

WHOLE GENOME SEQUENCING OF *MYCOBACTERIUM TUBERCULOSIS* AND ASSESSMENT OF TREATMENT OUTCOME IN TB PATIENTS FROM VHEMBE DISTRICT

by

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DECLARATION

I, **Ntshunxeko Thelma Banda** (Student number: **16013629**), hereby declare that the thesis titled; **Whole genome sequencing of *Mycobacterium tuberculosis* and assessment of treatment outcome in tb patients from Vhembe district** submitted to the University of Venda for the degree Doctor of Philosophy in Microbiology has not been previously submitted by me to this or any other University, and the work contained herein is my own work, and all other materials used are acknowledged accordingly.



20 March 2026

Signature

Date

DEDICATION

I dedicate this work to myself as a reminder that all things are possible, no matter the situation. One only needs to have faith in the LORD and a support system that only wants the best for you. **The delayed goal is not a goal denied.**

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LIST OF ABBREVIATIONS

ACP	ACYL CARRIER PROTEIN
AM	AMIKACIN
AIDS	ACQUIRED IMMUNODEFICIENCY SYNDROME
BCG	BACILLUS OF CALMETTE AND GUERINE
BDQ	BEDAQUILINE
BpaL	BEDAQUILINE + PRETOMANID + LINEZOLID
BPaLM	BEDAQUILINE + PRETOMANID + LINEZOLID + MOXIFLOXACIN
BSL	BIOSAFETY LEVEL
CCM	CENTRAL CARBON METABOLISM
CDC	CENTERS OF DISEASE CONTROL
CXR	CHEST X-RAY
Ddn	DEAZAFLAVIN-DEPENDENT NITROREDUCTASE
DLM	DELAMANID
DNA	DEOXYRIBONUCLEIC ACID
DR-TB	DRUG-RESISTANT TUBERCULOSIS
DST	DRUG SUSCEPTIBILITY TEST
DTH	DELAYED-TYPE HYPERSENSITIVITY
EMA	EUROPEAN MEDICINES AGENCY
EMB	ETHAMBUTOL
ESAT	EARLY SECRETED ANTIGENIC TARGET
ESX	ESAT-6 SECRETION SYSTEM
FDA	FOOD AND DRUG ADMINISTRATION
FQ	FLUOROQUINOLONES
HIV	HUMAN IMMUNODEFICIENCY VIRUS
INH	ISONIAZID
RIF	RIFAMPICIN
LAM	LIPOARABINOMANNAN
LED	LIGHT EMITTING DIODE
LM	LIPOMANNAN
LTBI	LATENT TUBERCULOSIS INFECTION
M	MONO-RESISTANT
MDR-TB	MULTIDRUG-RESISTANT TB

MGIT	MYCOBACTERIA GROWTH INDICATOR TUBE
MTB	<i>MYCOBACTERIUM TUBERCULOSIS</i>
MTBC	<i>MYCOBACTERIUM TUBERCULOSIS</i> COMPLEX
NAT	N-ACETYLTRANSFERASES
NDP	NATIONAL DEVELOPMENT PLAN
NGS	NEXT GENERATION SEQUENCING
NHLS	NATIONAL HEALTH LABORATORY SERVICE
NTM	NONTUBERCULOUS MYCOBACTERIA
PCR	POLYMERASE CHAIN REACTION
PDIM	PHTHIOCEROL DIMYCOCEROSATES
PGL	PHENOLIC GLYCOLIPIDS
PI	PHOSPHATIDYLINOSITOLS
PPD	PURE PROTEIN DERIVATIVE
POA	PYRAZINOIC ACID
PZA	PYRAZINAMIDE
R	RESISTANT
RNA	RIBONUCLEIC ACID
RRDR	RIFAMPICIN RESISTANCE DETERMINING REGIONS
RR-TB	RIFAMPICIN RESISTANCE TUBERCULOSIS
S	SUSCEPTIBLE
SA	SOUTH AFRICA
SDG	SUSTAINABLE DEVELOPMENT GOALS
SNP	SINGLE NUCLEOTIDE POLYMORPHISM
STR	STREPTOMYCIN
TB	TUBERCULOSIS
TB-SS	TUBERCULOSIS SIGN AND SYMPTOMS
WGS	WHOLE GENOME SEQUENCING
WHO	WORLD HEALTH ORGANISATION
XDR-TB	EXTREMELY DRUG-RESISTANT TUBERCULOSIS
ZN	ZIEHL-NEELSEN

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PUBLICATIONS AND CONFERENCES

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Banda N.T., Troare AN, and Potgieter N (2024). Rapid detection of *Mycobacterium tuberculosis* directly from sputum samples of patients from healthcare facilities in Venda, South Africa. SASBMB, 7-9 July, Polokwane, South Africa (Poster)

EXECUTIVE SUMMARY

BACKGROUND

Tuberculosis (TB) remains one of the most pressing global health challenges, contributing significantly to morbidity and mortality worldwide. The burden is particularly severe in regions with high HIV prevalence, where immunocompromised individuals are more susceptible to TB infection. South Africa, and specifically Limpopo Province, continues to face a dual epidemic of TB and HIV, with drug-resistant TB (DR-TB) posing a growing threat to public health. The emergence of multidrug-resistant TB (MDR-TB), pre-extensively drug-resistant TB (pre-XDR-TB), and extensively drug-resistant TB (XDR-TB) complicates treatment regimens, increases costs, and reduces treatment success rates. Despite national efforts to implement WHO-endorsed diagnostics such as Xpert MTB/RIF and Genotype MTBDRplus, limitations remain. These assays primarily detect mutations within the rifampicin resistance-determining region (RRDR) of the *rpoB* gene, but mutations outside this region may go undetected, leading to underestimation of resistance prevalence.

AIM

To investigate the genomic characteristics of *Mycobacterium tuberculosis* using whole-genome sequencing and their association with treatment outcomes among TB patients in the Vhembe District.

METHODOLOGY

This study employed a cross-sectional design incorporating both qualitative and quantitative components to comprehensively investigate tuberculosis (TB) within the Vhembe District of South Africa. Ethical approval was granted by the University of Venda Research Ethics Committee (SMNS/20/MBY/13/2104), with additional authorisation obtained from the Limpopo Department of Health (LP_2021_11_001). Institutional permission was secured from participating healthcare facilities prior to data collection. All participants received detailed information regarding the study objectives and procedures, and written informed consent was obtained in accordance with established ethical standards for research involving human participants. The

study population comprised adults aged 18 years and older who were diagnosed with TB and actively receiving treatment, either as inpatients or outpatients. A total of 70 sputum samples were collected using sterile containers pre-filled with 4% NaOH. Specimens were transported to the University of Venda TB laboratory under temperature-controlled conditions to preserve sample integrity. DNA extraction and Real-time PCR [Anyplex™ and Allplex™ (Seegene, Korea)] were performed according to the manufacturer's protocols to determine the MTB type in sputum samples. Further analysis was performed using various sequencing techniques to identify mutations.

RESULTS: The study enrolled more males (55%) and detected MTB as the predominant pathogen within the cohort, with a measurable prevalence of DR-TB [(4/100) 4%]. Resistance-associated mutations were detected in key loci, including *rpoB*, *katG*, and *inhA*, consistent with rifampicin and isoniazid resistance as determined by the Seegene assays. A highlight of an INH-R mono-resistant strain was identified, underscoring the importance of regular monitoring of TB epidemics. Importantly, genomic analyses revealed additional variants beyond regions routinely targeted by conventional diagnostics, highlighting the limitations of mutation-restricted assays. Notably, none of the DR-TB cases reported prior TB treatment, suggesting primary transmission of resistant strains rather than resistance acquired through therapeutic mismanagement. Shotgun metagenomics further revealed the presence of non-tuberculous mycobacteria (NTM) and other respiratory pathogens, underscoring the polymicrobial complexity of respiratory disease and the potential for diagnostic misclassification. The treatment outcome was more favourable [cure (46.34%) and treatment completion (18.29%)], with favourable outcomes outweighing unfavourable outcomes, and some factors were statistically significant ($p < 0.05$). Collectively, these findings indicate that, while favourable outcomes were achieved in the majority of cases, the presence of ongoing treatment and a small proportion of treatment failures highlight the continued need for effective monitoring and management strategies to improve treatment success rates within the study population.

CONCLUSION

The study highlights the importance of improving diagnostic techniques in low-income settings to prevent underdiagnosis and misdiagnosis, as NTM and INH-R mono-resistant strains were detected. Additionally, the study demonstrates the benefits of combining high-throughput genotyping, metagenomics, and structural modelling in TB research. This also highlights the effectiveness of molecular techniques in reducing turnaround time, preventing misdiagnosis, and detecting polymicrobial infections that may compromise TB treatment outcomes. By integrating molecular epidemiology and functional prediction, the results provide new insights into the dynamics of resistance transmission, diagnostic gaps, and targeted treatment approaches. The integration of genetic and statistical modelling provides a strong framework for enhancing DR-TB surveillance, improving treatment plans, and expanding individualized TB care in high-burden settings.

Keywords: Drug resistance TB, *Mycobacterium tuberculosis*, Treatment outcome, Whole genome sequencing (WGS).

CHAPTER 1

GENERAL INTRODUCTION

The introduction of this study, as well as the aim, problem statement, rationale, and objectives, are given in this chapter.

1.1 BACKGROUND

Tuberculosis (TB) is a curable infectious disease caused by the human pathogenic bacterium known as *Mycobacterium tuberculosis* (MTB). The bacterium belongs to the family Mycobacteriaceae and parasitizes host macrophages for survival (Miggiano et al., 2020; Lee et al., 2009). The *Mycobacterium* genus is hypothesized to have originated more than 150 million years ago (Barberis et al., 2017). This tubercle bacillus was discovered in 1822 by Robert Koch (a German physician and microbiologist), marking a turning point in understanding TB.

Despite being preventable and curable, this contagious bacterium is among the leading causes of morbidity and mortality worldwide and currently the leading cause of death as a single infectious agent (WHO, 2025). According to the WHO (2025), approximately 10.7 million cases and 1.23 million deaths were reported globally in 2024. A delayed public health response to the rapidly expanding Human immunodeficiency virus (HIV) epidemic in the late 1980s and early 1990s led to a sharp increase in TB rates, particularly in Sub-Saharan Africa (Abdool et al., 2010; Harries et al., 2001). Although TB mortality is strongly influenced by HIV co-infection in high-burden regions, a substantial proportion of deaths occur among HIV-negative individuals, highlighting the persistent global impact of the disease. These figures underscore the continued public health importance of TB and the need for improved diagnostic, treatment, and prevention strategies (WHO, 2025).

Tuberculosis is transmitted from one person to another through tiny droplets released into the air during coughing and sneezing (Swalehe et al., 2024). Individuals nearby may inhale the bacteria and become infected. This infection commonly affects the lungs; however, it has also been identified to affect other body parts, such as the brain, kidneys, and spine (Leowattana et al., 2023; Mejbek et al., 2023). The MTB pathogenesis is known to have two stages: the latent and the infectious stage. In the

latent stage, the bacterium is present; however, the immune system successfully contains it, preventing progression to the active stage (Verma et al., 2022). The latent infection may persist for years in the host and develop into an active stage when an individual is immunocompromised. In addition, the latent infection does not exhibit symptoms and is not contagious (Zhuang et al., 2024; Kiazzyk and Ball, 2017; Andrews et al., 2012). Once in the active stage (contagious), the bacillus begins to replicate and presents symptoms such as fatigue, coughing for several weeks, chest pain, sudden and unexplained weight loss, and night sweats (Law et al., 2020; Long et al., 2002).

Tuberculosis has been known to humankind for many decades, and the discovery of antibiotics in the 1940s led to the belief that TB occurrence would decrease, and it is considered a historical disease outbreak (Chakraborty and Jayawickrama, 2024; Saleem and Azher, 2013; Herzog, 1998). However, TB is still a substantial public health threat. In poverty-stricken populations, especially in those classified as low-income countries, TB is reported to cause significant illness. Studies have proven that poverty is the leading factor resulting in TB patients' non-compliance with treatment (Ramaliba et al., 2017; Ibrahim et al., 2014). This is supported by study findings showing a higher prevalence of TB in rural areas than in urban areas (Ramaliba et al., 2017; Ephrem et al., 2015). Haider et al. (2013) reported that poor housing conditions, such as overcrowding, increase TB transmission and weaken immunity. In 2019, TB remained the most common cause of death from a single infectious pathogen. An estimated 10.0 million individuals worldwide had TB infection, with an estimated 1.2 million TB fatalities among HIV-negative individuals and an additional 208,000 deaths of people living with HIV from this disease in the year 2019 (WHO, 2020).

Treatment for TB is divided into two main categories: first-line drugs (used for drug-sensitive TB) and second-line drugs (used for drug-resistant TB) (WHO, 2018). First-line drugs are essential for optimal effectiveness, and these include Isoniazid (INH), Rifampin (RIF), Ethambutol (EMB), Pyrazinamide (PZA), and Streptomycin (STR). Second-line drugs include fluoroquinolones and injectables such as kanamycin, capreomycin, and amikacin (WHO, 2018). TB treatment is effective. However, the side effects and organ toxicity are significantly reported to be associated with defaulting (Kuganab-lem et al., 2023).

As a result of the regimen's side effects and toxicity, this leads to antibiotic misuse, which is defined as failure to complete treatment, skipping of doses, and reusing

leftover antibiotics. Adherence to therapy requirements exposes the patient to minimal antibiotic doses, potentially resulting in insufficient antibiotic exposure to eradicate the bacterial infection. This behaviour has the potential to promote antibiotic resistance. Antibiotic misuse has consequences for individuals and communities, including antibiotic resistance, treatment failure, treatment cost, and environmental health hazards.

An additional factor in TB cases is misdiagnosis. A study done in Ethiopia reported detecting 2.4% false positives and 0.4% false negatives using the Ziehl-Neelsen (ZN) technique (Desalegn et al., 2018). In 2010, the WHO endorsed and implemented the molecular technique GeneXpert MTB/RIF to simultaneously detect MTB and RIF resistance, an indicator of MDR-TB. However, the technique has been proven to have its downfall. Studies have demonstrated the potential for misdiagnosis of some Nontuberculous mycobacteria (NTM) species as MTB at high bacterial loads in TB-endemic settings, highlighting the need to consider NTMs during pulmonary TB treatment (Karamat et al., 2021; Maiga et al., 2012). This is an essential note for NTM technicians in diagnostic laboratories when interpreting GeneXpert results in clinical practice (Pang et al., 2017). In the same study, it was noted that *M. abscessus* was misdiagnosed as a RIF-resistant MTB, resulting in an improper therapeutic regimen and treatment failure (Pang et al., 2017).

According to the research reported, the different TB diagnostic techniques have important advantages and limitations, particularly in terms of turnaround time, misreads, improper interpretation, and area-specific detections (Song et al., 2025; Acharya et al., 2020). This emphasizes that TB research must continue to improve diagnostic measures to achieve the Sustainable Development Goals (SDGs) target of ending TB in our communities by 2030. Noting factors arising from TB outbreaks, the development of drug-resistant TB (DR-TB), and the use of molecular technology for diagnosis and treatment determination, the way forward is to develop and implement strategies (Arinaminpathy et al., 2023; WHO, 2017). This can be addressed by conducting more research studies that examine a broader region of drug resistance genes and the prediction of regimen response. Resulting in patients adhering to regimen periods and discovering new regimens that may reduce the period.

1.2 PROBLEM STATEMENT

Over the past decades, MTB has developed drug-resistant strains, which adds to the challenges already being encountered in treating TB in South Africa and other parts of the world. Worldwide reports estimated 10.4 million new cases of tuberculosis (TB) in 2015, 1.3 million HIV-negative deaths from TB, and 374,000 HIV-positive fatalities (WHO, 2017). In African regions, TB prevalence remains particularly high due to the concurrent presence of HIV as well as poverty that influences malnutrition, overcrowding, doing odd jobs, and overall, the type of lifestyle in the rural communities (Wondmeneh and Mekonnen, 2023; Maja and Maposa, 2022; Millet et al., 2013).

The prevalence of drug-resistant tuberculosis (DR-TB) is rising and could jeopardize global attempts to eradicate the disease (WHO, 2014). The rise in MDR-TB incidence may be partly due to the lack of monitoring resistance development (Dowdy et al., 2008). A non-compliant TB patient who does not complete their treatment regimen contributes to the development of drug-resistant MTB mutations and to irrational antibiotic use (Chowdhury and Chowdhury, 2022; Ranawaka et al., 2021). The regimen does not eradicate the established resistance and provides a replicative environment. This poses a public health threat, as an unknown MTB strain may spread within a community. Individuals who experience active disease either fail to contain the initial infection or experience reactivation due to partial or complete immune suppression at some point after the initial infection (Espinal, 2001). An individual with MDR-TB requires a change in a regimen that is categorized as second-line drugs, such as ethionamide (Girard et al., 2005). This treatment is taken over a more extended period, is costly, and more toxic than first-line drugs used for routine treatment of TB (Shah et al., 2007).

The continued lack of treatment adherence leads to an environment where more mutations occur. This results in the emergence of XDR-TB; the MTB strain is resistant to at least one injectable second-line medication, such as amikacin, in addition to first-line regimen resistance and fluoroquinolone resistance (Jassal and Bishai, 2009). In 2009, it was reported that this would increase the statistics of mortality due to TB infection since TB had become more complex and formulation of an effective regimen would take time (Jassal and Bishai, 2009). Individuals with HIV or any other co-morbidities do not necessarily increase the drug resistance of TB. However, their immune system is already immunocompromised, making it much more likely that

MDR- and XDR-TB infections will be severe (Sumner et al., 2019; Zhang et al., 2019; Dowdy et al., 2008).

Historically, diagnosis relied on the gold standard, which involved culture-based methods that can take up to 8 weeks to confirm TB, leading to prolonged diagnosis and increasing the risk that the infected individual will spread the disease (WHO, 2015). In contrast, molecular diagnostic techniques provide results in 48 hrs from direct sputum samples. However, the current molecular diagnosis method endorsed by WHO and used in South African laboratories, usually located in Hospitals, is an area-targeted method. It focuses on the known “hotspots,” which can miss new mutations that may be endemic and may contribute to delays in ending TB in rural communities of developing countries. Notably, the GeneXpert (current method) assay primarily identifies rifampicin resistance within the *rpoB* rifampicin resistance-determining region, which, while clinically useful, does not capture the full spectrum of resistance-conferring mutations (Beckert et al., 2020; Makhado et al., 2018).

To achieve goal 3 of SDG, which strongly fights in accomplishing to end TB-caused deaths by 2030. WHO also developed the END TB strategy, which targets reducing TB-related suffering and stigma, a 90% reduction in death rates, an 80% reduction of TB deaths, and Universal access to TB care for all individuals. In addition, the South African National Development Plan (NDP) acknowledges TB as a major challenge in healthcare facilities and outlines strategies to control and eventually eliminate TB in our society. The strategies may be feasible with continuous improvement of diagnostic tools, as they are reported to have great potential to rapidly and accurately detect drug-resistant MTB strains, which pose a major health threat. This will help implement preventive measures to ensure access to efficient treatment and reduce the dissemination of potential novel mutations.

1.3 STUDY RATIONALE

Despite decades of research, TB remains a major global public health concern and is classified as one of the world's leading causes of death (Pan et al., 2020). The persistence of TB is largely due to the emergence of DR-TB forms, which complicates treatment and increases poor outcomes (Koch et al., 2018; Girard et al., 2005). In SA, TB continues to cause immense human suffering and economic loss (Foster et al., 2015). With an incidence rate of 615 per 100,000 of the population, TB in SA is exceptionally high compared to the global average of 130 per 100,000 people (WHO, 2020). The country is categorized as one of 20 countries that overlap with high burdens of TB, multidrug TB (MDR-TB), extensively drug-resistant TB (XDR-TB), and TB-HIV co-infection, with 58% prevalence of HIV among TB incident cases (WHO, 2020).

Currently, TB is curable with a standard six-month regimen of the first-line drugs [isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB)] for six months. However, in the 21st century, a public health threat emerged when Multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) cases started to be reported. Drug resistance arises from factors such as inappropriate use of antibiotics, the use of ineffective drug formulations (such as the use of single medications, low-quality medications, or improper storage conditions, and premature treatment termination with increased transmission in settings like mines, prisons, and hospitals (WHO, 2018). An individual with MDR-TB is prescribed a different regimen categorized as second-line drugs, which are usually longer, costlier, and more toxic (Lu et al., 2017). XDR-TB, defined as resistance to INH, RIF, and one fluoroquinolone, and a second-line injectable (amikacin, capreomycin, and kanamycin), poses an even greater threat, as treatment options are limited, costly, and often ineffective (CDC, 2013). In KwaZulu-Natal, XDR-TB transmission has been attributed more to person-to-person spread than to inadequate MDR-TB treatment (Shah et al., 2017).

The yearly incidence of MDR-TB cases was 19 per 100,000, with 71% treatment success for new and relapse TB, which was substantially lower than the global average of 14% (WHO, 2020). Additionally, in 2015, a study reported an increase in the incidence rate (3.5 per 100,000) of XDR cases (Lim et al., 2015). The country's TB epidemic is driven by several factors, such as low socioeconomic status and high TB-HIV coinfection, which continue to impose a substantial burden on the health system (WHO, 2023). South Africa's significant socioeconomic inequalities, together with TB,

flourish among vulnerable populations that live in poverty and high-density areas (WHO, 2020).

South Africa has been a national leader in deploying two molecular assays endorsed by WHO: Genotype MTBDRplus and Xpert MTB/RIF. Since the year 2012, the Xpert MTB/RIF assays have progressively replaced smear microscopy as a first-line test to rapidly diagnose TB and RIF resistance among people with presumptive tuberculosis in South Africa. These tests are complemented by culturing with the BACTEC mycobacteria growth indicator tube (MGIT) 960 cultured detection system, which was introduced as a gold standard for drug susceptibility testing in the early 2000s.

However, the assays used in the National Health Laboratory Service (NHLS), located in Hospitals in South Africa, are designed to detect mutations in the rifampicin resistance-determining regions (RRDR) of the *rpoB* gene, covering more than 95% of the known RR mutations among MTB strains. In addition, these tests are PCR-probe limited anti-TB drug susceptibility value, mainly when mutations occur outside the target regions; for example, they do not detect RR associated with *rpoB* mutations in codons 170 and 491, located outside the RRDR region. This may result in having undetected RR strains that may affect the control of drug-resistant TB in the future. Although the available data suggest that these mutations outside the RRDR are rare globally, they may be more prevalent locally.

Studies have shown that current diagnostic methods are slow to return results and are area-targeted; therefore, faster, more comprehensive diagnostics will enable faster turnaround times with detailed, accurate profiles, allowing earlier use of the most appropriate drug regimen. Thus, improving patients' outcomes and reducing regimen costs. The recent approach identified as a rapid and comprehensive method for viewing the organism's genotypes is Whole Genome Sequencing (WGS). Several studies have demonstrated that WGS provides high-resolution when investigating transmission events in potential outbreak scenarios. In addition, studies have proven WGS as a valuable tool to predict drug resistance to MTB (Wang et al., 2022; Doyle et al., 2018; Macedo et al., 2018.; Lee and Pai, 2017; Wollenberg et al., 2017; Brown et al., 2015; Witney et al., 2015). Faster, more comprehensive diagnostics will enable earlier use of the most appropriate drug regimen, thus improving patient outcomes and reducing overall healthcare costs (Witney et al., 2016). Therefore, strengthening

the quality and processes for screening, testing, and diagnosing patients within primary healthcare facilities is essential (Murdoch et al., 2021).

Novel diagnostics can substantially help reduce TB incidence and mortality and constantly monitor resistance development, contributing to increasing MDR-TB (Sun et al., 2013; Dowly et al., 2008). A study by Katale et al. (2020) used the WGS approach to uncover gene mutations currently used to predict phenotypic resistance to anti-TB drugs. Another study in Eswatini confirmed that clonal transmission of a particular MDR-TB outbreak is a driver of the MDR-TB epidemic, driven by a mutation missed by the Xpert assay (Beckert et al., 2020). This signifies the importance of this approach before decision-making for anti-tuberculosis treatment. Thus, WGS has the potential to inform the design of an optimally individualized treatment regimen for favourable treatment outcomes in endemic TB settings.

In addition, previous authors demonstrated that drug susceptibility test (DST) results from WGS can help clinicians manage patient care by recommending the most effective, individualized DR-TB treatments based on specific gene-derived profiles (Mahomed et al., 2017; Satta et al., 2017). This study aimed to better understand the regions responsible for drug-resistance mutations and investigate the effect of mutations within and outside the RRDR regions on diagnosis and treatment outcomes. Therefore, the study focused on sequencing the whole genome of *MTB* from sputum samples of active TB patients in healthcare facilities in the Vhembe district to correlate with treatment response and outcome, a linkage previously not reported. The results of this study might help improve patient outcomes, enhance personalized medical techniques, and strengthen TB control measures.

1.4 RESEARCH QUESTIONS

- What is the prevalence of MDR/XDR amongst diagnosed TB patients in Vhembe district?

Drug-resistant TB represents a significant public health threat. According to the WHO, drug resistance affects the global advancement in TB treatment and control. In comparison to other regions, Sub-Saharan Africa reported a low prevalence of multidrug-resistant TB. However, certain risk factors for MDR-TB may influence the

prevalence of MDR-TB in different regions. Risk factors include re-infections, HIV infection, or exposure to MDR-TB cases.

- Do bacterial co-occurrence play a role in drug-resistant TB treatment outcomes?

Bacterial co-occurrence may complicate TB management, particularly in resource-limited settings, where concurrent detection of MTB and other bacteria can contribute to diagnostic uncertainty and potentially influence treatment decisions. A study in the

- What is the success rate in treating drug-resistant TB in the study area?

In 2020, it was estimated that 9.9 million new cases were reported globally, of which about half a million were multidrug-resistant TB. Multidrug emergence threatens global public health efforts to control TB, which severely impacts treatment outcomes. Understanding the rate of treatment success at the local level (rural communities) will help determine the effectiveness of the regimens currently used in healthcare facilities in South Africa.

1.5 AIM AND OBJECTIVES

1.5.1 AIM

To investigate the genomic characteristics of *Mycobacterium tuberculosis* using whole-genome sequencing and their association with treatment outcomes among TB patients in the Vhembe District.

1.5.2 OBJECTIVES

- To determine the MDR/XDR-TB prevalence using Anyplex TM and Allplex TM assay.
- To sequence the whole genome of *Mycobacterium tuberculosis* using Next Generation Sequencing (NGS) and MassArray to identify mutations linked to drug resistance.
- To evaluate TB treatment outcomes by prediction as suggested by WHO guidelines and statistical analysis.

1.6 DISSERTATION OUTLINE

The following figure (Figure 1.1) summarizes the study outline.

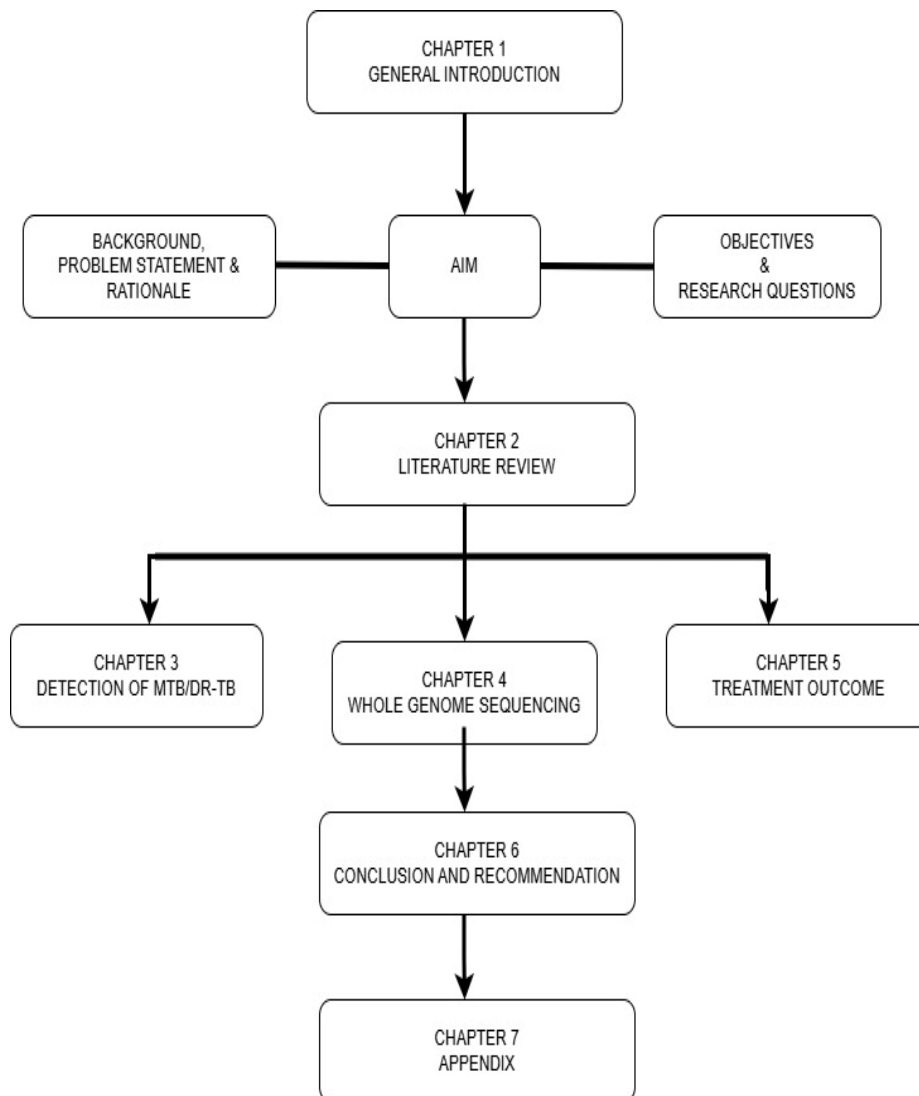


Figure 1.1: Dissertation outline (designed using draw.io)

The diagram outlines the flow from the general introduction—encompassing the background, problem statement, rationale, aim, objectives, and research questions—through the literature review, to the empirical chapters focusing on the detection of *Mycobacterium tuberculosis* and drug-resistant TB, whole-genome sequencing analysis, and treatment outcomes. The framework culminates in the conclusion and recommendations, with supporting materials provided in the appendix.

CHAPTER 2

LITERATURE REVIEW

This chapter summarizes the literature and provides an overview of tuberculosis, including the state of resistance, the causes of treatment failures, and the causative bacterium, MTB. The emphasis is placed on the Vhembe District, and information is drawn from previously published studies.

2.1 INTRODUCTION

Mycobacterium tuberculosis (MTB) was first described by Koch in 1882, and the tuberculin test for diagnosing the disease was developed in 1890. During this time, TB killed one out of every seven people living in the United States and Europe (Thomas, 2009). The first successful immunization against tuberculosis (TB) was developed using the attenuated bovine strain of tuberculosis by bacteriologists Albert Calmette and Camille-Guérin in 1996. The vaccine was named after the discoverers and is known as Bacillus of Calmette and Guérine (BCG). Understanding TB is crucial for seeking medical care in time. A delayed diagnosis may lead to an outbreak of TB in a community or population. South Africa was previously reported as having one of the highest incidences of TB worldwide. This may be influenced by demographic, socioeconomic, and environmental factors (Ramaliba et al., 2017).

Tuberculosis has developed a drug-resistant strain, which is a global issue and a significant public health threat. The emerging drug-resistant strains pose a severe global threat and pose a considerable challenge to public health. In 2018, the WHO reported that there were an estimated 10 million cases of TB infection and 1.3 million deaths in 2017. In 2005, drug-resistant TB was first detected in Tugela Ferry, and since then, the incidence of multidrug-resistant (MDR) and extensively drug-resistant TB has increased. Pietersen and colleagues (2014) reported South Africa as the second-highest country for MDR-reported cases. Since 2020, tuberculosis has been recorded as the second leading cause of death after COVID-19 (WHO, 2022). This single infectious disease has put many lives at risk, hence labelled as a global health concern. Millions of people die from TB regardless of their co-infection with other co-morbidities such as HIV, Diabetes, and Hypertension (Glaziou et al., 2018). Therefore,

developing, improving, and evaluating methods for detecting *MTB* is essential to help assess the treatment outcomes in rural settings.

2.2 GENERAL CHARACTERISTICS OF *MYCOBACTERIUM TUBERCULOSIS*

The bacterium *MTB* causes TB, a persistent infectious disease. The bacterium is classified under the family Mycobacteriaceae, genus *Mycobacterium* (Meehan et al., 2021). They are classified as high GC gram-positive bacteria belonging to the Actinobacteria phylum. Their cell envelope, as illustrated in Figure 2.1, is a distinctive characteristic that enables them to survive within host macrophages and to shield them from hostile ecological conditions. The mycobacteria have an inner and an outer membrane in their cell envelope, despite being Gram-positive bacteria.

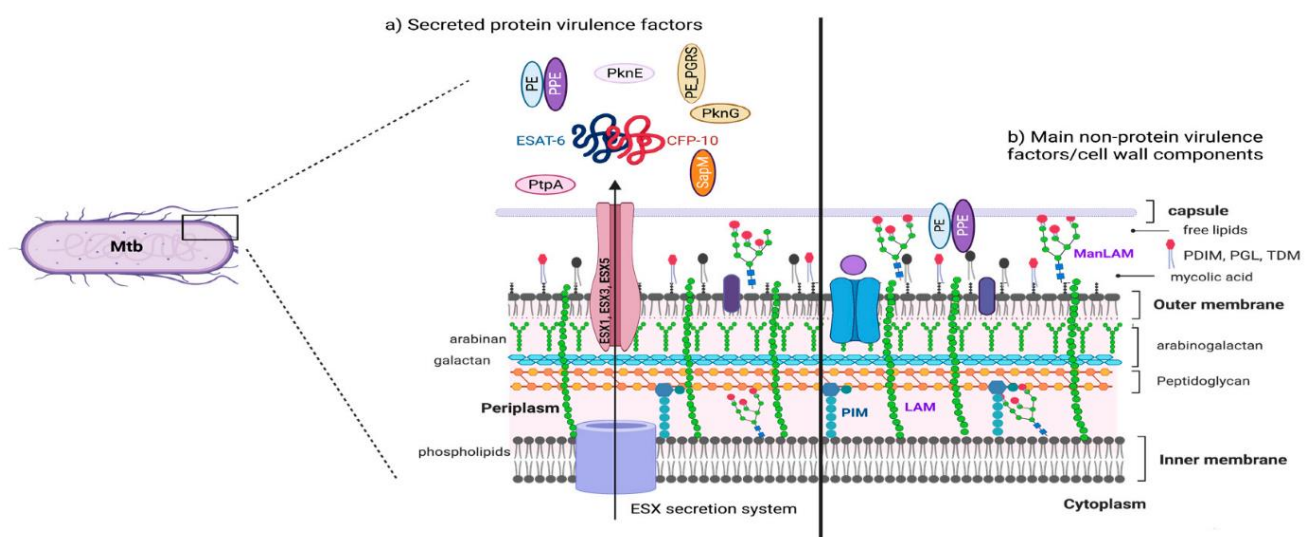


Figure 2.1: Structure of *Mycobacterium tuberculosis* (MTB). (adapted from Ramon-Luing et al., 2023). Schematic representation of the structural organisation and virulence determinants of *Mycobacterium tuberculosis* (MTB).

Mycobacterium tuberculosis is closely related to other species that are reported to cause TB in different hosts, for example, *Mycobacterium bovis* infects cattle (Phillips et al., 2003; Morris et al., 1994); *Mycobacterium caprae* infects goats (Bezous et al., 2010); *Mycobacterium suricattae* infects meerkat (Parsons et al., 2013); *Mycobacterium tuberculosis* in humans (Koch and Mizrahi, 2018; Deretiz and Fratti, 1999). All these species, along with others (e.g. *Mycobacterium tuberculosis*,

Mycobacterium africanum, *Mycobacterium microti*), form the *Mycobacterium tuberculosis* complex (MTBC) (Riojas et al., 2018). They niche in different environments such as the soil, water, plants and animals (Gagneux, 2018).

