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Cross-resistance among rifamycins in *Mycobacterium tuberculosis* clinical isolates.

Richard Maredi Mojapelo

(1319082)



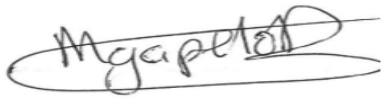
Degree of Master of Science in Medicine by research only

Dissertation submitted to the Department of Microbiology and infection diseases, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg 2019

DECLARATION

I, Richard Maredi Mojapelo, declare that this dissertation is my own work. The experimental work described was conducted under the supervision of Dr Andries Dreyer, Dr Halima Said and Prof Nazir Ismail at the Centre for Tuberculosis, National Institute for Communicable Diseases (NICD), Johannesburg. It is being submitted for the degree of Master of Science in Medicine to the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination to this or any other university.



_____

Date: 05 November 2019

DEDICATIONS

I would like to extend a special thank you to my parents (Melida and Christopher Mojapelo) for academic support throughout my academic career. Thank you both for giving me courage to chase my dream.

I am grateful to the Centre for Tuberculosis (CTB), National Institute for Communicable Diseases (NICD) for creating platform for me to carry out laboratory experiments for this project and also for all their financial supports.

PUBLICATIONS

Manuscript – In preparation

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Poster presentation

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Oral presentation

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ABSTRACT

Introduction: Rifamycins (RFMs) are a group of antimycobacterial drugs that belong to the large family of ansamycin. RFMs inhibit Mycobacterial growth by blocking the RNA polymerase subunit B (*rpo*B). High level cross-resistance among RFMs in *Mycobacterium tuberculosis (M. tuberculosis*) clinical isolates is commonly inferred. However, previous studies reported that the minimum inhibitory concentrations (MICs) of rifabutin (RFB) among rifampicin (RIF)-resistant *M. tuberculosis* carrying *rpo*B mutations varies depending on the mutation position.

Objective: -To determine the proportion of cross-resistance among rifamycins and to assess the use of the GenoType MTBDRplus Version 2.0 assay in predicting differential susceptibility to rifamycins in *M. tuberculosis* isolates.

Method: -A total of 300 unique baseline isolates which were collected between June 2015-April 2016 for routine laboratory based surveillance of RIF drug resistance in selected districts of South Africa were included. Drug susceptibility testing (DST) for RIF (1.0 µg/ml), RFB (0.5 µg/ml) and rifapentine (RFP) (0.5 µg/ml) was performed by the MGIT 960 system using World Health Organisation (WHO) recommended critical concentration (c.c). The MycoTB plate was used to determine MICs for RIF and RFB. To determine *rpo*B mutations, all the isolates were tested by Genotype MTBDRplus version 2.0 assay method and undefined isolates were sent for Sanger sequencing.

Results: -The proportion of cross resistance among RFMs were: across all three (216/300;72%), between RIF and RFB (217/300;72%) and RIF and RFP (292/300;98%). The S531L mutation was the mostly associated with cross resistance to all RFMs (144/153;94%), while the D516V mutation was associated with differential susceptibility to RFB (50/52;96%).

Conclusion: -The results show high levels of cross resistance across all rifamycins, however 28% of MDR/XDR-TB cases could potentially benefit from RFB as a substitute drug to the failing RIF. These findings provided additional evidence of the strong

association of specific *rpo*B mutations with the development of RFMs cross and differential susceptibility. The use of LPA and *rpo*B mutations specifically S531L and D516V can be beneficial in rapidly differentiating phenotypic differential susceptibility to RFB according to this study.

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The completion of this project could not have been possible without the help of the following individuals. For you, I am truly grateful.

- My supervisors, Dr Andries Dreyer and Dr Halima Said for the research topic and guidance when everything started.
- Prof Nazir Ismail and Dr Shaheed Omar Vally for their expert opinions during my protocol development as well as final write up
- Yasmin Gardee and Lavania Joseph for helping me in training me and carrying out all the laboratory work for my project.
- Dr Harry Moultrie and Linsay Blows for assistance with editing of the dissertation and statistical analysis of the results.
- I would also like to thank Zaheda Bhyat and Trisha Munsamy for helping me to carry out drug susceptibility testing work.
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LIST OF ABBREVIATIONS AND ACRONYMS

%	Percentage
>	Greater than
<	Less than
≤	Less than or equal to
≥	Greater than or equal to
°C	Degrees Celsius
µl	Microliter
AFB	Acid- Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
AMK	Amikacin
APM	Agar Proportion Method
ART	Antiretroviral Therapy
ATCC	American Type Culture Collection
BCE	Before the Common Era
BD	Becton Dickinson
BDQ	Bedaquiline
bp	Base pairs
BSC	Biological Safety Cabinet
CA	Categorical Agreement
c.c	Critical Concentration
C-TB	Centre for Tuberculosis

CAP	Capreomycin
CDC	Centers for Disease Prevention and Control
CFU/ml	Colony forming units per milliliter
CI	Confidence Interval
CIP	Ciprofloxacin
CO_2	carbon dioxide
CS	Cycloserine
DL	Delamanid
DNA	Deoxyribonucleic Acid
DR-TB	Drug Resistant Tuberculosis
DST	Drug Susceptibility Testing
EA	Expected Agreement
EMB	Ethambutol
et al.	Et alia (and others)
ETH	Ethionamide
FDA	Food and Drug Administration
FLDs	First Line Drugs
FQs	Fluoroquinolones
GC	Growth Control
GCRS	Guanine-Cytosine-Rich Repetitive Sequence
GERMS-SA	Group for Enteric Respiratory and Meningeal Disease Surveillance in South Africa
GU	Growth Units

HIV	Human Immunodeficiency Virus
HREC	Human Research Ethics Committee
ICT	Immunochromatographic Test
INH	Isoniazid
IS	Insertion Sequence
KAN	Kanamycin
LEV	Levofloxacin
LJ	Lowenstein–Jensen
LPA	Line Probe Assay
LTBI	Latent Tuberculosis Infection
LZD	Linezolid
M. tuberculosis	*Mycobacterium tuberculosis*
MABA	Microplate-Based Alamar Blue Assay
MAC	*Mycobacterium avium* complex
MBA	Molecular Beacon Assays
MDR-TB	Multidrug-resistant Tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MK	*Mycobacterium kansasii*
MIC	Minimum Inhibitory Concentration
ml	Millilitre
MODS	Microscopic Observation Drug Susceptibility
MTBC	*Mycobacterium tuberculosis* complex
MOX	Moxifloxacin

n	Number
NHLS	National Health Laboratory Services
NTM	Non Tuberculosis Mycobaterium
OADC	Oleic Acid-Albumin-Dextrose-Catalase
OFL	Ofloxacin
PAS	P-aminosalicylic acid
PCR	Polymerase Chain Reaction
POC	Point of Care
PZA	Pyrazinamide
QA	Quality Assurance
REDOX	Oxidation-Reduction
REMA	Resazurin Microtiter Assay
RFMs	Rifamycins
RFB	Rifabutin
RFP	Rifapentine
RFZ	Rifalazil
RIF	Rifampicin
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RRDR	Rifampicin Resistance Determining Region
SA	South Africa
SLDs	Second Line Drugs
SRL	Supranational Referral Laboratory

STR	Streptomycin
TAT	Turn-Around-Time
TB	Tuberculosis
USA	United States of America
WGS	Whole Genome Sequencing
WHO	World Health Organization
WT	Wild-Type
XDR-TB	Extensively Drug-Resistant Tuberculosis

CHAPTER 1

1.1: Background and rationale of the study

Tuberculosis (TB) remains a major global health problem with high associated morbidity and mortality. The 2018 Global Tuberculosis Report shows an estimated total of 10.0 million new cases of TB and 1.6 million attributable deaths in 2017. In addition, the emergence of drug-resistant TB (DR-TB) has hampered effective TB treatment and control (WHO, 2018).

Rifampicin (RIF) is the main first-line drugs (FLDs) taken as part of a standard treatment regimen for TB. Together with Isoniazid (INH), RIF forms the cornerstone of combination treatment for TB. It belongs to the large family of ansamycin. Other rifamycins (RFMs) derivatives, rifabutin (RFB), rifalazil (RFZ) and rifapentine (RFP), are also used for the treatment of susceptible TB. High-level cross-resistance among RFMs in *Mycobacterium tuberculosis* (*M. tuberculosis)* isolates is commonly inferred (Williams et al., 1998).

Although cross-resistance with other RFMs can occur, previous studies reported that the minimum inhibitory concentrations (MICs) of RFB among RIF-resistant *M. tuberculosis* carrying *rpo*B mutations varies depending on the mutation position. Mutations on codon 531 are reported to be associated with resistance to all RFMs (Chikamatsu et al., 2009, Jamieson et al., 2014, Yoshida et al., 2010). However, mutations on codon 516 are associated with resistance to RIF and RPT only, but still retain susceptibility to other RFMs, such as RFB or RFL, suggesting RFB/RFL could be alternative drug for treatment of RIF-resistant *M. tuberculosis* (Chikamatsu et al., 2009, Jamieson et al., 2014, Yoshida et al., 2010).

Thus, the present study was conducted to determine RFMs cross-resistance among RIF resistant MTB isolates and also to evaluate the usefulness of GenoType MTBDR*plus* version 2.0 for detection of RFMs differential susceptibility in RIF-resistant strains.

CHAPTER 2. LITERATURE REVIEW

2.1: Tuberculosis history

Tuberculosis (TB) remains a global health problem and is the second leading cause of death from infectious diseases worldwide, after human immunodeficiency virus (HIV) (WHO,2018). Throughout the recorded history of humankind, TB has claimed more lives than any other pathogen in the world. Tuberculosis (TB) reached epidemic proportions in Europe and North America during the 18th and 19th centuries, resulting in millions of deaths, especially in the low socio-economic populations (Daniel, 2006).

As a result of its ruthlessness, TB earned names such as ‘‘Captain Among these Men of Death” (Rubin, 1995) phthisis, and the great white plague (Connolly and Gibson, 2011, Min, 1994). Paleopathological methodology relating to TB dates back to 8000 Before the Common Era (BCE) and evidence of bony TB was found to be present during the Neolithic period in 5800 BCE and in Egyptian mummies dating to 2400 BCE (Zimmerman, 1979, Nerlich et al., 1997).

Tuberculosis (TB) is mentioned in the Biblical books of Deuteronomy and Leviticus, using the ancient Hebrew word called schachepheth (Daniel and Daniel, 1999). TB declined after the late 19th century, due to improved socio-economic conditions and since the mid-1900s, following the introduction of antimycobacterial drugs. Despite these favorable events, TB remained a global health issue to this day (Faustini et al., 2005).

In 1882 the German microbiologist, Robert Koch, isolated *M. tuberculosis* and established its pathogenicity, providing indisputable proof that TB is an infectious disease and that *M. tuberculosis* is the cause of TB (Migliori et al., 2007). In the 1800s,” just sleep and eat nutritious foods” was the advice given to the patient which are infected with TB (Keshavjee and Farmer, 2012).

During the 19th century, TB patients were isolated in sanatoria and given treatments such as injecting air into the pleural space to collapse diseased lung with TB cavitation

for better healing. Attempts were also made to decrease lung size by a surgery called thoracoplasty as a method for treating TB (Sauret Valet, 1998, Vicentini et al., 2010). Up to the turn of the 20th century, no effective TB treatment was available. The first antimycobacterial drug, streptomycin (STR) was introduced to fight TB in 1946 (Daniel, 2006, Wainwright, 1991).

Streptomycin (STR) was the first antimycobacterial agent that showed the potential for clinical use against TB. Streptomycin (STR) as a specific antimycobacterial drug was followed by isoniazid (INH), that became available in 1952. The rifamycin class of antimycobacterial drugs was discovered in *Nocardia mediterranei* in 1957, and was first evaluated in clinical trials in 1967 (Daniel, 2006).

In 1961 the Lederle Company announced the discovery of ethambutol (EMB). Ethambutol (EMB) is a first-line antimycobacterial drug which is used only in combination with other antimycobacterial drugs such as isoniazid (INH) and rifampicin (RIF) for treatment of susceptible TB (Thomas et al., 1961).

Pyrazinamide (PZA) is also a first-line antimycobacterial drug, but is used only in combination with other antimycobacterial drugs such as isoniazid (INH) or RIF. It has remarkable sterilizing activity in the clinical treatment setting, especially when used in regimens containing other standard antimycobacterial drug combinations (Salfinger et al., 1990).

2.2: Classification of *Mycobacteriaceae*

The microorganism that was identified as the causative agent of TB belongs to the genus *Mycobacterium*, which is the single genus within the family of *Mycobacteriaceae,* in the order *Actinomycetales* (Ryan and Ray,2004). Clinically, *Mycobacterium tuberculosis* complex (MTBC) refers to *Mycobacterium spp* which causes TB while the species which do not cause TB are labeled Non-Tuberculosis Mycobacteria (NTM) (Brosch et al., 2002, Malama et al., 2014). Figure 2.1 shows *Mycobacterium spp* belonging to MTBC and those belonging to NTM.

