu21Gln	114,5	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
OF4644_PM	gyrA	106	100	33,08	G280A	Asp94Asn	114,5	gyrA_Asp94Asn	Fluoroquinolone	Associated with R

OF4644_PM	gyrA	100	100	33,02	G284C	Ser95Thr	114,5	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OF4644_PM	gyrA	105	100	33,62	G2003A	Gly668Asp	114,5	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
OF4644_PM	mmpL5	103	98,06	33,09	T2842C	Ile948Val	114,5	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OF4644_PM	Rv0678	111	97,3	33,15	198delG	Ile67fs	114,5	Rv0678_Ile67fs	Bedaquiline	Associated with R-Interim-WHO ER
OF7650_PM	gyrA	109	99,08	33,24	G61C	Glu21Gln	112,29	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
OF7650_PM	gyrA	102	100	33,84	G284C	Ser95Thr	112,29	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OF7650_PM	gyrA	86	100	33,14	G739A	Gly247Ser	112,29	gyrA_Gly247Ser	Fluoroquinolone	Not associated with R
OF7650_PM	gyrA	107	100	33,23	G2003A	Gly668Asp	112,29	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
OF7650_PM	mmpL5	85	100	32,64	T2842C	Ile948Val	112,29	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OF7650_PM	mmpL5	107	100	33,44	A1792T	Trp598Arg	112,29	mmpL5_Trp598Arg	Bedaquiline	Uncertain significance
OF7650_PM	Rv0678	97	100	33,46	T451C	Ser151Pro	112,29	Rv0678_Ser151Pro	Bedaquiline	Associated with R-Interim-WHO ER
OM6733_PM	gyrA	105	100	33,3	G61C	Glu21Gln	115,1	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
OM6733_PM	gyrA	117	100	33,56	G284C	Ser95Thr	115,1	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OM6733_PM	gyrA	118	100	33,56	C1837G	Gln613Glu	115,1	gyrA_Gln613Glu	Fluoroquinolone	Not associated with R
OM6733_PM	gyrA	89	100	33,33	G2003A	Gly668Asp	115,1	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
OM6733_PM	mmpL5	119	100	32,94	T2842C	Ile948Val	115,1	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OS4813_PM	gyrA	151	100	33,19	G61C	Glu21Gln	119,67	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
OS4813_PM	gyrA	143	100	33,94	A281G	Asp94Gly	119,67	gyrA_Asp94Gly	Fluoroquinolone	Associated with R
OS4813_PM	gyrA	150	100	33,35	G284C	Ser95Thr	119,67	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OS4813_PM	gyrA	142	100	33,42	G2003A	Gly668Asp	119,67	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
OS4813_PM	mmpL5	82	100	33,17	T2842C	Ile948Val	119,67	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OS5480_PM	gyrA	134	99,25	33,31	G61C	Glu21Gln	119,53	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R

OS5480_PM	gyrA	134	100	33,39	G284C	Ser95Thr	119,53	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OS5480_PM	gyrA	112	100	32,98	G2003A	Gly668Asp	119,53	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
OS5480_PM	mmpL5	98	100	33,14	T2842C	Ile948Val	119,53	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OS5480_PM	mmpL5	99	98,99	32,43	G2381A	Thr794Ile	119,53	mmpL5_Thr794Ile	Bedaquiline	Not associated with R - Interim
OS5480_PM	mmpL5	98	98,98	29,28	C2299T	Asp767Asn	119,53	mmpL5_Asp767Asn	Bedaquiline	Not associated with R
OS5480_PM	Rv0678	92	94,57	32,8	144dup	Glu49fs	119,53	Rv0678_Glu49fs	Bedaquiline	Associated with R-Interim-WHO ER
OS8448_PM	gyrB	99	98,99	33,78	G873C	Met291Ile	101,94	gyrB_Met291Ile	Fluoroquinolone	Not associated with R
OS8448_PM	gyrB	84	100	33,29	G1337A	Arg446His	101,94	gyrB_Arg446His	Fluoroquinolone	Associated with R
OS8448_PM	gyrA	80	100	32,98	G61C	Glu21Gln	101,94	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
OS8448_PM	gyrA	73	98,63	33,69	G284C	Ser95Thr	101,94	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
OS8448_PM	gyrA	98	100	33,39	C1151T	Ala384Val	101,94	gyrA_Ala384Val	Fluoroquinolone	Not associated with R
OS8448_PM	gyrA	79	100	33,9	G2003A	Gly668Asp	101,94	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
OS8448_PM	mmpL5	71	98,59	32,89	T2842C	Ile948Val	101,94	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
OS8448_PM	mmpL5	64	100	32,47	G2381A	Thr794Ile	101,94	mmpL5_Thr794Ile	Bedaquiline	Not associated with R - Interim
OS8448_PM	Rv0678	93	93,55	32,28	198delG	Ile67fs	101,94	Rv0678_Ile67fs	Bedaquiline	Associated with R-Interim-WHO ER
OS8448_PM	Rv1979c	70	100	33,03	T857C	Asp286Gly	101,94	Rv1979c_Asp286Gly	Bedaquiline	Not associated with R - Interim
PI4323_PM	gyrA	96	100	32,6	G61C	Glu21Gln	113,89	gyrA_Glu21Gln	Fluoroquinolone	Not associated with R
PI4323_PM	gyrA	92	98,91	33,34	C269T	Ala90Val	113,89	gyrA_Ala90Val	Fluoroquinolone	Associated with R
PI4323_PM	gyrA	95	100	33,52	G284C	Ser95Thr	113,89	gyrA_Ser95Thr	Fluoroquinolone	Not associated with R
PI4323_PM	gyrA	85	100	33,48	G2003A	Gly668Asp	113,89	gyrA_Gly668Asp	Fluoroquinolone	Not associated with R
PI4323_PM	mmpL5	115	100	33,32	T2842C	Ile948Val	113,89	mmpL5_Ile948Val	Bedaquiline	Not associated with R - Interim
PI4323_PM	Rv0678	89	95,51	33,72	198dup	Ile67fs	113,89	Rv0678_Ile67fs	Bedaquiline	Associated with R-Interim-WHO ER