The cell wall is vital to *MTB*'s survival and pathogenicity (Kalscheuer et al., 2019). In almost all cases, TB is treatable and curable; however, if left untreated or if treatment is not completed, people die (Nguta et al., 2015). The outer membrane is a complex, asymmetrical structure comprising lipids, glycolipids, and proteins rather than the typical liposaccharide-containing membrane found in Gram-negative bacteria (Garcia-Vilanova et al., 2019). Unique lipids such as phenolic glycolipids (PGL), lipoarabinomannan (LAM), Lipomannan (LM), Phosphatidylinositols (PI) and phthiocerol dimycocerosates (PDIM) are abundant in the outermost layer of the outer membrane (Ghazaei, 2018). Furthermore, these unique lipids are virulent factors that influence the host immune system and enhance mycobacteria's pathogenicity. For example, Lipoarabinomannan (LAM), a major component of the mycobacterial cell envelope, acts as an immunomodulatory virulence factor by regulating macrophage activation and the downstream immune signalling pathway (Correia-Neves et al., 2019).

A hallmark feature of the mycobacterial cell envelope is mycolic acids, long-chain fatty acids with up to 90-carbon chain length (Minnikin et al., 2015). They contribute to the cell envelope's impermeability and are responsible for mycobacteria's acid-fast staining property. The bacteria are described as an acid-fast bacillus, typically measuring 0.5 μm by 3 μm , and they have a unique cell wall structure that is essential to their survival. A significant amount of the well-developed cell wall is covalently bound to the fatty acid mycolic acid. Providing an exceptional lipid barrier, arabinogalactan (a biopolymer made of arabinose and galactose monosaccharides) is attached to the underlying peptidoglycan-bound polysaccharide (Daffé and Marrakchi, 2019; Minnikin et al., 2015). Numerous physiological traits of tuberculosis that pose medical challenges are caused by this barrier, including resistance to medicines and host defence mechanisms. The cell wall components and their amounts affect the bacterium's virulence and growth rate. Another component of the permeability barrier in mycobacteria is the peptidoglycan polymer, which confers cell walls their stiffness and is located just outside the bacterial cell membrane (Maitra et al., 2019).

Mycobacterium tuberculosis uses specialized transport and secretion systems to interact with its host environment, spread infection, and evade immune responses (Zhai et al., 2019). According to these secretion mechanisms (Type VII-ESX 1-ESX 5, TAT, SEC, PE/PPE protein secretion), the bacterium can transport proteins, lipids, and other substances across its cell membrane or into the host (Malik et al., 2024; Rivera-Calzada et al., 2021). Furthermore, mycobacteria possess efflux pumps that are capable of removing toxic compounds, including antibiotics, from cells. It is reported that these pumps contribute to drug resistance (Kapp et al., 2018; Pule et al., 2016).

Additionally, mycobacterial cell envelopes contain porins that allow hydrophilic molecules to pass through the outer layer's impermeability, which is rich in lipids (Singh et al., 2018; Niederweis et al., 2008). For example, *MspA* promotes growth and increases antibiotic susceptibility (Mailaender et al., 2004). In conclusion, the impermeable cell membrane and mycobacterial complex act as a shield, providing defense against antimicrobials and protecting from harsh environmental conditions. Furthermore, the host-pathogen interactions are significantly shaped by this envelope.

In 80% of cases, *MTB* affects the lungs and causes severe symptoms such as cough, shortness of breath, fever, weight loss, hemoptysis, and intense night sweats. If left unrecognized and untreated, these symptoms can lead to serious health complications. TB is spread mainly through airborne droplets. The droplets containing *MTB* are released when contagious individuals cough, sneeze, or spit. These species may survive for weeks in household dust or as airborne droplets suspended in the atmosphere (Mehrota et al., 2014). Once inhaled by an immunocompromised individual, the bacteria will likely cause infection (Rohde et al., 2012).

2.3 MYCOBACTERIUM TUBERCULOSIS PATHOGENICITY

There are two types of TB: latent and active TB. It is of great importance to differentiate the two. In latent TB, the bacterium is in the body; however, the immune system can keep the bacteria under control (Kiazyk and Ball, 2017; Gupta et al., 2012). Active TB occurs when the pathogenic bacterium *Mycobacterium tuberculosis* (MTB) infects macrophages and adapts to the host's intracellular environment (Mehrota et al., 2014). By leveraging the adaptability of its central carbon metabolism (CCM) machinery, MTB enables the pathogen to quickly adapt to the macrophage intercellular environment

(de Carvalho et al., 2010). Nevertheless, growing evidence shows that the host cell's metabolic pathways also contribute to *MTB* pathogenicity. The adaptation of the bacterium to the host requires a switch in the bacterial CCM towards the hosts, resulting in the pathogen-induced promotion of liquid body accumulated by host macrophages (Rohde et al., 2012; Daniel et al., 2011; Pandey and Sasseti, 2008). The infected individual starts to experience the signs and symptoms.

For infection to develop (Figure 2.2), only a few droplets may initiate infection by reaching the alveoli in the lungs, where the bacterium is phagocytosed by alveolar macrophages (AMs) (Rahlwes et al., 2023). In addition, the bacterium has developed mechanisms to persist and replicate within the macrophages by resisting oxidative and nitrosative stress with the AMs. This results in active inhibition of phagosome fusion with lysosomes.

Intercellular survival is a hallmark of *MTB* pathogenesis and is facilitated by virulence factors that enable the bacterium to manipulate host-cell processes. For example, the secretion of a significant protein, early secreted antigenic target 6 (ESAT-6), is part of the Type VII secretion system, ESX-1 (Ramakrishnan, 2020). The literature reports ESAT-6 as a disruptor of phagosome maturation by inhibiting the fusion of lysosomes with phagosomes, resulting in *MTB* persistence within macrophages (Anes et al., 2023; Refai et al., 2018).

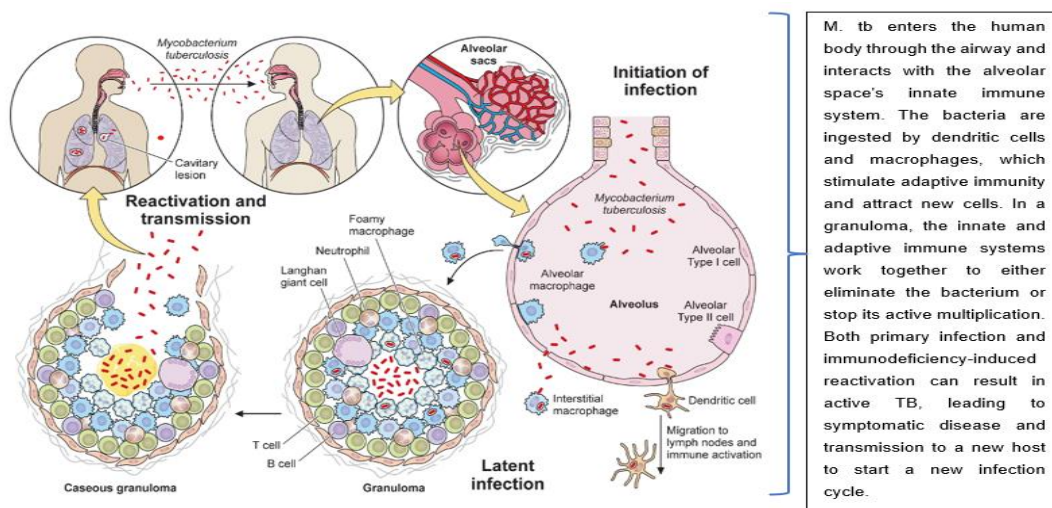


Figure 2.2: Overview of *MTB* infection (adapted from Rahlwes et al., 2023). Illustration of the pathogenesis and transmission cycle of *Mycobacterium tuberculosis* (*MTB*) in the human host. The figure demonstrates the initial entry of airborne bacilli into the respiratory tract, deposition within the alveoli, and subsequent phagocytosis by alveolar macrophages and dendritic cells, which trigger innate and adaptive immune responses.

However, not all individuals exposed to an infectious TB patient or individual become infected (Migliori et al., 2019). The probability that TB will be transmitted depends on several factors, such as Immune status, frequency and duration of the exposure, and infectiousness of the TB patient. An individual who spends more time in an enclosed area with an infected individual is most likely to contract the infection. The immune system generates unique immune cells called macrophages over 2–8 weeks, which encircle the tubercle bacilli. The cells form a shell that contains and controls the bacilli. However, if an individual is immunocompromised, the immune system cannot keep the tubercle bacilli under control; the bacilli begin to multiply rapidly. This results in infection, and the infected individual will experience signs and symptoms.

2.4 EPIDEMIOLOGY OF *MYCOBACTERIUM TUBERCULOSIS*

Although TB is a disease that is preventable and treatable, it continues to affect a substantial proportion of the world's population and is the 2nd most common cause of death after Human immunodeficiency virus / acquired immunodeficiency syndrome (HIV/AIDS). As a single infectious pathogen, TB was reported as a leading cause of death and a contributor to the death rate among people living with HIV. Globally, over 10 million people developed TB in 2024 and about 1.23 million deaths occurred among HIV-negative people, as well as 160,000 deaths among people living with HIV (WHO, 2025). These alarming figures highlight the persistent global burden of TB despite the availability of effective treatment.

Although progress has been slow, it is predicted that the End TB Strategy's goal of eliminating tuberculosis as a global public health issue by 2035 will still need to be met. For instance, the 2020 global TB report shows that there was only a 9% reduction in TB incidence during this period, with an annual decrease of only about 2%, despite the aim being to reduce TB incidence by 20% between 2015 and 2020. In comparison, just a 14% change in death rates was attained between 2015 and 2020, falling short of the 35% mortality reduction targets (WHO, 2022). The figure below (Figure 2.3) shows global trends in estimated TB deaths and mortality rates from 2000 to 2021.

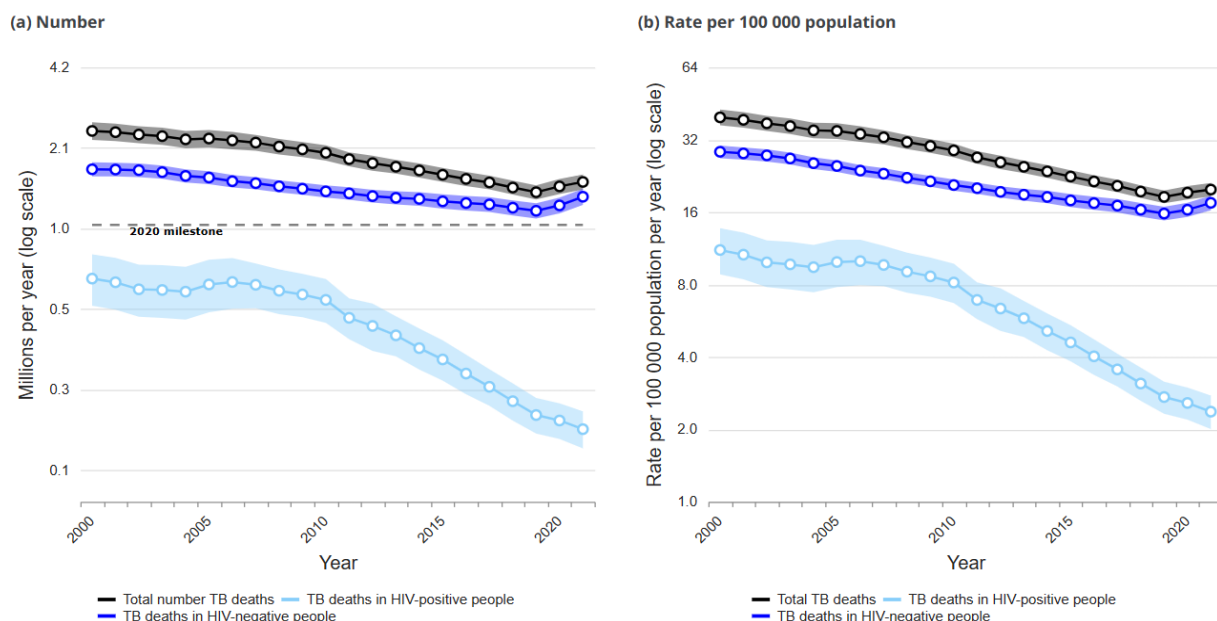


Figure 2.3: Global trends of TB deaths and mortality rate between 2000 and 2021 (adapted from WHO, 2022 Global Tuberculosis Report 2022 <https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2022/tb-disease-burden/2-2-tb-mortality>).

South Africa's *MTB* epidemiology is highlighted by alarmingly high incidence rates, substantial HIV co-infection, and increasing burden of drug-resistant strains (Naidoo et al., 2024; Chisompola et al., 2020; Gandhi et al., 2006). According to estimates, South Africa has one of the highest rates of tuberculosis (TB) in the world, with around 781 cases per 100,000 individuals (WHO, 2017). The high burden is worsened by the high prevalence of HIV, which significantly increases the risk of developing active TB. Approximately 60% of TB incidences in South Africa are co-infected with HIV. This highlights the close relatedness of these two public health issues (Todokera et al., 2020; Middelkoop et al., 2014).

The figure (Figure 2.4) below shows the estimated number of new TB cases (all forms) per 100,000 population per year in 2022. In this figure, it's evident that TB remains a burden due to its high incidence rates in sub-Saharan Africa, and varies widely among countries, from less than 10 to more than 500 new and relapse cases per 100,000 population per year (WHO, 2023).

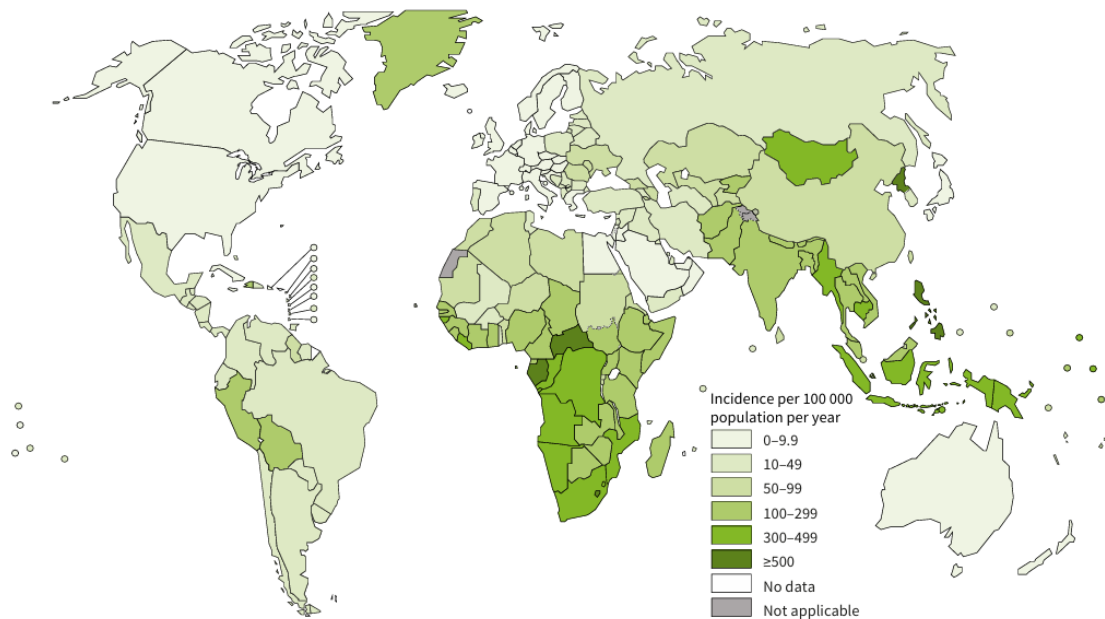


Figure 2.4: Global map of TB incidence rates (adapted from WHO, 2023 Global tuberculosis report 2023). Global distribution of tuberculosis incidence rates per 100,000 population per year, illustrating the geographic burden of the disease. Darker shades indicate higher incidence, with the greatest concentration observed in sub-Saharan Africa and parts of Southeast Asia, reflecting persistent public health challenges linked to socioeconomic disparities, high HIV prevalence, limited healthcare access, and ongoing transmission.

2.5 MYCOBACTERIUM TUBERCULOSIS TREATMENT

Tuberculosis drugs used to treat infection are classified as first- and second-line drugs. The first line of drugs used for the treatment of drug-sensitive tuberculosis includes about four regimens, namely isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA). The second line of drugs includes several fluoroquinolones such as moxifloxacin, gatifloxacin, levofloxacin, and other drugs such as rifabutin, rifapentine, cycloserine, and ethionamide. These second-line drugs are used to treat MDR-TB and XDR-TB.

2.5.1 FIRST-LINE DRUGS

- **Isoniazid**

Isoniazid (INH), also known as isonicotinic acid hydrazide, is the most common antibiotic used to treat active TB worldwide. This antibiotic was first described in 1912 and has been used as an anti-TB drug since 1952. The bactericidal activity of INH is

highly selective and specific to the mycobacterial species (Robitzek and Selikoff, 1952). In addition, INH has been used as a prophylactic drug for individuals with latent MTB infection to prevent the activation of the bacterium.

This first-line drug has a basic structure that inhibits the synthesis of mycolic acids and other essential components of the bacterial wall, thereby interfering with cell wall synthesis and exerting a bactericidal effect. The mechanism of action of INH is very complex and involves different concepts. The prodrug INH is activated by the catalase-peroxidase (*katG*), and the active INH targets the enzymes, namely, enoyl acyl carrier protein (ACP) reductase (*InhA*) and beta-ketoacyl ACP synthase (*KasA*). In contrast, INH is inactivated by arylamine *N*-acetyltransferases (NATs). Consequently, the molecular mechanisms of INH resistance involve multiple genes involved in multiple biosynthetic networks and pathways.

Mutations in the *katG* gene are the most common cause of INH resistance; the commonly reported mutation occurs in codon 315 (*katG315*), followed by *inhA*, *ahpC*, *kasA*, *ndh*, *iniABC*, and *fadE* (Seifert et al., 2015; Vilchèze et al., 2014; Ramaswamy et al., 2003). Substitutions have also been observed in the *fabG1*, *fabD*, *nat*, *accD6* and *fbpC*. Improved identification of INH resistance might be possible with a greater understanding of the mechanisms behind INH activity and resistance. This crucial information would aid novel drug design strategies (Unissa et al., 2016).

- **Rifampicin**

Rifampicin (RIF) is known to be the key component in TB treatment, reported and classified as one of the first-line regimens used to treat this disease (Asif et al., 2024). The rifamycin complex was first discovered in the late 1950s; rifampicin was synthesized in 1965, and the antimicrobial profile was described in 1966. This discovery was followed in 1967 by reports documenting the compound's activity against both gram-negative and gram-positive bacteria. Between the late 1960's and early 1970, researchers confirmed rifampicin antimycobacterial activity in vitro by testing the susceptibility of *MTB* strains to rifampicin. The majority minimum inhibitory concentration ranged between 0.006 and 0.05 ug/ml in liquid agar.

In 1999, Davies reported rifampicin to be able to kill the very slow-dividing mycobacteria in a way other drugs cannot do; this makes it one of the most important treatments for TB (Davies, 1999). Its mode of action involves inhibiting the bacterial

enzyme Ribonucleic acid (RNA) polymerase (β subunit), which is essential for the production of bacterial RNA. This ultimately leads to bacterial death by inhibiting their protein synthesis.

Several studies reported RIF as a vital antibiotic for TB treatment and demonstrated its safety and efficacy. However, rifampicin-resistant TB has emerged (mutation in the *rpoB* gene), and RIF is a hallmark for detecting multidrug-resistant TB (MDR-TB) (Zaw et al., 2018). A study by Ragonnet et al. (2017) found that the most significant contribution of MDR-TB/RR-TB identified at re-treatment was due to resistant amplification during first-line treatment of drug-susceptible TB (DS-TB). A study on molecular epidemiology indicated a significant clonal spread of numerous MDR-TB strains occurred in South Africa, where transmission of MDR/RR-TB caused around 64% of MDR/RR-TB patients diagnosed during re-treatment (Ragonnet et al., 2019; Marais et al., 2013).

About 96% of MTB isolates that are resistant to rifampicin have mutations in the 81-bp so-called "hot-spot region" (RRDR) of the *rpoB* gene, which spans codons 507–533 (Sinha et al., 2020; Unissa et al., 2016). In most studies, rifampicin resistance is reported to be most often associated with mutations at codons 516, 526, and 531 (Zaw et al., 2018). However, fewer alternations outside of the RRDR have also been reported. Possibility to develop cross-resistance with other rifamycins. Some codon mutations have been linked to low-level resistance to rifampicin while maintaining susceptibility to other rifamycins like rifabutin or rifalazil (Asif et al., 2024; Li et al., 2022; Cavusoglu et al., 2004). Recently, research in genome sequencing has revealed that rifampicin-resistant strains with *rpoB* mutations have acquired compensatory mutations in *rpoA* and *rpoC*, which encode the α and β' subunits of RNA polymerase (Shea et al., 2021; De Vos et al., 2013; Comas et al., 2012). The compensatory mutation would be responsible for restoring the fitness of stains in vivo and has also been associated with higher transmissibility in some settings. The figure below (Figure 2.5) illustrates mutation within the *rpoB* gene.

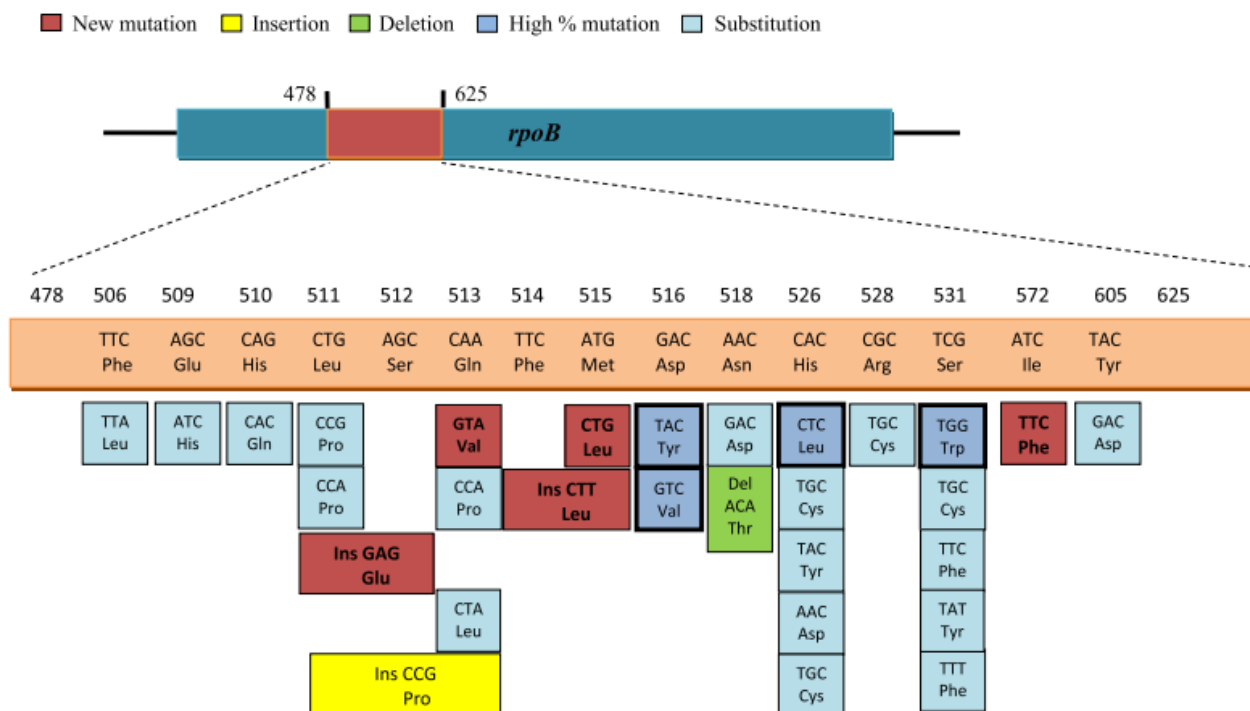


Figure 2.5: *rpoB* gene of MTB with different mutations (adapted from Sinha et al., 2020).

- **Ethambutol**

Ethambutol (EMB) is a derivative of N, N'-di-isopropyl ethylenediamine that was discovered through random selection and found to protect an infected mice *with MTB* strain H37Rv (Thomas et al., 1961) The EMB was then described to be a narrow-spectrum bacteriostatic that interferes with the biosynthesis of arabinogalactan in the cell wall of the MTB inhibiting the bacilli's multiplication and has been prescribed for TB treatment since 1966 (Palomino and Martin, 2014; Shepherd et al., 1966) . Ethambutol is only administered in the first two months of the initiation of treatment and is reported to be used worldwide for TB therapy in combination with the other three regimens, namely, isoniazid, pyrazinamide, and rifampicin. Its role is to prevent the build-up of other treatment agents and effectively treat opportunistic mycobacterial infections of patients with human immunodeficiency virus. The mechanism of this anti-TB drug is unclear, and it is unfortunate that resistance to EMB has been reported in as many as 4% of clinical isolates of *MTB* and is prevalent among multidrug-resistant strains.

- **Pyrazinamide**

Pyrazinamide (PZA), a nicotinamide analogue (Figure 2.5), is a unique antituberculosis (anti-TB) drug that plays a key role in shortening the duration of TB therapy. The compound was chemically synthesized in 1936, but its antituberculosis potential was recognized in 1952 (Yeager et al., 1952). The discovery of the TB drug was only based on a coincidental observation when nicotinamide had certain activity against mycobacterial in animal studies. PZA is prescribed for the first 2-month intensive phase of the 6-month therapy due to its ability to kill persistent bacilli and the presumption that inflammation leading to an acid environment in the lesion is decreased after 2 months (Zhang et al., 2013). Pyrazinamidase/nicotinamidase, which is expressed by the *pncA* gene in *MTB* transforms PZA, a prodrug, into the active form pyrazinoic acid (POA) (figure 2.6). The purified recombinant *MTB pncA* is a Mn²⁺- and Fe²⁺-containing enzyme that is a monomer (Petrella et al., 2011; Fyfe et al., 2009). The process by which PZA is converted to POA may resemble that of the nitriles superfamily, in which the active-site cysteine nucleophilically attacks to form a tetrahedral intermediate, which collapses upon ammonia loss and is then hydrolysed by water to form a thioester link (Via et al., 2015). The figure below (Figure 2.7) illustrates the conversion that occurs.

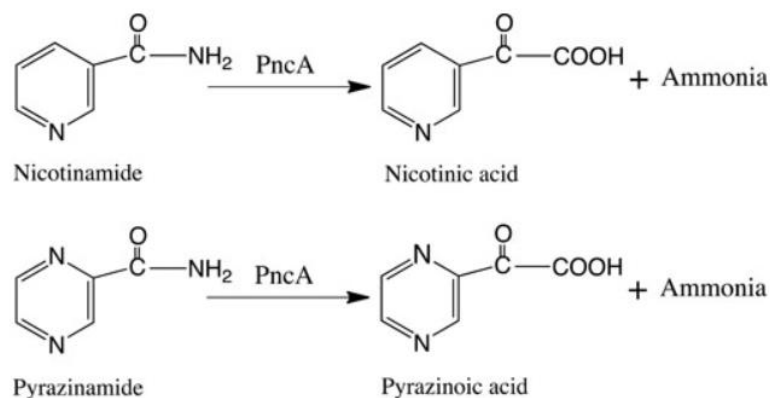


Figure 2.6: Conversion of Nicotinamide and Pyrazinamide to nicotinic acid and pyrazinoic acid, by an enzyme Nicotinamidase/Pyrazinamidase encoded by *pncA* gene of *MTB*, respectively (adapted from Zhang et al., 2013).

2.5.2 SECOND-LINE DRUGS

Second-line TB drugs are used to treat patients with MDR-TB/XDR-TB, and to ensure that appropriate drugs are provided to patients, it is critical to determine as much information about drug susceptibility as possible. Over the past 20 years, DR-TB treatment has undergone significant and continual improvements. In 2000, the treatment of MDR-TB was officially approved, and it was suggested that long-course injectable regimens lasting 18 months be used, either standardized or customized (WHO, 2000). In 2006, WHO revised the DR-TB treatment guidelines which concurred with reports on an outbreak of XDR-TB in KwaZulu Natal (Ghandi et al., 2006).

In 2008, the DR-TB treatment guidelines were revised and emphasized the use of a standardized regimen (WHO, 2008). In addition, a significant change in TB treatment occurred in 2016 when the WHO approved an injectable-based 9–12 month short-course regimen for the treatment of MDR-TB. This was after the regimen proved successful in a cohort trial conducted in Bangladesh (WHO, 2016; Aung et al., 2014). The study predicts consistent worldwide increases in the usage of shorter regimens between 2023 and 2026, with 126 792 patients receiving BPaLM (58% of DR-TB treatments), 43 716 patients receiving BPaL (20% of DR-TB treatments), and shorter all-oral bedaquiline-based regimens by 2026, reaching 13,119 patients. By 2026, it is anticipated that 19 262 patients will be using the longer all-oral regimen, while 15 344 will be using individualized treatment regimens (Gupta et al., 2024).

- **Fluoroquinolone**

Fluoroquinolones (FQ) are a class of antibiotics that play a critical role in the treatment of TB, particularly MDR-TB and XDR-TB. The included fluoroquinolones in the TB regimen are levofloxacin and moxifloxacin. These drugs may be effective in the DR-TB regimen; however, they must be managed cautiously to prevent the development of new resistance strains. They offer favourable pharmacokinetic profiles for the treatment of TB and target the Deoxyribonucleic acid (DNA) gyrase and topoisomerase IV; enzymes essential for bacterial DNA replication.

The development mechanism of fluoroquinolone resistance in *MTB* is a chromosomal mutation in the quinolone resistance-determining region of *gryA* or *gyrB* (Avalos et al., 2015). The most commonly found mutation in *gyrA* is located in positions 90 and 94;

however, mutations located in positions 74, 88, and 91 have been reported (An et al., 2023; Dixit et al., 2023). A study reported an interesting finding on the simultaneous occurrence of mutations in T80A and A90G in *gyrA* led to hypersusceptibility to several quinolones (Aubry et al., 2006).

- **Bedaquiline**

Bedaquiline (BDQ), a new-generation TB drug, is currently being used in more than 50 countries. This drug exhibits its anti-mycobacterial activity by preventing ATP synthesis, essential to *MTB* growth and survival in its non-growing dormant form. By interfering with mycobacterial F-ATP synthase activity, the medication inhibits ATP synthesis, thereby depleting bacterial ATP 25/26. Interestingly, BDQ has delayed bactericidal activity, meaning cell death does not happen immediately when ATP is depleted. In 2014, Koul et al. reported that BDQ inhibits protein and DNA synthesis in a dose-dependent manner, despite not directly targeting any component of these processes (Koul et al., 2014). Recently, BDQ was proven to affect nitrogen metabolism. Specifically, the activity of glutamine synthetase, as this enzyme is highly sensitive to changes in intra-bacterial ATP content (Wang et al., 2019). These findings suggest that BDQ's ATP synthesis inhibition causes differential inhibition of various ATP-dependent metabolic processes. The drug was approved by the Food and drug administration (FDA) in 2012, marking a significant milestone in fighting TB. In 2019, BDQ was approved for MDR-TB treatment in combination with pretomanid and linezolid while resistance to BDQ contributes to XDR-TB global health (Zhu et al., 2023). Bedaquiline has been shown to enhance DR-TB results in clinical studies and programmatic settings. Hence, the World Health Organization (WHO) has recommended that it be included in DR-TB regimens. However, a study reported limited improvement in DR-TB treatment results observed using BDQ on the continent (Traore et al., 2022).

- **Delamanid**

Delamanid (DLM) is a prodrug used to treat MDR-TB that requires activation by the mycobacterial enzyme deazaflavin-dependent nitroreductase (Ddn); once activated, it inhibits the synthesis of mycolic acids (an important component of the *MTB* cell wall),

resulting in disruption of the cell growth (Khoshnood et al., 2021; Lewis and Sloan, 2015).

The drug was approved in 2014 by the European Medicines Agency (EMA) to be used with other drugs to treat individuals diagnosed with MDR-TB (Liu et al., 2018). The drug was further approved by several countries including South Africa (Pontali et al., 2019). A study reported that a laboratory-induced resistance to delamanid has demonstrated that resistance to delamanid can be caused by mutations in one of the five genes involved in this bio-activation pathway: *ddn*, *fgd1*, *fbiA*, *fbiB*, and *fbiC*.

The synthesis of F420 involves the participation of FbiA, FbiB, and FbiC (Fujiwara et al., 2018). Therefore, conclusion can be that resistant to delamanid is resultant to loss of capability to activate it. The figure below (Figure 2.7) shows structural components of TB treatment.

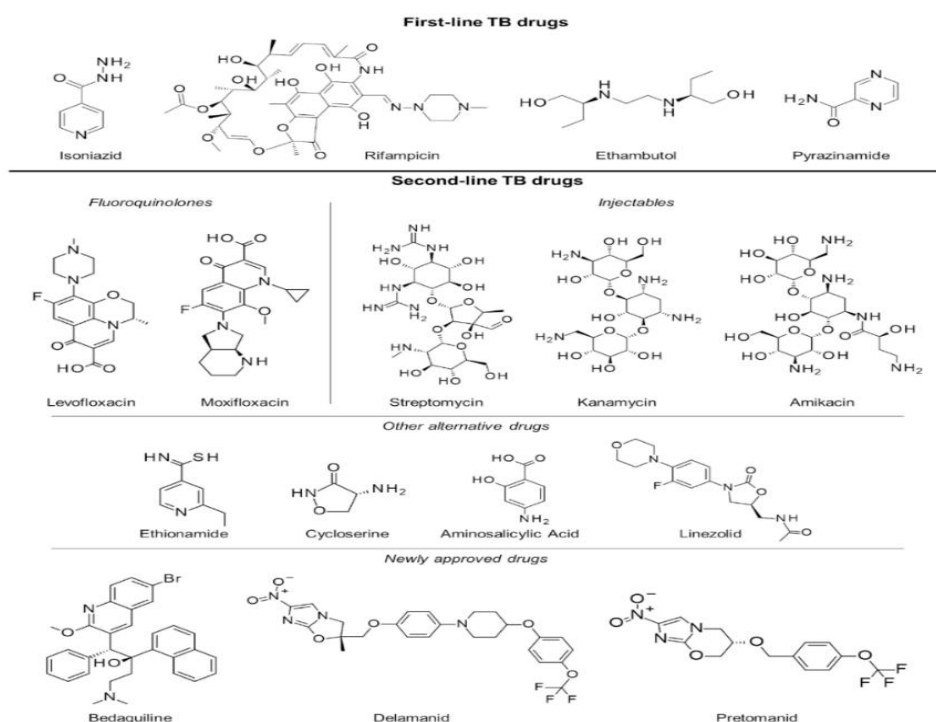


Figure 2.7: Chemical structures of approved first- and second-line drugs of tuberculosis (Adapted from an article written by Malicino et al., 2020).

To ensure the complete eradication of the infection, prevent drug resistance from developing, and reduce transmission, TB therapy is essential. Implementing the recommended action plan is essential for public and individual health outcomes. Drug-sensitive and drug-resistant TB regimens are administered differently, using the drugs classified under first-line and second-line drugs. The following table (Table 2.1) summarizes the drugs and their treatment durations.

In the 1980s and 1990, the HIV/AIDS epidemic reignited the urgency of TB research since TB was reported and recognized as an opportunistic infection in immunocompromised individuals. Multidrug-resistant (MDR) and extensive drug-resistant (XDR) strains started emerging and have resulted in TB becoming a major public health concern. Therefore, TB research has become one of the most important research projects that led to new drug development, such as Bedaquiline being approved in 2012, Delamanid being approved in 2014 and Pretomanid being approved in 2019. These drugs are more effective treatment options for drug-resistant TB. The figure (Figure 2.8) below illustrates dates for introducing TB regimens and the emergence of drug resistance.

Table 2.1: Recommended tuberculosis treatment regimens, drug combinations, and duration of therapy (Adapted from Cobongela and Mgwenya 2024).