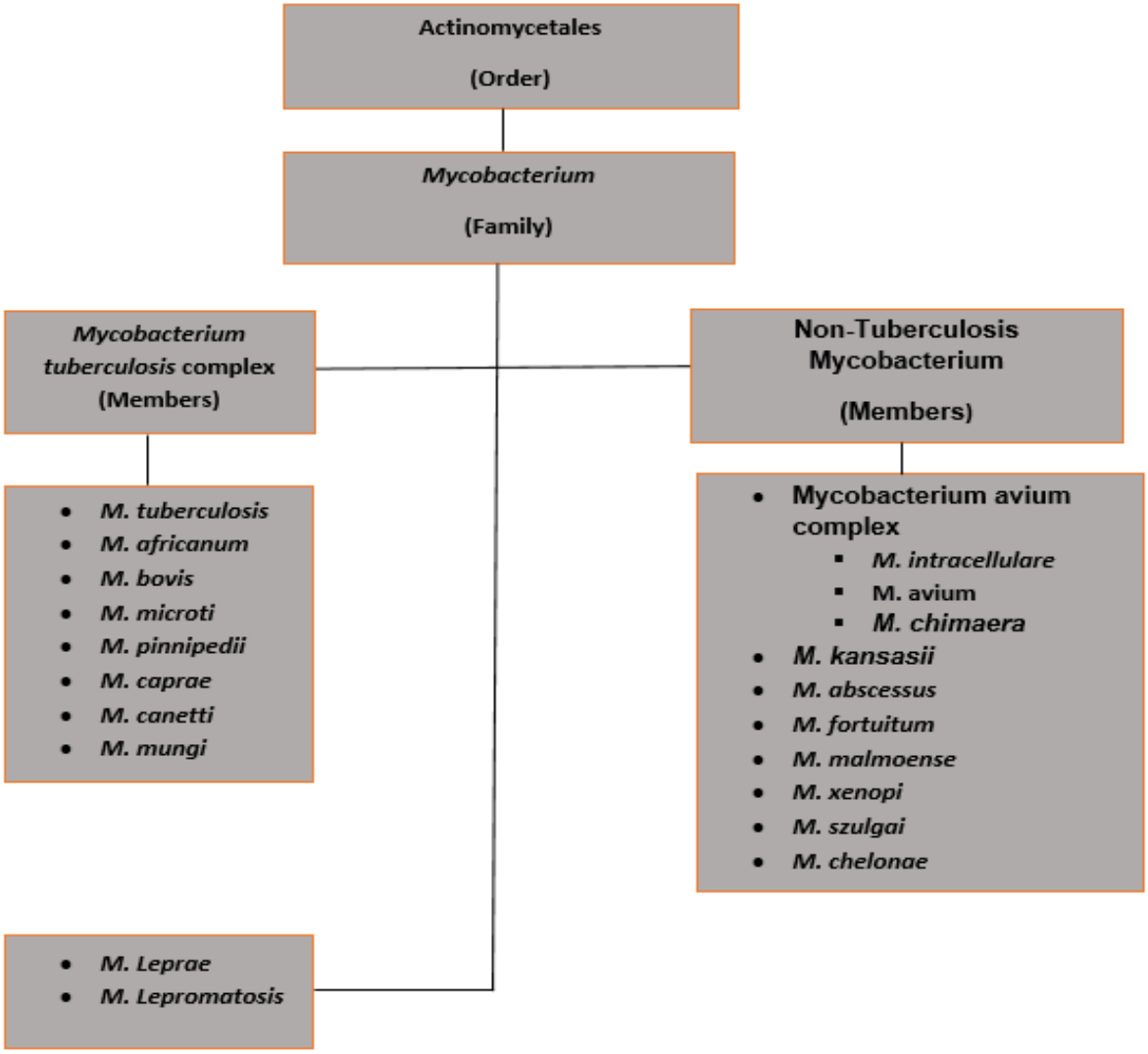


Figure 2.1: Taxonomic tree for *Mycobacterium species*

Mycobacterium tuberculosis (*M. tuberculosis)* and its very closely related *Mycobacterium species*, *M. africanum, M. bovis, M. microti, M. pinnipedii, M. caprae, M. canetti and M. mungi* together comprise what is known as the MTBC. *M. tuberculosis, M. canettii and M. africanum* are only associated with humans while *M. microti* is associated with rodents, whereas others have a wide host spectrum (Johnson and Odell, 2014, Malama et al., 2014). The majority of human TB cases are caused by *M. tuberculosis* (Hasegawa et al., 2002).

2.3: Microscopic morphology of *Mycobacterium species*

Mycobacterium are non-motile, acid-fast, non-spore-forming rod-shaped and obligate aerobes. The tubercle bacilli are 2-4 µm in length and have varying generation times with *M, tuberculosis* having a very slow generation time of between 15 and 20 hours. The cell wall of the *Mycobacterium* is unique in that it contains high concentrations of mycolic acids (Attorri et al., 2000).

Depending on environmental conditions and age of the culture, tubercle bacilli may differ in size and shape from coccobacilli to long rods. Previous studies have shown that tubercle bacilli have the ability to form rope-like structures called cording in a liquid medium (Attorri et al., 2000, Morris and Reller, 1993).

Mycobacterium avium complex (MAC) has been described as exhibiting dot-needle-like forms and loose aggregates, while *Mycobacterium kansasii* (MK) has been described as broad rods that exhibit marked cross-barring likened to ladders (Figure 2.2) (Morris and Reller, 1993).

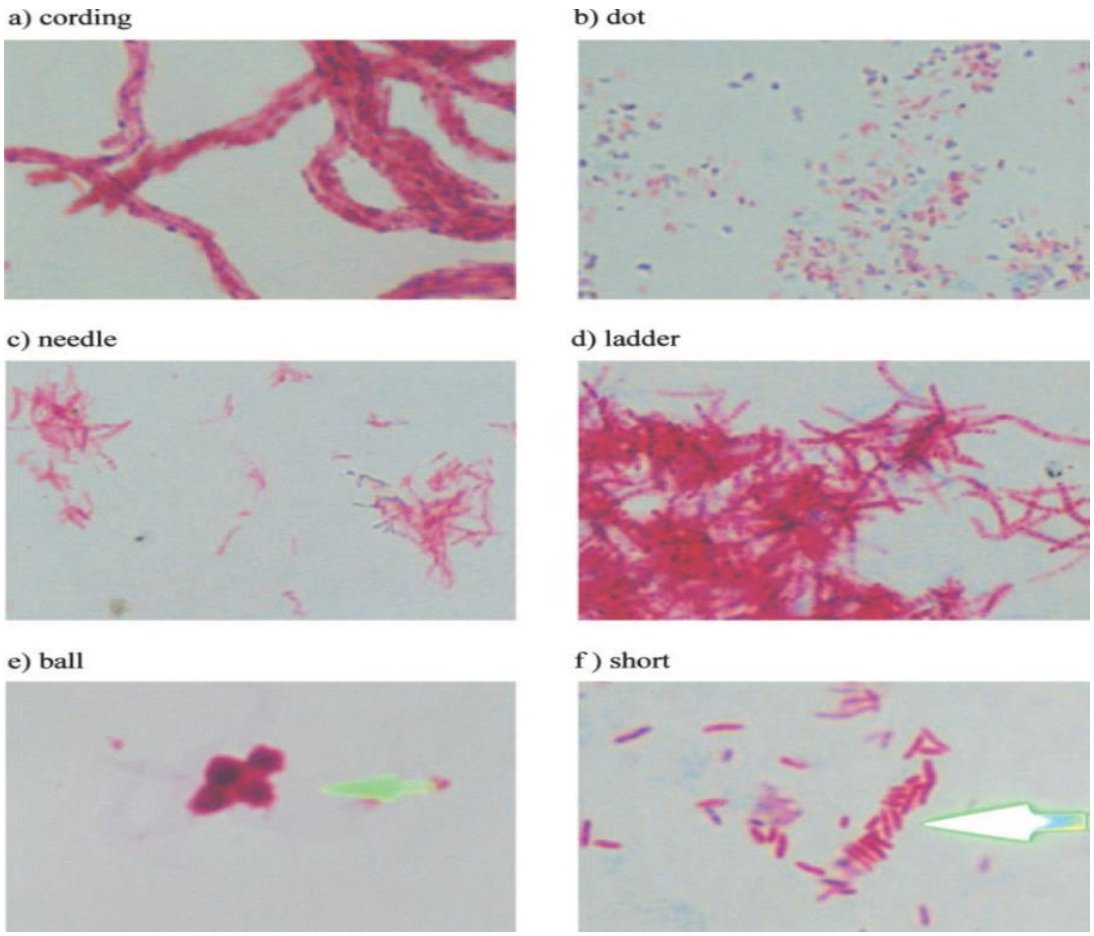


Figure 2.2: Different morphological patterns in Acid- Fast Bacilli smears

2.4: Pathogenesis of *Mycobacterium tuberculosis*

The bacterium is transmitted from an infected person to a new host through air particles. The chances of transmission from one individual to another depends on a number of factors including the number of infectious droplets (aerosols) discharged in bursts of forced expiration, such as coughing, sneezing and shouting, by the infected person, the effectiveness of ventilation and the level of immunity in the infected person prior to infection (Yates et al., 2016).

Droplet nuclei containing between 1-10 bacilli and a diameter close to 10 μm are discharged with a cough or sneeze, then spread in the surrounding air and transported by air currents. Normal conditions can keep the infective particles for prolonged periods

of time in the air and on microscopic dust particles and spread them throughout large areas (Turner and Bothamley, 2015).

The droplet nuclei that are larger than 10 μm are inhaled and held in the upper respiratory tract while smaller droplet nuclei proceed further down the bronchial tree to the respiratory alveoli where the initial TB infection is established. *M. tuberculosis* can cause disease at any tissue site; however, the lungs are the main port of entry and an important site for disease manifestation (Yates et al., 2016, Turner and Bothamley, 2015).

When inhaled, the tubercle bacilli reach the pulmonary alveoli where they become ingested by pulmonary macrophages (Kang et al., 2005). The macrophages will engulf the microorganisms which will either result in the containment of the disease when the macrophages, also called mononuclear phagocytes kill the intracellular tubercle bacilli, or to the progression of the disease when cellular immunity is inefficient and the macrophages fail to kill the intracellular organisms (Tailleux et al., 2005).

When not contained the microorganism will replicate at a slow rate and spread through the lymphatic system to the secondary lymph nodes. The cell-mediated immunity manifested by the activation of macrophages and helper T-lymphocytes occurs in 2-8 weeks after infection. Granulomatous lesions are formed around the microorganism by activated T lymphocytes and macrophages, minimizing the spread of MTBC by the phagocytic killing of the infecting bacilli or limiting their replication.

These interactive cellular events result in the development of a dynamic balance between mycobacterial persistence and host defense activation (Schluger and Rom, 1998). The survival of tubercle bacilli in this immunologically balanced micro-environment for prolonged periods results in a host-parasite situation referred to as latent TB infection (LTBI). When the immune system is suppressed the conditions will be favorable for active TB to occur (Pieters, 2008).

TB disease may become active, with clinical disease in about 1 in 4 people have symptoms which include: chest pain, and a productive bloody prolonged cough for more than three weeks, fever, night sweats, appetite loss, weight loss, pallor and fatigue (Centers for Disease Control and Prevention, 2012).

People who have LTBI are not infectious and are unlikely to spread the infection to others but their TB infection can turn into active TB and they may become infectious when their immune system is compromised (Akolo et al., 2010, Woldehanna and Volmink, 2004). The transmission and pathogenesis of *M. tuberculosis* are illustrated in figure 2.3.