Type of TB	Resistance	Regimen	Duration
Pulmonary TB	None	<i>First-line treatment</i> Intensive phase: HRZE regimen INH;RIF;PZA;EMB	6 months Initial phase: 2 months
		<i>Continuation phase: HR regimen</i> INH;RIF	Continuation phase: 4 months
		<i>New and shorter regimen</i> Intensive phase: With MFX RPT;MFX;INH;PZA	4 months Initial phase: 2 months
		Continuation phase RPT;MFX	Continuation phase: 2 months
		<i>New and shorter regimen</i> Intensive phase: Without MFX RPT;EMB;INH;PZA	4 months Initial phase: 2 months
		Continuation phase RPT;INH	Continuation phase: 2 months
MDR-TB	INH RIF	<i>Second-line treatment</i> Intensive phase: LFX;MFX,PZA; INHh (high-dose); EMB;CFZ;ETH;PTH; Injectable agents: AMK/KM/CM	9-12 months Initial phase 4-6 months

		Continuation phase: LFX/MFX;PZA;EMB;CFZ	Continuation phase 5-6Months
XDR-TB	INH RIF At least one of the fluoroquinolones At least one of the second-line injectables	Intensive phase: LFX/MFX; PZA;BDQ;LZD;CFZ;CS/TRD;ETH/PTH	18-24 Months Initial phase 6-8 months (or until culture conversion)
		Continuation phase: LFX/MFX; CFZ;LZD; CS/TRD	Continuation phase: 12-16 months

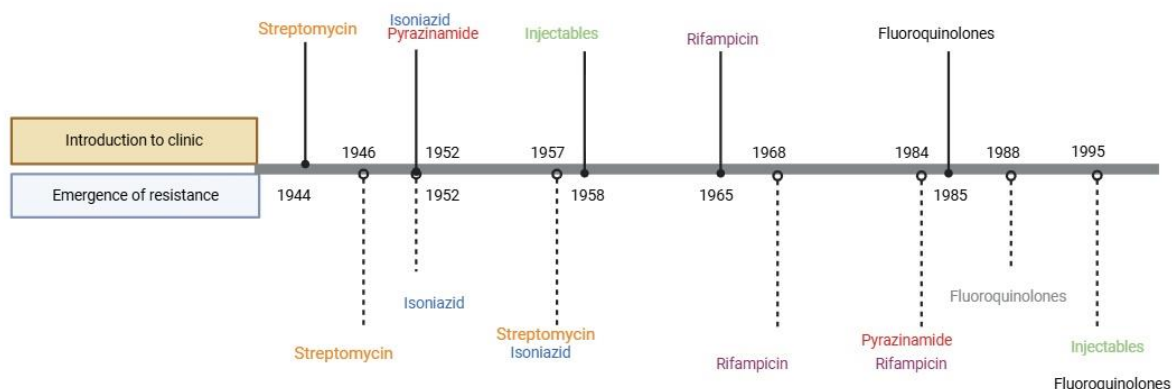


Figure 2.8: Timeline illustrating the clinical introduction of first- and second-line anti-tuberculosis drugs and the reported emergence of resistance. Created with BioRender.com based on published data from by Nimmo et al. (2022) and Ektefaie et al. (2021).

2.6 DRUG RESISTANCE MECHANISM OF TUBERCULOSIS

Drug resistance in TB occurs when *Mycobacterium tuberculosis* has developed the ability to survive and multiply despite the TB regimen taken by infected individuals (Hameed et al., 2018; Zhang et al., 2015). Worldwide, the emergence of MDR-TB and XDR-TB has increasingly threatened the global control of TB (Bu et al., 2023; Matteelli et al., 2007). Spontaneous chromosomal changes (insertions, substitutions, deletions, loss or alteration of RNA/Protein functions) in genes encoding drug targets or enabling enzymes are the sole contributors to resistance in *MTB*, illustrated in Figure 2.9 (Almeida Da Silva et al., 2011). Although *MTB* is expected to have a low mutation rate (0.3–0.5 substitutions per genome annually), antibiotic resistance increases rapidly. (Cohen et al,2015; Bos et al., 2014). The growing challenge of drug-resistant TB requires continuous monitoring, improved diagnostic techniques, and the development of new and more effective treatments.

Mycobacterium tuberculosis undergoes sequential mutations as it evolves under selection pressure, especially when exposed to antibiotics. The progressive

medication resistance caused by these mutations makes treating TB more difficult. Low-level resistance, conferred by a single mutation in a key gene, confers partial resistance but may not result in full clinical resistance. For example, *KatG* S315T leads to INH resistance; however, it remains susceptible to other TB drugs. Accumulation of mutations leads to full resistance to one or more drugs (*katG* S315T (INH-R) + *rpoB* S531L (RIF-R) leading to MDR-TB (resistant to isoniazid & rifampicin).

In 2006, an outbreak in Tugela Ferry highlighted the severity of XDR-TB, with 53 patients infected, with only 1 survivor (Gandhi et al., 2006). In 2022, an estimated 11,000 people fell ill with drug-resistant TB. Therefore, it can be concluded that in South Africa, drug-resistant TB persists as a serious health concern. To address this problem, comprehensive approaches are needed, such as enhancing early diagnosis, increasing access to efficient therapies, tackling the co-epidemic of HIV and TB, ensuring patients follow their treatment plans, and enhancing infection control. There is still more to be done to lessen the prevalence of drug-resistant TB in the nation despite advancements in TB care (Pai et al., 2022; Klopper et al., 2013; Streicher et al., 2012; Andrews et al., 2007). The table (Table 2.2) summarizes the anti-tuberculosis drugs used in healthcare facilities for TB patients and the resistance genes that confer drug resistance. The table was created based on information published in different articles (Alsayed and Gunosewoyo, 2023, Perveen et al., 2022).

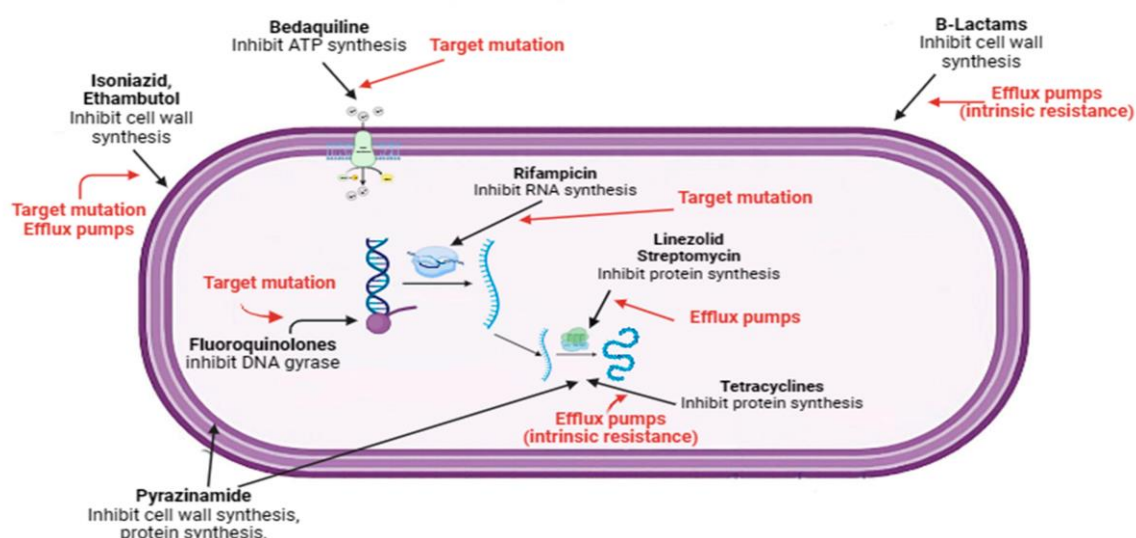


Figure 2.9: Mechanisms of action and resistance of first- and second-line anti-tuberculosis drugs in *MTB*. The schematic illustrates the primary cellular targets of major drug classes, including inhibition of cell wall synthesis (isoniazid, ethambutol, β -lactams), ATP synthase (bedaquiline), RNA synthesis (rifampicin), DNA gyrase (fluoroquinolones), protein synthesis

(linezolid, streptomycin, tetracyclines), and disruption of membrane energetics and cellular metabolism (pyrazinamide). Corresponding resistance mechanisms are highlighted, including target gene mutations and efflux pump-mediated intrinsic resistance. Adapted from Mancuso et al., 2023.

Table 2.2: Anti-tuberculosis drugs used in clinical practice, showing their class, route of administration, molecular targets, affected cellular processes, and major resistance-associated mutations in *MTB*.

Name of Drug	In Clinic	1 st /2 nd /3 rd line	Administration route	Compound class	Target	Affected cellular process	Resistance gene mutations
Streptomycin (SM)	1944	1 st	Injectable	Aminoglycoside	30S Ribosomal subunit	Protein translation	rpsL, rrs
Isoniazid (INH)	1952		Oral	Hydrazine	Enoyl-ACP Reductase (InhA)	Cell wall Synthesis	KatG inhA promoter
Ethambutol (EMB)	1961		Oral	Ethylene diamine	Arabinosyl Transferase (EmbB)	Cell wall synthesis	embB
Rifampicin (RIF)	1968		Oral	Rifamycin	RNA Polymerase (<i>RpoB</i>)	RNA synthesis	<i>rpoB</i>
Pyrazinamide (PZA)	1970		Oral	Nicotinamide			pncA
Kanamycin (KAN)	1957	2 nd	Injectable	Aminoglycoside	30S Ribosomal Subunit	Protein translation	Rrs, tlyA
Ethionamide (ETH)	1965		Oral	Thioamide derivative	Enoyl-ACP Reductase (InhA)	Mycolic acid synthesis	inhA, InhA promoter
Amikacin (AMK)	1976		Injectable	Aminoglycoside	30S Ribosomal Subunit	Protein translation	Rrs, tlyA
Ciprofloxacin (CFX)	1987		Oral	Fluoroquinolone	DNA Gyrase (GyrA)	DNA replication	gyrA
Levofloxacin (LVX)	1998		Oral	Fluoroquinolone	DNA Gyrase (GyrA)	DNA replication	gyrA
Moxifloxacin (MXF)	1999		Oral	Fluoroquinolone	DNA Gyrase (GyrA)	DNA replication	gyrA
Bedaquiline (BDQ)	2012		Oral	Diarylquinolone	ATP Synthase (AtpE)	ATP synthesis	atpE rv0678
Delamanid (DLM)	2014		Oral	Nitroimidazole	DprE2	Mycolic acid synthesis	biA/B/C fgd1,ddn
Linezolid (LZD)	2019	3 rd	Oral	Oxazolidinone	50S Ribosomal subunit	Protein translation	Rrl

Pretonamid (PRET)	2019		Oral	Nitroimidazole	DprE2	Cell wall synthesis	biA/B/C, fgd1,ddn
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2.7 RISK FACTORS ASSOCIATED WITH TUBERCULOSIS

2.7.1 HIGH-RISK AREA

Individuals residing in or traveling to regions with a high burden of TB and DR-TB are at a higher risk of infection owing to prolonged community exposure, persistent transmission dynamics, and inadequate infection prevention and control measures. High-incidence regions documented in the literature include parts of Africa, Asia, Eastern Europe, Russia, Latin America, and the Caribbean Islands. These settings are frequently characterized by socioeconomic deprivation, overcrowded living conditions, limited access to quality healthcare services, high rates of HIV co-infection, and poor treatment adherence, all of which collectively facilitate TB transmission and the development of drug resistance (WHO,2025).

The figure (Figure 2.10) below shows the summarized areas of TB, drug-resistant TB, and TB/HIV.

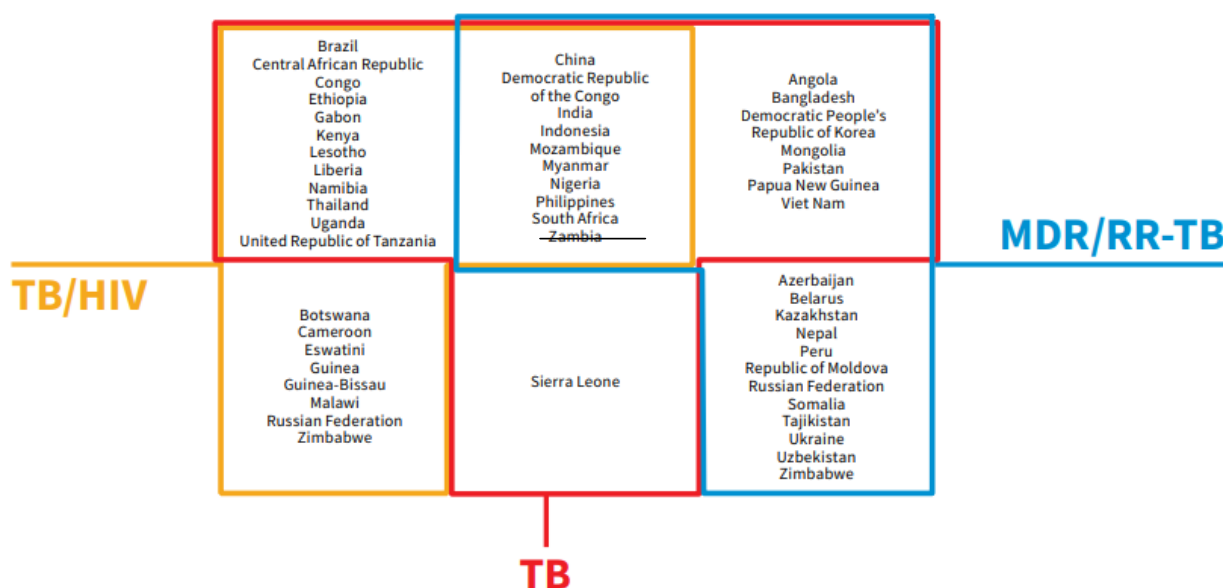


Figure 2.10: List of high-burden countries for TB, HIV-associated TB and DR- TB being used by WHO during the 2021-2025 period and their areas of overlapping (adapted from WHO, 2025).

2.7.2 IMMUNOCOMPROMISED SYSTEM

An effective immunocompetent system is reported to play a critical role in controlling *MTB* infection and successfully fighting TB bacteria. However, an immunocompromised system is less capable of mounting an adequate cellular response, resulting in ineffective bacterial clearance and successful infection. The individual will have signs and symptoms used to diagnose TB. Several medical conditions and medications are known to compromise the immune response, leading to reactivation of TB. The following medications and diseases are known to weaken the immune system:

Diseases and conditions associated with increased TB risk

- **Human Immunodeficiency Virus (HIV)** infection is the strongest known risk factor for TB, gradually reducing CD4+ T cells, which are essential for controlling *MTB*. It is the strongest known risk factor and remains highly prevalent in sub-Saharan Africa, including South Africa (Himani et al., 2024)
- **Diabetes mellitus** increases the risk of active TB by impairing innate and adaptive immune responses, particularly macrophage and lymphocyte function (Cheng et al., 2022).
- **Malnutrition** weakens immune defences and affects the response of anti-TB drugs and is associated with increased risk of relapse, resulting in poor treatment outcomes (Téllez-Navarrete et al., 2021).
- **Chronic kidney disease** is associated with immune dysregulation and a higher risk of both primary TB infection and reactivation of latent TB (Bandiara et al., 2022).
- **Cancer**, various types of leukaemia, lymphoma, and lung carcinoma are linked with reinfections of TB (Sundaram et al., 2025).
- **Chronic respiratory diseases**, such as asthma. Individuals receiving long-term corticosteroids may be more susceptible to TB (Castellana et al., 2019).

In addition to disease-related immunosuppression, several medications are known to increase the risk of TB infection or reactivation.

Immunosuppressive medications that are associated with increased TB risk

- **Corticosteroids** (e.g., prednisone and dexamethasone) suppress cellular immune responses and are associated with increased risk of TB reactivation, particularly with long-term use (Castellana et al., 2006).
- **Chemotherapeutic agents** Treatments for cancer increase vulnerability to opportunistic infections, such as tuberculosis, by suppressing bone marrow activity and immune cell synthesis (Sundaram et al., 2025).
- **Tumor necrosis factor-alpha (TNF- α) inhibitors**, including infliximab and adalimumab, substantially increase the risk of tuberculosis reactivation because TNF- α plays a crucial role in the formation and maintenance of granulomas that contain latent *MTB* infection (Kireev et al., 2025).

Controlling TB requires an understanding of the effect of immunosuppressive diseases and drugs, especially in high-burden environments like South Africa, where HIV co-infection and other comorbidities are still prevalent. Targeted screening, early diagnosis, and preventative treatment—all essential elements of tuberculosis control strategies—are made possible by the identification of immunocompromised patients (WHO, 2023)

2.7.3 LIFESTYLE

According to the WHO, low-income countries are at high risk of TB cases (WHO, 2021). Poverty has been associated with TB mortality as a vital risk factor (Kave et al., 2025). Individuals who are smokers, heavy alcohol consumers, drug abusers, imprisoned, miners, and have a close contact history with active TB individuals and chronic medical conditions have a higher risk of being infected with TB (Senanayake et al., 2018). Smoking is a TB-preventable risk factor (Yen et al., 2014). In 2009, Jee et al. conducted a prospective cohort study showing that smokers were identified to have a higher probability of developing TB compared to non-smokers. In addition, the risk is positively associated with the number of cigarettes an individual smoked daily.

Drug abuse has been reported to reduce the immune system, leading to challenges in screening, diagnosis, and treatment of TB. This also results in poor adherence to treatment, increasing drug interactions, and the emergence of drug-resistant TB, known as the major obstacle to TB control (Getahun et al., 2013; David et al., 1995).

A systematic review on alcohol, drugs, and substance abuse provides adequate evidence linking drug and harmful substance use to lead to inflammatory response modification, which is crucial in TB immunopathology. (Kiboi et al., 2016). This may lead to the conclusion that a healthy lifestyle dramatically impacts TB infection and control.

2.8 DIAGNOSTIC TECHNIQUES

TB and drug-resistant TB remain a public health threat even though they are common in most countries, including South Africa (SA), well-documented and under surveillance by the WHO. Misdiagnosis for drug-resistant TB still occurs due to target area diagnostic techniques.

For correct and effective TB control through proper administration and correct regimen for infected individuals, rely on the diagnostic techniques that are rapid, precise, and reliable. In 2013, WHO statistics reported that approximately half a million new cases of MDR-TB were diagnosed every year (WHO, 2013). Approximately 40,000 of these cases had extensively drug-resistant tuberculosis (XDR-TB). Despite this, limited laboratory capacity and lack of widespread drug susceptibility testing in resource-poor settings mean that less than 6% of cases are thought to have been correctly diagnosed (Calligaro et al., 2014; WHO, 2013). In most healthcare facilities from low-income settings such as SA, diagnostics are based on microscopy, culture-based, and GeneXpert from the nearest district hospital.

Diagnosing active tuberculosis can be challenging, particularly in young patients and those with immunocompromised systems; further testing is needed in addition to standard medical exams. If a patient has active tuberculosis, several (summarized in the figure below; Figure 2.11) tests could be performed to find out.

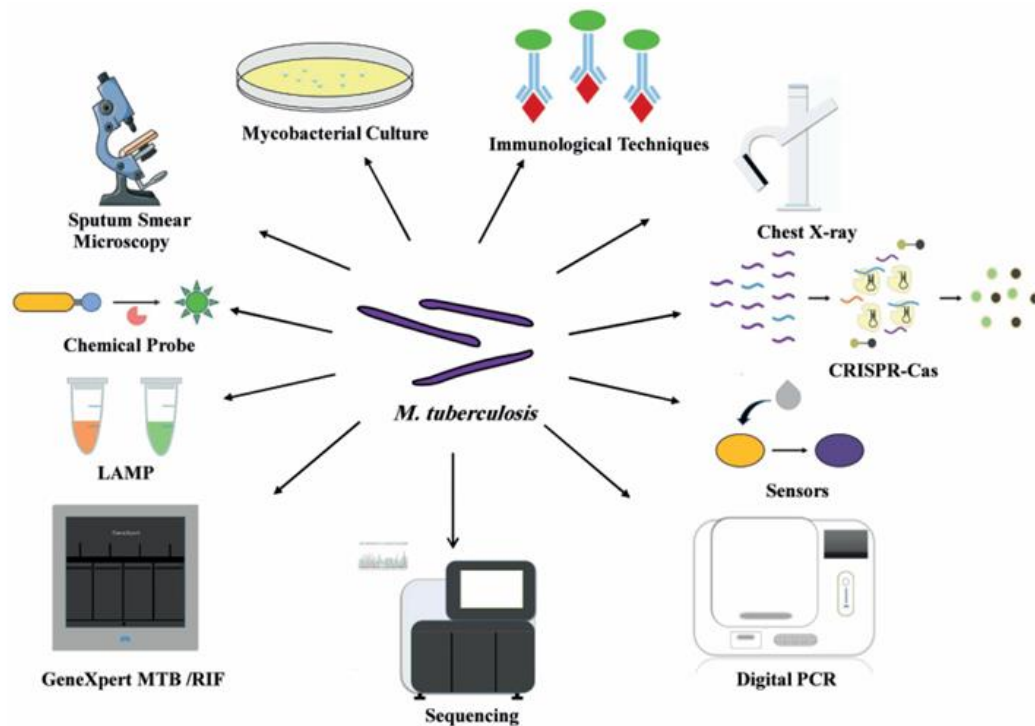


Figure 2.11: Overview of diagnostic methods for *MTB*, ranging from conventional approaches such as sputum smear microscopy, mycobacterial culture, immunological techniques, and chest X-ray, to advanced molecular and biosensing technologies, including GeneXpert MTB/RIF, LAMP, sequencing, digital PCR, CRISPR-Cas, chemical probes, and sensor-based platforms (adapted from Dong et al., 2022).

2.8.1 CHEST RADIOGRAPH (X-RAY)

Chest radiography (X-ray) is a common, inexpensive, rapid and non-invasive technique that produces an image used to examine the lungs and chest cavity (Caulfield and Wengenack, 2016). Experienced physicians examine this procedure for TB detection in clinical settings (Guo et al., 2020). Importantly, x-ray images of TB are often misclassified to other diseases of similar patterns, which leads to misdiagnosis and initiation of incorrect regimens, resulting in worsening the health condition. Using automated CXRs for TB detection and localizing suspected locations that may exhibit TB could significantly improve TB diagnosis quality. Crucially, there is a lack of

radiologists in low-income settings, and some areas have high TB prevalence (El-Gany et al., 2024; Rahman et al., 2020). The current research effort seeks to create an effective computer-aided detection system that will help physicians (and radiologists) become knowledgeable when diagnosing tuberculosis based on patients' x-ray results. The figure below (Figure 2.12) shows a healthy and TB-infected lung.

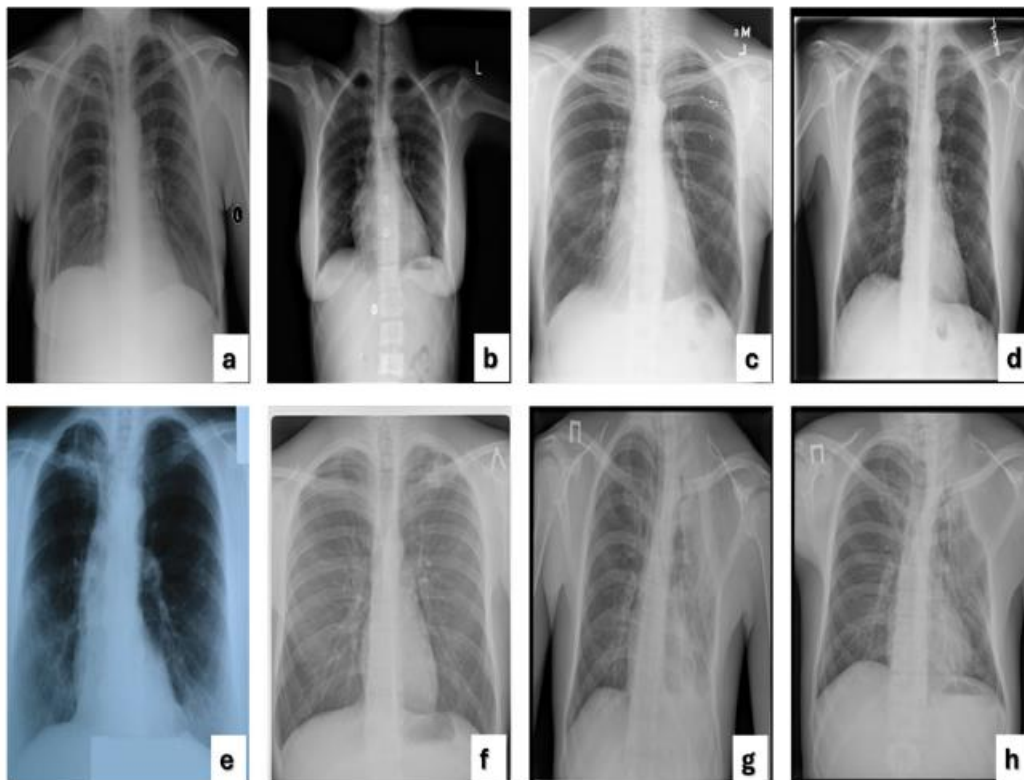


Figure 2.12: Comparative chest radiographs illustrating normal and infected lungs. Panels (a–d) show clear lung fields without pathological findings, representing healthy individuals. Panels (e–f) demonstrate radiographic evidence of infection, including opacities and abnormal densities. Panels (g–h) are included for further comparative analysis of lung morphology. Adapted from El-Ghany et al. (2024).

2.8.2 MICROSCOPY

Microscopy is the most commonly used AFB technique to detect TB in clinical samples such as sputum. Microscopy-positive sputum specimens are referred to smear positive specimens in clinical settings (Van Hung, 2007). Ziehl-Neelsen staining uses carbol fuchsin and methylene blue that help visualize the sputum under microscopy. This method has been extensively used since Robert Koch discovered *MTB* in the 19th century (Dong et al., 2022). However, this technique has limitations since it cannot

distinguish between viable and non-viable mycobacteria nor between *MTB* and non-*MTB* and does not provide information on drug resistance. In addition, research has shown that sensitivity for the diagnosis of TB is low (affected by sample quality and bacillary load) and variable compared with culture methods (the gold standard for TB diagnosis) (Dong et al., 2022; Caulfield and Wengenack, 2016). The outstanding points of this method are that it is inexpensive, 1 day a turnaround time, and non-complex analysis (Campelo et al., 2021)

Diagnosis methods have improved over the years, making TB diagnosis more efficient. A significant improvement in developing and endorsing the light-emitting diode (LED) microscope occurred in 2010. These microscopes were less expensive, required less maintenance, and had higher sensitivity for TB detection than the previously used microscopes. This meant that the diagnosis method of TB was less expensive, resulting in a relief in the budgets for TB. Despite microscopy's low sensitivity, it remains essential in areas where molecular techniques have not yet been implemented, possibly due to affordability or the low prevalence of TB.

2.8.3 MOLECULAR TECHNIQUES

The molecular diagnosis can also be called genotypic since the methods focus on genetic markers within and specifically for the *MTB* genome. This accelerates the time taken to diagnose TB and anti-TB drug resistance. The molecular diagnoses approach has several advantages over phenotypic-based testing (culture methods), namely: i) several points available to analyse, ii) determination of the phenotype is not dependent on culturing since *MTB* is a slow-growing bacterium, iii) high specificity and performed rapidly (shorter durations).

- **XPERT MTB/RIF (ALSO KNOWN AS GENE XPERT)**

The Xpert MTB/RIF is an automated cartridge-based system that offers ease of use and a workflow that reduces the risk of cross-contamination between specimens. The test is reported to be simple for laboratory technicians to perform with no requirements for advanced biosafety equipment (Caulfield and Wengenack, 2016). The test provides results on TB- and RIF-associated drug resistance in about 2 hours and is performed directly from sputum specimens (Scott et al., 2011). For these reasons, the

WHO recommended the use of Xpert MTB/RIF (illustrated in Figure 2.13) in developing countries with a high TB prevalence. The use of this Xpert MTB/RIF assay has expanded significantly from 550,000 in 2011 to 4.8 million test cartridges purchased by low- and middle-income countries in 2014. By 2015, 69% of the countries recommended using Xpert MTB/RIF as the initial diagnostic test for people at risk of drug-resistant TB (WHO, 2015).

The most advantageous benefit added by Xpert MTB/RIF is the ability to simultaneously detect the RIF resistance by detecting mutation on the 81bp region of the *rpoB* gene that confers approximately 96% of the cases reported for RR in TB (Sailo et al., 2022; Jamieson et al., 2014; Miller et al., 1994). RIF resistance is a predictor of MDR-TB, as reports have shown that isolates resistant to RIF were also resistant to INH (WHO, 2019). However, there are reports of false RIF resistance (Claessens et al., 2017, Williamson et al., 2012).



Figure 2.13: The Cepheid Xpert® system and MTB/RIF test cartridge (adapted from Caulified and Wengenack, 2016).

- **ANYPLEX™ MTB/NTM (SEEGENE)**

Anyplex MTB/NTM is a real-time assay suitable for *MTB* and NTM detection directly from specimens. It has been validated across various sample types and can be used on multiple amplification platforms. Results interpretation is automated and can be released within approximately 4 hours of sample receipt (Perry et al., 2014). This method showed high sensitivity and specificity in detecting MTB with favourable cost and time efficiency (Sawatpanich et al., 2022; Choe et al., 2015). A study's findings indicated that the Seegene Anyplex™ MTB/NTM detection assay is appropriate and practical for the identification and differentiation of MTBC and NTM infections when selective PCR testing for TB is conducted in a low-incidence area. According to prospective performance statistics and analyses of improved TB surveillance data, more research is necessary to determine the clinical and financial benefits of switching from smear testing to PCR in comparable low-incidence situations (Lee et al, 2014; Perry et al., 2014). The figure below (Figure 2.14) shows the Anyplex™ MTB/NTM results and interpretation.

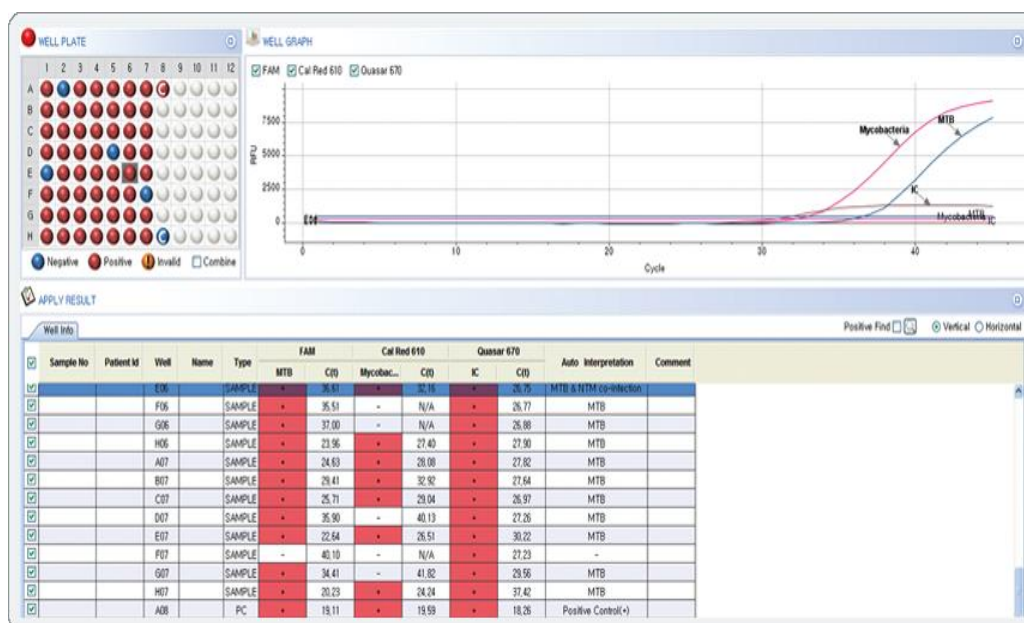


Figure 2.14: Anyplex™ results (MTB infection in Fam Channel and NTM Cal Red channel) (adapted from Seegene website)

- **ALLPLEX™ MTB/MDR/XDRE (SEEGENE)**

The Anyplex™ *MTB/MDR/XDR* detection assay is an advanced multiplex real-time PCR technique, was developed by Seegene (South Korea) for the simultaneous detection of MTB and MDR/XDR resistance alleles. This assay was released to the market in 2012 and its recommended by WHO (Boyle and Pai, 2013) Seegene's Dual Priming Oligonucleotide (DPO) and Tagging Oligonucleotide Cleavage Extension (TOCE) technologies are applied without further downstream processing, while the MTB target sequences and each drug-resistant target mutation are amplified and detected specifically by the corresponding oligos in the Anyplex™ *MTB/MDR/XDR* detection assay. DPO™ is a tool used to block the extension of non-specifically primed templates, while TOCE is a novel real-time readout technology based on melting temperature analysis that provides enhanced multiplexing capability. Combining the two enables a simultaneous real-time detection of multiple point mutations with high specificity (Elfeky and Elshimy, 2015; Perry et al., 2014). The figure below (Figure 2.15) illustrates the interpretation of Allplex *MTB/MDR/XDR* results.

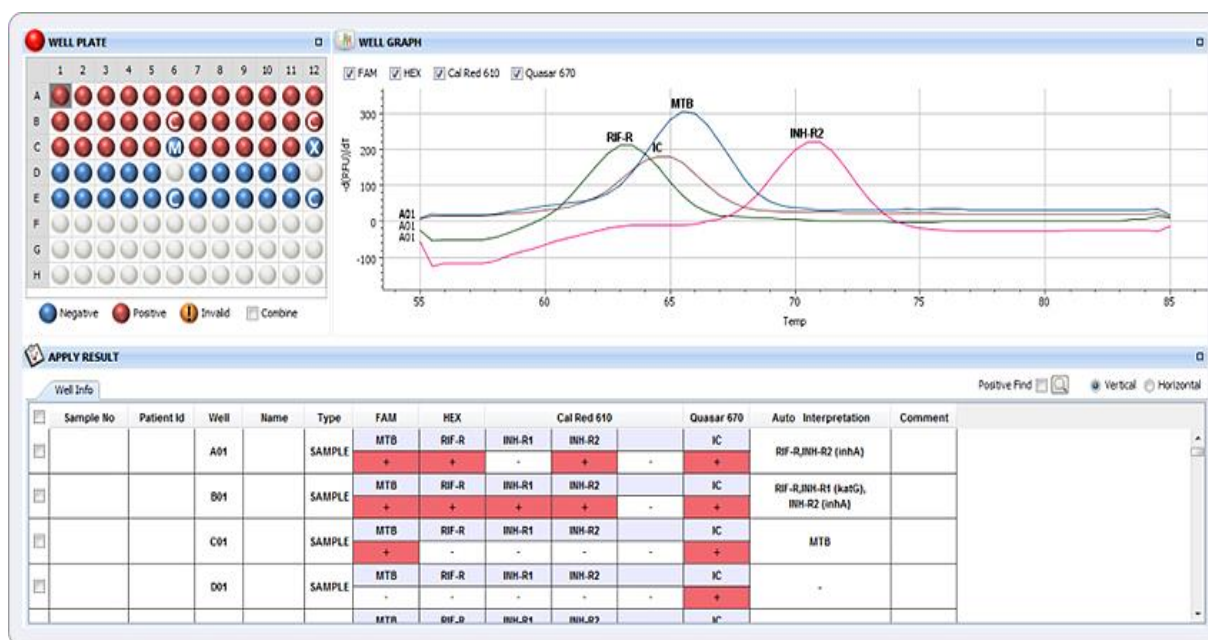


Figure 2.15: Allplex™ results (adapted from Seegene website)

- **MASSARRAY**

MassArray technology, which is based on MALTDI_TOD mass spectrometry, is a powerful technique for genotyping and mutation analysis in MTB research. It enables rapid, high-throughput detection of single-nucleotide polymorphisms (SNPs) and genetic variants associated with drug resistance (Su et al., 2017). MassArray provides a cost-effective approach to screening large sample sets for known mutations in genes like *rpoB*, *katG* and *inhA*, which are crucial in determining first-line TB drug resistance for RIF and INH (Wu et al., 2025; Yang et al., 2023; Li et al., 2021). Compared to traditional sequencing methods, this targeted approach enables researchers to quickly identify resistant strains, leading to faster treatment decisions and improved patient management.

Besides drug resistance detection, MassArray plays a vital role in TB molecular epidemiology. This technique allows TB researchers to identify TB strains, monitor transmission trends and investigate the genetic diversity of MTB populations by using specific genetic markers (Yang et al., 2023). This information is essential for understanding disease outbreaks across communities and for guiding public health interventions. MassArray's ability to process multiple samples efficiently makes it particularly useful in large-scale epidemiological studies that require rapid, accurate genotyping. In Addition, MassArray can complement whole-genome sequencing (WGS) by validating key SNPs identified in broader sequencing efforts. This is supported by a study that demonstrated MALTI-TOF exhibits excellent diagnostic performance (sensitivity and accuracy) for both TB and drug-resistant TB (Shi et al., 2025). Another study enrolled 170 cases and found that MassArray had higher sensitivity than GeneXpert (Liang et al., 2024).

- **METAGENOMICS**

Metagenomic next-generation sequencing (mNGS) represents a groundbreaking method for diagnosing infectious diseases, utilising unbiased, high-throughput sequencing to directly identify and analyse microbial genomes from clinical specimens such as cerebrospinal fluid, sputum and blood (Elbehiry and Abalkail, 2025). In contrast to conventional culture techniques and targeted molecular assays, mNGS serves as a complementary diagnostic tool that can identify previously unrecognized,

fastidious, or polymicrobial infections while simultaneously detecting antimicrobial resistance (AMR) determinants. These capabilities are particularly valuable in complex clinical situations, including infections affecting immunocompromised individuals, sepsis cases, and infections with negative conventional culture results (Zhao et al., 2024; Gao et al., 2024; Miller and Chiu, 2022).

Metagenomics is a promising tool in TB research, as it bypasses culturing, reducing turnaround time for diagnosis and for implementing treatment outcomes. A study by You et al. (2024) demonstrated that mNGS had comparable sensitivity and specificity to Xpert-MTB/RIF and higher sensitivity than traditional mycobacterial culture methods. This tool exhibited promising potential in detecting a diverse range of PTB clinical samples. Additionally, a study reported that mNGS may be a promising technology for early diagnosis of active TB, especially sputum culture-negative PTB and tuberculous serous effusions (Jin et al., 2020).

- **WHOLE GENOME SEQUENCING TECHNIQUE**

Whole genome sequencing (WGS) is a powerful technique used to determine the complete information encoded in an organism's DNA (Vourli et al., 2021). The process determines the full DNA sequence of an organism's genome at a single time (Ruan et al., 2020). The comprehensive information allows researchers to identify genetic variations, mutations, or even structural changes that can influence health, disease, and evolutionary traits.

In TB research, WGS has emerged as a powerful, high-throughput molecular approach for comprehensive characterization and managing TB infections (Cohen et al., 2019; Votintseva et al., 2017; Walker et al., 2017; Coll et al., 2015). By interrogating the entire bacterial genome and identifying SNPs with high sensitivity and specificity, WGS provides detailed insights into the genetic diversity, transmission dynamics, and evolutionary history of *MTB* strains. This technology has revolutionized outbreak investigations by facilitating the precise identification of transmission chains and distinguishing between recent transmission events and reactivation of latent infections.

Furthermore, WGS enables rapid and accurate detection of genetic mutations associated with anti-tuberculosis drug resistance, thereby supporting individualized treatment strategies and improving patient management (Satta et al., 2018; Walker et al., 2017). The technology also serves as a robust tool for strain genotyping and phylogenetic analysis, enabling researchers to classify circulating lineages, investigate their geographical distribution, and assess their epidemiological significance (Negrete-Paz et al., 2021; Nikolayevskyy et al., 2016).

In addition, WGS plays a critical role in national and global surveillance programs by monitoring the emergence and spread of drug-resistant strains, informing public health interventions, and strengthening TB control efforts. Moreover, by providing genome-wide data, WGS contributes significantly to the understanding of pathogen evolution, adaptation, and population structure, thereby enhancing knowledge of the molecular mechanisms underlying the persistence and transmission of *MTB* within diverse host and environmental contexts (Cohen et al., 2019; Walker et al., 2017; Coll et al., 2015).

Initial studies of *MTB* revolved around restriction fragment length polymorphic (RFLP) analyses, and this allowed, for the first time, the application of molecular epidemiology, showing clearly that TB epidemics in the USA and South Africa were due to the spread of clonal isolates (Victor et al., 2007; Gomez Marin et al., 1995). It suggested that certain strains of *MTB* had become better adapted to causing disease in humans, or that strains became reactivated in TB patients (reactivation disease) more easily. This view was supported by the finding of genetic disruption of various genes involved in cell wall lipid and lipoprotein synthesis, which are associated with hypervirulent strains of *MTB* (Moopanar and Mvubu, 2020). Sequencing the entire genome of the *MTB* strain H37Rv identified about 4,000 genes (Cohen et al., 2019). However, more than 10 percent of these are pseudogenes, which helped to highlight the process by which pathogenicity islands had developed within *MTB*.

2.9 APPLICATION OF WHOLE GENOME SEQUENCING IN TUBERCULOSIS RESEARCH

Whole-genome sequencing (WGS) identifies single-nucleotide variations and provides fast, cost-effective detection. It also divides patient groups over time and space, which is in close proximity to clusters. It encompasses approaches that enable high-

resolution surveillance of disease outbreaks and provide the information required to manage and control their spread. Whole-genome sequencing improves the definition of tuberculosis research and enables future studies on transmission dynamics, pathogens, and host genetic determinants of disease outcomes in both diseases. Clinical and public health management processes are controlled through the use of genomic data and modeling of theoretical infection dynamics, transmission, and drug response mechanisms. This research is planned at a time of low tuberculosis incidence worldwide, and continuous genomic analysis of *MTB* globally will play a key role in fulfilling the WHO's ambitious goal of eliminating tuberculosis as a serious public health problem.

The first WGS study of *MTB* was published in 1998 by Cole et al. in *Nature*. This landmark study sequenced the entire genome of the reference strain *MTB* H37Rv, providing the foundation for understanding the genetic basis of TB pathogenesis, drug resistance, and evolution. Recently, studies demonstrated the significant potential of WGS in predicting treatment outcomes for MDR-TB and XDR-TB. A study done He et al. (2020) evaluated the feasibility of WGS in forecasting treatment outcomes for MDR-TB patients. The study found that specific genetic mutations causing high-level fluoroquinolone resistance and mutations in the *ethA* gene, were independent risk factors for unfavourable treatment outcomes. Studies have confirmed and also highlighted the practicality of WGS in clinical settings to provide a drug resistance profile with a possible turnaround time of a week (Liu et al., 2022; Zignol et al., 2018; Walker et al., 2015).

A systematic review explored the association between WGS-identified drug resistance mutations and treatment outcomes in MDR/XDR-TB cases. The review reported a high level of fluoroquinolone resistance mutations associated with 94AAVC and 94GGC, which are linked to unfavourable treatment outcomes. Other reported drug resistance mutations include kanamycin high-level resistance mutations (*rrs* 1401G), *rpoB* Ile491Phe, and *ethA* mutations, which confer prothionamide resistance (Hazra et al., 2023). The study concluded that WGS has a significant capacity to provide accurate and comprehensive drug resistance data for MDR/XDR-TB, which can inform personalized drug therapy to optimize treatment outcomes.

Additionally, a study by Cox et al. (2020) assessed how WGS affects treatment regimens for RR-TB in high-burden settings. This is supported by a study done in India

where mutations outside the RRDR, Ile491Phe and Val170Phe, were seen in 1.3% of cases; disputed mutations in *rpoB* (Asp435Tyr, His445Asn, His445Leu, and Leu430Pro) were seen in 6% of cases, and mutations to newer drugs such as bedaquiline (1%) and linezolid (7.5%) were also detected (Bhanushali et al., 2024). The high percentage of pre-XDR cases and developing resistance to newer medications are highlighted in this study on clinical samples; continuous transmission of these strains can pose a major risk to public health, and WGS can efficiently detect and support precision medicine for tuberculosis. Therefore, the studies provide insight into the conclusion that incorporating WGS-derived drug susceptibility testing could lead to more effective, individualized treatment regimens, potentially improving patient outcomes.

2.10 TREATMENT OUTCOME

An individual is classified as a TB patient either clinically (TB symptoms and/or X-ray but there's no lab confirmation) or bacteriologically (one sample is positive by smear microscopy, culture or molecular tests). Once a patient starts treatment, they are classified according to their response. Evaluation is performed clinically and bacteriologically, generally with smear microscopy for DS-TB and culture for DR-TB (WHO, 2014). Previously, a time threshold was used to align treatment outcomes and align with the two (intensive and continuous) phases of treatment schemes. However, introducing a new regimen with a shortened period, changes to the conversion steps to follow, and a reduction in injectables led to the revised definitions tabulated in Table (WHO, 2021). The changes in TB treatment are positive, as the treatment regimen has been shortened, which increases adherence. In addition, developing a new treatment regimen also allows the conversion period to be shorter (from a month to a week).

Table 2.3: Updated definitions of treatment outcomes for DS-TB and DR-TB (WHO, 2021)

Outcome	Definition
Treatment success	Treatment completed and patient cured
Treatment failed	A patient whose treatment regimen needs to be terminated or permanently changed ^a to a new regimen or treatment plan
Cured	A patient with pulmonary TB with bacteriologically confirmed TB at the beginning of treatment and has completed treatment as recommended by the National policy with evidence of bacteriological response ^b and no evidence of failure

Treatment completed	A patient who completed treatment as recommended by National policy but whose outcome does not meet the definition for cure or treatment failure
Died	A patient who died ^c before starting the treatment plan or during the course of treatment
Lost to follow-up	A patient who did not start treatment or whose treatment was interrupted for 2 consecutive months or more
Not evaluated	A patient for whom no treatment outcome was assigned ^d
<i>A proposed definition for the use of operational research only</i>	
Sustained treatment	An individual assessed at 6 months (DS-TB and DR-TB) and at 12 months (DR-TB only) after successful TB treatment, who is alive and TB-free

a Reasons for the change include:

- no clinical response and/or no bacteriological response (see note 'b');
- adverse drug reactions; or
- evidence of additional drug resistance to medicines in the regimen.

b "Bacteriological response" refers to bacteriological conversion with no reversion.

- "bacteriological conversion" describes a situation in a patient with bacteriologically confirmed TB where at least two consecutive cultures (for DR-TB and DS-TB) or smears (for DS-TB only), taken on different occasions at least 7 days apart, are negative.
- "bacteriological reversion" describes a situation where at least two consecutive cultures (for DR-TB and DS-TB) or smears (for DS-TB only), taken on different occasions at least 7 days apart, are positive after the bacteriological conversion or in patients without bacteriological confirmation of TB

Tuberculosis (TB) remains a global health issue. Currently, South Africa has made significant progress in TB diagnostics through the rollout of WHO-endorsed molecular assays, such as Xpert *MTB*/RIF and Genotype MTBDRplus, with Xpert replacing smear microscopy as the first-line test at National Health Laboratory Service (NHLS) laboratories since 2012. These tools enable rapid detection of TB and rifampicin resistance and are supported by MGIT 960 culture-based drug susceptibility testing. However, current molecular assays are probe-limited and primarily target mutations within the rifampicin resistance-determining region (RRDR) of the *rpoB* gene, leaving clinically relevant mutations outside this region, including codons 170 and 491, undetected. This incomplete mutation coverage may result in missed rifampicin-resistant cases, delayed appropriate treatment, and continued transmission.

In high-burden settings such as the Vhembe district in Limpopo Province, where TB and HIV co-infection are prevalent, data on non-RRDR mutations are scarce. Investigating these mutations is therefore essential to improve diagnostic accuracy, understand local resistance patterns, and strengthen TB control and treatment outcomes. Therefore, the conceptual framework guiding this study is based on the

interactions among TB burden in the district, prevalence of drug resistance, diagnostic availability and capacity, and treatment outcomes. The figure below (Figure 2.16) shows the study's conceptual framework.

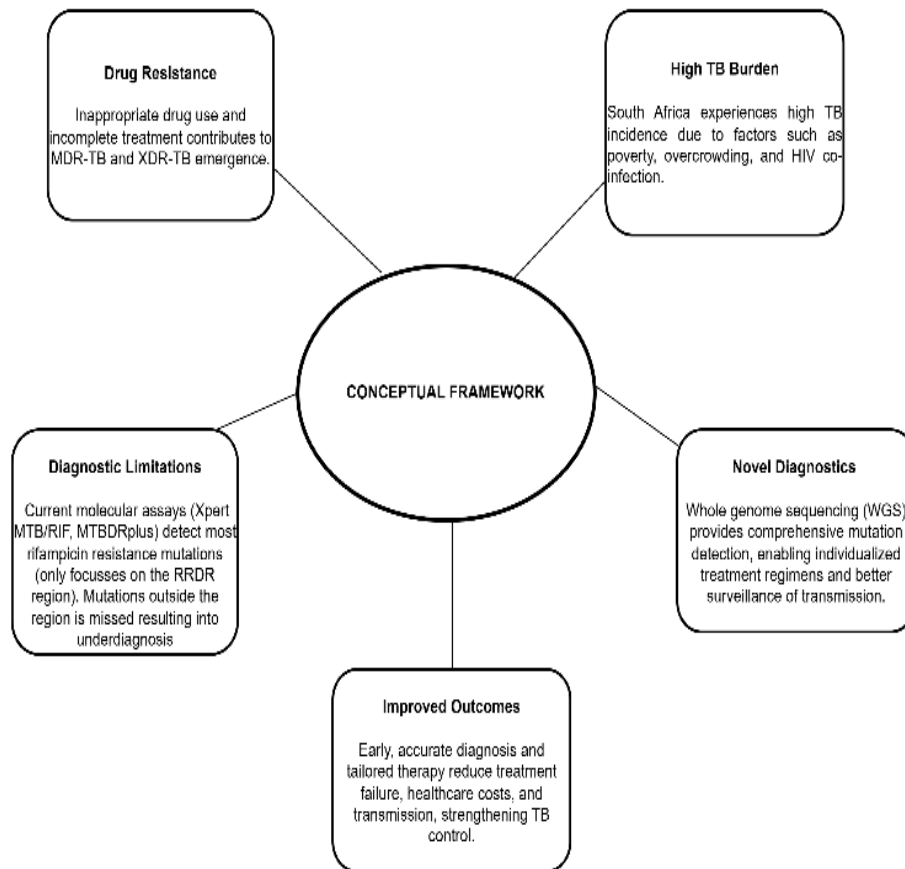


Figure 2.16: The study conceptual framework (created using draw.io www.drawio.com). Conceptual framework outlining key factors influencing tuberculosis (TB) control, including the high disease burden linked to socioeconomic conditions and HIV, the rise of drug resistance due to inadequate treatment, and the limitations of current diagnostics.

CHAPTER 3: OBJECTIVE 1

This chapter describes the reports on objective 1. The narrative outlines the approach from sample collection to laboratory analysis, including the results obtained. In summary, the methods cover the detection of target bacteria and the prevalence of resistance in the Vhembe District.

This part of the research incorporated information collected from 100 patients with tuberculosis to illustrate public health patterns, inform intervention strategies, and influence policy-making decisions.

CHAPTER 3

PREVALENCE OF MDR/XDR-TB IN THE VHEMBE DISTRICT

3.1 INTRODUCTION

Tuberculosis (TB) remains a significant public health problem in the WHO African Region, with a high number of cases and morbidity. In 2021, this region accounted for approximately 23% of new TB cases and 31% of TB-related deaths; this is equivalent to 2.5 million people falling ill and an estimated half a million deaths, respectively, due to TB infections (WHO, 2022). Approximately 20% of the reported new TB cases were people with HIV/AIDS co-infection. Despite TB remaining a significant public health problem, drug-resistant TB is a global burden due to the increasing number of infections and deaths reported. An estimated 450,000 new cases of rifampicin-resistant TB (RR-TB) were reported globally, with an estimated 77,000 cases of

multidrug-resistant TB (MDR-TB), of which Nigeria and South Africa account for 53% of the burden (WHO, 2023; WHO, 2022).

Previously, 150,329 cases of MDR-TB/RR-TB were enrolled in treatment, accounting for only approximately a third of the estimated burden (WHO, 2021; WHO, 2020). This highlights the capacity gap for either improvement in diagnoses or treatment regimens for DR-TB. In comparison with susceptible TB, DR-TB presents a lower probability of treatment success even though it requires more resources for treatment (WHO, 2021). In 2020, the treatment success rate for MDR-TB/RR-TB was reported to be 59% globally in comparison with 86% for new and relapse drug-susceptible TB (DS-TB) (WHO, 2021). The diagnostic landscape of TB has significantly advanced over the past decade, notably leading to the widespread adoption of molecular diagnostic technologies.

Tuberculosis is one of the most challenging bacterial infections to treat in South Africa. Researchers are conducting studies to develop solutions to fight TB and meet the SDG 2030 goals of eradicating this disease in the communities. Therefore, this chapter focuses on using molecular techniques to determine the prevalence of DR-TB circulating in the Vhembe district, Limpopo, South Africa.

3.2 MATERIALS AND METHODS

This study used qualitative and quantitative methods to strengthen and validate the findings. A structured approach was used to answer the research questions, employing a cross-sectional design. This study design was chosen because it provides sufficient, rapid answers to the community's TB-related challenges. In addition, it has the advantage of highlighting the approach to address the public health threat.

3.2.1 ETHICAL CLEARANCE

The study protocol was developed and submitted to the University of Venda Ethics Committee (UREC) for ethical consideration and approved under reference number SMNS/20/MBY/13/2104. (Appendix 1). An additional ethical clearance was applied at the provincial level under the Limpopo Department of Health [(LP_2021_11_001) Appendix 2A and 2B] to access the study site of interest. Permission was requested

and granted to access healthcare facilities at the Vhembe District Health Department, subject to provincial approval (Appendix 3). Thereafter, various healthcare facilities were visited to introduce the study through verbal and written presentations. Participating participants were briefed about the study, and formal consent was obtained for signing.

3.2.2 STUDY SITE

The study was conducted across several healthcare facilities (Appendix 4) in the Vhembe district that were approached and had active TB patients currently on TB treatment. The Vhembe District Municipality was established in the early 2000 and has geographic coordinates 22.7696 °S, 29.9741 °E, located in the northern region of Limpopo Province (DCOGTA, 2020). The province shares a boundary with Mozambique in the south-east through the Kruger National Park, and with Zimbabwe in the North and Botswana in the West. The Limpopo province is named after the Limpopo River, which serves as a demarcation between the district and its international surroundings (DCOGTA, 2020). It is endowed with a wide expanse of land covering 25,596 km² and is subdivided into four municipalities: Collins Chabane, Makhado, Thulamela, and Musina. In 2019, the total population was 1,402,779. It covers a predominantly rural area. It is a prominent cultural hub and a catalyst for agricultural and tourism development (National School of Government, 2018).

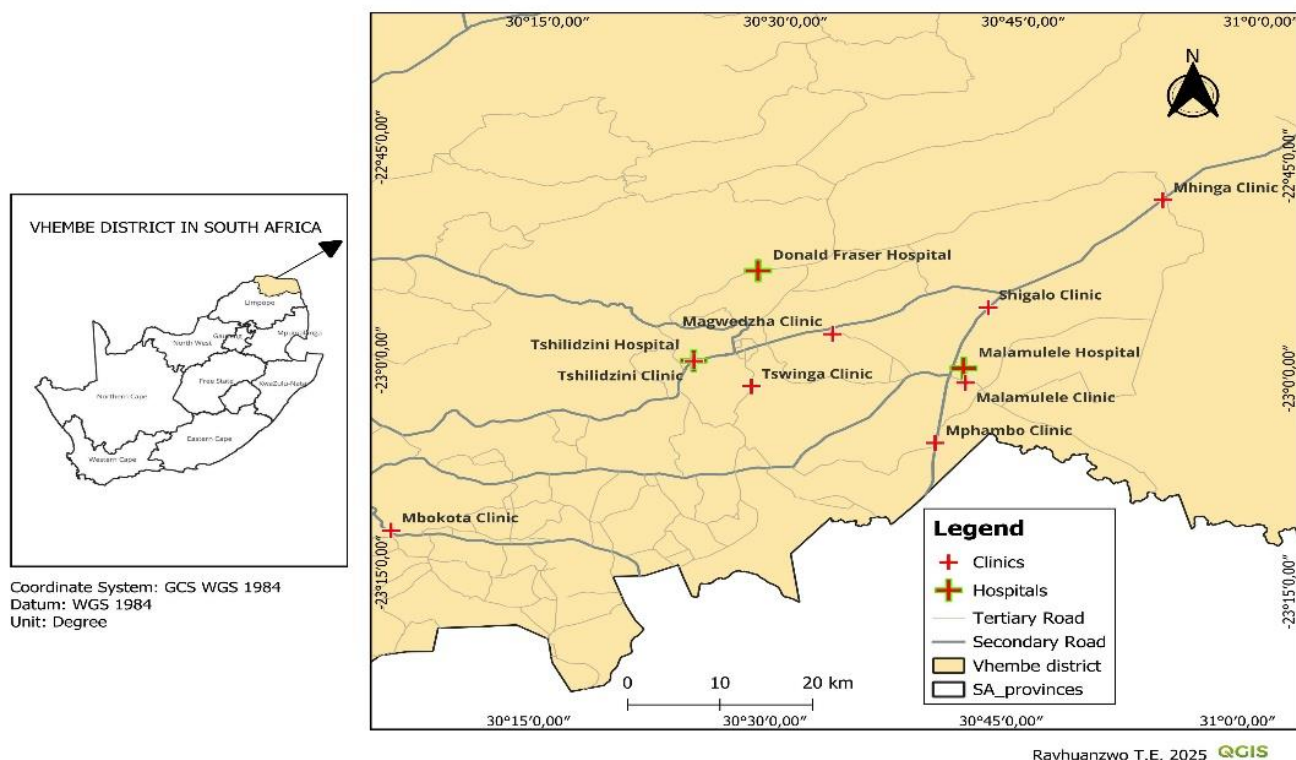


Figure 3.1: A map showing the study site in Vhembe district, South Africa (Created by Ravhuanzwo, 2025 from University of Venda)

3.2.3 STUDY POPULATION

Selection criteria

Participants included in this study were selected based on the inclusion criteria below:

- 18 years or older
- Diagnosed with TB
- On TB treatment
- Inpatient or outpatient

Participants who were excluded were based on the exclusion criteria below:

- Younger than 18 years of age
- Does not provide consent.
- Patients who are critically ill
- Prisoners

Sample size

This study forms part of a bigger study led by Prof AN Traore (Supervisor). For this section of the study, a total of 100 samples is reported. The samples were randomly selected from the batch collected at different sites within the overall study population.

3.2.4 SAMPLE COLLECTION

A signed consent form (Appendix 5) was obtained from the participant. A structured questionnaire (Appendix 6) was used to collect participants' background information. A total of 100 sputum samples were obtained between August 2022 and August 2025. To ensure participant confidentiality and anonymity, each participant was assigned a unique study identification code at enrolment. A 100ml container containing 4% NaOH (Sigma-Aldrich, St. Louis, MO, USA). was given to the participant and directed to the marked zone (outside, open area of the facility building with air circulation). The participant had to cough, spit into the container, and close it tightly. The samples were placed into the cooler box with ice blocks and carried to the University of Venda, Life Science Building, TB laboratory.

3.2.5. SAMPLE ANALYSIS

Sample pre-treatment

Upon arrival, samples were incubated at room temperature under a biosafety cabinet for approximately 15 min. A total of 1.5 ml of the sputum sample was transferred to a new 2ml tube (Eppendorf, Hamburg, Germany) and centrifuged (Heraeus Megafuge 16, Thermo Scientific, Waltham, USA) at 13 000 rpm for 5 min. The supernatant was discarded, and 1 ml of 1X PBS solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added to the pellet. The mixture was vortexed briefly and centrifuged at 13 000 rpm for 5 min. The supernatant was discarded at this step, and the procedure was repeated twice: first with 1X PBS and second with sterile water. The supernatant was discarded, and the pellet was used for DNA extraction.

DNA extraction

The manufacturer's protocol (Seegene, Seoul, Korea) was followed for DNA extraction as follows: a total of 100 µl DNA Extraction solution was added to the pellet, and 10 µl of Internal control (IC) was added to the tube. The tube cap was locked using a cap-lock, vortexed for 30 sec, and placed in a 100 °C heat block to boil for 20 min. After

boiling, the sample was centrifuged at 15,000 x g (13,000 rpm) for 5 min. A 100 µl supernatant (PCR template) was pipetted into a new 1.5 ml tube and stored at -20 °C until further analysis. The extracted DNA was used as a template for the Anyplex™ and Allplex™ assays.

ANYPLEX™ and ALLPLEX™ ASSAYS

ANYPLEX™ MTB/NTM

A real-time detection assay, Anyplex™ MTB/NTM V2.0 (Seegene Inc., Republic of Korea), was used to identify the mycobacterial species in sputum samples. The protocol was adapted from a previous study by Sali et al. (2016). For Real-time PCR: A 15 µl PCR amplification mixture was combined with 5 µl of extracted DNA (PCR template) to obtain a 20 µl PCR reaction volume, following the manufacturer's instructions (Seegene, Seoul, Korea). The PCR reactions were run on the CFX 96™ Real-Time PCR system (Bio-Rad Laboratories Ltd, USA) with thermocycles conditioned as follows: Initial hold for 5 min at 50 °C, followed by 95 °C for 15 min and 45 cycles of 95 °C for approximately 30 secs and 60 °C for a min with fluorescence detection on the FAM (MTBC) Cal Red 610 (genius *Mycobacterium*) and Quasar 670 (internal control) channel. Seegene Viewer 2.0 software was used to automatically interpret results, following the manufacturer's thresholds and cutoffs (FAM CT < 40 for MTBC detection, Cal Red 610 CT < 38 with negative MMT NC for NTM detection).

ALLPLEX™ MTB/MDR/XDR ASSAY

A total of 5 µL of the supernatant (extracted DNA) was used as template DNA for polymerase chain reaction (PCR) amplification using the Allplex™ system (Seegene, Seoul, South Korea). The assay targets specific resistance-associated mutations within the *Mycobacterium tuberculosis* genome.

For drug resistance, included run on mutations on Seven isoniazid (INH) resistance-associated mutations in *MTB* were targeted, including *katG* variants S315I (ATC), S315N (AAC), S315T (ACC), and S315T (ACA), as well as mutations in the *inhA* promoter at positions -15 (T), -8 (A), and -8 (C). For, rifampicin (RIF) resistance, eighteen mutations within the *rpoB* gene were analysed: L511P (CCG), Q513K (AAA),

Q513L (CTA), Q513P (CCA), a three–amino acid deletion spanning codons 513–516, D516V (GTC), D516Y (TAC), S522L (TTG), S522Q (CAG), H526C (TGC), H526D (GAC), H526L (CTC), H526N (AAC), H526R (CGC), H526Y (TAC), S531L (TTG), S531W (TGG), and L533P (CCG).

Additionally, seven fluoroquinolone (FQ) resistance mutations in the *gyrA* gene were assessed: A90V (GTG), S91P (CCG), D94A (GCC), D94G (GGC), D94H (CAC), D94N (AAC), and D94Y (TAC). Additionally, six mutations associated with resistance to injectable drugs are targeted, including *rrs* gene mutations at positions 1401(G), 1402(T), and 1484(T), as well as mutations within the *eis* promoter region at –37(T), –14(T), and –10(A) (Seegene, Seoul, South Korea) The figures (Figure 3.2) below show the steps followed in the analysis and the interpretation of the results.



Created using AI

Figure 3.2: Schematic representation of the molecular diagnostic workflow for the detection of *Mycobacterium tuberculosis* (MTB) from sputum specimens.

3.3 RESULTS

This study is part of a larger study; therefore, the total number of participants included is only 100. Responses from the questionnaires were converted to percentages and

presented in figures and tables to better understand the findings in the study population. The result section is divided into subsections.

3.3.1 PREVALENCE OF TUBERCULOSIS IN PARTICIPANTS

Rapid detection of MTB is crucial for early diagnosis, enabling patients to start treatment early and for preventing or controlling transmission in high-burden regions or densely populated areas such as squatter camps. Molecular assays are among the most effective tools for quick and accurate detection of *MTB* and its resistance profile. Sputum samples were directly analysed for the type of *Mycobacteria* they contained. The prevalence of *Mycobacteria* species within the study population was determined using the Anyplex™ assay and is demonstrated in Figure 3.3. Overall, *Mycobacteria* were detected in 70% of participants (MTB and NTM), indicating a substantial burden of infection in the sampled population. When combined, TB-positive cases (MTB with or without NTM co-infection) represented 47% of the study population, highlighting the continued dominance of TB-associated pathogens in this setting. While 30% had no detection of any type of mycobacteria and 23% had NTM detected.

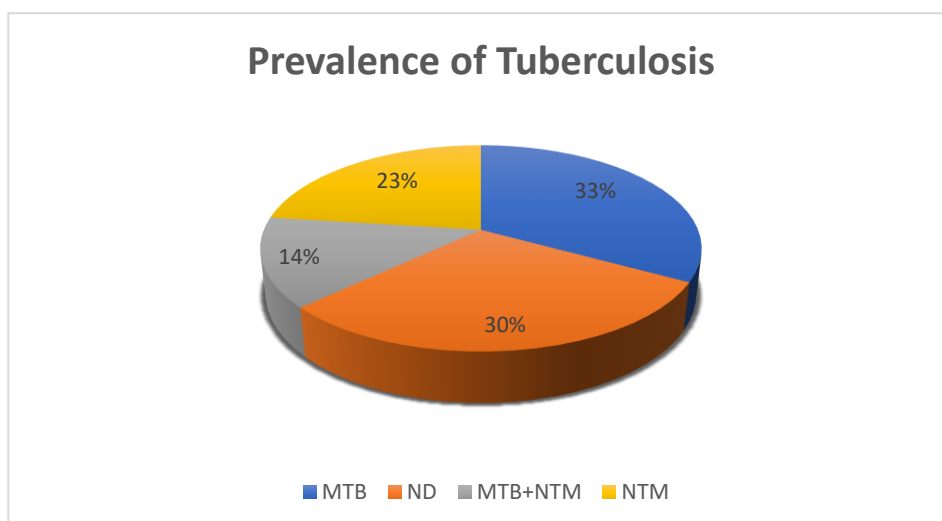


Figure 3.3: Prevalence of *Mycobacterium tuberculosis* (MTB) and non-tuberculous mycobacteria (NTM) among study participants (n=100).

The pie chart illustrates the distribution of cases: 33% MTB-positive, 14% *MTB* with NTM co-infection, 23% NTM-only, and 30% with no detection (ND). Data were

obtained using the Anyplex™ assay, highlighting the burden of TB and related mycobacterial infections in the sampled population.

Among the 100 samples, 4 (4%) were found to be resistant to anti-tuberculosis agents using the Allplex™ assay. Compared with the percentages in Figure 3.4, the 4 in Figure 3.4 represent DR-TB participants who had MTB, and some had co-occurrence of MTB and NTM. The figure (Figure 3.4) below shows the prevalence of drug-resistant tuberculosis (DR-TB) among the study population.

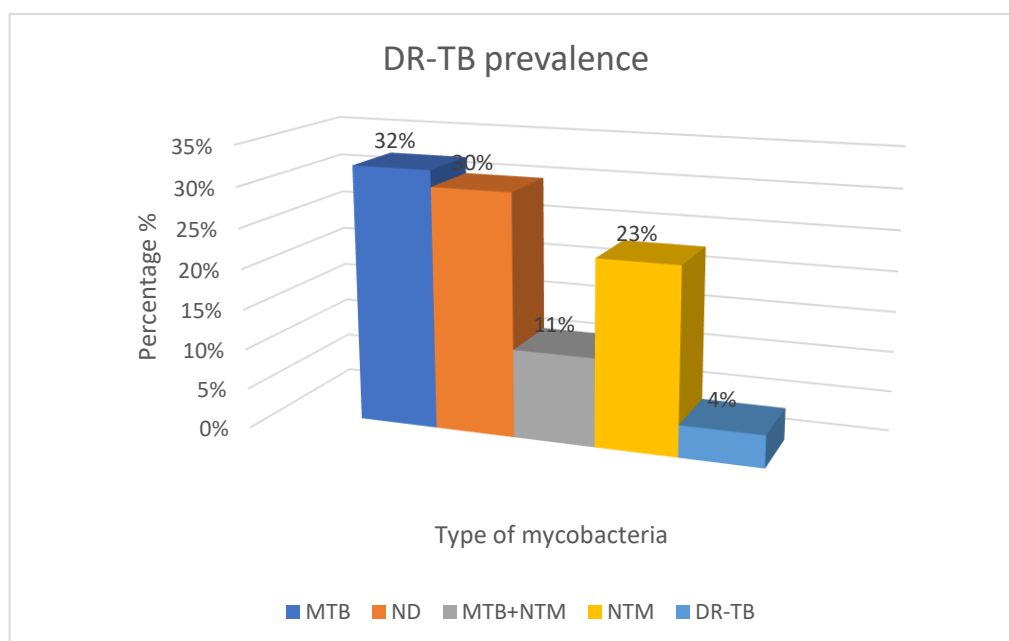


Figure 3.4: Prevalence of drug-resistant tuberculosis (DR-TB) among study participants (n=100). The bar chart illustrates the distribution of mycobacterial categories: 32% *Mycobacterium tuberculosis* (MTB), 10% MTB with NTM co-infection, 23% NTM-only, 30% not determined (ND), and 4% DR-TB. Detection and resistance profiling were performed using the Alloplex™ assay, which highlighted the presence of DR-TB cases in the population.

The prevalence of drug-resistant TB among all TB cases (*MTB* only inclusive with *MTB*+ NTM co-occurrence cases). The Allplex™ *MTB*/MDR/XDR assay identified that the majority of isolates (91.49%) were susceptible to first- and second-line anti-tuberculosis agents. Monoresistance to rifampicin, isoniazid, and fluoroquinolones was detected at low frequencies (2.13% each), while 2.13 % of isolates exhibited combined resistance across multiple drug classes. These findings suggest a relatively low prevalence of drug resistance within the study cohort; however, the detection of multidrug resistance underscores the continued necessity for rapid molecular diagnostics to inform timely therapeutic interventions and limit transmission of resistant *MTB* strains.

Table 3.1: Prevalence and distribution of drug-resistant tuberculosis (DR-TB) detected using the Allplex™ *MTB*/MDR/XDR assay among study participants (n=47)

Assay	No drug-resistant	RIF-R	INH-R	FQ-R	Combined resistance [RIF-R+INH-R+FQ-R]
Allplex <i>MTB</i> /MDR/XDR N=47	43 (91.49%)	1 (2.13%)	1 (2.13%)	1 (2.13%)	1 (2.13%)

Rifampicin (RIF), Isoniazid (INH), Fluoroquinolone (FQ), Resistance(R)

Characteristics of the DR-TB participants

The demographic and clinical profiling of participants diagnosed with DR-TB within the study cohort are presented in Table 3.2. This structured presentation enables comparative interpretation of male and female participants affected by DR-TB and facilitates identification of potential demographic or clinical trends associated with resistance.

The age distribution indicates that DR-TB cases were predominantly observed within the economically active age groups [18–44 years (3%)], with minimal representation among older participants (1%). Employment data suggest heterogeneity in socioeconomic positioning, with unemployment and student status represented among affected individuals. HIV status is included to contextualise the well-established epidemiological interaction between TB and HIV, particularly in high-burden settings.

Importantly, the DR-TB profiling differentiated between the different resistant strains in the study population. Even though they occur at low incidence rates [INH-R (1%), RIF-R (1%), XDR (2%)], the presence of the DR-TB underscores the clinical severity and public health implications in advanced patterns within the cohort.

Table 3.2: Demographic and Clinical Profile of Participants Diagnosed with Drug-Resistant Tuberculosis (DR-TB) (N = 100)

Parameters	Female N=100 (%)	Male N=100 (%)
Age		
18-24	1 (1%)	0 (0%)
25-44	1 (1%)	1 (1%)
45-60	0 (0%)	1 (1%)
Employment status		
Student	1 (1%)	0 (0%)
Employed	0 (0%)	2 (2%)
Unemployed	1 (1%)	0 (0%)
Self-Employed	0 (0%)	0 (0%)
HIV-status		
Negative	2 (2%)	1 (0%)
Positive	0 (0%)	1 (0%)
Drug-resistance profile		
Mono-resistant TB: RIF-R	1 (1%)	0 (0%)
Mono-resistant TB: INH-R	1 (1%)	0 (0%)
XDR-TB: (FQ-R+RIF-R+INH-R)	0 (0%)	2 (2%)

Rifampicin (RIF), Isoniazid (INH), Fluoroquinolone (FQ), Resistance(R)

To comprehensively determine the influence of contextual and participant-related factors on tuberculosis outcomes within the study population, questionnaire data were systematically organised into thematic subsections to facilitate structured analysis and clear interpretation. The following section presents an integrated overview of participant characteristics, encompassing demographic variables (including age, sex, education, employment status, and housing conditions), socio-environmental determinants, and cultural beliefs that may influence health-seeking behaviour and treatment adherence. These factors are analysed in relation to microbiological outcomes, including drug-resistant tuberculosis (DR-TB), drug-susceptible (normal)

tuberculosis, non-tuberculous mycobacteria (NTM), mixed MTB+NTM infections, and cases with no detectable mycobacterial infection.