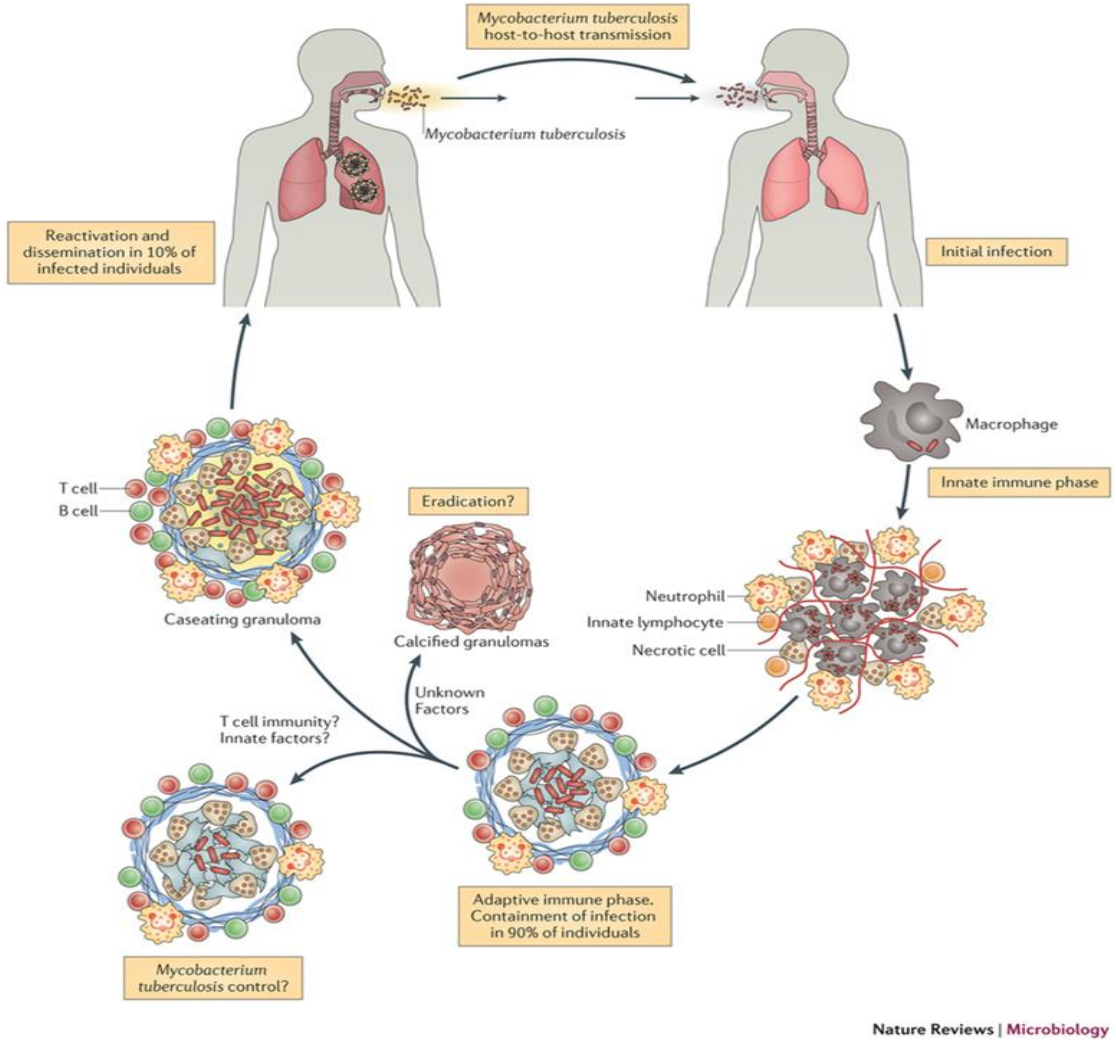


Figure 2.3: Mode of transmission and pathogenesis of Mycobacteria

2.5: Risk factors of pulmonary Tuberculosis

There are many factors that make people susceptible to TB infection and active TB disease. The most important risk factor globally is HIV infection. According to WHO, in a TB endemic region, 10% of all TB cases were co-infected with HIV, while in SA about 59% were co-infected with HIV. This is a serious problem in Africa, especially in sub-Saharan Africa, where rates of HIV are high (WHO,2018).

Together with well-established risk factors (such as HIV, malnutrition, and young age), emerging variables such as diabetes, indoor air pollution, alcohol, use of immunosuppressive drugs, and tobacco smoke play a significant role at the individual as well as at the community level (Narasimhan et al., 2013).

Socioeconomic and behavioral factors have also been shown to increase the susceptibility to infection. Specific groups like health care workers are at an increased risk of TB infection and active TB disease (Narasimhan et al., 2013). The risk factors that increase pulmonary TB are shown in figure 2.4 below.

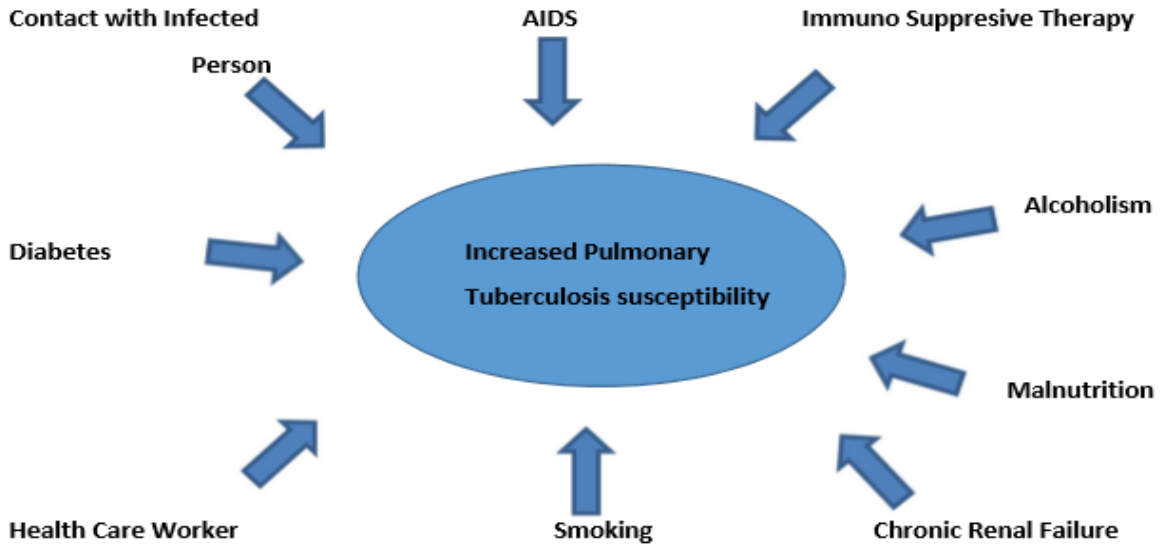


Figure 2.4: Risk factors of pulmonary Tuberculosis

2.6: Treatment of Tuberculosis

There are more than 20 antimycobacterial drugs that are currently used for the treatment of susceptible TB. These antimycobacterial drugs are used in different combinations based on individual patient's clinical response to standard TB treatment and/or in-vitro drug-susceptibility test results for each patient's *M. tuberculosis* isolate. TB disease must be treated for at least 24 weeks and or more (WHO, 2016).

Most of the *M. tuberculosis* of patients with active disease are killed during the first 8 weeks of the treatment. But there are patients with persistent MTBC organisms including those with drug resistance that require much longer treatment. If treatment is not continued for the required time or drugs for treatment are taken erratically, the surviving MTBC may cause the individuals to become sick and infectious again, potentially with a drug-resistant disease (WHO, 2010).

2.6.1: First-line antimycobacterial drugs (FLDs)

First-line drugs (FLDs) for standard antimycobacterial drug treatment include INH, RIF, PZA, EMB and, until recently STR. These are the TB antimycobacterial drugs that are very potent against MTBC, are predominantly tuberculocidal and are the cornerstone of combination treatment for susceptible TB (WHO, 2016).

2.6.2: Second-line antimycobacterial drugs (SLDs)

Second-line antimycobacterial drugs (SLDs) are divided into different categories including: (i) Fluoroquinolones: ofloxacin (OFX), levofloxacin (LEV), moxifloxacin (MOX) and ciprofloxacin (CIP), (ii) injectable aminoglycoside drugs: kanamycin (KAN), amikacin (AMK) and capreomycin (CAP) and (iii) Other second-line antimycobacterial drugs: ethionamide (ETH) cycloserine (CS), p-aminosalicylic acid (PAS), delamanid (DL), bedaquiline (BDQ)/ TMC207, and linezolid (LZD) (WHO,2013).

The SLDs are used for the treatment of MDR-TB. These drugs are difficult to procure and are more toxic and expensive than FLDs. Therefore, the detection and treatment of drug-susceptible or single drug-resistant TB (mono-resistant TB) is an important strategy for preventing the emergence of MDR-TB (Nahid et al., 2016).

2.7: Emergence of Drug-Resistant Tuberculosis

Drug-resistant tuberculosis (DR-TB) occurs when *M. tuberculosis* acquires spontaneous chromosomal mutations that render the organism resistant to one or more antimycobacterial drugs (Zhang and Young, 1994). Multidrug-resistant tuberculosis (MDR-TB) is defined as resistance to both rifampicin (RIF) and isoniazid (INH), with or without resistance to other first-line antimycobacterial drugs (WHO,2010).

Extensively drug-resistant tuberculosis (XDR-TB) is defined as resistance to RIF, INH plus any fluoroquinolone (FQ) and resistance to one or more of the following injectable aminoglycosides, kanamycin (KAN), amikacin (AMK) and capreomycin (CAP) (WHO,2010).

In the world, there are only a few thousand patients with MDR and XDR TB which are successfully treated each year (WHO, 2016). Drug-resistant TB disease can develop in two different ways, namely disease associated with primary drug resistance or disease caused by strains with acquired resistance.

Primary resistance occurs in persons who are initially infected with resistant organisms. Acquired resistance develops during TB therapy, either due to fact that the patient was treated with an inadequate TB regimen, or because of other conditions or factors such as drug malabsorption or drug-drug interactions that led to low blood levels or because of non-compliance of treatment (Ershova et al., 2012).

Acquired drug resistance in *M. tuberculosis* was noted after the introduction of STR, the first drug to treat TB. Exposure to the antimycobacterial drug will induce stress responses inside the infected cell that favors both genetic and physiological mechanisms that lead

to colony survival. Current antimycobacterial drugs are commonly associated with specific resistance mutation. Mechanisms of action of the antimycobacterial drug and the target genes on *M. tuberculosis* are summarized in Table 1.1. The capacity to study resistance mechanisms has been aided by the use of WGS (Cohen et al.,2014, Walker et al., 2015).

Table 1.1: Mechanisms of action of the antimycobacterial drug and the target genes on *M. tuberculosis*

Antimycobacterial drug	Mechanism of Action	Target genes
Rifampicin (RIF)	Inhibits transcription initiation	*rpo*B
Streptomycin (STR)	Inhibits Protein Synthesis	*rps*L and *rrs*
Ethambutol (EMB)	Inhibits arabinogalactan synthesis	*emb*B
Isoniazid (INH)	Inhibits mycolic acid synthesis	*kat*G *inh*A
Pyrazinamide (PZA	Unknown	*pnc*A and *rps*A
Ethionamide (ETH)	Inhibits mycolic acid synthesis	*eth*A and *inh*A
Fluoroquinolones (FQs)	Inhibits DNA gyrase	*gyr*A and *gyr*B
Capreomycin (CAP)	Inhibits protein synthesis	*tly*A and *rrs*
Kanamycin (KAN) and Amikacin (AMK)	Inhibits protein synthesis	*rrs*
Bedaquiline (BDQ)	Inhibits mitochondrial ATP synthesis	*atp*E
Cycloserine (CS)	Inhibits Peptidoglycan biosynthesis	*alr*A
p-aminosalicylic acid (PAS)		*thy*A *and fol*C
Linezolid (LZD)	Inhibits protein synthesis	*rpl*C
Delamanid (DL)	Inhibits mycolic acid synthesis	Rv3547

2.8: Epidemiology of TB in the World and South Africa

In the last decade, WHO published several Global TB reports the aim of which was to provide a reliable and up-to-date assessment of the TB epidemic and progress in the

prevention, diagnosis and treatment of the disease at global, regional and country levels. There were an estimated 10.0 million new TB cases in 2017, according to the 2018 Global TB report (WHO, 2018).