By stratifying the findings across these diagnostic categories, this section aims to elucidate potential associations between socio-demographic context, cultural perceptions, and the observed distribution of mycobacterial disease within the cohort, thereby providing a foundation for subsequent epidemiological and clinical interpretation.

3.3.2 SOCIODEMOGRAPHIC CHARACTERISTICS OF THE STUDY PARTICIPANTS

Table 3.3 summarises the sociodemographic characteristics of the study participants (N=100) by mycobacterial detection status. A statistically significant association was observed between gender and mycobacterial category and was non-significant ($p=0.684$), with males predominating in the *MTB* (65.6%) and *MTB*+NTM (54.5%) groups, whereas females were represented in the NTM (52.2%) group. Age distribution was also not significantly associated with infection category ($p=0.586$). Individuals aged 25-44 years accounted for the majority of DR-TB (50%), *MTB* (68.8%), *MTB*+NTM (54.5%), and NTM (56.5%). Nationality did not reach statistical significance ($p=0.847$), although migrant representation appears higher in the no-detection cases than in the others. Education ($p=0.943$) and employment status ($p=0.304$) were not statistically significantly associated with mycobacterial categories; however, unemployment was the most prevalent across most groups.

Table 3.3: Sociodemographic characteristics of the study participants stratified by Mycobacterial detection status (N=100)

Variable	DR-TB (n=4)	MTB (n=32)	MTB+NTM (n=11)	NTM (n=23)	No detection (n=30)	Total (N=100)	p- value*
Gender							
Male	2 (50.0%)	21 (65.6%)	6 (54.5%)	11 (47.8%)	15 (50.0%)	55 (55.0%)	0.684
Female	2 (50.0%)	11 (34.4%)	5 (45.5%)	12 (52.2%)	15 (50.0%)	45 (45.0%)	

Age group							
18–24	1 (25.0%)	1 (3.1%)	0 (0.0%)	1 (4.3%)	2 (6.7%)	5 (5.0%)	0.586
25–44	2 (50.0%)	22 (68.8%)	6 (54.5%)	13 (56.5%)	16 (53.3%)	59 (59.0%)	
45–60	1 (25.0%)	7 (21.9%)	5 (45.5%)	6 (26.1%)	7 (23.3%)	26 (26.0%)	
>60	0 (0.0%)	2 (6.3%)	0 (0.0%)	3 (13.0%)	5 (16.7%)	10 (10.0%)	
Nationality							
Native	4 (100%)	30 (93.8%)	10 (90.9%)	21 (91.3%)	26 (86.7%)	91 (91.0%)	0.847
Migrant	0 (0.0%)	2 (6.3%)	1 (9.1%)	2 (8.7%)	4 (13.3%)	9 (9.0%)	
EDU Level							
NFE	0 (0.0%)	2 (6.3%)	1 (9.1%)	1 (4.3%)	3 (10.0%)	7 (7.0%)	0.943
Grade 1–7	1 (25.0%)	6 (18.8%)	0 (0.0%)	6 (26.1%)	4 (13.3%)	17 (17.0%)	
Grade 8–12	2 (50.0%)	18 (56.3%)	8 (72.7%)	13 (56.5%)	17 (56.7%)	58 (58.0%)	
Tertiary	1 (25.0%)	6 (18.8%)	2 (18.2%)	3 (13.0%)	6 (20.0%)	18 (18.0%)	
EM Status							
Student	1 (25.0%)	2 (6.3%)	0 (0.0%)	1 (4.3%)	1 (3.3%)	5 (5.0%)	0.304
Unemployed	3 (75.0%)	21 (65.6%)	6 (54.5%)	15 (65.2%)	21 (70.0%)	66 (66.0%)	
Employed	0 (0.0%)	4 (12.5%)	5 (45.5%)	3 (13.0%)	4 (13.3%)	16 (16.0%)	
Self-employed	0 (0.0%)	5 (15.6%)	0 (0.0%)	4 (17.4%)	4 (13.3%)	13 (13.0%)	

3.3.3 ENVIRONMENTAL CHARACTERISTICS OF THE STUDY PARTICIPANTS

Environmental factors are reported to contribute to TB infections. It is easier to get infected with TB if an individual lives in a dark, dusty, and damp environment because the mycobacteria survive longer in dusty rooms with no fresh air circulating, even in dark spaces with no sunlight that can kill them. Table 3.4 presents the distribution of environmental characteristics among the study population according to mycobacterial detection status.

Environmental factors assessed included housing type, sharing of bedrooms, dust exposure, mask use, mode of transport, and open-space exposure. Most participants across all groups stayed in modern houses (75%), and about 6.25% of *MTB*-detected participants stayed in squatter camps. More than 50% of the overall population shared a bedroom with household members. Half of the participants (50.00%) with *MTB*+NTM (54.54%) co-occurrence and those with NTM (69.56%) detection were working or staying mostly in dusty areas, which was almost significant ($p=0.098$).

Ventilation plays an important role in TB cases, and we observed that 80% of the study population were in open places, which showed statistical significance ($p=0.002^*$). While the majority (60%) of participants use a mask only sometimes.

Table 3.4: Environmental and social factors associated with study participants (N=100)

Parameters	DR-TB N=4 (%)	MTB N=32 (%)	MTB+NTM N=11 (%)	NTM N=23(%)	No detection N=30 (%)	Total N=100 (%)	p- Value
Type of House							
Modern	3 (75.00%)	23 (71.86%)	9 (81.81%)	17 (73.91%)	24 (80.0%)	75 (75.00%)	0.688
RDP	0 (0.00%)	6 (18.75%)	1 (9.09%)	4 (17.39%)	4 (13.33%)	16 (16.00%)	
Traditional	1 (25.00%)	1 (3.13%)	0 (0.00%)	2 (8.70%)	1 (3.33%)	5 (5.00%)	
Squatter camp	0 (0.00%)	2 (6.25%)	1 (9.09%)	0 (0.00%)	0 (0.00%)	3 (3.00%)	
Rental	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (3.33%)	1 (1.00%)	
Bedroom Sharing							
Yes	2 (50.00%)	16 (50.00%)	5 (45.45%)	14 (60.87%)	18 (60.00%)	55 (55.00%)	0.913
No	2 (50.00%)	15 (46.88%)	6 (54.54%)	8 (34.78%)	10 (33.33%)	41 (41.00%)	
Sometimes	0 (0.00%)	1 (3.12%)	0 (0%)	1 (4.35%)	2 (6.67%)	4 (4.00%)	
Dust Exposure							
Yes	2 (50.00%)	9 (28.14%)	6 (54.54%)	16 (69.56%)	12 (40.00%)	45 (45.00%)	0.098
No	1 (25.00%)	15 (46.86%)	3 (27.27%)	4 (17.39%)	7 (23.33%)	30 (30.00%)	
Sometimes	1 (25.00%)	8 (25.00%)	2 (18.18%)	3 (13.04%)	11 (36.67%)	25 (25%)	
Open Space							
Yes	2 (50.00%)	26 (81.25%)	10 (90.91%)	14 (60.87%)	29 (96.67%)	81 (81.00%)	0.002*
No	1 (25.00%)	1 (3.13%)	0 (0.00%)	6 (20.09%)	0 (0.00%)	8 (8.00%)	
Sometimes	1 (25.00%)	0 (0.00%)	1 (9.09%)	1 (4.35%)	1 (3.33%)	4 (4.00%)	
No response	0 (0.00%)	5 (15.63%)	0 (0.00%)	2 (8.70%)	0 (0.00%)	7 (7.00%)	
Wear masks							
Yes	1 (25.00%)	11 (34.38%)	4 (36.36%)	4 (17.39%)	5 (16.67%)	25 (25.00%)	0.270
No	1 (25.00%)	6 (18.75%)	3 (27.27%)	3 (13.04%)	2 (6.67%)	15 (15.00%)	
Sometimes	2 (50.00%)	15 (46.86%)	4 (36.36%)	16 (69.56%)	23 (76.67%)	60 (60.00%)	
Mode of transport							
Walking	1 (25.00%)	12 (37.50%)	3 (27.27%)	6 (26.09%)	10 (33.33%)	32 (32.00%)	0.955
Own car	0 (0.00%)	4 (12.50%)	2 (18.18%)	2 (8.70%)	3 (10.00%)	11 (11.00%)	
Public	3 (75.00%)	15 (46.86%)	6 (54.54%)	15 (65.22%)	17 (56.67%)	56 (56.00%)	
House bound	0 (0.00%)	1 (3.13%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (1.00%)	

Overcrowding remains a critical route of transmission in high-burden settings. Increased household density reduces effective air volume per individual and prolongs exposure to infectious droplet nuclei. The majority of participants in the study group

had family sizes of 5-9, with 1% having 10 or more, indicating overcrowding. Figure 3.5 illustrates family member sizes across the study cohort.

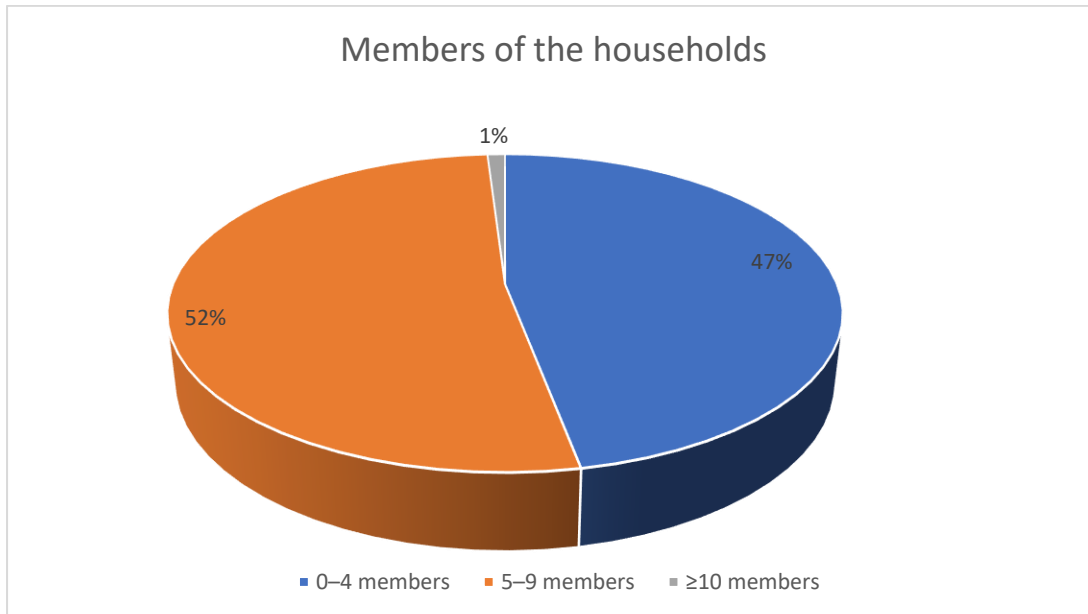


Figure 3.5: Family members across the study cohort

3.3.4 CULTURAL FACTORS

In low-income communities, people use medicinal plants or concoctions to aid illnesses they may encounter. Some individuals use traditional herbs as part of their daily diet (Observation and understanding of the study area from prior sample collection). The majority of the study population were Christians (78%), with the majority (93%) not consulting a traditional healer, and 3% used a remedy from the traditional healer, believing it would aid their sickness. None of the cultural characteristics is significant ($p > 0.05$) for the mycobacterial species. Table 3.5 summarizes the cultural beliefs by the Mycobacteria species classification.

Table 3.5: Cultural characteristics by Mycobacteria classification (n=100)

Parameters	DR-TB N=4 (%)	MTB N= 32 (%)	MTB+NTM N=11 (%)	NTM N=23(%)	Not detectable N=30 (%)	Total N=100	P value
Religion							
African	0 (0%)	7 (21.86%)	3 (27.27%)	2 (8.7%)	5 (16.67%)	17 (17%)	0.340
Christianity	3 (75%)	24 (75.0%)	8 (72.73%)	19 (82.61%)	24 (80%)	78 (78%)	
Christianity + African	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (3.33%)	1 (1%)	
Other	1 (25%)	1 (3.13%)	0 (0%)	2 (8.7%)	0 (0%)	4 (4%)	
Consulted a traditional healer.							
Yes	0 (0%)	2 (6.25%)	0 (0%)	1 (4.35%)	4 (13.33%)	7 (7%)	0.516
No	4 (100%)	30 (93.75%)	11 (100%)	22 95.65%	26 (86.67%)	93 (93%)	
Used a traditional remedy							
Yes	0 (0%)	0 (0%)	0 (0%)	1 (9.09%)	2 (6.67%)	3 (3%)	0.561
No	4 (100%)	32 (100%)	11 (100%)	10 (90.91%)	28 (93.33%)	97 (97%)	

3.3.5 MEDICAL HISTORY, ATTITUDE, AND BEHAVIOURAL FACTORS

An individual's attitude and behaviour related to health and disease are a significant component of clinical encounters. Society also influences an individual. This shapes lifestyle, symptom presentation, adherence to medical advice, and response to treatment. The health attitude and behaviour may range from anxiety to worry, stigmatization, and denial of the illness, thus delaying seeking professional healthcare and leading to a lack of treatment adherence. Figure 3.6 Majority of the participants had their BCG vaccines, and a few are recurrent TB patients. Participants demonstrated a higher level of TB status disclosure to family members compared to non-relatives, potentially due to stigmatization associated with TB. Among DR-TB participants, all participants (100%) reported contact with relatives, while no contact with non-relatives was observed (0%).

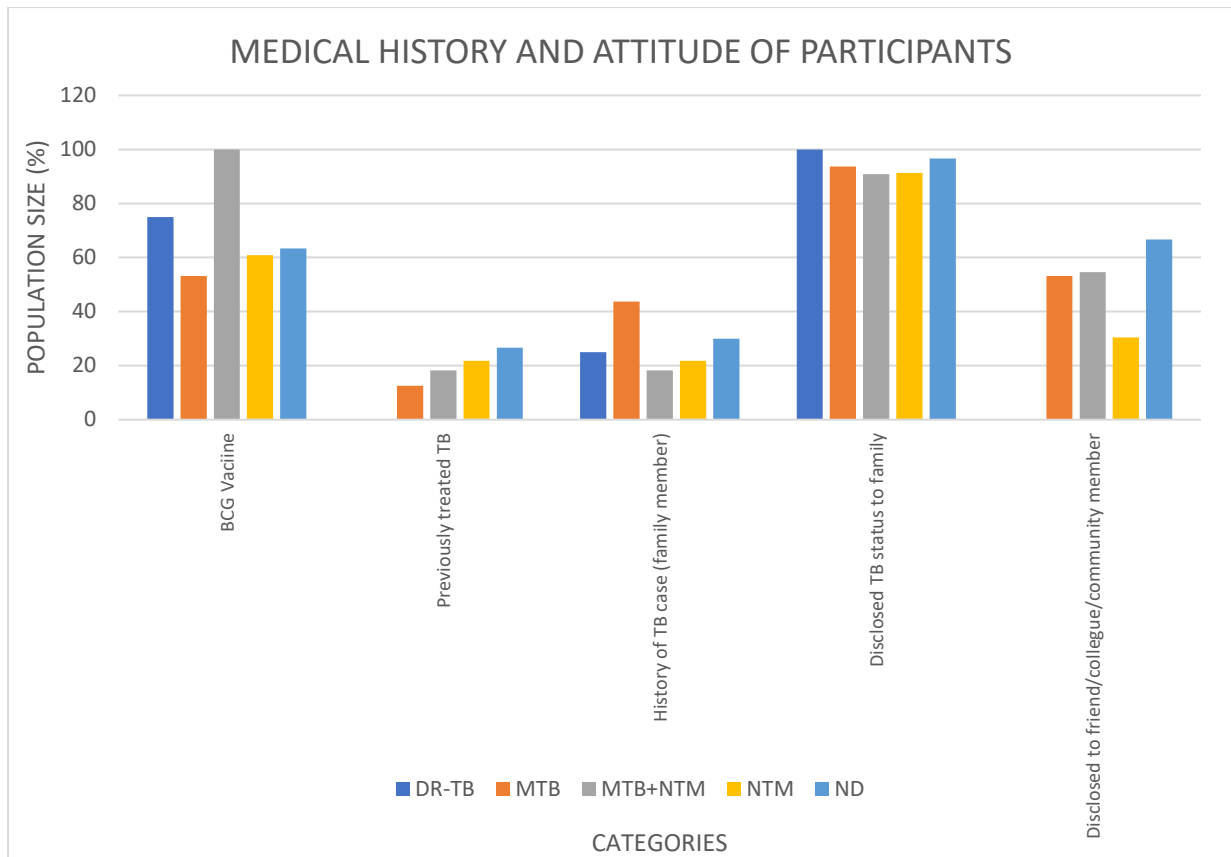


Figure 3.6: Participants' medical history, attitude, and behavioural factors

Early healthcare-seeking behaviour is essential for controlling tuberculosis (TB), since delayed presentation leads to less favourable treatment outcomes, increased illness severity, and ongoing community transmission. In the beginning, TB frequently manifests as vague respiratory symptoms that are either first misdiagnosed or mistaken for milder ailments, which causes some people to put off seeking a formal medical evaluation. Cultural views, a lack of health literacy stigma, socioeconomic limitations, and dependence on alternative treatment paths can all have an impact on whether people seek help. Figure 3.7 shows that most participants seek help in 1-3 weeks of experiencing the signs and symptoms.

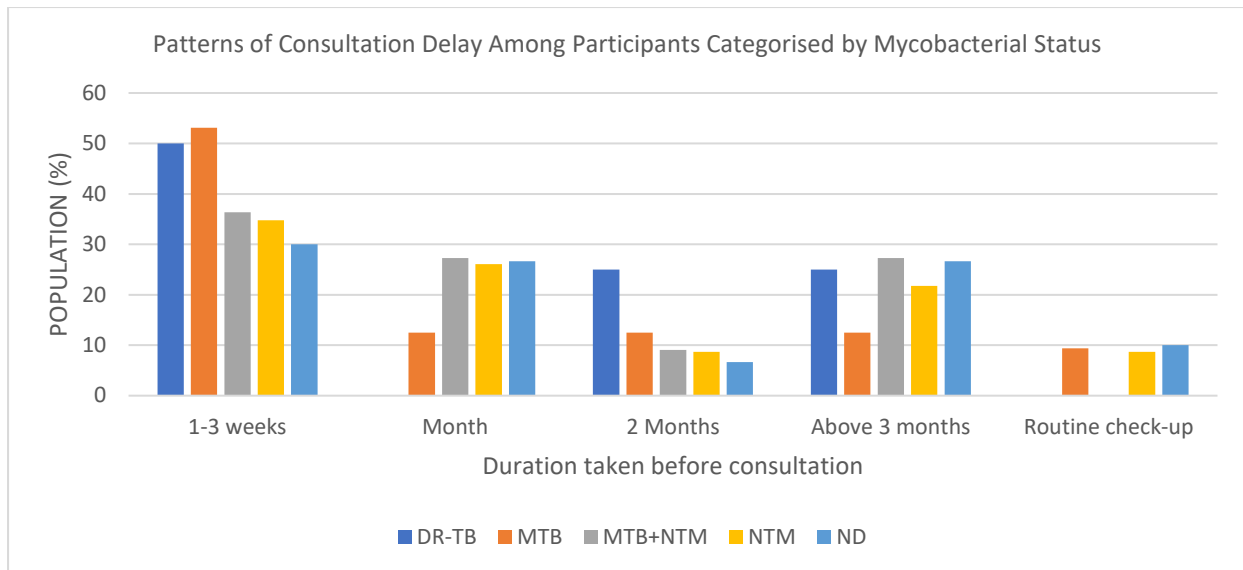


Figure 3.7: Consultation delay among participants categorized by Mycobacterial status (N=100)

Participants were also checked for co-morbidities that may influence their well-being and make TB, as it is known to be an opportunistic infection in immunocompromised individuals. HIV is the highest co-morbidity among all co-morbidities the participants have in the study cohort, with MTB+NTM having the highest, and the DR-TB have no other co-morbidity than HIV and are the lowest (25%), and NTM participants have almost all co-morbidities found in the study population. Figure 3.8 demonstrates the comorbidities of the study cohort.

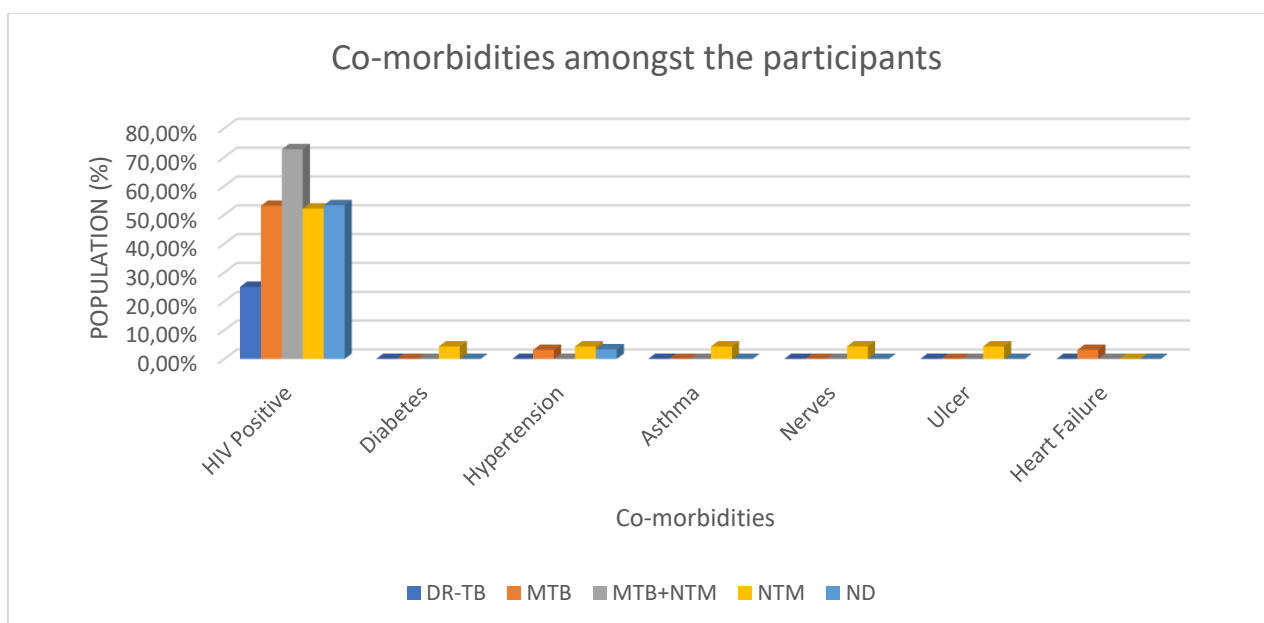


Figure 3.8: Co-morbidities amongst the study participants (N=100)

3.4 DISCUSSION

Tuberculosis (TB) remains a significant health challenge in South Africa, with high prevalence and mortality rates (Maja and Maposa, 2022; Kubjane et al., 2022). Several factors influence TB transmission and treatment outcomes. Demographic factors such as age, gender, and race play a role, with young and older individuals from black and coloured ethnic groups at higher risk (Maja & Maposa, 2022; Dhlakama et al., 2022). Socioeconomic factors, including education level and poverty, contribute to delayed hospital care uptake and poor treatment adherence (Dhlakama et al., 2022; Koomen et al., 2019). Health-related factors like HIV co-infection, smoking, alcohol consumption, and comorbidities increase TB risk (Maja & Maposa, 2022; Dhlakama et al., 2022). Healthcare system challenges, including inadequate infection prevention and control measures, insufficient isolation facilities, and excessive workload among healthcare workers, further complicate TB management (Tan et al., 2020; Sissolak et al., 2011). Cultural beliefs such as stigma and the reliance on traditional medicine also influence TB transmission and treatment outcomes (Matakanye et al., 2021).

Prevalence of drug-resistant TB using molecular diagnostics

The current diagnostic measures may have misdiagnosed individuals with Nontuberculous mycobacteria (NTM), or they may have had a co-infection, and MTB was successfully treated. Findings demonstrated that the GeneXpert assay can misdiagnose some NTM species as MTB at high bacterial load, highlighting important considerations for interpreting GeneXpert results in clinical practice (Pang et al., 2017). Therefore, it is important to improve diagnostic measures as the standard TB therapy may not be effective against NTMs. Notably, delays in establishing a diagnosis are possibly associated with an increase in morbidity and mortality of NTM pulmonary diseases (Ahmed et al., 2020).

Around 200 NTM species have been reported (Shahraki et al., 2015). The majority of non-pathogenic NTM species are found in the environment, except for *Mycobacterium leprae* and *Mycobacterium ulcerans*, which are pathogenic, except for the *Mycobacterium tuberculosis* complex (MTBC) (Chavarro-Portillo et al., 2019). Other species are opportunistic in immunocompromised individuals (Swenson et al., 2018).

Some NTM species mimic TB symptoms. In this current study, 23% were NTM and 14% were co-occurrence with MTB. These cases pose challenges for both diagnosis and TB regimens. A study in Zambia reported NTM prevalence (15,1%) and a marked difference in NTM+MTB (0.2%) co-infections compared to the current study (Chanda-Kapata et al., 2015). Another study in Iran detected NTM in 15.1% of the study population (Nasiri et al., 2018). The two studies are closely related to the current study.

Drug-susceptibility results from the Allplex MDR/XR assay indicated that most isolates were susceptible to all tested drugs. However, drug resistance was also detected (2.13% INH, 2.13 % RIF, 2.13% FQ, 2.13% INH+RIF+FQ). This indicates the presence of multidrug resistance. The detection of multidrug-resistant organisms may be in a small proportion. However, INH-R mono-resistance was detected in the study population. A study by Ranjan et al. (2023) detected 9.6% of INH-M in their study population using an MTBDRplus line probe assay. A study done in the Western Cape, South Africa, demonstrated poor treatment outcomes (16%) in individuals with INH-M and about 61 % progressed to MDR-TB (Karen et al., 2011). A study provides evidence for an association between INH mono-resistance and a lower likelihood of TB treatment success. Increased attention should be paid to the timely detection and management of INH mono-resistant TB (Karo et al., 2019).

Molecular rapid MDR detection methods that can simultaneously identify INH- and RIF-resistance separately will allow diagnosis of RIF and INH monoresistance and MDR-TB, which is crucial, as each type of resistance has different treatment recommendations (WHO, 2023). Currently, the method used in the study population is GeneXpert MTB/RIF, which only tests for RIF resistance. This is due to previous studies that reported RIF and INH as interlinked (most RIF-R individuals are also INH-R). In addition, RIF is considered the primary indicator of MDR-TB. However, INH-M is being detected and may be a global public threat in the future if measures are not taken. WHO recently recommended modifying treatment for individuals who are INH-R but RIF-susceptible, but this decision can only be made with further testing, since GeneXpert reports only RIF (WHO, 2022).

The study highlights that the first-line drugs are effective because the dataset includes MTB strains that are susceptible to them. However, fluoroquinolones are of great concern because they are essential for a second-line TB regimen. Therefore, molecular diagnostic methods used to detect TB in our communities should include

more drugs available to close up future issues that may arise and prevent reaching the SDG goals due to resistance not detected in time or inappropriate treatment.

Sociodemographic patterns

In the present study, males accounted for the *MTB* (65.6%) and *MTB*+NTM (54.5%) group, aligning with global epidemiological patterns of male predominance in TB incidence (Kubjane et al., 2023). Globally, males demonstrate higher TB incidence and mortality rates, with male-to-female incidence ratios ranging from 1.1 to 2.5 across geographic regions (Ledesma et al., 2022; WHO, 2020). (WHO, 2020). Comparable trends were reported, 50.2% males in Ethiopia (Asemahagn and Amsalu, 2018) and in Uganda, reporting 57% of males (Nidoi et al., 2021). Male predominance in TB has been attributed to behavioural exposures such as smoking, occupational risk, delayed consultation at healthcare, and biological susceptibility differences.

Conversely, regional variations remain evident, with some areas reporting results that differ from the literature, showing that females have higher TB incidence rates than males. For example, Brust et al. (2018) reported 64% females in KwaZulu-Natal, South Africa, and Adane et al. (2020) reported 50.6% females in Ethiopia, highlighting regional variability in TB epidemiology. Notwithstanding heterogeneity in study populations and methodological approaches, the reviewed literature consistently demonstrates a higher TB prevalence among males. These variations suggest that local socioeconomic, HIV prevalence, and healthcare access patterns significantly influence gender distribution.

The highest burden of *MTB* occurred among individuals aged 25–44 years (68.8%), and 56.5% of the NTM group comprised the economically productive segment of the population. The 45-60 age group accounted for 45.5% of co-occurrence between *MTB* and NTM. This finding aligns with TB epidemiology across sub-Saharan Africa, where transmission is often concentrated among socially active adults with higher contact rates. From a public health perspective, infections among these demographics carry amplified socioeconomic consequences, including reduced workforce productivity and increased household vulnerability (Daniel et al., 2025). This is observed and supports the current study's findings from a study conducted in Harare, where the age group 25-44 accounted for 64.5% of TB cases (Humayun et al., 2022).

Even though Nationality stands alone isn't a direct factor for TB, it correlates with other determinants such as healthcare access, socioeconomic conditions, and prevalence of co-infections like HIV. The current study's nationality didn't reach significance; however, the migrant group had a higher proportion of *MTB*+NTM co-occurrence (9.1%) than the NTM group (8.7%) in *MTB*-only cases (6.3%). The prevalence of tuberculosis infection reported in Lesotho and South Africa was much higher than in Tanzania, suggesting that the risk of tuberculosis infection is higher in settings with a higher force of infection (Ntshiqqa et al., 2025). Nonetheless, migrant populations remain epidemiologically important in TB control due to documented barriers in healthcare access and treatment continuity (Pareek et al., 2016).

Unemployment and low educational level are significant determinants of TB. They are directly linked to higher infection risk, poor treatment adherence, and limited knowledge of the disease (Kave et al., 2025). Lower educational attainment is associated with limited awareness of disease transmission, whereas unemployment and low-skilled occupations often compel individuals to reside or work in overcrowded settings with inadequate ventilation (Apolinário et al., 2017; Choi et al., 2016). This current study demonstrates a high prevalence of TB among the unemployed, with 66.67% *MTB* and 50% on NTM. The employed group shows a high prevalence (57.14%) of coexistence between *MTB* and NTM.

Environmental conditions

Environmental living conditions play an important role in TB transmission. Factors including overcrowding, lack of access to piped water, and inadequate sanitation are associated with increased TB risk (Kapwata et al., 2024). While occupational silica dust exposure is linked to pulmonary TB, environmental exposure to gold mine tailings showed no significant association (Kootbodien et al., 2019). Historically, forced removals have indirectly affected TB transmission by requiring longer commutes from peri-urban areas to urban workplaces (Richardson et al., 2016). While public transportation plays a crucial role in sustaining TB transmission, minibus taxis pose the highest risk due to poor ventilation and close passenger contact (Andrews et al., 2014). The annual risk of TB infection for daily commuters is estimated at 3.5%-5.0%, highlighting the importance of addressing environmental factors and improving public

transportation conditions to combat TB in South Africa (Hella et al., 2018; Andrews et al., 2014).

Transmission risk is increasingly influenced by behavioural and exposure dynamics rather than housing alone (Srivastava et al., 2015). This conduct might be explained by a lack of awareness and comprehension of the significance of face mask use in reducing the spread of tuberculosis. Additionally, discomfort from wearing a mask may contribute to non-compliance. According to the WHO, it is critical to stress the importance of face masks in stopping the transmission of infectious diseases (Driessche et al., 2021; WHO, 2009). A study by Dharmadhikari et al. (2012) found that surgical masks effectively reduce the transmission of drug-resistant TB. Notably, mask-wearing to prevent tuberculosis transmission met scepticism and low uptake due to stigma, restricted access, discomfort, and perceived liberty deprivation (Howard et al., 2021). This may explain why the study population had a lower proportion of participants who wore masks at all times.

The most highlighted environmental factors are the open space and dusty areas. The study observed a significant association ($p=0.002$) between open space availability and the detection of mycobacterial species, underscoring the critical role that environmental factors play in the epidemiology of mycobacterial infections. Variables categorized under open space around households, including house density and airflow patterns within residential environments, are widely recognized as proxy indicators of ventilation (Azad et al., 2018). The infectious droplet containing *MTB* remains suspended for prolonged periods and increases the probability of transmission among close contacts in areas with limited open space (lack of ventilation). Consequently, individuals residing in densely built environments with minimal outdoor ventilation are more likely to experience a higher exposure risk to DS-TB as well as DR-TB, particularly in communities where there is high TB prevalence. The findings in this study are supported and align with studies done that established that overcrowded housing, poor ventilation, and inadequate environmental design are key drivers to TB transmission in high-burden settings, including sub-Saharan Africa (Escombe et al., 2019; Taylor et al., 2016; Lygizos et al., 2013; Escombe et al., 2007).

However, the observed association cannot be attributed exclusively to *MTB* transmission. Unlike *MTB*, which is primarily transmitted through airborne person-to-person contact, NTM species are ubiquitous environmental organisms commonly found in soil, dust, and water sources (Honda et al., 2018; Herfst et al., 2017). Consequently, the relationship between open space and mycobacterial detection may reflect multiple exposure pathways. Households with greater interaction with outdoor environments may experience increased exposure to environmental mycobacteria, whereas households with limited open space may be more susceptible to *MTB* transmission through crowding and poor ventilation. The near-significant association observed in dusty areas provides some support for the contribution of environmental reservoirs, although the present study's evidence suggests that housing-related factors exert a stronger influence on mycobacterial detection.

Therefore, environmental exposure pathways associated with outdoor spaces, such as soil contact or environmental aerosols, may partly explain the detection of NTM in some individuals within the cohort. This provides an explanation of how dusty areas are almost significant ($p=0.098$). However, limited open space and overcrowding likely exert a stronger influence on *MTB* and DR-TB transmission dynamics than on NTM acquisition in TB-endemic settings. The significance of open space in this study suggests that environmental conditions influence mycobacterial infections in different ways. Limited living space may increase the risk of *MTB* transmission due to crowding and poor ventilation, while exposure to broader environmental sources, such as soil and water, may contribute to NTM infections (Modrá et al., 2019).

An alternative explanation is that open space serves as a proxy for broader socioeconomic and structural determinants of health. Limited residential space is frequently associated with higher household occupancy, poorer housing quality, and reduced access to environmental health resources, all of which have been independently associated with increased TB risk. Therefore, the association observed in this study may reflect the cumulative effects of interconnected environmental and socioeconomic factors rather than the independent effect of open space itself.

The interpretation of this finding is further constrained by the inability to distinguish whether the association was driven predominantly by *MTB*-positive or NTM-positive

participants. Species-stratified analyses were not performed, preventing assessment of the relative contribution of transmission-related and environmental exposure pathways. Consequently, while the findings support the importance of environmental conditions in the epidemiology of mycobacterial infections, the mechanisms underlying this association remain uncertain. Future studies incorporating direct measures of ventilation, household crowding, environmental sampling, and species-specific analyses would provide greater insight into the pathways linking residential environments and mycobacterial exposure.

Cultural factors associated with study participants

In many rural communities, traditional healing practices are often integrated into healthcare-seeking behaviour, especially due to accessibility, cultural beliefs, and trust in indigenous medicine. Research reported a high number of diagnosed patients consulted traditional healers before seeking professional help at healthcare facilities. Mathibela et al. (2015) indicate that approximately 80% of people in Africa rely on traditional medicine for most of their health needs. In this study, about 7% consulted traditional healers, of whom 3% used their medication. A study by Ramaliba et al. (2017) found that 18.92% of TB patients first visited a traditional healer, while 8.3% used self-medication rather than seeking treatment at a clinic or hospital. A study reported that people who have been infected with TB tend to consult a traditional healer before seeking Western medication (Viney et al., 2014). Additionally, a study in Limpopo revealed that even though participants acknowledged that TB is caused by a bacteria and that it can be cured if treated correctly, about 76% of respondents indicated that they still had strong beliefs in their culture and religion, and as a result, they acknowledged visiting traditional healers and faith healers for help even after their TB diagnosis as they still linked their disease to a curse or punishment and did not attribute it to a bacteria (Matakanye et al., 2021).

Medicinal plants are widely used due to their availability, affordability, and cultural significance, especially in rural areas where modern healthcare is limited. Previous studies done in the study site of the current study (Vhembe district, Thohoyandou) showed the availability and effectiveness of different medicinal plants found in the area to treat different illnesses (have anti-inflammatory, anti-bacterial and anti-fungal

properties) (Sigidi et al., 2017; Samie & Mashau, 2013; Samie et al., 2005). Some individuals in the Vhembe district may use medicinal plants as part of their daily diet because they believe it is good for their health, without consulting traditional healers.

For example, *Moringa oleifera* has gained popularity in nutritional and health benefits. Consequently, it has increased consumption in various forms, including herbal tea and a powdered additive incorporated into food preparations (knowledge gained from discussion and self-observation). This observation, along with reports that Moringa is rich in antioxidants, vitamins, and bioactive compounds (Mehwish et al., 2022; Singh and Chaturvedi, 2021), may help boost immunity, which is crucial for TB patients. However, its influence on TB outcomes would depend on further clinical studies to determine whether it has direct antibacterial effects on *MTB* or primarily supports overall health and recovery. Additionally, widespread use of moringa in communities with a high TB burden might encourage a shift towards more natural, nutrition-based supportive therapies alongside conventional TB treatment (Mashamaite et al., 2023).

Medical history, attitude and behavioural factors

Tuberculosis (TB) is still a public health threat in communities. Research highlights that medical history, patient attitudes, and behavioural factors are critical, which often act as barriers to timely diagnosis, treatment adherence, and infection control. The interplay of these factors significantly affects the success of Directly Observed Treatment Short-Course (DOTS) programs, especially in high-burden, low-income, or overcrowded settings. It has been discovered that BCG is likely to prevent *MTB* infection rather than only the progression of infection to TB illness. The durability of the impact, susceptibility to high-dose pathogen infection, and protection against various *MTB* strains are important areas for improving BCG protection against *MTB* infection.

About 100% of *MTB*+NTM co-occurrence cases and 75% of the DR-TB group were vaccinated with BCG. Although the Bacille Calmette–Guérin (BCG) vaccine remains the only licensed vaccine against *MTB*, its protective efficacy is variable, particularly against pulmonary TB in adults and in high-burden endemic settings. Consequently, substantial research efforts have focused on developing new vaccines, including recombinant and subunit vaccines, to address the complications of conventional BCG.

Prior history of tuberculosis is an important risk factor, and patients who have been treated for TB before are more likely to have discontinued therapy or develop MDR-TB (Soedarsono et al., 2023). Individuals who have received previous TB therapy are more prone to develop resistance due to incomplete adherence, inappropriate drug regimens, subtherapeutic concentrations, or treatment failure (WHO, 2023). In this study, participants across all groups had a history of TB in fewer than 30%. Retreatment cases have consistently been shown to harbour higher rates of resistance to first-line agents such as rifampicin and isoniazid compared to newly diagnosed patients, reflecting the selective pressure imposed by prior drug exposure (WHO, 2023; Dookie et al., 2022). Furthermore, prior treatment interruptions have been strongly associated with resistance amplification, particularly in high-burden settings where treatment monitoring may be inconsistent (Seung et al., 2015).

However, in the present study, none of the patients diagnosed with DR-TB reported a prior history of TB infection or treatment. This observation suggests that drug resistance in this cohort is more likely attributable to primary transmission of already DR-TB rather than resistance acquired during previous therapy. The finding is epidemiologically significant, as primary DR-TB reflects ongoing community transmission of resistant strains, indicating potential gaps in infection control, early detection, or contact tracing strategies (WHO, 2023). It underscores the need for strengthened surveillance systems and rapid molecular diagnostics to interrupt transmission chains and prevent further dissemination of resistant TB within the population.

The fear of TB stigma may cause infected individuals to hide their TB status from family members. Additionally, TB stigma creates feelings of shame or guilt, resulting in self-isolation as those infected internalize negative perceptions from their community regarding the illness (Chen et al., 2021). The socioeconomic effects of TB stigma vary between men and women. Overall, men tend to focus more on how TB stigma affects their economic opportunities, such as losing jobs and decreasing income. As a note, in this study, disclosure to friends and colleagues is much lower than that to family. Though TB stigma influences their financial situation, women are generally more worried that it will negatively impact their chances of marriage or lead to rejection from their families. In certain regions, though, men express greater concern than women regarding the impact of TB stigma on their marriage prospects (Kwedi nolna et al.,

2016; Nair et al., 1997). In addition, TB and HIV/AIDS share common physical signs and symptoms, such as weight loss, which also often leads to its association with HIV/AIDS (Huq et al., 2022; Dodor and Kelly, 2009).

Co-morbidities play a significant role in TB outcomes, increasing treatment failure, relapse, or death (Nowiński et al., 2023). The leading co-morbidity is HIV, responsible for one in five HIV-related deaths globally. In the current study, HIV is the leading comorbidity when compared to other diseases. In 2024, an estimated 54,000 people died from TB-related causes in South Africa, with 29,000 of these deaths occurring among people living with HIV. While TB incidence has dropped significantly (61% reduction between 2015 and 2024), mortality has only declined by 17% over the same period, highlighting the persistence of TB-HIV deaths (WHO, 2025).

An important predictor of adverse treatment outcome is diabetes, which is reported as a significant risk factor for TB (Foe-Essomba et al., 2021). Generally, diabetes has been found that the coexistence of diabetes and TB increases the risk of death, treatment failure, and disease relapse (Baker et al., 2011). A study done in the Eastern Cape, South Africa, underscores the importance of implementing integrated management strategies that address both DR-TB and its comorbidities, particularly in resource-limited settings where such patients are prevalent (Hosu et al., 2024).

In conclusion, the study highlights that addressing factors associated with TB in our communities and contributing to the SDG goal requires a comprehensive approach that includes improved diagnostic techniques, patient education, psychosocial support to reduce stigma, community-based care to improve access, and nutritional support.

Chapter 4: OBJECTIVE 2

This chapter describes the reports on objective 2. The narrative outlines the approach from sample collection to laboratory analysis and the results obtained. In summary, the methods cover the evaluation of resistance mutations in bacterial DNA using MassARRAY and NGS.

After completing the initial year of research, we submitted 10 samples for sequencing via MassARRAY; however, only 4 samples that exhibited resistance provided clear outcomes. The same samples were subsequently forwarded to the SAMRC Genomics Lab but failed to pass the quality control (QC) criteria. To resolve this issue, we dispatched an additional 10 samples to the ARC for metagenomic analysis, during which comprehensive sequencing was performed and the outcomes of all measures implemented were highlighted, highlighting point mutations that could play a role in TB.

CHAPTER 4

EVALUATION OF THE GENETIC POLYMORPHISMS IN BACTERIAL DNA FROM TB PATIENTS

4.1 INTRODUCTION

Despite the global concerted efforts to reduce TB-related incidence and morbidity, the reported TB cases are still alarming, and TB remains classified as one of the world's top killers among infectious diseases. In 2018, approximately 10 million people developed TB; 7 million were diagnosed and successfully treated, and 1.5 million people died from this disease. In 2020, an estimated 10 million people fell in, with approximately 8% accounted for by people living with HIV. Globally, the incidence of TB has reduced by 11% (WHO, 2022).

Drug-resistant tuberculosis (DR-TB) remains a major global public health concern, with approximately 500,000 cases of multidrug-resistant or rifampicin-resistant tuberculosis (MDR-TB/RR-TB) reported annually. Despite this substantial burden, only nearly one-third of diagnosed cases receive appropriate treatment (Migliori and Tiberi, 2022). With the increasing incidence of *MDR- and XDR-TB* strains, there is a rise in

demand for novel personalized treatment plans (Dheda et al., 2017). The origin of *M. tuberculosis* resistance is attributed primarily to a single nucleotide polymorphism (SNP) in core genes or promoters; however, the mechanism of resistance has not been well described in 10–40% of strains in the past decade (Farhat et al., 2013; Almeida Da Silva and Palomino, 2011).

SNPs comprise every variant of a single nucleotide in the *MTB* genome as compared to the reference genome (H37Rv), and mutations represent a subset of SNPs that are relevant or functional in terms of resistance. This genetic variation provides insight into the differences and phylogenetic relationships among strains (Stucki and Gagneux, 2013). One of the known reported SNPs that result in drug resistance is on the *katG* gene, codon 315, where the nucleotides normally read as AGC but have changed to ACC, changing the amino acid translation from Serine (Ser) to Threonine (Thr) (Khumwan et al., 2022; Motavaf et al., 2021; Van Doorn et al., 2006).

Early diagnostic measures and correct regimens may reduce the spread and development of drug-resistant TB. This may be achieved by validating molecular techniques and implementing them to replace the gold-standard method, which takes longer to determine strains' phenotypic profiles. A new technique is revolutionizing the genotypic approach to a microorganism's drug-susceptibility profile: whole-genome sequencing (WGS) using Next-Generation Sequencing (Votintseva et al., 2017).

Recently, the WGS has become an increasingly preferred molecular-genetic diagnostic method for diagnosing TB and resistant strains. Clinical laboratories are currently validating this technique for implementation in routine practice for the diagnosis and management of TB (Meehan et al., 2019; Cirillo et al., 2017). In addition, WGS has been reported by researchers as a tool that provides insight into the anti-TB drugs (first- and second-line) when compared to other genotypic methods such as the GeneXpert MTB/RIF (Cepheid, Inc., Sunnyvale, CA, USA) and phenotypic tests (WHO, 2019; Witney et al., 2016). Analysis of sequenced data can also be used to evaluate molecular epidemiology, including transmission, clustering, and taxonomy of *MTB* strains. Several studies confirmed the effectiveness of WGS, demonstrating its ability to determine resistance with high sensitivity, leading to more appropriate selection of anti-TB drug regimens and higher treatment efficacy (Chatterjee et al., 2017; Arnold et al., 2016; Nikolayevskyy et al., 2016).

4.1.1 THE ACQUIRED RESISTANCE BY BACTERIAL DNA FROM TB PATIENTS (CAUCASIAN VS ASIAN VS AFRICANS)

Acquired drug resistance in *MTB* refers to the development of resistance to anti-TB drugs, which is a growing global concern, and it varies across different populations, including Caucasians, Asians, and Africans (Chisompola et al., 2020). The development of drug resistance occurs during the treatment period. Ethnic and geographic differences influence the patterns of resistance, which may be driven by various factors such as treatment regimens, healthcare infrastructure, and genetic susceptibility (Correa-Macebo et al., 2019; Hayward et al., 2019).

Resistance can develop due to inadequate treatment regimens, inconsistent drug use, poor adherence, or insufficient drug concentration at the infection site (Alara and Alara, 2024).

Factors Contributing to Ethnic Differences in Resistance

Differences in the prevalence and distribution of DR-TB observed across global populations are primarily attributable to variations in healthcare systems, treatment practices, disease burden, socio-economic conditions, and epidemiological factors rather than ethnicity itself. While population-specific genetic factors may influence susceptibility to TB infection and disease progression, current evidence suggests that the emergence and transmission of drug-resistant strains are predominantly driven by treatment-related and public health factors.

a. Healthcare Access and Treatment Adherence

Access to timely diagnosis, effective treatment, and patient support services plays a critical role in preventing the emergence of drug resistance. Countries with well-resourced healthcare systems generally demonstrate lower rates of acquired resistance due to improved treatment monitoring, uninterrupted drug supply chains, and stronger patient follow-up mechanisms. In contrast, regions with high TB burdens often face challenges related to delayed diagnosis, treatment interruption, inadequate

healthcare infrastructure, and limited access to drug susceptibility testing, all of which contribute to the development and transmission of DR-TB (WHO, 2024).

The high burden of MDR-TB reported in countries such as India, China, and parts of Eastern Europe has largely been attributed to historical treatment practices, health system constraints, and ongoing transmission of resistant strains rather than population-specific biological characteristics (Matteelli et al., 2025; Tan et al., 2025).

In sub-Saharan Africa, the TB epidemic is further complicated by the high prevalence of HIV co-infection, which increases susceptibility to active TB disease and can adversely affect treatment outcomes. Additional challenges, including limited healthcare access, delayed diagnosis, and inconsistent treatment adherence, may further contribute to the emergence and spread of drug-resistant strains.

b. Host, pathogen and environmental factors

Host genetic factors may influence susceptibility to TB infection, disease severity, and immune response; however, their contribution to the development of drug resistance remains less clearly defined. Current evidence suggests that genetic variation among human populations may affect host-pathogen interactions, but these effects are likely secondary to healthcare and treatment-related determinants of resistance (Schurz et al., 2024).

Recent genomic studies have demonstrated that interactions between human hosts and *MTB* lineages may differ across geographical regions due to evolutionary adaptation and pathogen lineage diversity. These interactions may influence disease presentation, transmission dynamics, and treatment outcomes, although their direct role in driving drug resistance remains incompletely understood (Naidoo et al., 2023).

Environmental and socio-economic factors also play important roles in shaping DR-TB epidemiology. Poverty, malnutrition, overcrowding, poor housing conditions, and co-existing infectious diseases increase TB transmission and may indirectly contribute to the spread of resistant strains. Such factors are particularly important in high-burden settings where ongoing community transmission sustains the circulation of MDR-TB and XDR-TB strains (Li et al., 2025; WHO, 2024).

c. Resistance Profiles

The prevalence of MDR-TB and RR-TB remains a major global public health challenge. According to the WHO, approximately 3.2% of new TB cases and 16% of previously treated TB cases are affected by MDR/RR-TB, highlighting the strong association between previous treatment exposure and the development of resistance (WHO, 2024).

Variations in DR-TB prevalence across regions are largely explained by differences in TB burden, healthcare infrastructure, treatment practices, HIV prevalence, and the effectiveness of TB control programs. Regions with high TB incidence and substantial healthcare challenges generally report higher rates of MDR-TB and XDR-TB. Conversely, lower-burden settings often report fewer cases, although resistant TB remains a concern due to migration, ongoing transmission, and imported infections.

Overall, the available evidence indicates that differences in DR-TB prevalence are better explained by healthcare, epidemiological, and socio-economic factors than by ethnicity. Therefore, interventions aimed at improving treatment adherence, strengthening healthcare systems, expanding access to rapid diagnostics, and reducing TB transmission remain the most effective strategies for controlling drug-resistant tuberculosis globally

4.2 MATERIALS AND METHODS

4.2.1. SAMPLE COLLECTION AND PRE-TREATMENT OF SAMPLES

A total of 10 sputum samples were gathered from TB-positive patients aged 18 and older. Clear instructions were provided to patients during specimen collection. They were required to collect their samples outdoors in a private area, away from others, and were prohibited from using confined spaces such as toilets. Before collecting sputum, patients rinsed their mouths with water without brushing their teeth. They were instructed to cough forcefully to expectorate sputum into the container. Each patient provided a sputum sample of 3-5 mL into a 100 mL collection bottle containing 5 mL of NaOH (Sodium hydroxide, Sigma, St. Louis, MI, USA) for decontamination. The collected samples were stored in a cooler box with an ice pack (maintaining a temperature of 2-8 °C) and transported to the Microbiology Laboratory at the University of Venda for further analysis.

The samples were incubated at room temperature for 15 minutes before undergoing pre-treatment. Subsequently, 1.5 mL of each sample was transferred to a new sterile tube and centrifuged using a Thermo Scientific Heraeus Pico 17 Benchtop centrifuge (Waltham, MA, USA) at 15,000 x g (13,000 rpm) for 5 minutes. The supernatant was discarded, and 1 mL of 1X PBS (Sigma) was added, then thoroughly mixed. The samples were centrifuged again at 15,000 x g (13,000 rpm) for 5 minutes, after which the supernatant was removed with a pipette. This step was repeated, adding another 1 mL of 1X PBS solution, mixing well, and then centrifuging at the same speed for 5 minutes, with the supernatant discarded once more. Finally, the resulting pellet was stored at -20°C for further analysis.

4.2.2. DNA EXTRACTION

The Allplex™ MTB/XDRe kit (Seegene, Seoul, South Korea) was utilized for DNA extraction according to the manufacturer's instructions. Prior to extraction, the prepared sediments were removed from -20°C and allowed to thaw. The sediments were then mixed with 1 mL of sterile water and centrifuged at 15,000 x g (13,000 rpm) for 5 minutes, after which the supernatant was discarded using a pipette. Before adding 100 µL of the DNA Extraction Solution provided in the kit, 10 µL of the MTB/DR internal control (IC), which confirms the DNA extraction procedure and identifies PCR inhibition, was added and vortexed for 30 seconds. The tubes were sealed with a cap-lock and boiled for 20 minutes on a heat block. Following this, the samples were centrifuged again at 15,000 x g (13,000 rpm) for 5 minutes. Finally, 5 µL of the supernatant was utilized as the PCR (polymerase chain reaction) template.

The target sequences of *Mycobacterium tuberculosis* included seven mutations associated with fluoroquinolone (FQ) resistance [*gyrA* A90V(GTG), S91P(CCG), D94A(GCC), D94G(GGC), D94H(CAC), D94N(AAC), D94Y(TAC)] and six mutations related to injectable drug resistance [*rrs* 1401(G), 1402(T), 1484(T), *eis* promoter -37(T), -14(T), -10(A)] (Seegene, Seoul, South Korea).

4.2.3. GENOTYPING OF POLYMORPHISMS USING MassARRAY ASSAY

The protocol outlined by Yang et al. (2023) was adhered to for this study. In summary, MTB and the locations of MTB resistance gene mutations were identified using

MassArray technology. Table 4.1 presents the anti-tuberculosis medications along with the corresponding gene resistance loci. Two PCR reaction systems were operated simultaneously. Each reaction included HPLC H₂O (0.8 µL), 10X PCR buffer (0.5 µL of 20 mM MgCl₂ and 0.4 µL of 25 mM MgCl₂), 25 mM dNTP Mix (0.1 µL), 0.5 µM Primer Mix (1 µL), PCR Enzyme (0.2 µL), and 2 µL of template DNA, resulting in a final volume of 5 µL. The reaction program consisted of an initial step at 72°C for 5 minutes, followed by a hold at 4°C; then 95°C for 30 seconds; 60°C for 30 seconds; 72°C for 60 seconds, and repeated as necessary.

Post-amplification cleanup was achieved by adding 2 µL shrimp alkaline phosphatase (SAP) per well to remove residual deoxynucleotides. The incubation of plates was 40 min at 37°C for 40 minutes, then the enzyme inactivation at 85°C for 5 minutes before being held at 4°C. For allelic discrimination, 2 µL of iPLEX extension mixture was added to each reaction. The extension mix consisted of 0.62 µL nanopure water, 0.2 µL iPLEX buffer, 0.2 µL iPLEX termination mix, 0.94 µL extension primer mix, and 0.04 µL iPLEX enzyme (Agena Bioscience, San Diego, CA, USA). Extension cycling parameters included an initial denaturation at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and five internal cycles of 52°C for 5 seconds and 80°C for 5 seconds. The final extension step was performed at 72°C for 3 minutes, after which the reactions were maintained at 4°C. Table 4.1 summarizes the anti-TB agents and their corresponding resistance loci.

The MassARRAY analysis was performed at Inqaba Biotechnical Industries (Pty) Ltd using the Agena Bioscience MassARRAY platform. Genotype calls were generated and analysed using Typer 4.0 and Plate Manager 1.0 software. The software assigns confidence levels to each genotype call based on peak intensity, signal-to-noise ratio, clustering patterns, and the quality of the mass spectra obtained from matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

The quality classifications were interpreted as follows:

- **No-Alleles:** No reliable allele call could be assigned due to the absence of detectable extension-product peaks or poor-quality spectra.
- **Low Probability:** A genotype call was generated, but the confidence level was below the software's recommended threshold owing to weak signal intensity, overlapping peaks, or ambiguous clustering.

- **Aggressive:** A genotype call assigned under less stringent calling criteria, allowing the software to make calls from lower-quality spectra, thereby increasing call rates but with a potentially higher risk of misclassification.
- **Moderate:** A genotype call assigned using intermediate stringency criteria, providing a balance between call rate and genotype accuracy.
- **Conservative:** A high-confidence genotype call assigned under stringent quality-control criteria, requiring strong peak resolution, clear cluster separation, and high-quality spectra, thereby minimizing the likelihood of incorrect allele assignment.

Only genotype calls meeting the predefined quality-control criteria established by the MassARRAY analysis software and laboratory protocols were considered for downstream analyses.

Table 4.1: Description of MassARRAY-targeted gene detection sites and associated genetic markers (adapted from Yang et al., 2023)

Drug/ Identification	Resistance Locus	Single-base extension primer	Wild type	Mutation
RIF	<i>rpoB</i> _516 761 110	Reverse-ACGTTGGATGCGATCAAGGAGTTCTTCGGC	A	G/T
	<i>rpoB</i> _531 761 115	Forward-ACGTTGGATGCGATCAAGGAGTTCTTCGGC	C	T/G
	<i>katG</i> _315 2 155 168	Forward-ACGTTGGATGGGATCTCGAGGAACTGTTG	G	C/A
	<i>katG</i> _315 2 155 169	Reverse-ACGTTGGATGTGGAAGAGCTCGTATGGCAC	C	A
INH	<i>inhA</i> -15C 1 674 481	Forward-ACGTTGGATGCTCGTGGACATACCGATTTC Reverse-ACGTTGGATGACTGAACGGGATACGAATGG	C	T
	<i>embB</i> _306 4 247 429	Forward-ACGTTGGATGATATTCGGCTTCCTGCTCTG	A	G/C

FQ	<i>gyrA</i> _94 7 581	Forward-ACGTTGGATGGAGCCGAAGTTGCCCTGG	G	A/C/T
	<i>gyrA</i> _94 7 582	Reverse-ACGTTGGATGATGCAATGTTTCGATTCCGGC	A	G/C
	<i>gyrB</i> _499 6 620	Forward-ACGTTGGATGCGACCGCTTTTGCAGAACC Reverse-ACGTTGGATGCGTAAGGCACGAGAGTTGGT	C	A
SM	<i>rpsL</i> _43 781 687	Forward-ACGTTGGATGAAGGGTCGTCGGGACAAGAT Reverse-ACGTTGGATGAAGGGTCGTCGGGACAAGAT	A	G/T
	<i>rrs</i> 1 472 359	Forward-ACGTTGGATGCGGATTGACGGTAGGTGGAG	A	C
	<i>rrs</i> 1 473 167	Forward-TTCGGATCGGGGTCTGCAACTCGACCCCGT	T	C

RIF: Rifampicin; INH:Isoniazid; EMB:Ethambutol,FQ:Fluoroquinolone; SM:Streptomycin

Data analysis was performed using Typer 4.0 and Plate Manager 1.0 software, with test results classified as having no alleles, low probability, aggressive, moderate, or conservative quality.

4.2.4. METAGENOMICS

Whole-genome sequencing (WGS) was initially selected as the primary genomic approach to characterize *MTB* directly from sputum samples and to identify potential genetic determinants associated with drug resistance and treatment outcomes. Genomic DNA was extracted from the clinical specimens using standardized nucleic acid extraction protocols designed to optimize the recovery of mycobacterial DNA from sputum samples. The extracted DNA was quantified using fluorometric methods and evaluated for purity and integrity prior to library preparation. Sequencing libraries were prepared according to the manufacturer's protocol and subjected to quality

assessment to ensure that the DNA concentration, fragment size distribution, and library yield met the thresholds required for high-throughput sequencing.

Despite these preparatory steps, the samples did not meet the minimum quality-control (QC) criteria for reliable WGS analysis. Several libraries exhibited suboptimal DNA concentrations, uneven fragment distributions, and reduced library complexity. Preliminary sequencing metrics further revealed insufficient read depth and limited genome coverage for *MTB*. In addition, a substantial portion of the sequencing reads was found to be human DNA from the host, which considerably decreased the percentage of reads specific to the infection. Collectively, these elements produced inadequate genomic assemblies and hindered the generation of high-quality whole-genome sequences suitable for subsequent investigations, such as resistance gene profiling, phylogenetic reconstruction, and variant calling.

One recognized drawback of performing WGS directly from clinical samples is the inability to achieve acceptable sequencing quality metrics, especially in cases with low bacterial load or significant quantities of contaminating host DNA. In sputum samples, the abundance of human DNA and other microbial flora can substantially dilute pathogen-derived nucleic acids, thereby reducing sequencing efficiency and genome coverage. Additionally, the samples were stored and transported to Cape Town; nucleic acid degradation during storage and handling may have occurred, further compromising sequencing performance. The generated sequencing data did not meet the minimum coverage and depth standards required for reliable genomic analysis due to these limitations.

The collaborating laboratory recommended culturing the samples to improve DNA quality, as the current facility lacked access to a Biosafety Level 3 (BSL-3) laboratory required for the safe handling of *MTB*; a different method was therefore opted for. To address these limitations and improve the detection of microbial genetic material, a metagenomic next-generation sequencing (mNGS) approach was subsequently implemented. Metagenomic sequencing enables the unbiased analysis of all nucleic acids in a sample, allowing simultaneous detection of multiple microbial species without prior culture or targeted amplification. This strategy is particularly advantageous in complex clinical matrices where pathogen DNA may be present at low abundance relative to host DNA.

Using a metagenomic sequencing approach enabled the detection of a broader range of microbial genomic fragments in the samples. The identification of MTB sequences and other microbial species that may have been present in the clinical specimens was made possible by bioinformatic analysis of metagenomic data. In addition to providing more information about the microbial makeup of the specimens and any co-infections, this method increased the likelihood of retrieving useful genomic data from samples that did not meet WGS quality requirements. Consequently, metagenomic sequencing was used as an alternative strategy to support downstream genomic and microbiological analyses within this study.

Sample processing and sequencing

A total of 10 DNA samples were used in this section, considering the associated analysis costs. The extracted DNA samples were sequenced using paired-end shotgun metagenomic sequencing on the MGI G400 platform. The raw sequencing reads were subjected to comprehensive quality control using FastP v0.20.0, including Q20 quality filtering, adapter trimming, removal of poly-G tails, and a minimum read length of 50 bp. This process generated an average of 28.9 million high-quality reads per sample (ranging from 4.7 million to 130.5 million reads), providing sufficient sequencing depth for thorough microbial profiling.

Bioinformatics analysis pipeline

- **Quality control and read trimming**

Raw sequencing reads were subjected to comprehensive quality assessment using FastQC (Andrews, 2010) to evaluate base quality scores, GC content, sequence length distribution, and adapter contamination. Trimming was performed using Trimmomatic (Bolger et al., 2014) with the following parameters: adapter removal, sliding-window trimming (4:20), leading and trailing quality thresholds of 20, and a minimum read length of 50 bp. Quality-trimmed reads were re-evaluated using FastQC to confirm successful processing.

- **Taxonomic classification**

Taxonomic classification was performed using Kraken2 v2.0.8 with four specialized reference databases: the standard RefSeq database, a bacteria-specific database, a fungal database, and a viral database to maximize microbial detection. Species-level abundance estimation was further refined using Bracken with a 150-bp read length, enabling a quantitative assessment of microbial community composition.

4.2.5. STATISTICAL ANALYSIS

Descriptive statistics were calculated for sequencing depth, read classification rates, and taxonomic abundances. Abundance calculations were performed both as a percentage of total reads and as a percentage of bacterial reads to provide an appropriate context for interpretation. All analyses were performed in Python 3.8 using pandas, NumPy, and matplotlib libraries.

4.3 RESULTS

This section presents the findings generated from the molecular and genomic analyses conducted in this study. The results are organised thematically to align with study objective 2: using molecular diagnostics (MassARRAY and Metagenomic sequencing) to highlight their importance in TB research and their contribution to identifying resistance-associated mutations associated with drug-resistant TB. Where appropriate, statistical analyses are provided to support the observed patterns. Interpretation of these findings is reserved for the subsequent discussion section.

4.3.1. IDENTIFICATION OF SNPs BY MassARRAY SEQUENCING

The section reports on 4 samples, found to have drug resistance with Allplex™ (Seegene, Korea), reported in Chapter 3 and these positive samples were sent to Inqaba Biotec for further analyses. The MassARRAY genotyping identified several resistance-associated mutations across first-line and selected second-line anti-tuberculosis drug targets, as presented in Table 4.2. The most frequently detected mutation occurred in the *inhA* promoter region (–15C>T), observed in 37.5% of successfully genotyped wells, suggesting a predominance of low-level isoniazid resistance within the analysed cohort. In contrast, no mutations were detected at the

*katG*_315 locus, indicating the absence of high-level isoniazid resistance among samples with valid genotype calls.

A mutation within the *rpoB*_516 locus was identified in 25.0% of wells, providing molecular evidence suggestive of rifampicin resistance. Given the established role of *rpoB* mutations within the rifampicin resistance-determining region (RRDR), this finding raises concern about the potential for multidrug-resistant tuberculosis (MDR-TB), particularly when considered alongside concurrent detection of *inhA* promoter mutations. However, incomplete locus coverage across the RRDR necessitates cautious interpretation, as additional resistance-conferring mutations may not have been captured by the assay.

Similarly, a resistance-associated mutation in *rpsL*_43 was detected in 25.0% of wells, suggesting streptomycin resistance in a subset of samples. No mutations were observed in the *rrs* loci, suggesting that the successfully genotyped specimens retained aminoglycoside susceptibility. Although an allele was detected at the *pncA* locus, the extensive genetic diversity associated with pyrazinamide resistance and the limited mutation coverage of targeted assays preclude definitive phenotypic inference.

Table 4.2: Frequency and Distribution of Mutations Associated with Drug-Resistant Tuberculosis

DRUG	GENE/LOCUS	OBSERVED ALLES	INTERPRETATION	NUMBER OF WELLS	FREQUENCY
RIF	<i>rpoB</i> _516 (761110)	T	DR-associated	2	50%
	<i>rpoB</i> 761 115	-	No call	0	0%
INH	<i>inhA</i> -15 promoter (1674481)	A	DR-associated	3	75%
INH	<i>katG</i> _315 (215516)	T	DR-associated	2	50%
SM	<i>rpsL</i> _43 (781687)	G	Wild-type	2	50%
AM	<i>rrs</i> (1472359)	T	Wild-type	2	50%
	<i>rrs</i> (1473316)	A	Wild-type	3	75%
	<i>pncA</i> _2288964	T	Wildtype	2	50%
EMB	<i>embB</i> _	-	No call	0	0%

The MassARRAY platform successfully detected several SNPs associated with the DR-TB regimen among *MTB* samples in this current study. These SNPs were identified within genes known to confer resistance to both 1st- and 2nd-line TB drugs. These findings provide important molecular insights into the genetic basis of resistance and circulating strains within the study population. Table 4.3 presents the distribution of SNPs identified per sample, highlighting the specific mutations detected in resistance-associated genes implicated in the TB treatment regimen.

Table 4.3: Distribution of Genomic Mutations Identified per *Mycobacterium tuberculosis* Sample

Analysed SNP	Ref allele	Alternative Allele	Sample 1	Sample 2	Sample 3	Sample 4
Emb_4247429	A	G/C	-	-	-	-
inhA_1674481	C	T	A	-	T	A
Kat_G_2155168	G	A/C	G	G	-	-
pncA_2288964	T	C	T	T	-	-
rpoB_7761110	A	G/T	T	T	-	-
rpoB_761155	C	T/G	-	-	-	-
RpsI_781687	A	G	T	T	-	-
Rrs_1472359	A	G	T	T		
Rrs_1473167	C	T/A	A	A	-	A

4.3.2 DETECTION OF SPECIES USING METAGENOMICS

Due to budget constraints, we have limited our sequencing analysis to 10 samples. This decision was made to ensure cost-effectiveness while still obtaining valuable data for our study.

Shotgun metagenomic sequencing successfully detected MTB in all 10 sputum samples, achieving 100% sensitivity within the study cohort. Across all samples, a total of 22,574 TB-specific reads were identified, with an average of 2,257 reads per sample (range: 1,004-8,180). These findings demonstrate the robust capability of metagenomic approaches for TB detection directly from respiratory specimens. Figure 4.1 illustrates the TB detection summary and read distribution.

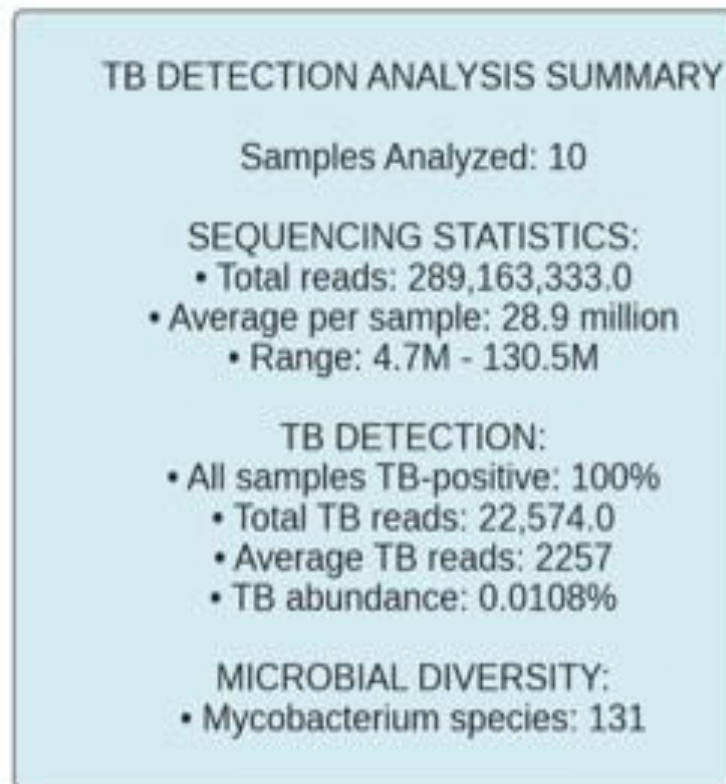


Figure 4.1: *Mycobacterium tuberculosis* detection analysis

All samples achieved TB detection (100% positivity rate) with sequencing depth ranging from 4.7M to 130.5M reads (average: 28.9M). Total TB reads across all samples: 22,574 (average: 2,257/sample).

The metagenomic sequencing data demonstrated the characteristic compositional complexity of respiratory specimens, with host-derived DNA accounting for 74.6–89.0% of total reads. This predominance of human nucleic acid is a well-recognized feature of sputum- and bronchoalveolar lavage-derived metagenomic libraries and represents a major technical constraint in culture-independent pathogen detection. The overwhelming abundance of host DNA substantially reduces sequencing efficiency by limiting the proportion of reads available for microbial characterization, thereby increasing the sequencing depth and associated costs required to achieve adequate pathogen coverage. Consequently, bacterial sequences accounted for only 9.4–23.9% of the total sequencing output in the present study, underscoring the low microbial signal typically encountered in respiratory metagenomics. These findings highlight the importance of pre-analytical optimization strategies, including host DNA depletion, selective microbial enrichment, and targeted capture approaches, to

improve pathogen recovery and enhance the diagnostic performance of metagenomic workflows in pulmonary tuberculosis and other respiratory infections (Chiu & Miller, 2019; Gu et al., 2019; Votintseva et al., 2017).

Within the bacterial fraction, *MTB* abundance ranged from 0.05% to 0.33% of bacterial reads, corresponding to only 0.01–0.27% of total sequencing output. The extremely low proportion of mycobacterial DNA relative to host and commensal microbial nucleic acids underscores one of the principal analytical challenges of direct metagenomic sequencing of respiratory specimens. Nevertheless, *MTB* was successfully identified in all samples, indicating that untargeted sequencing approaches can achieve reliable pathogen detection even when target DNA constitutes only a minute fraction of the total nucleic acid pool. This finding is particularly relevant in tuberculosis diagnostics, where low bacillary burden is frequently observed in patients with paucibacillary disease, early infection, HIV co-infection, extrapulmonary tuberculosis, and during treatment monitoring. The ability to detect *MTB* under such conditions suggests that metagenomic sequencing may offer advantages over conventional diagnostic approaches that rely on higher organism loads for optimal performance. The figure below illustrates the TB read counts vs. Total Sequencing Depth.