In 2017, there were an estimated 1.3 million TB deaths among HIV negative people and an additional 0.3 million deaths among HIV-positive people. South Africa is one of the countries with the highest burden of TB, and the 2018 WHO report showed an incidence of 322,000 cases. Although TB is declining in SA, it is still ranked among countries with high rates of incident cases occurring in a country which is part of the high TB prevalent sub- Saharan region (Figure 2.5)

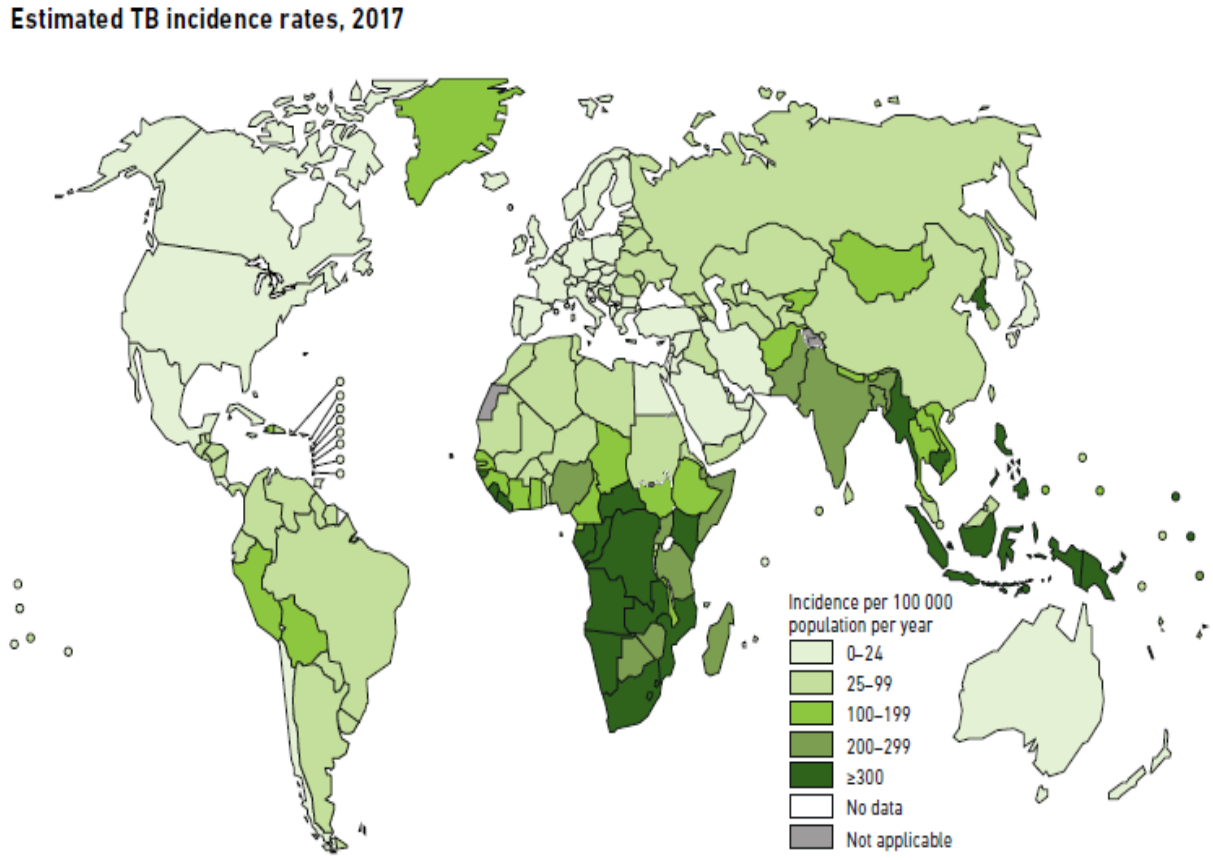


Figure 2.5: Estimated TB incidence rates, 2017.

2.9: Diagnosis of Tuberculosis

Tuberculosis (TB) has been historically diagnosed by demonstrating *M. tuberculosis* in clinical specimens taken from patients suspected of suffering from TB. Diagnosis of TB include: clinical diagnosis (Lewinsohn et al.,2017), chest radiographs (Marciniuk et al, 1999), immunological (tuberculin skin test (TST) and the IFN-γ release assays (IGRAs)), microscopy, culture and molecular techniques (WHO,2018, Cerezales and Benítez ,2011, Loh, 2011).

Clinical diagnosis is mainly by the screening of symptoms of TB such as: coughing for more than two weeks, unexplained weight loss, night sweats, loss of appetite, fever and chest pain.

Diagnosis using immunological methods rely on specific cellular responses of the host, based on delayed hypersensitivity reactions following exposure of patients to *M. tuberculosis* organisms. These methods detect the presence of TB infection but do not distinguish between TB disease and infection. Immunological methods use various antigens such as secretory proteins, heat shock proteins, and mycobacterial lipopolysaccharides and these antigens together with peptides have been researched as candidates for TB diagnosis (Bothamley, 1995, Amicosante et al., 1995).

The laboratory diagnosis of TB in the vast majority of high burden countries remains microscopic examination using either Auramine or Ziehl-Neelsen (ZN) staining (Morris and Reller, 1993, Ba and Rieder, 1999). Compared with fluorescence microscopy with Auramine, light microscopy of Ziehl-Neelsen (ZN) is the most widely available test for diagnosing TB in most countries. ZN microscopy is highly specific, but its sensitivity is variable (20%-80%). The fluorescence microscopy is more sensitive (10%) than the Ziehl-Neelsen and takes less time (Steingart et al., 2006).

Although microscopy is affordable and fast, it has low sensitivity especially among patients co-infected with HIV (Getahun et al.,2007). Because they cannot produce enough sputum and this will result in lower case detection rate (Colebunders and Bastian, 2000).

Culture is WHO recommended gold standard for diagnosis of TB. Mycobacterial culture can be performed on either a solid (Lowenstein-Jensen [LJ]) or an agar-based medium [e.g., Middlebrook 7H11]) or a liquid medium (e.g., Middlebrook 7H9). The yield of *M. tuberculosis* isolated from a liquid medium is greater than that from a solid egg-based medium. Automated liquid culture systems such as the BACTEC™MGIT 960 (BD Diagnostics, Sparks, MD, USA), is a rapid, automated method, which also has the advantages of being capable of bacterial identification and antimicrobial susceptibility testing with high sensitivity and specificity (ref). The method is based on fluorometric technology for the detection of oxygen consumption due to bacterial growth in MGIT tubes (Garrigó et al, 2007).

Methodological advances in molecular biology which provide alternative rapid approaches to diagnose TB. The PCR method was introduced in the early 1990's for identification of MTBC (Springer et al., 1996). Using'' in house'' PCR targets such as IS6110 (an insertion element found exclusively within the members of the MTBC),65 kDa (medically important antigen of MTBC), TRC4 (a conserved repetitive element with specificity for MTBC), GCRS (guanine-cytosine-rich repetitive sequence) and 16S rRNA (transcriptional regulator) has been shown to have the potential to play an important role in the diagnosis of mycobacterial disease (Eisenach et al., 1990, Eisenach et al., 1991, Saiki et al., 1988).

Some of the commercial kits available are the Gen-Probe amplified MTD test (*Mycobacterium tuberculosis* Direct Test; Gen-Probe Incorporated, 9080 Campus Point Drive, 92121 California, USA) (Lyra et al., 2014). COBAS® Amplicor is a test based on amplification, hybridization and detection of the *M. tuberculosis* multicopy IS6110 insertion element (Lyra et al., 2014).

The Detect-TB Ampligenix Biotech Company kit (Ampligenix Biotech, Brazil) is a test which associates the amplification of the *Mycobacteria* IS6110 sequence with the reversed hybridisation of the amplification product with probes complementary to internal sequences of the PCR-product (Lyra et al., 2014).

The Xpert MTB (Cepheid, Sunnyvale, CA, United States) is a fully automated assay based on real-time PCR for the detection of TB and RIF resistance performed directly on untreated sputum. The Assay was endorsed by WHO in 2010 for use at the point of care (hospital) (Steingart et al., 2014).

2.10: Diagnosis of drug-resistant Tuberculosis

In 2013, the WHO estimated that less than half of all DR-TB cases were detected globally. Undetected resistance cases result in treatment failure and increase the risk of TB transmission and death (WHO, 2014). The diagnosis of MDR-TB and XDR-TB is hampered by the lack of effective and affordable rapid diagnostic techniques to detect resistance.

Accurate drug susceptibility testing (DST) is considered highly important for both guidance of the therapy and surveillance of drug resistance for MTBC (Aziz et al., 2007, Abuali et al., 2012, Andrews, 2001).

2.10.1: Phenotypic drug susceptibility testing methods

The commonly used DST method for *M. tuberculosis*, involving the preparation of a concentration series of drugs in LJ medium or Middlebrook 7H10/7H11 medium. Inoculation of the bacterial cultures on the Middlebrook 7H10/7H11 plates or LJ slants, and reading of the inhibition of growth by drugs at different concentrations (Canetti et al., 1969).

Although many settings still use this method, other alternative DST methods are become available, including those that use impregnated discs (Wayne and Kraus,1966) or the

Etest (Hazbon et al, 2000), the radiometric BACTEC 460TB method (Siddiqi,1995), the automated non-radiometric Mycobacteria Growth Indicator Tube (MGIT) 960 system (Siddiqi et al., 2012), the colorimetric test, and the microtiter-based Alamar Blue assay (Franzblau et al,1998)

2.10.1.1: Phenotypic susceptibility testing using solid agar

Conventional DST on solid media is the gold standard for DST of *M. tuberculosis* isolates and is still utilized in many countries worldwide. The methodologies used for conventional DST on egg-based LJ medium are the proportion method, the absolute concentration method and the resistance ratio method. But for agar-based Middlebrook 7H10 or 7H11 medium, only the proportion method is used.

The proportion method is commonly used globally and it is the only method validated for use and approved by WHO for against FLDs and SLDs. These methods require plates with wells or plates which contain test concentrations of drugs and controls and are restricted to discriminating between resistance (R)/susceptible (S) rather than determining the exact MICs on cultured isolates (Canetti et al., 1969, Abuali et al., 2012).

2.10.1.2: Phenotypic susceptibility testing using liquid media

A commercial broth-based proportion method based on the same principle as the solid agar method was introduced and endorsed by WHO, and is the BACTEC MGIT 960 system. It is based on fluorometric technology for the detection of oxygen consumption generated by mycobacterial growth inside the tubes.

Detection of fluorescence in the drug-containing tubes determines a strain as resistant. Critical concentration (cc) of each antimycobacterial drug is prepared from kits provided by the supplier (Siddiqi et al., 2012). Liquid culture is more rapid and sensitive than culture in Middlebrook 7H10 or 7H11 medium /LJ medium but is costly and is more susceptible to contamination. Middlebrook 7H10 or 7H11 medium /LJ medium culture

medium is too slow to have an impact on patient management (Rusch-Gerdes et al., 2006).

2.10.1.3: Minimum inhibitory concentration (MIC) testing using the MYCOTB Plates

The Sensititre MYCOTB MIC plate (MYCOTB; Trek Diagnostic Systems, Cleveland, OH) is a 96 well microtitre plate containing 12 antimycobacterial drugs at different dilution (Figure 2.6). The MYCOTB plate is configured for determination of MICs of FLDs and SLDs. Compared with other *M. tuberculosis* complex susceptibility methods, which test one critical concentration of a drug, the MycoTB plate examines a range of antimycobacterial drug concentrations and produces an MIC result. For each antimycobacterial drug the lowest concentration with no visible growth is considered to be the MIC (Abuali et al., 2012).

	1	2	3	4	5	6	7	8	9	10	11	12
A	OFL 32	MXF 8	RIF 16	AMI 16	STR 32	RFB 16	PAS 64	ETH 40	CYC 256	INH 4	KAN 40	EMB 32
B	OFL 16	MXF 4	RIF 8	AMI 8	STR 16	RFB 8	PAS 32	ETH 20	CYC 128	INH 2	KAN 20	EMB 16
C	OFL 8	MXF 2	RIF 4	AMI 4	STR 8	RFB 4	PAS 16	ETH 10	CYC 64	INH 1	KAN 10	EMB 8
D	OFL 4	MXF 1	RIF 2	AMI 2	STR 4	RFB 2	PAS 8	ETH 5	CYC 32	INH 0.5	KAN 5	EMB 4
E	OFL 2	MXF 0.5	RIF 1	AMI 1	STR 2	RFB 1	PAS 4	ETH 2.5	CYC 16	INH 0.25	KAN 2.5	EMB 2
F	OFL 1	MXF 0.25	RIF 0.5	AMI 0.5	STR 1	RFB 0.5	PAS 2	ETH 1.2	CYC 8	INH 0.12	KAN 1.2	EMB 1
G	OFL 0.5	MXF 0.12	RIF 0.25	AMI 0.25	STR 0.5	RFB 0.25	PAS 1	ETH 0.6	CYC 4	INH 0.06	KAN 0.6	EMB 0.5
H	OFL 0.25	MXF 0.06	RIF 0.12	AMI 0.12	STR 0.25	RFB 0.12	PAS 0.5	ETH 0.3	CYC 2	INH 0.03	**POS**	**POS**

Figure 2.6: Schematic of the MYCOTB plate showing antimycobacterials and their concentrations in micrograms/milliliter.

2.10.1.4: Minimum inhibitory concentration (MIC) testing using Microplate-based Alamar Blue assay (MABA)

The Alamar Blue® Assay incorporates a fluorometric or colorimetric growth indicator based on detection of metabolic activity of mycobacteria. The system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from the growth of mycobacteria.

The resazurin microtiter assay (REMA) plate method is performed in 7H9-S medium containing Middlebrook broth, 0.1% Casitone, and 0.5% glycerol and supplemented with oleic acid, albumin, dextrose, and catalase (Becton-Dickinson). The antimycobacterial drugs are diluted in Middlebrook 7H9-S medium. Serial two-fold dilutions of each drug are prepared and distributed in wells in a 96-well plate (Palomino et al., 2002).

2.10.1.5: Microscopic-observation drug-susceptibility assay (MODS)

The MODS assay is a liquid culture method which is based on the microscopic detection of characteristic *M. tuberculosis* morphology indicating growth. It can be performed from cultures and directly from sediment samples. The samples are inoculated into wells of tissue culture plates containing liquid Middlebrook 7H9 medium and antimycobacterial drugs with subsequent visualization of growth using an inverted light microscope (Moore et al., 2006).