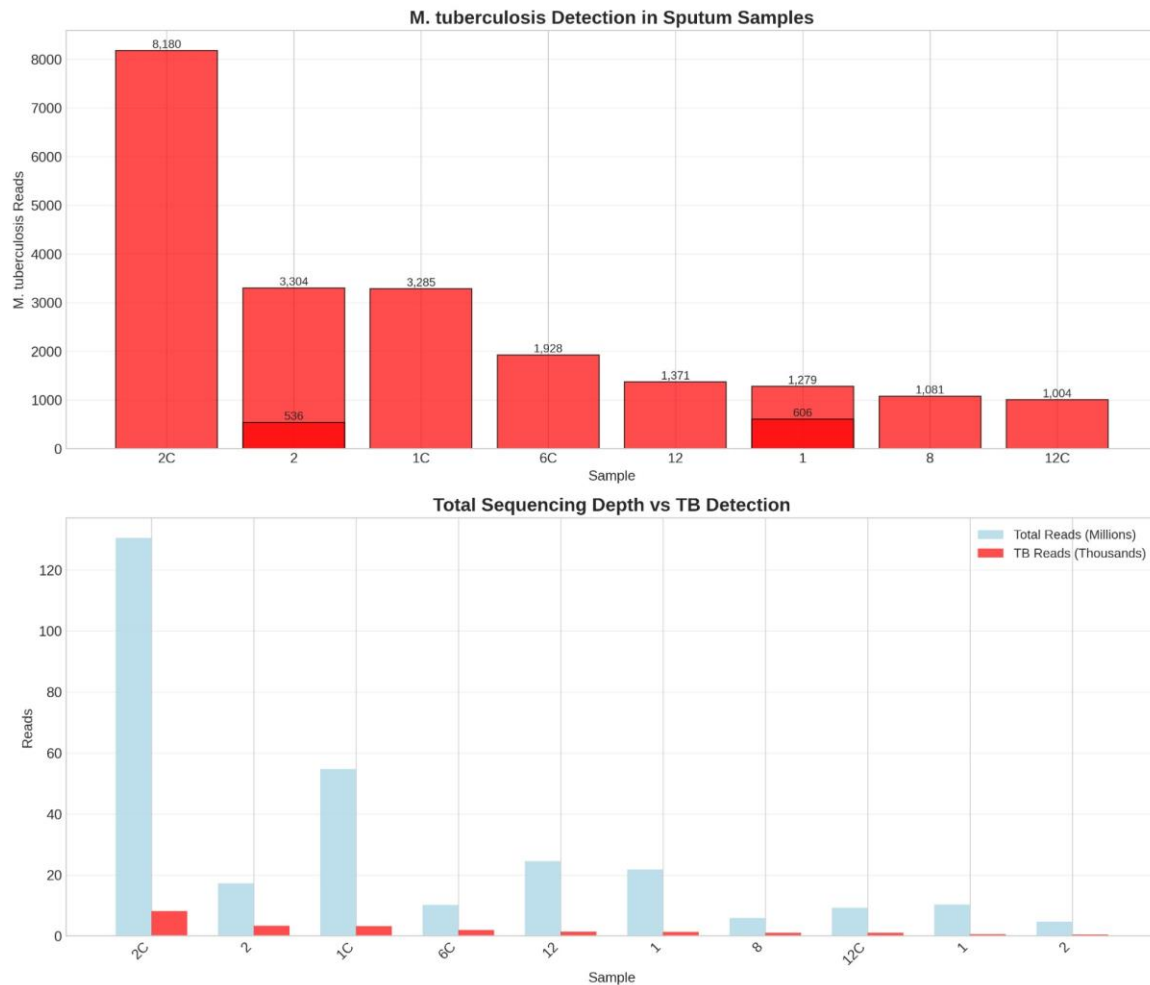


Figure 4.2: TB Read counts vs. Total Sequencing Depth

Sample 2C showed the highest TB read count (8,180 reads) while sample 12C had the lowest (1,004 reads). The lower panel illustrates the stark disparity between total sequencing depth (blue bars, millions) versus TB-specific reads (red bars, thousands), reflecting the challenge of pathogen detection within host DNA background.

Individual sample analysis revealed varying TB read recovery rates. Despite this variability, all samples exceeded the detection threshold, demonstrating robust performance across different sample qualities. The correlation between total sequencing depth and TB read recovery ($R^2 = 0.31$) suggests that factors beyond simple sequencing depth influence TB detection sensitivity, including bacterial load, sample quality, and host cell content. Figure 4.3 illustrates the abundance of TB relative to bacterial reads.

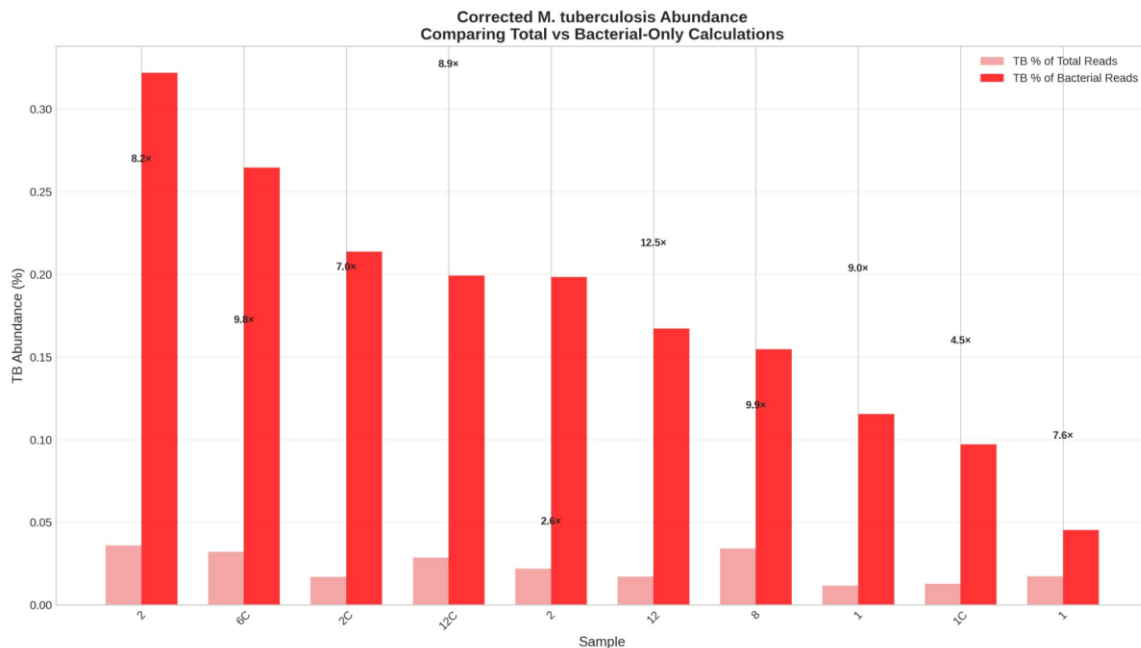


Figure 4.3: TB abundance relative to bacterial reads

When calculating TB abundance relative to bacterial reads only (excluding human host DNA), percentages increased substantially: 0.05-0.33%. This demonstrates the importance of reporting context; TB comprises ~0.01% of total reads but ~0.1-0.3% of bacterial communities

The metagenomic analysis in this current study identified 131 distinct *Mycobacterium* species across all samples, demonstrating the extensive diversity of mycobacteria present in respiratory specimens. This comprehensive characterization provides unprecedented insight into the mycobacterial community composition of TB patient sputum samples. The most frequently detected species included *MTB* (present in 100% of samples), along with various NTMs, commonly found in environmental and human-associated microbiomes.

In addition to *MTB*, we detected other members of the MTBC in some samples. *M. canettii*, an environmental species closely related to *M. tuberculosis*, was identified in multiple samples. *M. africanum*, a member of the TB complex causing disease primarily in West Africa, was also detected. These findings demonstrate the ability of metagenomic sequencing to resolve closely related mycobacterial species.

The analysis also revealed diverse NTM species, including rapid-growing mycobacteria (*M. abscessus*, *M. fortuitum*) and slow-growing species (*M. avium* complex, *M. gordonae*, *M. kansasii*). Some species, such as *M. smegmatis* and *M.*

phocaicum, are commonly found in environmental sources and are likely components of the normal respiratory microbiome or reflect environmental exposure. This comprehensive profiling demonstrates the capability of metagenomic approaches to simultaneously detect pathogenic and commensal mycobacteria. Figure 4.4 is a heatmap illustrating the distribution of *Mycobacterium* species across the 10 sputum samples.

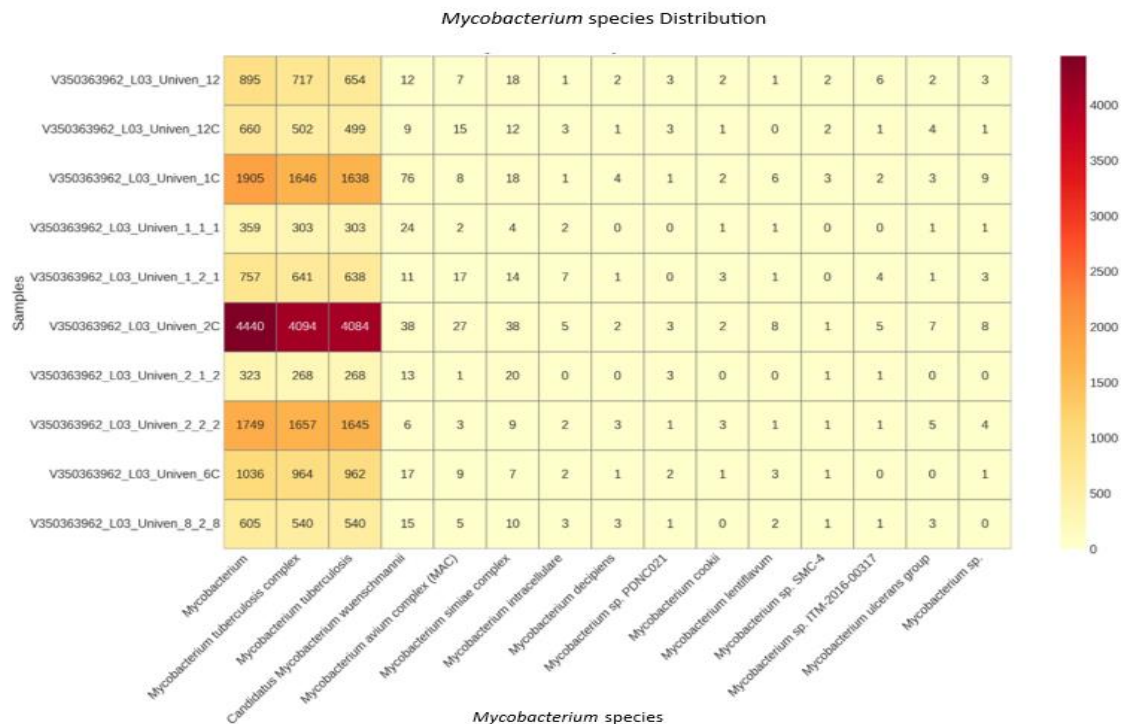


Figure 4.4: Distribution of *Mycobacterium* species

Heatmap showing detection of 131 *Mycobacterium* species across 10 samples. *M. tuberculosis* was detected in all samples (100%), alongside diverse non-tuberculous mycobacteria, including *M. canettii*, *M. africanum*, *M. abscessus*, *M. avium* complex, and environmental species

4.3.3 BACTERIAL COINFECTION (CO-OCCURRENCE OF RESPIRATORY PATHOGENS)

Metagenomic analysis successfully identified multiple clinically relevant respiratory pathogens co-occurring with *M. tuberculosis* from 7 (70%) samples. Figure 4.5 shows the bacterial co-occurrence of respiratory pathogens. The most commonly detected bacterial pathogens included:

- ***Streptococcus pneumoniae*** (detected in 3 samples): A leading cause of community-acquired pneumonia, detected in multiple samples with varying abundance
- ***Staphylococcus aureus*** (detected in 6 samples): Present in several samples, relevant for polymicrobial respiratory infections
- ***Haemophilus influenzae*** (detected in 1 sample): Detected across multiple samples, a known respiratory pathogen.

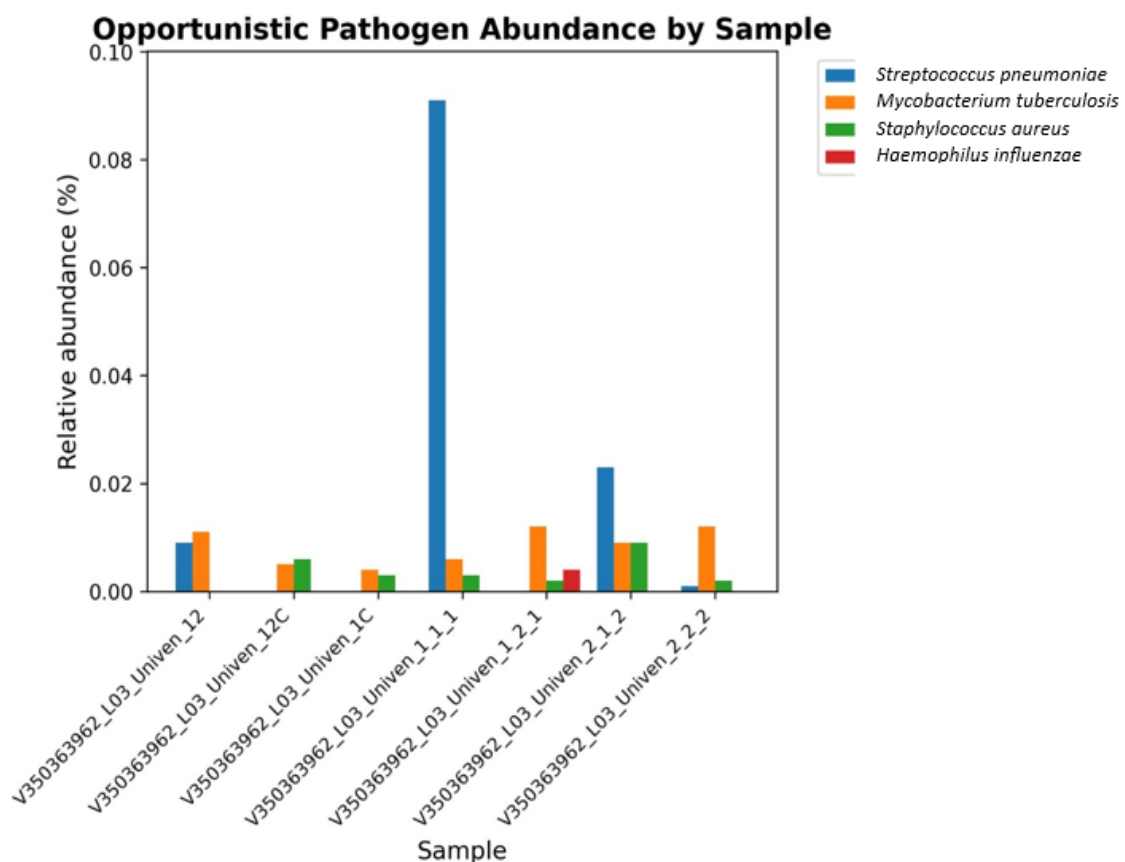


Figure 4.5: Co-occurring respiratory pathogens

The bar graph shows detection of clinically relevant respiratory pathogens, including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Klebsiella pneumoniae*, across TB-positive samples, indicating polymicrobial infection patterns.

4.4 DISCUSSION

This discussion is divided into categories based on the molecular diagnostic method reported in the results section.

MassARRAY

The present study demonstrates that the MassARRAY platform is a valuable molecular tool for the rapid identification of clinically significant drug resistance-associated mutations in MTB. The technology combines multiplex polymerase chain reaction (PCR) amplification with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), enabling simultaneous interrogation of multiple resistance-associated loci within a single assay (Su et al., 2017). This high level of multiplexing represents one of the principal advantages of MassARRAY over conventional molecular assays, as it allows concurrent detection of mutations associated with resistance to several first- and second-line anti-tuberculosis drugs while maintaining high analytical throughput.

An important strength of the present study is its rapid turnaround time for mutation detection (Table 4.2 and 4.3). Unlike conventional phenotypic DST, which requires culturing the slow-growing MTB organism and may take several weeks to obtain resistance profiles, MassARRAY provides molecular results within a considerably shorter timeframe. Prompt identification of resistance-associated mutations supports the early initiation of appropriate anti-tuberculosis therapy, thereby reducing delays in effective treatment, limiting the transmission of DR-TB, and improving clinical outcomes. The clinical value of rapid molecular diagnostics is particularly evident in South Africa, where the sustained burden of MDR-TB continues to undermine tuberculosis control and prevention strategies.

The predominance of *inhA* promoter mutations, detected in 50% of the analyzed resistant samples, suggests that this resistance mechanism may contribute substantially to isoniazid resistance in the study population and may be associated with low-level isoniazid resistance. Mutations in the *inhA* regulatory region promote overexpression of the InhA enzyme, thereby diminishing the bactericidal activity of isoniazid and frequently resulting in cross-resistance to ethionamide (Ushtanit et al., 2022). From a clinical perspective, this resistance pattern is particularly significant because low-level resistance may remain undetected by conventional phenotypic drug susceptibility testing at standard critical concentrations, potentially compromising treatment effectiveness if molecular findings are not integrated into therapeutic decision-making. Pitso et al. (2019) conducted a study in South Africa (Free State

province) that detected lower levels compared to the current study; 13.4% of the *inhA*-only mutation and 22.7% of the *katG* + *inhA* mutation, reviewing LPA tests.

The concurrent presence of *rpoB* and *inhA* mutations raises concern for the emergence of multidrug resistance within the study population. Research has recognised rifampicin resistance as a proxy marker for multidrug-resistant tuberculosis (MDR-TB), as it often co-occurs with INH-R. Studies have demonstrated that detection of a mutation in the *rpoB* gene, specifically within the rifampicin resistance-determining region (RRDR), is indicative of probable rifampicin resistance. For example, a study conducted in Zambia found that about 90% of the isolates harboured mutations in both *rpoB* and *katG*, with a common mutation at *rpoB*_531 and *katG*_315 (Solo et al., 2020). Similarly, research in the Eastern Cape (South Africa) found that the most prevalent rifampin resistance-conferring mutation was *rpoB* codon S531L in INH-resistant strains; the *katG* gene at codon S315TB and the *inhA* gene at codon C-15 TB had the most mutations, 54.5% and 24.7%, respectively (Faye et al., 2023). These findings underscore the importance of detecting both *rpoB* and *inhA* mutations for the accurate identification of MDR-TB and highlight the utility of molecular diagnostic tools in guiding effective treatment strategies

The detection of a mutation in the *rpsL* gene would strongly suggest the presence of streptomycin resistance, which may reflect prior antibiotic exposure or the transmission of previously resistant strains (Wang et al., 2019). Although SM is no longer routinely used as a first-line agent in many tuberculosis treatment programs, resistance to this drug remains epidemiologically relevant and may provide insight into historical treatment practices and the evolution of resistance. Additionally, in some low-resource settings, SM is used as an affordable alternative or substitute for AM in longer regimens for MDR-TB (WHO, 2022; Dai et al., 2021; Serio et al., 2018). Mutations in *rpsL*, particularly codons 43 and 88 are among the well-established genetic determinants of high-level streptomycin resistance and are frequently associated with strains that have undergone substantial antibiotic selective pressure (Smittipat et al., 2016; Zhang and Yew, 2015).

In contrast, the absence of mutations in the *katG* locus implies that high-level isoniazid resistance was not the dominant mechanism in this cohort, supporting the hypothesis that resistance is primarily mediated through alterations in the *inhA* promoter region. Similarly, no resistance-associated mutations were detected in the *rrs* gene,

suggesting that aminoglycoside susceptibility was maintained among samples that produced valid genotype calls. While an allele was observed in the *pncA* gene, the extensive mutational heterogeneity associated with pyrazinamide resistance, coupled with the limited mutation coverage characteristic of targeted assays, precludes definitive conclusions regarding phenotypic resistance. This is supported by a sputum-based study comparing MassARRAY with phenotypic drug DST, which reported strong concordance for RIF and INH, whereas agreement KAN was notably poor (Wu et al., 2022).

A study by Liang et al. (2024) using MassARRAY demonstrated greater diagnostic sensitivity than the Xpert assay for etiological diagnosis of tuberculosis, even though many Xpert results were derived from combined findings across multiple tests. Su et al. (2017) reported that MassARRAY has approximately LOD of 5 CFU/mL (Hsu et al., 2015), markedly lower than that of the conventional Xpert assay (114 CFU/mL) and even the next-generation Xpert MTB/RIF Ultra (16 CFU/mL) (Chakravorty et al., 2017). Notably, more than one-third of the cases initially tested negative by Xpert were subsequently identified by MassARRAY, underscoring its enhanced analytical sensitivity. Nevertheless, because MALDI-TOF MS is mutation-targeted, MassARRAY may not achieve complete coverage of resistance genes that lack well-defined mutation hotspots.

Overall, the mutation patterns identified in this study illustrate the complex and heterogeneous nature of DR-TB. These findings reinforce the utility of multiplex molecular platforms for the rapid detection of clinically relevant resistance markers while simultaneously highlighting the inherent limitations of targeted genotyping approaches. Consequently, integrating broader genomic technologies, such as whole-genome sequencing, may enhance mutation detection, provide a more comprehensive resistance profile, and ultimately support the optimization of individualized tuberculosis treatment strategies. These results support the integration of comprehensive genomic approaches, such as whole genome sequencing, to improve resistance detection and guide individualized tuberculosis treatment strategies.

Metagenomics

In high-burden settings such as South Africa, rapid diagnosis and comprehensive characterization of drug resistance are essential for effective disease control. Metagenomic approaches are increasingly recognized as transformative tools in TB research and clinical management. The present study successfully demonstrated the application of shotgun metagenomic sequencing for the culture-independent detection of *MTB* directly from respiratory specimens. The successful detection of *MTB* in all analysed samples confirms the technical feasibility and analytical robustness of direct sputum metagenomics for TB diagnosis. Furthermore, the average recovery of 2,257 *MTB*-specific reads per sample provided sufficient genomic information for reliable species-level identification despite the highly complex microbial and host background characteristic of respiratory specimens.

These findings contribute important empirical evidence regarding the performance characteristics of direct sputum metagenomics. *MTB* represented only 0.01–0.27% of total sequencing reads and 0.05–0.33% of bacterial reads, highlighting the considerable challenge posed by the overwhelming abundance of host-derived and commensal microbial DNA in respiratory samples. Nevertheless, successful detection was achieved across all specimens, demonstrating that clinically meaningful pathogen identification is possible even when *MTB* constitutes a minute fraction of the total metagenomic dataset. These quantitative estimates provide valuable baseline information for future study design, particularly regarding sequencing depth requirements, analytical sensitivity, and cost optimization for large-scale implementation of metagenomic TB surveillance programs.

The predominance of MTBC members over NTM observed in this study further supports the biological validity of the sequencing approach. The identification of a specimen containing more than 4,000 MTBC-specific reads indicates a high bacillary burden and is consistent with active pulmonary disease. Importantly, shotgun metagenomics' ability to distinguish between MTBC and NTM species provides a significant diagnostic advantage, particularly in TB-endemic regions where clinical and radiological manifestations frequently overlap. Misclassification of NTM disease as TB can result in inappropriate treatment regimens, prolonged morbidity, and the emergence of antimicrobial resistance. Consequently, accurate species discrimination has direct implications for patient management and antimicrobial stewardship.

The present findings are consistent with the growing body of literature supporting the application of mNGS in TB diagnostics. Li et al. (2023) suggested that the clinical use of mNGS is likely to expand substantially to address the limitations of conventional diagnostic approaches for MTBC. Similarly, Li et al. (2022) predicted that diagnostic mNGS would play an increasingly important role in routine clinical practice because of its ability to simultaneously detect MTB and differentiate it from NTM species. Zhou et al. (2019) further demonstrated that mNGS exhibited significantly greater diagnostic sensitivity than several conventional diagnostic modalities. More recently, You et al. (2024) reported that mNGS achieved diagnostic performance comparable to Xpert MTB/RIF while outperforming mycobacterial culture with respect to sensitivity across diverse pulmonary TB specimens. Collectively, these studies support the growing consensus that metagenomic sequencing has the potential to complement or, in selected clinical scenarios, replace conventional diagnostic workflows.

The capability of mNGS to discriminate between *MTB* and NTM is particularly relevant in high HIV prevalence settings, where immunocompromised individuals are at increased risk of opportunistic mycobacterial infections. NTM infections often present with clinical features that closely resemble pulmonary tuberculosis but require fundamentally different treatment approaches and exhibit distinct disease trajectories. Failure to accurately distinguish these pathogens may result in inappropriate therapy, delayed clinical improvement, and poorer patient outcomes (Zhang et al., 2024). The species-level resolution provided by metagenomic sequencing therefore represents an important advancement in precision diagnostics for respiratory disease.

In addition to *MTB* detection, metagenomic sequencing identified several additional clinically relevant bacterial pathogens associated with respiratory tract infections. This observation highlights one of the principal advantages of unbiased sequencing approaches, namely the simultaneous detection of multiple pathogens within a single assay. Such comprehensive pathogen profiling may be particularly valuable in patients with HIV infection, diabetes mellitus, hypertension, and other comorbidities that predispose individuals to polymicrobial infections and adverse clinical outcomes. Failure to recognize concomitant bacterial infections may contribute to suboptimal therapeutic responses, prolonged hospitalization, and increased mortality. Consequently, the broad-spectrum pathogen detection capability of mNGS has

important implications for individualized patient management and antimicrobial selection.

The clinical value of mNGS for identifying unexpected or difficult-to-diagnose pathogens has been demonstrated previously. Wilson et al. (2014) reported the first successful clinical application of mNGS for the diagnosis of neuroleptospirosis in a critically ill adolescent patient, in which sequencing results directly informed targeted antimicrobial therapy and contributed to clinical recovery. Similarly, Ding et al. (2021) demonstrated that the pulmonary microbiome of TB patients is often characterized by the predominance of *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, highlighting the complex microbial ecology associated with TB.

A notable finding of the present study was the identification of *Staphylococcus aureus* in 6 samples, representing 60% of the analyzed cohort. *S. aureus* is a well-recognized opportunistic pathogen and an established cause of respiratory tract infections, bacteremia, and methicillin-resistant *S. aureus* (MRSA) disease (Tong et al., 2015; Hassoun et al., 2017). Previous studies have similarly identified *S. aureus* as a frequent co-pathogen among patients with tuberculosis (Lamas et al., 2019; Hassoun et al., 2017). The identification of such co-infections underscores the clinical utility of metagenomic sequencing in informing antimicrobial therapy and improving patient management through comprehensive pathogen detection and resistance profiling.

Collectively, these findings position metagenomic sequencing as a potentially transformative technology for TB diagnosis and surveillance in high-burden settings characterized by overlapping respiratory infections and substantial diagnostic complexity (Chen et al., 2025; Zhou et al., 2019). By overcoming the limitations of targeted hotspot-based molecular assays, metagenomic approaches provide broader pathogen coverage, improved species resolution, and the capacity to characterize comprehensive antimicrobial resistance (Chen et al., 2025; Govender et al., 2021). These capabilities have the potential to enhance diagnostic accuracy, improve patient stratification, and provide deeper insights into the microbial ecology underlying TB pathogenesis and treatment response. The findings therefore support the emerging paradigm shift towards integrating genomic surveillance into routine TB control programs (Rong et al., 2026, Elbehiry and Abalkhail, 2025).

Despite these advances, several challenges must be addressed before metagenomic sequencing can be implemented widely in routine clinical practice. A fundamental limitation of sequencing-based diagnostics is their inability to distinguish viable organisms from residual microbial DNA, transient colonization, or environmental contamination (Zhao et al., 2024; Simner et al., 2018). Consequently, metagenomic findings must be interpreted within the context of clinical presentation, radiological findings, and complementary microbiological investigations (Govender et al., 2021; Miller and Chiu, 2022). Future studies incorporating quantitative thresholds, host-response biomarkers, and viability-based sequencing approaches may improve the clinical interpretation of metagenomic data and facilitate the transition of these technologies from research settings to routine diagnostic practice (Chen et al., 2025).

Chapter 5: OBJECTIVE 3

To fulfill this part of the research, a comprehensive review of the primary study's complete database was conducted to verify that both the treatment initiation date and the sampling date were included. From the analyzed data, only 82 samples met the established criteria and are addressed in this chapter in order to determine the most suitable therapies and customize treatments to meet the specific requirements of individual patients

CHAPTER 5

STATISTICAL ANALYSIS OF FACTORS ASSOCIATED WITH TB TREATMENT OUTCOMES IN VHEMBE DISTRICT

5.1 INTRODUCTION

Despite global efforts to manage and eliminate the disease, tuberculosis (TB) remains a major public health concern (Monde et al., 2023; Chakaya et al., 2021). There are many factors, such as patient non-adherence to TB treatment standards and pre-treatment health systems and community deals, that influence treatment outcomes in low and middle-income countries where TB is endemic (McNabb et al., 2021; Zetola et al., 2021). The WHO has set ambitious TB treatment targets to measure the performance of treatment programs in low- and middle-income countries (Nour and Nour, 2024; Biruk et al., 2016). Being one of the countries with high TB prevalence, South Africa continues to face significant challenges in meeting the TB treatment targets (Oliver and Luies, 2023). The global treatment success rate for HIV-negative individuals was 85% in 2019 and 76% for HIV-positive individuals, despite the fact that TB prevention, diagnosis, treatment, and care services are readily accessible (WHO, 2021). The success rate for HIV/TB co-infection is significantly lower in Africa, where it is 78.9% (Teferi et al., 2021; Chaves Torres et al., 2019).

Treatment outcome data are important for monitoring the effectiveness of currently used drugs and patient adherence (Tadesse et al., 2021; Munro et al., 2007). Areas with high TB rates and limited resources often choose the WHO-recommended standardized treatment strategy. In South Africa, the management of MDR-TB involves

referring patients to specialized provincial centers where the WHO-standardized program is followed (Loveday et al., 2018; Marais et al., 2013). However, certain factors, such as stigma, play an important role in whether the patients undergo treatment at referral facilities (Addo et al., 2022; Cremers et al., 2015).

Quantitative, univariate, and bivariate methods were used in previous studies addressing TB treatment in the Vhembe district (Mkhavele et al., 2025; Mamba, 2022). In some studies, prediction models were developed separately for cure and non-cure outcomes by performing diagnostic cross-tabulations and evaluating accuracy using sensitivity, specificity, and positive and negative predictive values (Peetluk et al., 2022; Madan et al., 2018). However, they did not evaluate the factors contributing to the decision-making process or the relative importance of the risk elements. This resulted in a lack of information on assessing the practical importance of the outcome predictions and the role of the valuable variables in those predictions (Houben et al., 2016).

Understanding the factors influencing TB treatment outcomes is important, including their primary effects and connections (Shilongo, 2022). The treatment outcome, which involves the health state classification of TB patients at the end of an anti-TB treatment regimen, determines the effectiveness of the treatment strategy (Chandru and Varma, 2023; Günther et al., 2021). Currently, the South African National TB control programs use TB therapy compliance to assess treatment (Makhado et al., 2020; Ershova et al., 2014; Tumbo and Ogunbanjo, 2011). An effective method for monitoring TB treatment outcomes is to assess culture conversion or microbiological activity, which reflects instantaneous cure during an anti-TB antibiotic regimen (Heykencdorf et al., 2022; Lee and Pethe, 2022; Phillips et al., 2016). However, the model approach acknowledges individual variation, especially in longitudinal data, and not only represents the therapeutic efficacy of microbiological activities but also depicts more complex processes (Lyu et al., 2023). Moreover, exploring TB treatment outcomes should account for other related factors that may contribute to or attenuate bias in the estimated effect (Fan et al., 2024; Balogun et al., 2021; Hussain et al., 2019).

Concerning treatment outcomes, previous studies used only a small number of patient demographics, healthcare services, TB classification, and medication history factors. This work examines all tuberculosis symptoms and many potential risk factors by listing categorical and continuous covariates, which could be used in subsequent data

analysis to identify factors influencing TB treatment outcomes using a classification approach (Villalva-Serra et al., 2022; Agyare et al., 2021; Baluku et al., 2021). The final goal is to predict TB treatment outcomes in our province using the Bayesian model averaging approach across the sequence of TB anti-TB treatment processes. Some of the existing prediction models for TB treatment outcomes have been specifically developed for the TB treatment outcome in the HIV or TB/HIV co-infection subpopulation. A predictive model for a TB treatment outcome by HIV status (Hosu et al., 2024). The features that are predictive of treatment success for patients with TB and HIV are cavitory, treatment default, and absence of close social support, which are important for improvement to meet the End TB Strategy goal (Nyasulu et al., 2022).

5.2 METHODOLOGY

A total of 175 participants enrolled in the study; only 82 had a start date and a sample date recorded. The treatment outcome was recorded following the WHO standards. Data was captured and sorted in Excel. Statistical analyses were done using IBM SPSS. The table below (Table 5.1) shows how the outcome was predicted.

Table 5.1: Adapted and modified WHO Definitions of Tuberculosis Treatment Outcomes (WHO,2025).

Outcome	Definition
Treatment success	Treatment completed and patient cured
Treatment failed	A patient whose treatment regimen needs to be terminated or permanently changed to a new regimen or treatment plan (>5 months and MTB is detectable)
Cured	A patient with pulmonary TB with bacteriologically confirmed TB at the beginning of treatment who has completed treatment as recommended by the National policy, with evidence of bacteriological response and no evidence of failure
Treatment completed	A patient who completed treatment as recommended by National policy, but whose outcome does not meet the definition for cure or treatment failure (>6 months of treatment and ND of MTB)
Died	A patient who died before starting the treatment plan or during the course of treatment

Lost to follow-up	A patient who did not start treatment or whose treatment was interrupted for 2 consecutive months or more
Not evaluated	A patient for whom no treatment outcome was assigned
<i>A proposed definition for the use of operational research only</i>	
Sustained treatment	An individual assessed at 6 months (DS-TB and DR-TB) and at 12 months (DR-TB only) after successful TB treatment, who is alive and TB-free

5.3 RESULTS

The results indicate clear differences in treatment status among diagnostic categories. Participants with confirmed *MTB* infection were predominantly still undergoing treatment, reflecting the prolonged duration of standard anti-tuberculosis therapy. Similarly, individuals with mixed *MTB*+NTM infections showed a high proportion of ongoing treatment, alongside the only recorded cases of treatment failure, suggesting potential challenges in managing co-infections or possible drug resistance.

The treatment outcomes of the study population (n=82) demonstrate varying levels of therapeutic success. Approximately half of the patients were cured, accounting for 46.34% (38/82) of the cohort. This indicates the most frequent favourable outcome in the population. In contrast, treatment failure was very low, accounting for 7.32% (6/82). Collectively, these findings indicate that the medication and adherence of the study population is good, as the findings indicate more favourable outcomes than unfavourable outcomes. Table 5.2 presents the distribution of treatment outcomes stratified by mycobacterial diagnostic category: *MTB*, *MTB*+NTM (co-occurrence), NTM, and ND. Treatment outcomes were categorized as cured, treatment in progress, treatment failure, or treatment completed, enabling comparative assessment of clinical response across microbiological groups.

Table 5.2: Distribution of Tuberculosis Treatment Outcomes Among Study Participants (n=82)

Treatment outcome	Frequency N=82 (%)	95% CI of Proportions
Cured	38 (46.34%)	35.55-57.13
Treatment in progress	23 (28.05%)	18.32-37.78

Treatment completed	15 (18.29%)	9.92-26.66
Treatment failure	6 (7.32%)	1.69-12.95

The age distribution across treatment outcomes shows that the 25-44-year age group accounts for the majority of outcomes, while treatment failure was predominantly concentrated among individuals aged 45-60 years. Males made up a slightly higher percentage of patients in most groups, although gender disparities were very small across outcome categories. The majority of patients across treatment outcomes were HIV-positive, which is consistent with the well-established relationship between HIV infection and the burden of tuberculosis in high-prevalence settings.

The mycobacterial diagnostic category and treatment outcome were found to be statistically significantly correlated ($p = 0.001$). While mixed *MTB*+NTM infections were closely linked to treatment failure and continued therapy, patients with NTM infections were primarily represented among cured and treatment-completed cases. Treatment time also showed diversity among outcomes, with longer treatment durations more usually linked to treatment failure or completion and shorter treatment durations typically seen among cured patients.

Overall, these results demonstrate how intricately clinical, microbiological, and demographic factors interact to influence the course of tuberculosis treatment in the study group. The distribution of important clinical, microbiological, and sociodemographic factors by tuberculosis treatment outcomes is shown in Table 5.3: cured ($n = 38$), treatment completed ($n = 15$), treatment in progress ($n = 23$), and treatment failure ($n = 6$). Age group, gender, HIV status, identified mycobacterial species, and treatment duration were among the variables analysed. To show the relative importance of each item within the treatment outcome group, column percentages are provided.

Table 5.3: Distribution of Sociodemographic, Clinical, and Microbiological Characteristics according to Tuberculosis treatment outcomes in the study cohort (N = 82)

Variable	Cured N=38 (%)	Treatment completed N=15 (%)	Treatment in progress N= 23 (%)	Treatment Failure N= 6%)	Total N=82 (%)	P value
Age						
18-24	1 (2.63%)	2 (13.33%)	1 (4.35%)	0 (0.00%)	4 (4.88%)	0.217
25-44	16 (42.11%)	5 (33.33%)	12 (52.17%)	1 (16.67%)	34 (41.46%)	
45-60	9 (23.68%)	6 (40.00%)	7 (30.43%)	5 (83.33%)	27 (32.93%)	
>60	12 (31.58%)	2 (13.33%)	3 (13.04%)	0 (0.00%)	17 (20.73%)	
Gender						
Female	15 (39.47%)	3 (20.00%)	10 (43.48%)	3 (50.00%)	31 (37.80%)	0.430
Male	23 (60.53%)	12 (80.00%)	13 (56.52%)	3 (50.00%)	51 (62.20%)	
HIV status						
Positive	26 (68.42%)	9 (60.00%)	15 (65.22%)	3 (50.00%)	53 (64.63%)	0.001*
Negative	12 (61.58%)	6 (40.00%)	8 (34.78%)	3 (50.00%)	29 (35.37%)	
<i>Mycobacteriu spp</i>						
ND	8 (21.05%)	2 (13.33%)	0 (0.00%)	0 (0.00%)	10 (12.20%)	0.001*
NTM	30 (78.95%)	13 (86.67%)	0 (0.00%)	0 (0.00%)	43 (52.44%)	
MTB	0 (0.00%)	0 (0.00%)	10 (43.48%)	0 (0.00%)	10 (12.20%)	
MTB+NTM	0 (0.00%)	0 (0.00%)	13 (56.52%)	6 (100.00%)	19 (23.17%)	
Duration						
> 1 month	1 (2.63%)	0 (0.00%)	4 (17.39%)	0 (0.00%)	5 (6.10%)	<0.001*
1-3 months	31 (81.58%)	0 (0.00%)	17 (73.91%)	0 (0.00%)	48 (58.54%)	
4-6 months	6 (15.79%)	7 (46.67%)	2 (8.70%)	0 (0.00%)	15 (18.29%)	
7-9 Months	0 (0.00%)	8 (53.33%)	0 (0.00%)	4 (66.67%)	12 (14.63%)	
>9 months	0 (0.00%)	0 (0.00%)	0 (0.00%)	2 (33.33%)	2 (2.44%)	

5.4 DISCUSSION

Treatment outcomes are a critical indicator for evaluating the effectiveness of TB control programs, as they reflect the success of diagnosis, treatment adherence, and, overall, the management of patients within a healthcare system (Heyckendorf et al., 2022; Churchyard et al., 2014). To identify gaps in care delivery, it is important to monitor outcomes, such as whether patients are cured, complete treatment, or fail, as this provides valuable insight into the performance of TB strategies. The WHO states that a comprehensive evaluation of treatment outcomes is crucial for determining whether national TB programs are meeting global control targets and for targeting public health actions to reduce TB mortality and transmission (Chakaya et al., 2021).

Successful treatment outcomes, particularly cure and treatment completion, contribute significantly to interrupting TB transmission by reducing infectious cases within

communities and to achieving the SDG goals aimed at reducing the TB epidemic. Conversely, unfavourable treatment outcomes, including treatment failure or prolonged treatment duration, may indicate an underlying challenge that includes DR-TB, poor adherence, delayed diagnoses, or co-occurrence of conditions such as HIV infections and NTMs, as observed in the study (Omara et al., 2025; Van Ingen et al., 2018). These factors may contribute to the development of MDR-TB and to *MTB* persisting in affected individuals. Thus, examining treatment outcomes in relation to clinical, microbiological, and demographic factors provides a crucial epidemiological framework for understanding disease dynamics and improving TB management.

The current study reports overall treatment success rates of 46.34% (cured) and 28.05% (treatment completed) among TB patients, which are much lower than the 92.5% reported in a study conducted in Eastern Ethiopia. Another study in Poland also obtained an overall treatment success rate in the study population of 88.0%, with 9.2% of patients failing to achieve treatment success and 2.8% dying. Additionally, this study's treatment success rate of 74.39% (combined cured and treatment-completed categories) is still lower than the South African rate of 81-83 % in drug-susceptible TB (DOH, 2025). This remains below the WHO 90% target to clear the TB epidemic in our communities. Globally, treatment success was 88% in 2023 and 2022, an increase from 87% in 2021 and 86% in 2020 (WHO, 2025). Overall, these findings reveal a substantial discrepancy between the treatment outcomes in this cohort and both national and international TB control targets, underscoring the need for more robust TB management strategies, enhanced adherence support, greater diagnostic capacity, and closer monitoring of treatment response in the study setting.

Age and treatment outcomes

The 25–44-year age group accounted for the largest proportion of patients cured (42.11%) and those still in treatment (52.17%). In contrast, treatment failure was observed mainly among individuals aged 45–60 years, who accounted for 83.33% of unsuccessful outcomes. This distribution mirrors established epidemiological patterns of tuberculosis, in which the economically productive adult population bears the greatest disease burden, largely due to increased exposure associated with occupational environments, mobility, and frequent social interactions. The increased

incidence of underlying comorbidities in older people, such as diabetes mellitus, chronic pulmonary disease, and other disorders that impair immune function, may be the reason for the higher percentage of treatment failure among patients between the ages of 45 and 60. These comorbidities raise the risk of poor clinical outcomes and have a detrimental effect on therapy response. Similar observations have been reported in other countries, where the treatment outcomes of active TB in older people have been found to be unfavourable, including Japan (Hase et al., 2021), India (Murali et al., 2021), and China (Liu et al., 2025). This age-related immune deterioration may contribute to persistent infection, delayed bacterial clearance, and an increased risk of treatment complications

Gender and Treatment Outcomes

The study cohort was predominantly male, with 60.53% of cured cases and 80.00% of treatment-completed cases occurring among males. This finding is consistent with global TB epidemiology, where men consistently show higher TB incidence and notification rates than women (WHO, 2023). Several studies have reported on factors contributing to this gender disparity, which include occupational exposure (most working in mines, construction), higher smoking rates, alcohol consumption, and delayed healthcare-seeking behaviour among men (Rickman et al., 2025; Humayun et al., 2022; Horton et al., 2016).

Interestingly, treatment failure in this cohort was equally distributed between males and females (50% each). Although males often have poorer adherence patterns, the equal distribution observed here may suggest that clinical or microbiological factors, rather than gender alone, influenced treatment failure in this population. Most studies often report poorer adherence and higher loss-to-follow-up among male TB patients, and the equal distribution observed in the current study suggests that treatment failure may be influenced more strongly by clinical and microbiological determinants, such as drug resistance, mixed mycobacterial infections, HIV co-infection, or delayed diagnosis, rather than gender alone.

HIV Status and Treatment Outcomes

HIV co-infection was prevalent in the sample, with HIV-positive people accounting for 65.22% of treatment-in-progress cases and 68.42% of cured patients. It is commonly acknowledged that among those living with HIV, especially in sub-Saharan Africa, tuberculosis is the most common opportunistic illness and cause of death (Wondmeneh and Mekonnen, 2021). These findings are consistent with a large and well-established body of epidemiological and clinical research demonstrating that TB remains a predominant opportunistic infection and a major cause of death among individuals living with HIV, particularly within sub-Saharan Africa. The null hypothesis is strongly invalidated by the observed level of statistical significance ($p = 0.001$), which indicates a highly significant association between HIV co-infection and TB disease outcomes within this cohort.

Mycobacterium Species and Treatment Outcomes

The type of mycobacterial infection plays a crucial role in the therapeutic response, as a statistically significant association ($p=0.001$) was observed between the *Mycobacterium* species and treatment outcome. Among cured cases, non-tuberculous NTM accounted for 78.95%, whereas mixed MTB+NTM infections were dominant among treatment-in-progress (56.52%) and treatment-failure cases (100%). This finding highlights the clinical complexity of polymicrobial mycobacterial infections, which may complicate diagnosis and treatment decisions. Clinically, NTM infections frequently resemble tuberculosis (TB), but they require different antibiotic regimens, and some NTM species may not respond to normal TB therapy (Kangabam et al., 2025; Kalpana et al., 2022). Therefore, incorrect treatment and poor clinical results may result from misdiagnosis or delayed detection of NTM (Bhanushali et al., 2023). Moreover, competing microbial dynamics and differential drug susceptibility may arise from mixed MTB and NTM infections, leading to treatment failure (Huang et al., 2022).

Treatment Duration and Clinical Outcomes

Treatment duration showed clear patterns across outcome categories. The majority of cured patients (81.58%) completed treatment within 1–3 months, while treatment-completed cases were mainly within the 7–9-month range (53.33%). Conversely, treatment failure occurred predominantly after prolonged treatment durations (7–9

months and >9 months). The treatment duration was strongly associated with treatment outcomes ($p < 0.001$). Patients who achieved a cure were predominantly treated within 1–3 months, while treatment failure was observed mainly in individuals with prolonged treatment durations exceeding 7 months, indicating that delayed response to therapy may reflect underlying drug resistance, mixed infections, or treatment complications

A duration of six months of medication is usually needed for standard first-line TB treatment regimens, which consist of an intensive phase and a continuation phase (WHO, 2023). Prolonged treatment may be a sign of medication resistance, poor adherence, delayed sputum conversion, or co-infection problems. Extended therapy has been associated with a higher likelihood of treatment discontinuation and the establishment of drug-resistant strains of tuberculosis, especially when adherence is inconsistent (Jadhav et al., 2024; Heidary et al., 2022). Rapid molecular diagnostics and drug susceptibility testing are crucial for managing TB, as the higher rate of treatment failure in longer treatment-duration groups may indicate underlying drug resistance or diagnostic difficulties.

In conclusion, the study findings demonstrate that the most powerful predictors of treatment outcome in this cohort were microbiological characteristics, specifically the type of mycobacterial infection, as evidenced by a statistically significant correlation ($p = 0.001$). In contrast, demographic variables such as age, gender, and HIV status were among the demographic variables that displayed the anticipated epidemiological trends, but they did not seem to have an independent impact on treatment results. The presence of NTM and mixed MTB+NTM infections underscores the need for improved diagnostic techniques, such as metagenomic sequencing, to ensure accurate pathogen identification and appropriate treatment. These results highlight the importance of implementing comprehensive diagnostic techniques within the study's framework, which incorporates modern molecular diagnostics, microbial genomes, and clinical outcome assessment. Methods such as metagenomic sequencing enable accurate pathogen identification, detection of mixed infections, and better characterization of microbial communities, supporting more accurate treatment decisions. In the end, using this concept in tuberculosis research and clinical practice could increase public health responses to both drug-resistant TB and newly emerging

NTM-associated disease, improve patient outcomes, and improve treatment stratification.

CHAPTER 6: CONCLUSION & RECOMMENDATIONS

This chapter provides a general conclusion of the study and offers recommendations for future research.

CHAPTER 6

CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

6.1 CONCLUSION

In high-incidence settings such as South Africa (SA), Tuberculosis (TB) continues to be a major global public burden. The emergence of drug-resistant TB strains in a setting with a dual epidemic of TB and HIV poses a significant challenge to disease control efforts. The present study investigated the genomic characteristics of

Mycobacterium tuberculosis (MTB), determined the prevalence and molecular basis of drug-resistant TB in a rural setting district of Limpopo province. The study used MassARRAY, whole-genome shotgun metagenomics, and molecular docking to advance TB diagnostics and therapeutic research.

The study demonstrated that the first- and second-line drugs were effective because the drug-susceptible MTB strain was dominant in the cohort; however, cases of DR-TB, mixed co-occurrence (MTB and NTM), and NTM were observed. The prevalence of DR-TB was low; however, the detection of INH monoresistance and the XDR-TB cases highlights the need to improve diagnostic methods, as TB continues to resurface as the leading infectious disease globally. The other most important finding in the study is the number of NTMs detected. Diagnostic methods within our facilities must be able to differentiate NTM from MTB at the initial diagnostic stage to avoid misdiagnosis, as NTM symptoms often mimic those of tuberculosis. In settings with limited resources, NTM colonization or infection may be falsely diagnosed as drug-susceptible or drug-resistant TB, which can lead to extended morbidity, apparent treatment failure, and unnecessary exposure to second-line anti-TB regimens.

Despite a complex respiratory microbiome background, whole-genome shotgun sequencing effectively detected MTB directly from sputum samples and generated sufficient MTB-specific reads to enable final species identification. Beyond MTB detection, metagenomics revealed co-occurrence of NTM, polymicrobial respiratory pathogens, and broader resistome profiles beyond classical TB genes.

In circumstances including polymicrobial diseases, metagenomics is preferable to single-target diagnostics, as evidenced by this thorough pathogen detection. Additionally, a significant drawback of targeted assays is addressed by the capacity to detect resistance-conferring mutations outside traditional hotspot regions, enabled by full-genome sequencing.

Most importantly, this study contributes to the increasing body of evidence demonstrating that direct-from-sputum sequencing is a practical clinical technique in high-burden situations. These advanced molecular methods shorten the wait time for growing the TB, which can take 6-8 weeks, before determining its drug-resistance profile.

The study's treatment outcomes for TB patients revealed significant variation, influenced by genetic factors, particularly drug resistance mutations identified through Metagenomic sequencing. These findings underscore the importance of personalized treatment approaches based on genetic insights, which can enhance the effectiveness of TB management. Overall, the study highlights the critical need for ongoing monitoring and adaptation of treatment strategies to improve outcomes for TB patients in the Vhembe District.

6.2 LIMITATIONS / CHALLENGES

STUDY LIMITATIONS

Several limitations should be considered when interpreting the findings of this study.

First, the study was conducted in a predominantly rural district of Limpopo Province, South Africa. The socio-demographic, healthcare access, and epidemiological characteristics of this population may differ from those of urban populations and other districts within the province or elsewhere in the country. Consequently, the findings may not be fully generalizable to all tuberculosis (TB) patients in South Africa.

Second, the study included only adult participants aged 18 years and older, as stipulated by the ethical approval conditions. As a result, the prevalence of MTB, DR-TB, and associated genetic mutations among pediatric populations could not be assessed. The findings should therefore be interpreted as representative only of the adult TB population.

Third, the study's cross-sectional design provided a single-point assessment of participants and did not allow longitudinal follow-up. Consequently, temporal relationships between clinical, behavioral, and microbiological factors could not be established. In addition, treatment adherence, treatment response dynamics, and the development of drug resistance over time could not be evaluated. A longitudinal cohort design would have provided a more comprehensive assessment of treatment outcomes and the development of resistance.

Fourth, participant recruitment was limited to patients who attended healthcare facilities during the study period and consented to participate. Patients who were absent from clinic visits, declined participation, or were otherwise inaccessible were not represented. This may have introduced selection bias, particularly if non-participating individuals differed systematically from participants in terms of treatment adherence, disease severity, or treatment outcomes.

Fifth, treatment outcome analyses were restricted by incomplete clinical records. Although 175 participants were enrolled, only 82 had complete information regarding treatment initiation dates and sample collection dates, which were required for outcome classification. Exclusion of participants with incomplete records may have introduced selection bias if the excluded individuals differed from those included in the analysis. Furthermore, the reduced sample size may have limited the statistical power to detect significant associations between treatment outcomes and microbiological or demographic variables.

Finally, sequencing was performed on a subset of eligible samples. Consequently, the detected resistance-associated mutations and genomic diversity may not fully represent the complete spectrum of circulating MTB strains within the study population. The reduced sequencing coverage may have limited the ability to identify uncommon mutations and genetic variants associated with drug resistance.

OPERATIONAL AND LOGISTICAL CHALLENGES ENCOUNTERED DURING THE STUDY

Several operational and logistical challenges were encountered during the implementation of this study.

Limited laboratory infrastructure and equipment availability. The University of Venda did not possess all the specialized equipment required for the planned molecular and genomic analyses. Consequently, certain laboratory procedures had to be conducted at collaborating institutions. This necessitated transporting samples and coordinating between laboratories, resulting in delays in sample processing and increased project costs.

Financial constraints. The study was conducted under limited research funding, which affected the number of samples that could be processed and sequenced. Budgetary limitations also influenced sample transportation, laboratory consumables, and access to specialized molecular diagnostic platforms.

Delays in administrative and ethical approval processes. Data and sample collection commenced later than initially anticipated due to the sequential approvals required from institutional, provincial, district, and facility-level authorities. Permission to conduct the study required compliance with multiple administrative procedures, which extended the study initiation period.

Healthcare facility management and staff turnover. Changes in facility management and healthcare personnel during the study period necessitated repeated engagement and orientation regarding the study objectives and procedures. These changes occasionally disrupted recruitment activities and sample collection schedules.

Participant accessibility and communication challenges. A substantial number of participants could not be contacted on scheduled sample collection or follow-up dates because of incorrect, outdated, or unavailable contact information. In some instances, next-of-kin details were also unavailable or non-functional, making participant tracing difficult.

Participant recruitment challenges. Recruitment was affected by participants who declined enrolment after being approached. In addition, concerns related to tuberculosis-associated stigma appeared to influence willingness to participate among some eligible individuals.

Impact of the COVID-19 pandemic. Residual healthcare service disruptions and infection-control measures associated with the COVID-19 pandemic affected participant attendance at healthcare facilities and reduced recruitment opportunities during certain periods of data collection.

Sample availability and quality constraints. Some eligible participants were unable to provide sputum specimens of sufficient volume or quality for all planned laboratory analyses. This occasionally reduced the number of samples available for downstream molecular investigations.

Data management and record retrieval challenges. Difficulties were encountered in retrieving complete clinical records for some participants due to missing, incomplete, or inconsistently documented information across healthcare records and laboratory databases. Considerable effort was required to verify and reconcile information from multiple sources.

Despite these challenges, the study objectives were achieved, and adequate measures were implemented to maintain data quality, laboratory standards, and adherence to approved research protocols.

6.3 RECOMMENDATIONS

The primary aim of this study was to determine the prevalence of drug-resistant tuberculosis (DR-TB) within the study population. The findings underscore the need for larger, well-powered studies to strengthen surveillance of emerging isoniazid monoresistance identified in this cohort. Expanded investigations should also include longitudinal tracking of treatment duration, particularly given the substantial proportion of non-tuberculous mycobacterial (NTM) infections and the documented co-occurrence of *Mycobacterium tuberculosis* (MTB) and NTM. Such monitoring would provide greater clarity regarding treatment response dynamics and diagnostic complexity in polymicrobial infections.

Future research should refine sampling strategies by adopting a longitudinal design. Ideally, specimens should be collected at baseline (before treatment initiation), during the mid-treatment phase, and at treatment completion. This structured approach would facilitate assessment of treatment adherence and enable differentiation between primary DR-TB (resistance present at baseline) and acquired resistance emerging during therapy. Establishing this distinction is critical for informing clinical management and guiding public health interventions.

This study employed multiple molecular techniques to identify single-nucleotide polymorphisms (SNPs) associated with MTB drug resistance. The findings recommend routine implementation of advanced molecular diagnostic platforms to accurately distinguish mycobacterial species and detect resistance-conferring mutations before treatment initiation. Notably, participants were newly diagnosed with TB yet (first time being diagnosed with TB in their lifetime) harboured drug-resistant

strains in the absence of previously identified comorbidities, highlighting ongoing community transmission of resistant pathogens.

To support progress toward the WHO End TB Strategy, strengthened community-level interventions are essential. This can be accomplished by expanded public awareness campaigns and educational initiatives, which may reduce stigma and promote early health-seeking behaviour. During sample collection, it was observed that some individuals travelled considerable distances to access treatment services. In contrast, others delayed seeking care for more than three months, both of which may compromise disease control efforts.

Furthermore, there is a pressing need for an integrated TB information system within the Department of Health to address gaps in patient follow-up. Patients who relocate before completing therapy may re-present at different facilities without an accurate treatment history, resulting in unrecognised defaulting and ongoing transmission risk. A coordinated, interoperable system would enhance continuity of care, reduce loss to follow-up, and strengthen national TB control efforts.

Finally, the following recommendations are worth mentioning for the treatment outcomes of the study:

- ✓ *Personalized Treatment Plans:* Develop individualized treatment regimens based on genetic profiling of *Mycobacterium tuberculosis* strains. This approach can help tailor therapies to address specific drug-resistance patterns, thereby improving overall treatment efficacy.
- ✓ *Regular Monitoring:* Implement routine monitoring of treatment outcomes and genetic changes in MTB to identify emerging resistance and adjust treatment protocols accordingly. Regular follow-ups can help in timely interventions.
- ✓ *Strengthening Laboratory Capacity:* Enhance laboratory capabilities for rapid and accurate whole genome sequencing. This will facilitate the prompt identification of drug-resistant strains and enable informed decision-making in
- ✓ *Public Awareness Campaigns:* Launch awareness campaigns to educate patients and communities about the importance of adherence to treatment regimens and the implications of drug resistance. Improving patient knowledge can enhance compliance and treatment success.

- ✓ *Collaborative Research Initiatives:* Foster partnerships between research institutions and healthcare organizations to conduct ongoing studies on treatment outcomes. Collaborative efforts can yield valuable insights into effective treatment strategies and resistance patterns.
- ✓ *Policy Development:* Advocate for policies that support the integration of genomic data into public health strategies for TB control. Policies should prioritize funding for research and resources aimed at improving treatment outcomes for TB patients

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ANNEXURES

Appendix 1: Ethical consideration from University of Venda

ETHICS APPROVAL CERTIFICATE

RESEARCH AND INNOVATION
OFFICE OF THE DIRECTOR

NAME OF RESEARCHER/INVESTIGATOR:
Prof AN Traore

STAFF NO:
3854

PROJECT TITLE: **ADME polymorphism in tuberculosis: Pharmacogenetic analysis of samples from patients in hospitals in the Vhembe district of Limpopo, South Africa.**

PROJECT NO: SMNS/20/MBY/13/2104

SUPERVISORS/ CO-RESEARCHERS/ CO-INVESTIGATORS

NAME	INSTITUTION & DEPARTMENT	ROLE
Prof AN Traore	UNIVEN, Biochemistry and Microbiology	Principal Investigator Staff
Dr NE Madala	UNIVEN, Biochemistry and Microbiology	Co- investigator
Prof N Potgieter	UNIVEN, Biochemistry and Microbiology	Co- investigator
Prof KS Heysel	University of Virginia	Co- investigator
Dr D van Der Westhuizen	Ingabe Biotechnology	Co- investigator
Mashilo MS (11640422)	UNIVEN, Biochemistry and Microbiology	PHD Student Co- investigator
Banda NT (16013629)	UNIVEN, Biochemistry and Microbiology	PHD Student Co- investigator
Mphaphuli AM (15018175)	UNIVEN, Biochemistry and Microbiology	Masters Student Co- investigator
Patel SA (16021768)	UNIVEN, Biochemistry and Microbiology	Masters Student Co- investigator
Mahamud HA (16021767)	UNIVEN, Biochemistry and Microbiology	Masters Student Co- investigator
Chueu MS (17003376)	UNIVEN, Biochemistry and Microbiology	BSc HONS Student

Appendix 2A: Provisional ethical clearance



LIMPOPO

PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH

Ref : LP_2021-11-001
Enquires : Ms PF Mahlokwane
Tel : 015-293 6028
Email : Phoebe.Mahlokwane@dhsd.limpopo.gov.za

Prof Traore AN

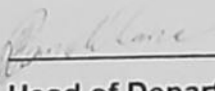
PERMISSION TO CONDUCT RESEARCH IN DEPARTMENTAL FACILITIES

Your Study Topic as indicated below;

ADME POLYMORPHISM IN TUBERCULOSIS: PHARMACOGENETIC ANALYSIS OF SAMPLES FROM PATIENTS IN HOSPITALS IN THE VHEMBE DISTRICT OF LIMPOPO, SOUTH AFRICA

1. Permission to conduct research study as per your research proposal is hereby Granted
2. Kindly note the following:
 - a. Present this letter of permission to the office of District Executive Manager a week before the study is conducted.
 - b. This permission is for Vhembe PHC facilities only as selected in the NHRD.
 - c. In the course of your study, there should be no action that disrupts the routine services, or incur any cost on the Department.
 - d. After completion of study, it is mandatory that the findings should be submitted to the Department to serve as a resource.
 - e. The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.
 - f. The approval is only valid for a 1-year period.
 - g. If the proposal has been amended, a new approval should be sought from the Department of Health
 - h. Kindly note that, the Department can withdraw the approval at any time.

Your cooperation will be highly appreciated



Head of Department

18/05/2022

Date

pp

Appendix 2B: Provisional ethical clearance renewal



LIMPOPO

PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF

HEALTH

Ref : LP_2023-05-013
Enquires : Dr Ramalivhana NJ
Tel : 015-293 6028
Email : Phoebe.Mahlokwane@dhsd.limpopo.gov.za

Prof Traore AN et al

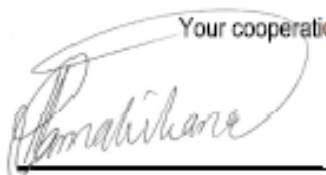
PERMISSION TO CONDUCT RESEARCH IN DEPARTMENTAL FACILITIES

Your Study Topic as indicated below;

ADME POLYMORPHISM IN TUBERCULOSIS: PHARMACOGENETIC AND PHARMACOKINETICS ANALYSIS IN TB PATIENTS FROM HEALTHCARE FACILITIES IN THE VHEMBE DISTRICT OF LIMPOPO, SOUTH AFRICA

1. Permission to extend your research study as per your research proposal is hereby Granted.
2. Kindly note the following:
 - a. Present this letter of permission to the Office of District Executive Manager a week before the study is conducted.
 - b. In the course of your study, there should be no action that disrupts the routine services or incur any cost on the Department.
 - c. After completion of study, it is mandatory that the findings should be submitted to the Department to serve as a resource.
 - d. The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.
 - e. **The approval is only valid for a 1-year period.**
 - f. If the proposal has been amended, a new approval should be sought from the Department of Health
 - g. Kindly note that, the Department can withdraw the approval at any time.

Your cooperation will be highly appreciated.



pp

Head of Department

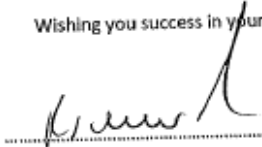
14/07/2023

Date

Private Bag X9302, Polokwane 0700
Fidel Castro Ruz House, 18 College Street, Polokwane 0700
Tel: 015 293 6000. Fax: 015 293 6211. Website: www.doh.limpopo.gov.za

The heartland of Southern Africa - *development is about people!*

Appendix 3: District Permission letter

	LIMPOPO PROVINCIAL GOVERNMENT REPUBLIC OF SOUTH AFRICA
DEPARTMENT OF HEALTH	
Ref : s5/6	
ENQ : Makhado T.L	
TEL : 0159621001 ext 1070	
Date : <u>11 May 2022</u>	
Dear Sir/ Madam <u>Ntshunxeko Thelma Banda</u>	
 RE: Permission to conduct research on the <u>Vhembe District Health facilities as per</u> <u>approval.</u>	
 1. The above matter has reference 2. Your letter received on the <u>2022-05-11</u> requesting for permission to conduct an investigation is hereby acknowledged. 3. The district has no objection to your request 4. Permission is therefore granted for the study to be conducted within Vhembe District 5. You are however advised to make the necessary arrangements with the facilities concerned,	
Wishing you success in your endeavors	
 CHIEF DIRECTOR	<u>12/5/2022</u> DATE
 Private Bag X5009 TSOHOYANDOU 0950 Old Parliamentary Building Tel:(015) 962 1848, (015) 962 1852, (015) 962 1754, (015) 962 1001/2/3/4/5/6 Fax(015) 962 2373,(015) 962 2274,(015) 962 4623	
<i>The heartland of Southern Africa – Development is about people!</i>	

Appendix 4: List of facilities in Vhembe district

Primary health care centers in Vhembe district, Limpopo province South Africa that will be recruited to participate in the study.

NO.	NAME of health care facility
1	Bungeni health centre
2	Folovhodwe clinic
3	Gondeni clinic
4	Donald Fraser Hospital
5	Dzingahe clinic
6	Lwamondo clinic
7	Makonde clinic
8	Madombidzha clinic
9	Mukula clinic
10	Mashamba clinic
11	Mashau clinic
12	Damani clinic
13	Malamulele clinic
14	Masisi clinic
15	Musina clinic
16	Rabali clinic
17	Muledane clinic
18	Mutale health centre
19	Malamulele hospital
20	Phiphidi clinic
21	Manenzhe clinic
22	Mphephu clinic
23	Mhinga clinic
24	Masakona clinic
25	Shigalo clinic
26	Shayamdima clinic
27	Thengwe clinic
28	Tshino clinic
29	Tshiombo clinic
30	Tshakuma clinic
31	Tshaulu clinic
32	Sibasa clinic
33	Tshilwavusiku health centre
34	Khomele clinic
35	Vhambelani Maelula clinic
36	Vhufuli Tshitekere clinic
37	Lwamondo clinic
38	Madimbo clinic

39	Tshilidzini Hospital
40	Vhuri-vhuri clinic
41	Lambani clinic
42	Levubu clinic

Appendix 5: Consent form

Research and Innovation
Office of the Director

RESEARCH ETHICS COMMITTEE

UNIVEN Informed Consent

Appendix B

LETTER OF INFORMATION

Title of the Research Study : ADME polymorphism in tuberculosis: Pharmacogenetic analysis of samples from patients in Hospitals in the Vhembe District of Limpopo, South Africa

Principal Investigator/s : Prof Afsatou Ndama Traore (PhD Biochemistry; UJ)

Co-Investigator/s/supervisor/s : Prof Natasha (PhD Medical Virology; UP)
Prof Scott Heyssel (MD, MPH: Oregon Health & Sciences)
Dr D van der Westhuizen (PhD Molecular Biology; UP)
Dr NE Madala (PhD Biochemistry; UJ)

Brief Introduction and Purpose of the Study:

The study will include a cross-sectional study that will be conducted among TB patients admitted in 3 referral hospital in the Vhembe District (rural) of Limpopo (South Africa) and will include 275 participants (225 TB patients and 50 healthy controls) aged 7 years and above. Interviews will be conducted to collect socio-demographic information and other factors related to TB and samples (Blood, Saliva and Urine) of the participants will be collected. DNA isolated from Sputum and Blood samples will be analysed using sequencing/NGS to understand the risk associated with treatment failures and predisposition to TB.

This project aims at evaluating the pharmacogenetics of South African tuberculosis patients in the Vhembe region of the Limpopo province, South Africa. The findings of the study will provide information on the risk associated with treatment failures and predisposition to TB.

General information will be obtained from participants via a questionnaire, informed consent will be obtained and then samples will be collected. The information obtained will not expose the identity of the participants.

Outline of the Procedures : See attached proposal

Risks or Discomforts to the Participant: There will be no risks involved in participating. Collection of samples will be done once.

Benefits : No monetary compensation is offered for participation.

Reason/s why the Participant May Be Withdrawn from the Study: Participation in this study is completely voluntary. There will be no adverse consequences for the participant should they choose to withdraw

Remuneration : None

Costs of the Study : None

Confidentiality : Information obtained will be captured under a code and

UNIVEN Informed Consent

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will not be made public and for publication purpose, the information will be referred by a code number. Identities will be kept confidential.

Research-related Injury : None

Persons to Contact in the Event of Any Problems or Queries:

(Prof Afsatou Ndama Traore (Department of Microbiology/ University of Venda) Please contact the principal investigator (074 493 5836), the co-investigator (015-962-8474 or 015-962-8107) or the University Research Ethics Committee Secretariat on 015 962 9058. Complaints can be reported to the Director: Research and Innovation, Prof GE Ekosse on 015 962 8313 or Georges.Ivo.Ekosse@univen.ac.za

General:

Potential participants must be assured that participation is voluntary and the approximate number of participants to be included should be disclosed. A copy of the information letter should be issued to participants. The information letter and consent form must be translated and provided in the primary spoken language of the research population

CONSENT

Statement of Agreement to Participate in the Research Study:

- I hereby confirm that I have been informed by the researcher, (Afsatou Ndama Traore), about the nature, conduct, benefits and risks of this study - Research Ethics Clearance Number: _____.
- I have also received, read and understood the above written information (*Participant Letter of Information*) regarding the study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.
- I understand that significant new findings developed during this research which may relate to my participation will be made available to me.

Full Name of Participant Date Time Signature

I, ...

(Name of researcher) herewith confirm that the above participant has been fully

Informed about the nature, conduct and risks of the above study.

Full Name of Researcher

..... Date..... Signature.....

Full Name of Witness (If applicable)

..... Date Signature.....

Full Name of Legal Guardian (If applicable)

..... Date..... Signature.....

Please note the following:

Research details must be provided in a clear, simple and culturally appropriate manner and prospective participants should be helped to arrive at an informed decision by use of appropriate language (grade 10 level- use Flesch Reading Ease Scores on Microsoft Word), selecting of a non-threatening environment for interaction and the availability of peer counseling (Department of Health, 2004)

If the potential participant is unable to read/illiterate, then a right thumb print is required and an impartial witness, who is literate and knows the participant e.g. parent, sibling, friend, pastor, etc. should verify in writing, duly signed that informed verbal consent was obtained (Department of Health, 2004).

If anyone makes a mistake completing this document e.g. a wrong date or spelling mistake, a new document should be completed. The incomplete original document has to be kept in the participant's file and not thrown away, and copies thereof must be issued to the participant.

References:

Department of Health: 2004. *Ethics in Health Research: Principles, Structures and Processes*

<http://www.doh.gov.za/docs/factsheets/quidelines/ethnics/>

Department of Health. 2006. *South African Good Clinical Practice Guidelines*. 2nd Ed. Available at:

http://www.nhrec.org.za/?page_id=14

University of Venda School of Maths and Natural Sciences Department of Microbiology Tel: 015 962 8474	Name Code _____ Area _____ _____ Participant code: _____
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TUBERCULOSIS (TB) PROJECT QUESTIONNAIRE		
SECTION A DEMOGRAPHIC DATA		
Date of birth _____	Gender: F ☐ M ☐	Age _____
1. Employment status		
Student ☐	Unemployed ☐	Self-employed ☐ Employed ☐
2. Educational level		
No Education ☐	Grade 1 -7 ☐	Grade 8 - 12 ☐ Tertiary ☐
3. Religion		
African ☐	Christianity ☐	Islam ☐ Other ☐
SECTION B ENVIRONMENTAL FACTORS		
4. Place of residence		
Native ☐	Migrant ☐	Other ☐
5. Type of house		
Squatter camp ☐	RDP house ☐	Rental ☐ Modern house ☐ Traditional ☐
6. Working space		
Is it dusty? NO ☐ YES ☐ Sometimes ☐		
Do you wear protective mask? NO ☐ YES ☐ Sometimes ☐		
Is it open space? NO ☐ YES ☐ Sometimes ☐		
7. Mode of transport to work/school		
Walking ☐	Own car ☐	Public trans ☐ Bicycle ☐ Other ☐
8. How many are you in your family?		
0-4 ☐	5-9 ☐	10 and above ☐
9. How many bedrooms do you have in your house?		
1 ☐	2 ☐	3 ☐ 4 ☐ 5 and above ☐
10. Are you sharing a room?		
NO ☐	YES ☐	Sometimes ☐
11. Is there a family member who has been diagnosed with TB?		
NO ☐	YES ☐	

SECTION C: CULTURAL FACTORS		
12. Have you ever consulted a traditional healer? NO ☐ YES ☐ Sometimes ☐		
13. Did the traditional healer gave you a TB remedy? NO ☐ YES ☐ Sometimes ☐		
14. After how long did you go to the health care center for consultation?		
1-3 weeks ☐ Month ☐ Two months ☐ Above Three months ☐		
SECTION D: ATTITUDES AND BEHAVIOUR		
	Circle Answers	
15. Have you ever had a vaccine to prevent tuberculosis (BCG vaccine)? (Usually given as infant or child. You may have scar on your arm from the vaccine)	NO	YES
16. Have you ever had a positive/reactive TB skin test?	NO	YES
17. When you tested positive for TB, did you tell your family member?	NO	YES
18. How long did you take to tell them?		
1-7 days ☐ 2 weeks ☐ 3 weeks ☐ Months ☐		
19. Do your family seem to be supportive in term of encouraging you		
NO ☐ YES ☐ Sometimes ☐		
20. Did you inform your colleagues at work, community members or friends after tested positive to TB?		
NO ☐ YES ☐		
21. Do they seem to be supportive in terms of encouraging you to go to the health care centers?		
NO ☐ YES ☐ Sometimes ☐		
22. Have you ever been treated for either active or latent TB?	NO	YES
23. Do you have any chronic illnesses (for example: diabetes, asthma, ulcerative colitis, lupus, leukaemia, lymphoma, chronic renal failure)? Please circle the illnesses	NO	YES
Thank you for your time.		