2.10.2: Molecular susceptibility testing methods

Since resistance is caused by genetic mutations, another approach is to detect the mutations themselves. Many mutations associated with resistance have been identified and molecular assays have been developed for their detection (Zhang and Young, 1994).

The advantages of molecular assays for DST include rapid turn-around times, but the limitations include a low sensitivity for demonstrating resistance determining mutations involving some compounds, and a major issue is a cost. However, some assays such as the GeneXpert are extremely easy to use and can even be taken out of the laboratory setting and used as a “near” point of care test (Zhang and Young, 1994, Abuali et al., 2012).

There are currently two types of genotypic assays that have been endorsed by the WHO for use in high incidence settings. These are the Molecular Beacon Assays (MBA) and Line Probe Assays (LPA) (Steingart et al., 2014, Nikolayevskyy et al., 2009).

2.10.2.1: Molecular Beacon Assays (MBA)

Beacon assays detect MTBC and associated RIF resistance directly from sputum samples using PCR. The GeneXpert TB assay is automated from DNA extraction to the PCR and post-PCR analysis. The WHO encouraged the use of the instrument due to its rapid TAT and can be used at the point of care (POC), but it has a number of limitations including cost and only able to detect resistance to RIF (Steingart et al., 2014).

2.10.2.2: Line Probe Assay (LPA)

GenoType MTBDRplus assay (Hain Lifescience, Germany) is based on DNA-Strip technology. The procedure is divided into the following steps: DNA extraction from a decontaminated clinical specimen, a multiplex Polymerase Chain Reaction (PCR) and amplicon detection by reverse hybridization.

All reagents needed for PCR (polymerase and primers) are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized specifically for the test. The membrane strips are coated with specific probes complementary to the amplified nucleic acid. After chemical denaturation, single-stranded amplicons bind to the probes (hybridization).

Highly specific binding of complementary DNA strands is ensured by stringent conditions which result from the combination of buffer composition and a certain temperature. The probes will discriminate several sequence variations in the gene regions examined. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. The binding is detected colorimetrically, usually as visible bands corresponding to the presence of TB and a sensitive or resistant genotype (WHO,2008).

Currently, the main commercial assays for the rapid diagnosis of TB which are endorsed by WHO are GenoType® MTBDR/MTBDRplus and GenoType MTBDRsl Version 2.0 Assay (both Hain Lifescience), as well as the Xpert® MTB/RIF (Cepheid, Sunnyvale, CA, United States) (Nikolayevskyy et al., 2009, Steingart et al., 2014).

2.10.2.3: Chip-based technology

The CombiChip Mycobacteria™ Drug-Resistance Detection DNA chip (Pusan, South Korea) is an oligonucleotide microchip coupled with PCR for the detection of mutations associated with resistance to INH and RIF. This oligonucleotide chip is based on detection of point mutations in the *kat*G codon 315, i*nh*A15 and *rpo*B gene region determining INH and RIF resistance.

The PCR-amplified products are hybridized to an oligonucleotide microchip composed of probes that detect various mutants of the *kat*G codon 315, *inh*A15 and target sequences if the *rpo*B gene (Kim et al., 2006).

2.10.2.4: Whole Genome Sequencing (WGS)

Whole genome sequencing (WGS) can provide a rapid and comprehensive view of the complete genotype of *M. tuberculosis*, and thus enables reliable prediction of the phenotypic DST within a clinically relevant timeframe. Identification of known mutations that confer resistance within few days provides the prospect for personalized rather than empirical treatment of drug-resistant tuberculosis, this will lead to improved outcomes (Yu et al., 2015).

2.11: AIM

To evaluate cross-resistance among rifamycins in *M. tuberculosis* clinical isolates

2.12: STUDY OBJECTIVES

- To perform rifabutin and rifapentine drug susceptibility testing (DST) using liquid culture.
- To determine the minimum inhibitory concentration (MIC) using Sentitre MYCOTB Plate for both rifabutin and rifampicin.
- To identify mutations that confer cross-resistance using DNA sequencing.
- To evaluate the use of known line probe assay mutations to predict the susceptibility of rifamycins among rifampicin resistant isolates.

2.13: HYPOTHESIS

All *M. tuberculosis* isolates that are rifampicin resistant will show cross resistance to other rifamycins.

2.14: ALTERNATIVE HYPOTHESIS

Not all *M. tuberculosis* isolates which are rifampicin resistant are resistance to other rifamycins.

2.15: RESEARCH QUESTION

Is there complete cross resistance between rifamycins and can specific *rpo*B mutations be used to predict susceptibility to the individual antimycobacterial drugs in the rifamycin class?

CHAPTER 3

3.1: METHODOLOGY

3.1.1: Bacterial strain selection and isolate preparation

The isolates were collected as part of ongoing laboratory-based surveillance of rifampicin-resistant (RIF-R) Tuberculosis (TB) named Group for Enteric Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA). Samples identified as RIF resistant isolates by GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, United States) from sentinel surveillance sites (hospitals) in selected high-burden districts in South Africa are included.

The surveillance was implemented at the district level, with one district targeted per province known to be hot spot areas of TB. For the current study, 300 isolates collected between 2015 to 2016 were used. These isolates were from six districts including the Nelson Mandela Metro (Eastern Cape), Ehlanzeni (Mpumalanga), Dr Kenneth Kaunda (North West)**,** Frances Baard (Northern Cape), Umgungunglovo (KZN) and City of Johannesburg (D) (Gauteng). The above mentioned districts are shown in Figure 3.1.

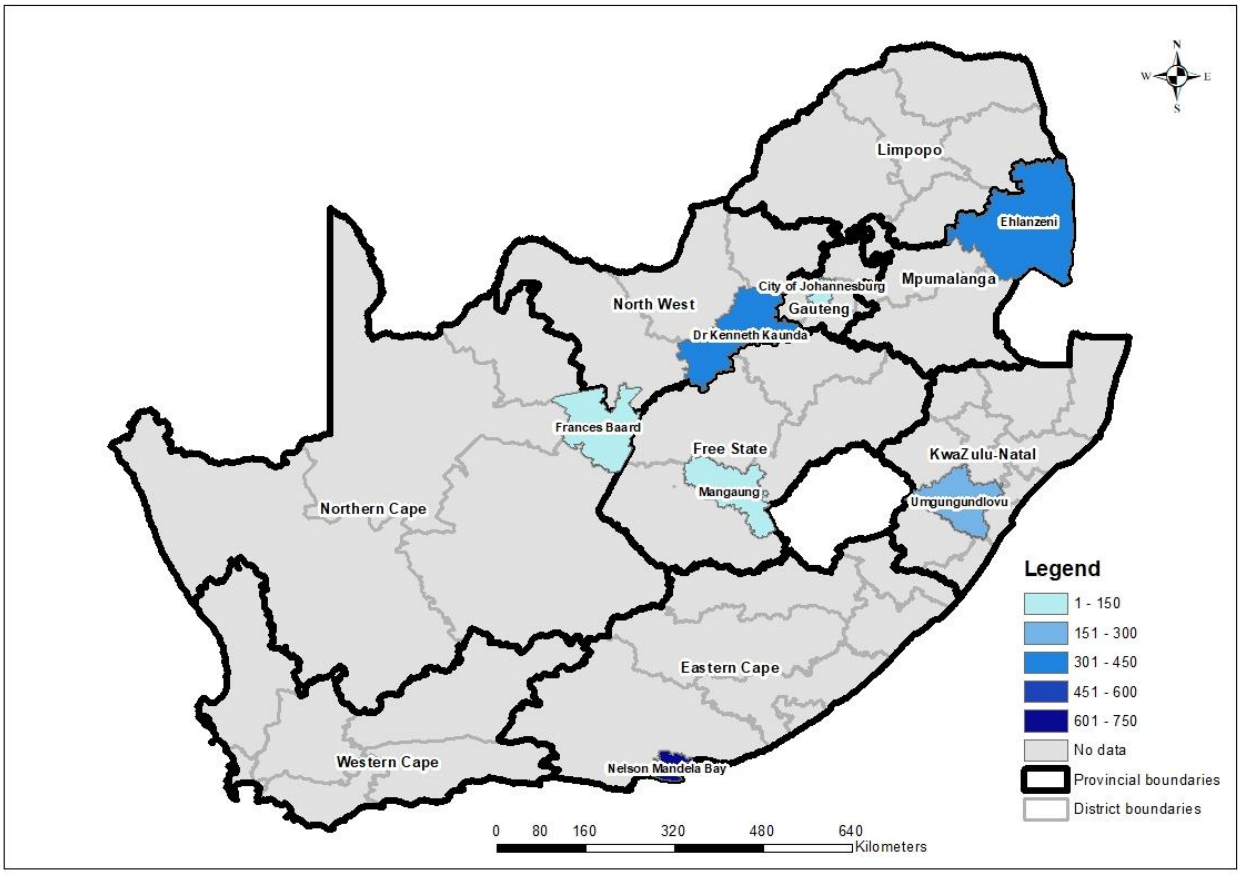


Figure 3.1: Map showing six selected districts of South Africa

The study was conducted at National Health Laboratory Services (NHLS); Centre for Tuberculosis (CTB) which is the Supranational Referral Laboratory (SRL), all the experiments were performed under biological Safety Cabinet (BSC)-level III, the cabinet was class II Type A1 (Figure 3.2). All the clinical isolates were stored in Middlebrook 7H9 broth containing glycerol at -70°C.

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Figure 3.2: Photos taken outside Centre for Tuberculosis, Sandringham, South Africa were Laboratory work was conducted.

All the laboratory experiments and the work flow of the present study are illustrated in Figure 3.3

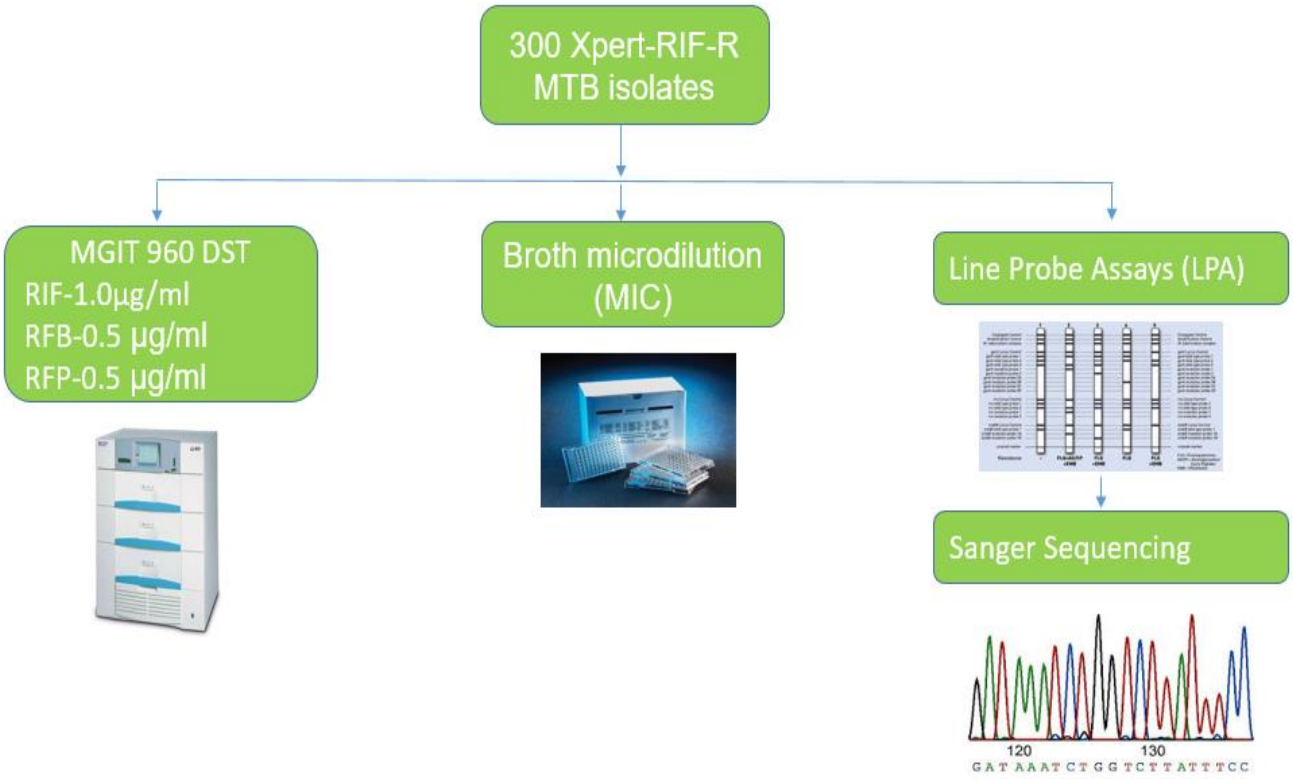


Figure 3.3: The workflow of the Project

3.1.2: Drug susceptibility testing (DST) using the MGIT 960 system

All the frozen isolates were thawed and sub-cultured on liquid medium with the MGIT960 system (BD Diagnostics, Sparks, MD, USA), prior to testing. After flagging positive the isolates were removed and prepared for Drug susceptibility testing (DST). Drug susceptibility testing (DST) was performed according to the manufacturer's instructions (Siddiqi et al., 2012).

Drug concentrations used for rifampicin (RIF), rifabutin (RFB) and rifapentine (RFP) testing were 1.0 µg/ml, 0.5 µg/ml and 0.5 µg/ml respectively. Briefly, 7 ml MGIT tubes containing Middlebrook 7H9 broth were supplemented with 0.8 ml of supplement and were inoculated with 0.1 ml of the antimycobacterial drug solution and 0.5 ml of the test

microorganism suspension (NCCLS,2000). For the preparation of the drug-free growth control tube (GC), the microorganism suspension was diluted 1:100 with sterile saline, and then 0.5 ml was inoculated into the tube (Figure 3.4).

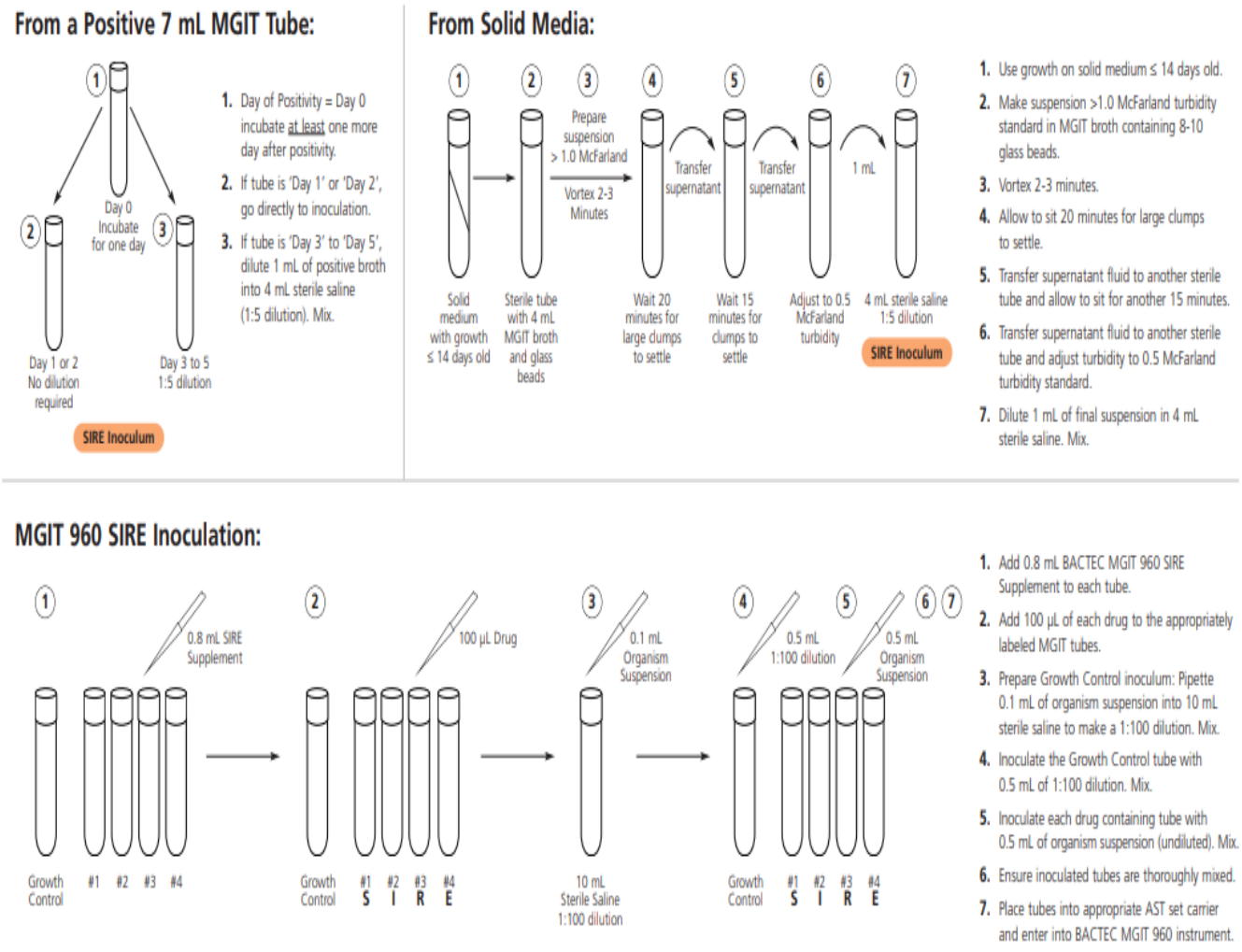


Figure 3.4: BD BACTEC™ MGIT™ 960 SIRE Preparation and Inoculation

The susceptibility testing sets were mixed by inverting them 3-4 times and were placed in the MGIT 960 instrument and continuously monitored using EpiCenter (version 6.10 A) TB eXiST software. For all the isolates which were run per batch, a fully drug-susceptible *M. tuberculosis* ATCC27294 H37Rv was included to evaluate the potency of the antimycobacterial drugs. Results were interpreted as follows. At the time when the

GU of the drug-free GC tube was >400, if the GU of the drug-containing tube to be compared was ≥100, the strain was resistant (R). If the GU of the drug-containing tube was <100 the strain was susceptible (S) after 4 days (Siddiqi et al., 2012).

3.1.3: Minimum inhibitory concentration (MIC) testing using the MYCOTB Plates

The Minimum inhibitory concentration (MIC) testing using Sensititre MYCOTB MIC plate (MYCOTB; Trek Diagnostic Systems, Cleveland, OH) was performed using the manufacturer's instructions. The primary MGIT tubes were sub-cultured by pipetting of 0.5 ml of *M. tuberculosis* on Middlebrook 7H10 agar plate. The Middlebrook 7H10 agar plates were tilted 3-4 times for the microorganism to cover almost the whole plate and were sealed with parafilm and incubated at 37°C for up to 6 weeks.

Colonies were scraped from a Middlebrook 7H10 agar plate and vortexed in a sterile saline–tween tube with glass beads. The inoculum was allowed to settle for 15-30 min and then adjusted to match a 0.5 McFarland standard. Then 100µl of suspension was transferred into a tube containing 11 ml Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (OADC), to yield $1x10^5$ CFU/ml. A 100 µl aliquot of the final inoculum was transferred into each well (drug-containing and two GC wells), and the plate was covered with a seal and incubated at 37°C.

The MYCOTB plates were read between 7-10 days manually and then recorded with the computerized Vizion system (Trek Diagnostic Systems, Cleveland, OH, USA). The Vizion system projects an image of the plate onto a computer screen, allowing the operator to select the MIC value for each antimycobacterial drug.

For quality assurance (QA), a fully drug-susceptible *M. tuberculosis* ATCC27294 H37Rv was always included to evaluate the potency of antimycobacterial drugs in the MYCOTB Plates. When reading the plate, the GC wells were reported as being valid or invalid based on visible growth, and the MIC value for each drug was recorded onto a data

sheet as the first antimycobacterial drug-containing well with no visualized *M. tuberculosis* growth (Abuali et al., 2012).

- For determinations of resistance by the MycoTB plate method, an isolate was considered resistant if the MIC was higher than the APM critical concentration (1.0 µg/ml for RIF and 0.5 µg/ml for RFB)
- For determinations of susceptibility by the MYCOTB plate method, an isolate was considered susceptible if the MIC was lower than or equivalent to the APM critical concentration (1.0 µg/ml for RIF and 0.5 µg/ml for RFB).

3.1.4: The GenoType MTBDRplus Version 2.0 assay

The GenoType MTBDR plus Version 2.0 assay (Hain Lifescience GmbH, Nehren, Germany) was performed on all isolates to detect *rpo*B gene mutations and to determine if specific *rpoB* mutations can predict differential phenotypic susceptibility to RIF and RFB.

DNA extraction: DNA extraction was performed using NucliSENS® easyMAG® (Durham, NC) according to the manufacturer's instruction. The DNA was extracted from the MGIT primary tube. Briefly, 3 ml from the base of primary MGIT culture tube was transferred into 5ml sterile round-bottomed tubes (BD falcon).

The specimens were loaded into the aerosol free centrifuge and they were centrifuged at 4000 rpm for 20 minutes. After the centrifuging 2 ml of the supernatant was aspirated and discarded. Three glass beads were added into each BD falcon tube and vortexed all the tubes for 10 minutes on MultiReax (Heidolph).

The specimen tubes were then placed in the 80°C oven for 20 minutes to heat kill the bacilli. One ml of sample was loaded into the cartridge and the generic protocol selected was with a sample input of 1.0 ml and elution volume of 50 µl. After DNA extraction, microtubes were labelled and 50µl was aliquoted into each tube and stored in the -20°C for later use (Loens et al., 2007).

The Genotype MTBDR Version 2.0 assay was performed as recommended by the supplier. The Amplification Mixture A (AM-A) and Amplification B (AM-B) were removed from the package and the PCR tubes were labeled. Ten microliters of AM-A and 35µl of AM-B were transferred to the labeled PCR tubes. A total of 10µl of DNA was transferred to all the PCR tube. Positive (*M. tuberculosis* ATCC27294 H37Rv) and negative (DNA free water) controls were included in each test. The amplification parameters which were used are shown in Table 3.1.

Table 3.1: LPA amplification parameters

15 min	95°C	1 cycle
30 min	95°C	10 cycles
2 min	65°C	
25 sec	95°C	
40 sec	50°C	20 cycles
40 sec	70°C	
8 min	70°C	1 cycle

A fully automated instrument, the GT-Blot 48 (Hain Lifescience GmbH, Nehren, Germany) was used for hybridization. Briefly, the GT-Blot tray was put in a tilted position, 20 µl of denaturation solution and 20 µl of amplicons was added into each well and incubated at room temperature for 5 min. The tray was placed into the GT-Blot 48.

The instrument dispensed pre-warmed hybridization reagent (HYB) into the tray and the strips were added. The door was closed and the machine was started. After aspiration was completed, the door was opened and the tray was put on the bench. After hybridization was completed the tray was inverted into a paper towel to get rid of excess water.

The tray was inserted in the drawer of the Genoscan (Hain Lifescience, Nehren, Germany) reader and strips were scanned. Eight wild-type (WT) probes and four mutations (MUT) probes were used to analyze the RRDR *rpo*B region (Figure 3.5).

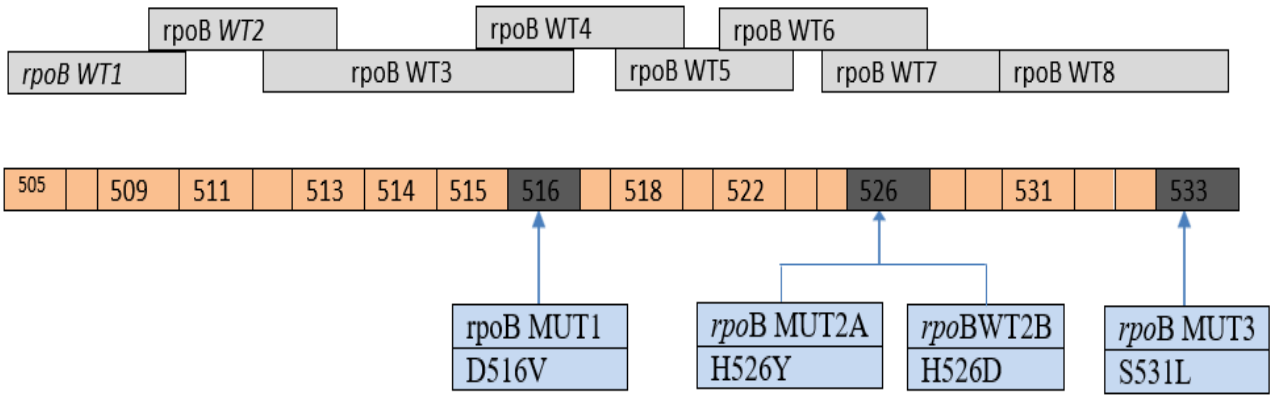


Figure 3.5: Rifampicin resistant determining region of the *rpo*B with wild-type probes and mutation probes of GenoType® MTBDR/MTBDRplus (Hain Life science package insert,2009).

The probes for detecting target sequences were as follows: WT1 (codons 505 to 509), WT2 (codons 510 to 513), WT3 (codons 513 to 517), WT4 (codons 516 to 519), WT5 (codons 518 to 522), WT6 (codons 522 to 525), WT7 (codons 526 to 529), WT8 (codons 530 to 533), MUT1 (codon D516V), MUT2A (codon H526Y), MUT2B (codon H526D), and MUT3 (codon S531L).

When all the WT probes gave a positive signal and all the MUT probes reacted negatively, the *M. tuberculosis* isolate was considered susceptible to RIF. When one or two negative signal was obtained with the WT probes, the isolate was considered RIF resistant.

3.1.5: Sanger Sequencing on the *rpo*B gene sequencing

Due to the overlapping design of probes for detection of RRDR *rpo*B mutations using Genotype MTBDR assay at codons 513, 516, and 522, absence of each of the WT2 and WT3, WT3 and WT4, and WT5 and WT6 pairs could represent a single mutation.

The isolates with missing WT without a defined mutation probe, DNA was extracted by NucliSENS® easyMAG® (Durham, NC) and were subsequently sent for Sanger sequencing to Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). The PCR and Sanger sequencing was performed as previously described by Rahman et al, 2016. The forward and reverse primers are shown in Table 3.2.

Table 3.2: *rpo*B primers used to amplify the RRDR region of the *rpo*B gene.

Primer Name	**Description**	**5'->3' sequence**
rpoB-1F	Forward	CTTGCACGAGGGTCAGACCA
rpoB-2R	Reverse	ATCTCGTCGCTAACCACGCC

3.1.6: Data analysis

The percentage of categorical agreement between MycoTB and BACTEC MGIT 960 system for RIF and RFB was calculated. Sensitivity, specificity, positive and negative predictive values of MycoTB compared to the BACTEC MGIT 960 system were also calculated for RFB.

- Categorical agreement (CA) between MYCOTB plate method and MGIT 960 system. The isolate was considered to be in agreement if both MGIT and MYCOTB characterized the isolate as susceptible or both MYCOTB and MGIT 960 system characterized the isolate as resistant

- Sensitivity of MycoTB plate method. For determination of sensitivity of MycoTB for detection of drug resistance. Sensitivity = (number of MGIT-resistant isolates identified as resistant by MycoTB) / [(number of MGIT-resistant isolates identified as resistant by MycoTB) + (number of MGIT-resistant isolates identified as susceptible by MycoTB)] x100
- Specificity of MycoTB plate method. For determination of specificity of MycoTB for detection of drug resistance. Specificity = (number of MGIT-susceptible isolates identified as susceptible by MycoTB)/ [(number of MGIT-susceptible isolates identified as susceptible by MycoTB + number of MGIT-susceptible isolates identified as resistant by MycoTB)] x 100
- Positive Predictive Value (PPV). For determination of Positive Predictive Value of MycoTB plate method for detection of drug resistance. PPV = (number of MGIT-resistant isolates identified as resistant by MycoTB) / [(number of MGIT-resistant isolates identified as resistant by MycoTB + number of MGIT susceptible identified as resistant by MycoTB)] x100
- Negative Predictive Value (PPV). For determination of Negative Predictive Value of MycoTB plate method for detection of drug resistance. NPV = (number of MGIT-susceptible isolates identified as susceptible by MycoTB) / [(number of MGIT-susceptible isolates identified as susceptible by MycoTB + number of MGIT resistant identified as susceptible by MycoTB)] x 100

Kruskal–Wallis test was used to determine whether RRDR *rpo*B mutations were associated with differences in RIF and RFB MICs. The eclplots were constructed using STATA 14.

Kappa is an index that measures the level of agreement between two independent assays evaluating the same thing. Kappa value is calculated for determining concordance between MycoTB and BACTEC MGIT 960 system for RFB. The kappa calculation is based on the difference between how much agreement is actually observed compared to how much agreement would be expected to be present. The

perfect agreement would equate to a k of 1, and the chance agreement would equate to 0.

Table: 3.3: Guidelines for Strength of Agreement

Guidelines for Strength of Agreement Indicated with *k* Value	
k Value	strength of agreement
<0	poor
0-.020	slight
0.21-0.40	fair
0.41-0.60	moderate
0.61-0.80	substantial
0.81-1.00	almost perfect
Note.—Data are from Landis and Koch (Landis and Koch, 1977)	

3.1.7: Ethics Approval

Ethics approval for this study was granted by the University of the Witwatersrand Human Research (Medical) Ethics Committee. The Ethics Clearance Certificate Number was M150754.

CHAPTER 4

4.1: RESULTS

4.1.1: Proportion of cross resistance among rifamycins

A total of 300 rifampicin (RIF) resistant isolates were tested by BACTEC™ Mycobacteria Growth Indicator Tube (MGIT) 960 System (BD Diagnostics, Sparks, MD, USA) and MYCOTB plate. Among the isolates tested by MGIT 960 System, 216 (72%) were cross-resistant to all three rifamycins while 78 (26%) showed resistance to RIF and rifapentine (RFP) but not to rifabutin (RFB). Only 1 (0.3%) isolate was susceptible to RFP but resistant to RIF and RFB and 5 (2%) isolates were RIF resistant but found to be susceptible to RFB and RFP.

4.1.2: Comparison of MycoTB plate and MGIT 960 method

According to the MycoTB plate 212 (71%) isolates were cross-resistant to both RIF and RFB while 88 (29%) were susceptible to RFB. RFP was not tested as it is not included in the MycoTB MIC panel. The categorical agreement (CA) between MGIT and MycoTB methods was 94% (Table 4.1).

The sensitivity, specificity, positive predictive value and negative predictive value of MycoTB plate against MGIT 960 was 94% (CI;90,54-97,11%), 92% (CI;83,39-96,54%), 97%(CI;93,51-98,35%) and 86% (CI;78,45-91.68%), respectively.

Table 4.1: Comparison of MycoTB plate MIC results to MGIT critical concentration results

	resistant on MGIT	Susceptible on MGIT	total
resistant on MycoTB	205 (a)	7 (c)	212
Susceptible on MycoTB	12 (b)	76 (d)	88
Total	217	83	300

a-True positives: b-False negatives; c-False positives; d-True negatives

4.1.3: The kappa value calculations between MGIT and MycoTB methods for RFB

The Cohen's kappa value was found to be 0.85 (calculations are shown below).

$$\text{Cohen's kappa value} = \frac{\text{categorical agreement (CA)- Expected agreement (EA)}}{\text{1-Expected agreement (EA)}}$$

$$k = \frac{CA - EA}{1 - EA}$$

The categorical agreement (CA) between MGIT and MycoTB methods for RFB:

$$CA = \frac{a+d}{a+b+c+d}$$

CA= 0.94

The expected agreement (EA) between MGIT and MycoTB methods for RFB:

The expected probability (EP) that both would call Resistant at random is:

$$\text{EA-Resistant} = \frac{a+b}{a+b+c+d} \cdot \frac{a+c}{a+b+c+d}$$

EA-Resistant=0.72 x 0.70 =0.54

The expected probability (EP) that both would call Susceptible at random is:

$$\text{EA-Susceptible} = \frac{c+d}{a+b+c+d} \cdot \frac{b+d}{a+b+c+d}$$

EA-Susceptible =0.27 x 0.29 =0.078

Expected agreement (EA)= EA-Resistant + EA-Susceptible =0.54+0.078

Expected agreement (EA)=0.618

$$k = \frac{CA - EA}{1 - EA}$$

$$k = \frac{0.94-0.618}{1-0.618}$$

k=0.85

Table 4.2: MycoTB plate MIC results compared to MGIT critical concentration for RFB.

Statistic	Formula	Percentage	95% CI
sensitivity	$\frac{a}{a+b}$	94.47%	90.54-97.11%
specificity	$\frac{d}{c+d}$	91.57%	83.39-96.54%
Positive Predictive value	$\frac{a}{a+c}$	96.70%	93.51-98.35%
Negative Predictive Value	$\frac{d}{b+d}$	86.36%	78.45-91.68%
Accuracy	$\frac{a+d}{a+b+c+d}$	93.66%	90.29-96.14 %

a-True positives: b-False negatives; c-False positives; d-True negatives

4.1.4: Frequency of the mutations among 300 RIF resistant isolates.

Among 300 isolates tested, the most frequent *rpo*B RRDR mutation was S531L (n = 153, 51%), followed by D516V (n=52, 17%), H526Y (n=32, 11%) and H526D (n=20,7%). Mutations L533P and H526L were found in 5 isolates each which is 2%. While mutations H526R, S531W, S522L and L511P were present in 3 isolates each (1%). The mutation D516Y was observed in 4 (1%) isolates.

The remaining mutations were only observed in single isolates (i.e., D516del, D516G-L533P and D516delinsHis) which collectively accounted for <1%. There were 14 (5%) isolates that had no mutation in the *rpo*B RRDR (Table 4.3). Out of the 216 isolates that showed cross-resistance to all three rifamycins by MGIT 960 method, S531L was the most frequently observed *rpo*B RRDR mutation, found in 145 (66%) isolates, followed by H526Y and H526D which were 30 (14%) and 20 (9%) respectively (Table 4.3).

All isolates with H526R, (n=3) and S531W (n=3) were associated with cross resistance to all rifamycins. A total of 6 (3%) isolates resistant to all 3 rifamycins had a wild-type suggesting mutations outside the RRDR region the presence of another mechanism of resistance.

4.1.5: Contribution of *rpo*B mutations to rifamycin differential resistance

Among the 83 RIF-resistant/RFB-susceptible isolates, D516V was the most frequently observed *rpoB* RRDR mutation, found in 50 (60%) isolates, followed by S531L and H526L in 8 (9%) and 5 (6%) respectively. Seven out of the 83 (8%) isolates that were RIF-resistant/RFB-susceptible had no mutations in the RRDR. The mutations L533P and D516Y were observed in 4/83 (5%) and 4/83(5%) respectively. Mutations H526Y, D516delinsHis, S522L and L511P were only observed once among RIF-resistant/RFB-susceptible isolates (Table 4.3).

Table 4.3: Distribution of *rpoB* mutations and of drug susceptibility testing of rifampin, rifabutin and rifapentine

		MGIT 960 Resistance profile against RIF-RFB-RFP				MYCOTB Resistance profile against RIF-RFB	
Mutations	n	R-R-R	R-S-R	R-R-S	R-S-S	R-R	R-S
D516del	1	1				1	
D516G,L533P	1	1				1	
H526R	3	3				3	
H526D	20	20				18	2
H526Y	32	30	1		1	31	1
L533P	5	1	4			3	2
S531L	153	145	6	1	1	141	12
Wild-type	14	6	7		1	7	7
D516delinsHis	1		1				1
D516V	52	2	49		1	1	51
H526L	5		5			1	4
S522L	3	2	1			1	2
L511P	3	2	1			2	1
S531W	3	3				2	1
D516Y	4		3		1	-	4
Total	300	216	78	1	5	212	88

RIF=rifampicin, RFB=rifabutin, RFP=rifapentine, R=resistant; S susceptible

4.1.6: Comparison of RIF and RFB MIC ranges and medians by rpoB mutations

This study showed a wide range of RFB and RIF MICs seen in isolates with the same RRDR *rpo*B mutations. When considering the level of resistance, strains with mutation S531W had RIF MICs ≥8 µg/ml while RFB MICs were ≤8 µg/ml. The S531L mutation had a median MIC of 16 µg/ml for RIF and 2µg/ml for RFB.

In addition, both H526Y and H526D mutations had a median MIC of 16 µg/ml for RIF, while for RFB was 4 µg/ml. The mutation H526R was found in 3 isolates, all of them had RIF MICs which are ≥16 µg/ml and RFB MICs ranging between 1-16 µg/ml with median MIC of 8µg/ml for RFB and 16 µg/ml for RIF.

The majority of D516V mutations (51/52) were found among the RFB susceptible isolates with MICs of ≤0.5µg/ml. The median MIC for RIF with mutation D516V was found to be 12 µg/ml while for RFB was 5 µg/ml (Figure 4.1).

Both mutation L511P and L533P had RFB median of 1 µg/ml and 8 and 16 µg/ml for RIF respectively. The mutations D516Y (4/4) and H526L (4/5) were found to confer medium levels of resistance with RFB MICs of ≤2 µg/ml while for RIF was ≥2 µg/ml. The isolates with mutation S522L had RIF MICs ranging between 4-16 µg/ml which for RFB was ranging between 0.12-2 µg/ml.

The mutation H526L had RIF MIC ranging between 4-16 µg/ml while for RFB 0.12-2 µg/ml while the median MIC for RIF was 4 µg/ml and 1 µg/ml for RFB. One isolate with mutation D516delinsHis had RIF MIC ≤0.5µg/ml and RIF MIC ≥16 µg/ml. One isolate with mutation D516del had MIC which is ≥16 µg/ml for both RIF and RFB (Figure 4.1).

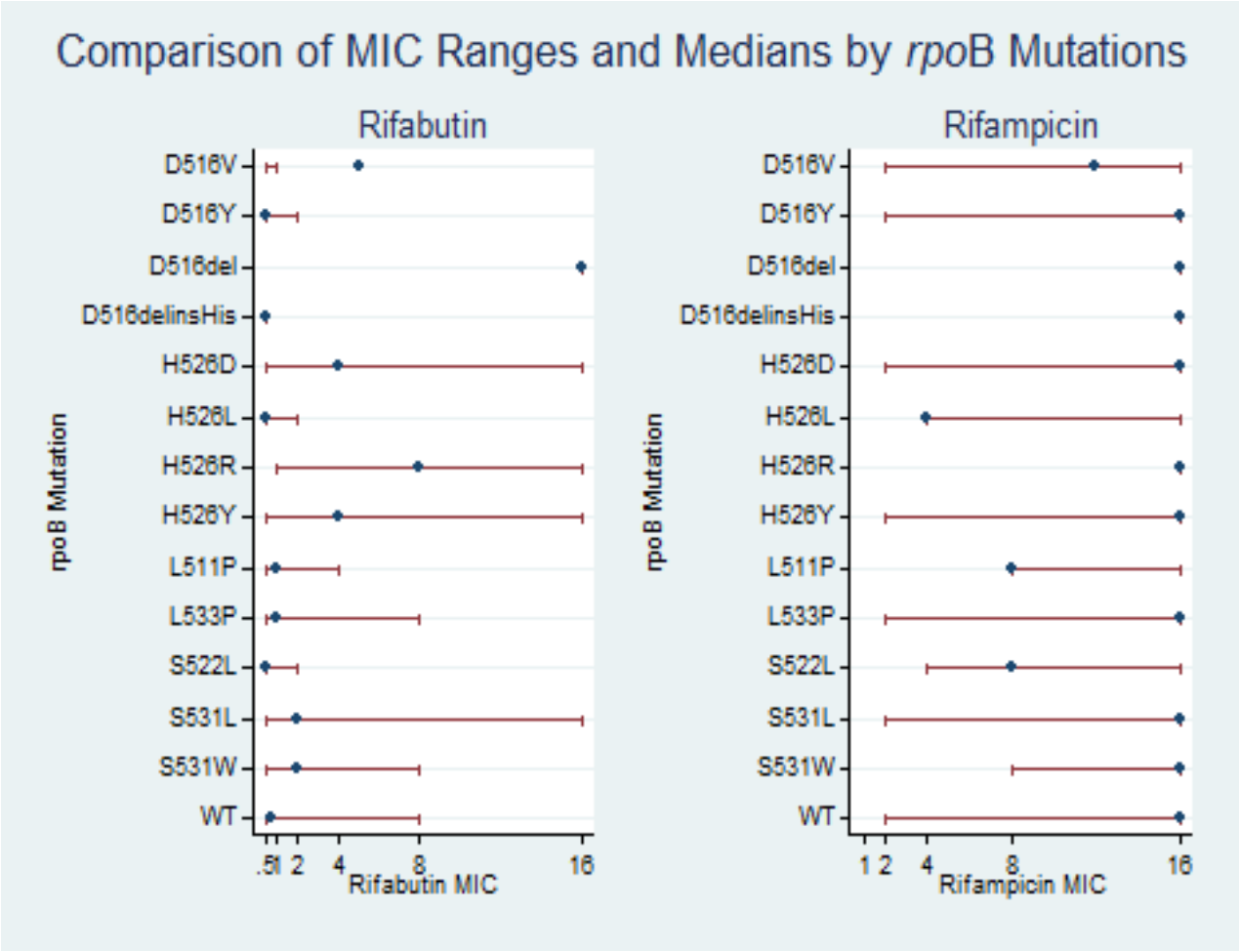


FIGURE 4.1: Comparison of RIF and RFB MIC ranges and medians by *rpo*B mutations

CHAPTER 5

DISCUSSION

Rifamycins are a group of structurally similar drugs including rifampin (RIF), rifapentine (RFP), rifabutin (RFB) and rifalazil (RFL). Of these rifamycins, RIF is the most potent antimycobacterial drug in the current treatment of active tuberculosis regimen. Due to the structural similarity, it is usually assumed that RIF-resistant *M. tuberculosis* isolates are also resistant to all rifamycins.

Although cross-resistance with other rifamycins can occur, other studies showed that some *rpo*B mutations, confer resistance to RIF but still retain susceptibility to other rifamycins, such as RFB or RFL, suggesting RFB/RFL could be alternative drug for treatment of *M. tuberculosis* (Chikamatsu et al., 2009, Jamieson et al., 2014. Williams et al., 1998). Therefore, the present study evaluated cross resistance among RIF, RFB and RFP and if specific *rpo*B mutations can predict differential phenotypic resistance to RIF and RFB.

The study also investigated the association of *rpo*B mutation and resistance levels of RFB based on the determination of MICs with Sensititre plate method. Such information could have implications for the selection of drug treatment regimens in the management of RIF resistant TB.

In this study, overall cross resistance among the three rifamycins was found to be 72% for all the isolates tested by MGIT 960 method. The cross resistance was higher between RIF and RFP (98%) as compared to RIF and RFB (74%). Although there is no enough literature regarding cross resistance between RIF and RFP, a study performed in the USA showed 100% cross resistance between RIF and RFP (Williams et al., 1998).

The high cross resistance between RIF and RFP suggests that the use of RFP among RIF resistant strains will not make any significant difference. The cross resistance between RIF and RFB was slightly lower with MycoTB plate method when compared to MGIT (71%). Previous studies have reported differential resistance between RIF and RFB (Jamieson et al., 2014, Berrada et al., 2016, Williams et al., 1998) and showed

about one-fourth of RIF-resistant *M. tuberculosis* isolates to be RFB-susceptible. In this study, 26% and 29% of RIF resistant isolates were RFB-susceptible by MGIT method and MycoTB plate method, respectively.

This is in line with previous studies conducted in countries such as Canada, USA and Sweden, where RIF-resistance/RFB-susceptible isolates were found in 15-30% of MTB cases (Jamieson et al., 2014, Schön et al., 2013, ElMaraachli et al.,2015). This finding indicates that RFB may be active against 26-29% of RIF resistant TB cases and could be beneficial for the treatment of RIF resistant cases. The study done in South Africa (SA) and Belgium showed that the 11 RRDR *rpo*B mutations associated with RFB susceptibility representing 33.2% of South African patients and 16.6% of Belgian patients determined as RIF resistant by Xpert MTB/RIF (Whitfield et al,2018).

The study conducted in South Korea found higher treatment success rates in RFB-susceptible MDR-TB patients receiving treatment regimen that contains RFB as an additional drug as compared to RFB resistant MDR-TB patients receiving a drug susceptibility testing (DST) guided treatment regimen (85.7% against 52.4% success rates) (Jo et al., 2013).

In this study, the Genotype MTBDR assay was used for rapid detection of mutations in the rifampicin resistance determining region (RRDR) region of the *rpo*B gene and strains with inconclusive results were also sequenced. The Genotype MTBDR assay was able to detect the majority (85%) of *rpo*B gene mutations, with only 15% of the isolates with uninterpretable (with missing WT and no corresponding mutation) results. This shows that Genotype MTBDR assay could be used as first line method. Isolates with mutations outside the RRDR of the *rpo*B gene or in cases of phenotypically RIF resistant isolates with wild type profile can be sequenced by WGS. The use of WGS for routine purposes is not practical as it is expensive, requires computational resources and experts to analyse the data.

All the 13 *rpo*B mutations detected within (RRDR) have been reported previously in the TB Drug Resistance Mutation Database (Sandgren et al., 2009). Mutations on codons

516,526 and 531 accounted for more 90% of RIF resistant strains in this study. These mutations S531L, H526D, H526Y and D516V are the most frequently reported mutations in most of the studies and the LPA contains probes which are used to identify them (Gamboa et al., 1998, Chen et al., 2012, Williams et al., 1998, Jamieson et al., 2014, Yoshida et al., 2010).

The S531L was the predominant mutation (67%) among isolates with cross resistance to all rifamycins. Although the previous studies (Chen et al., 2012, Yoshida et al., 2010) showed that the S531L mutation was mostly linked with cross resistant isolates, similarly in this study only 5% with this S531L mutation was RFB susceptible. Considering the scarcity of finding the S531L mutation among RIF and RFB differential resistant strains, the mutation should mainly be associated with RIF and RFB cross resistance.

Other studies show that the mutation S531L can also be found among RIF and RFB differential resistant strains (Jamieson et al., 2014, Rukasha et al., 2016). The mutation S531W was found to affect RIF and RFB susceptibility, there are studies that also support these findings (Chen et al., 2012, Cavusoglu et al., 2004, Yang et al., 1998, Williams et al., 1998, Jamieson et al., 2014).

Certain mutations at codon 526, such as H526Y, H526D and H526R mutation were mostly associated with RIF and RFB cross resistance. However only 6% of strains with H526Y mutation were RFB susceptible. The mutations in codon 516 have been associated with low-level resistance to RIF but susceptibility to RFB (Jamieson et al., 2014, Williams et al., 1998). In this study, the majority (63%) of RIF resistant/RIF susceptible isolates had the D516V mutation.

Yoshida et al, reported 85% of isolates with RIF and RFB differential resistance had the D516V mutation. Another study which was done in the USA also showed 75% of RIF and RFB differential resistance had D516V mutation. The study also showed that 13 % of isolates with the D516V mutation that we predicted would be RIF-R/RFB-S were found to be cross resistant to both and RFB (ElMaraachli et al.,2015).

Although frequency of the D516V mutation among isolates with differential resistance differs, it was found to be the mostly observed mutation in all the studies (Chen et al., 2012, Cavusoglu et al., 2004, Yang et al., 1998, Williams et al., 1998, Jamieson et al., 2014).

In the study done by Whitfield et al, 2018, the D516V mutation was the most frequent, occurring in 23.2% of SA and 12.6% of Belgian RR-TB patients, and representing 69.7% and 76.0% of rifabutin-susceptible RRDR *rpo*B mutations in South Africa and Belgium, respectively. In the present study, most of RIF and RFB differential resistant strains had a single nucleotide substitution at codon 516 (66%). Therefore RFB can be an appropriate substitute for RIF in MDR/XDR-TB cases

All mutations on codon 526 were found among cross resistant isolates with the exception of the H526L mutation. It has been reported that resistance/susceptibility to RFB in isolates with *rpo*B mutations at codon 526 were found to be residue dependent. The previous papers show that a change of amino acid histidine (H) with either tyrosine (Y), arginine (R) or aspartic acid (D) conferred resistance to both RIF and RFB, while a change of the histidine (H) residue with leucine (L) was characterized by RFB susceptibility (Chen et al., 2012, Jamieson et al., 2014, Williams et al., 1998).

The mutation L511P is often referred as ‘’disputed mutation’’ because of the fact that is found in both RIF resistant and susceptible isolates in other studies (Jamieson et al., 2014, Williams et al., 1998). The mutations L533P, S522L and H526L have been reported to confer highly discordant RIF results by phenotypic DST.

In this study, the L511P mutation was found in two of three cross resistant isolates to RIF and RFB and in one RIF-resistant/RFB-susceptible. The other mutation that was found among in both cross and differential resistant isolates was S522L. Williams et al also reported similar discordant results among isolates with this mutation (S522L) (Rigouts et al., 2013, Van Deun et al., 2009, Van Deun et al., 2013).

Although other studies show that the L533P mutation is found among RFB resistant and susceptible strains (Yang et al., 1998, Chen et al., 2012, Williams et al., 1998) however majority (80%) of strains were found to be RFB susceptible. These findings are similar to the study which was conducted in the same setting (NICD) with 75% of L533P being RFB susceptible (Rukasha et al., 2016). The study done in SA and Belgium classified mutations S531L, S531W and H526R as high confidence RFB resistant while mutations H526Y and H526D were minimal and moderate confidence RFB resistant, respectively (Whitfield et al,2018).

Due to concerns over failure to detect RIF-resistance by MGIT 960 Instrument in isolates with these “disputed mutations”, suggestions of lowering the c.c of RIF to 0.5 µg/ml to bring genotype and phenotype into concordance have been made. Lowering the c.c of RIF will improve detection of these isolates. But there is a risk of generating false RIF-resistance, because c.c will be closer to the MIC of WT isolates. False RIF-resistance may lead to the removal of RIF from Tb treatment regimens (Berrada et al., 2016).

The study in Taiwan showed that 70% of isolates with L533P were cross resistant while 30% were RIF and RFB differential resistant from MDR-TB cases (Chen et al., 2012). The strains that had no RRDR *rpo*B mutations (wild type) were also found among RIF and RFB cross resistant group (43%). This could be due to mutations which are outside the target region or due to a different mechanism of resistance such as the efflux pumps (molecules are exported outside the mycobacteria cell) (Pasca et al., 2004).

In this study, the MICs varied among the different *rpo*B mutations. The S531L mutations were found to be associated with high levels of RIF resistance (≥8 µg/ml), as compared to variable levels of resistance to RFB MICs ranging from low to high (0.25-16 µg/ml). A similar finding was reported by a study done in the USA, all isolates with S531L mutation had RIF MIC≥8 µg/ml and RFB MIC ranging between 2-8 µg/ml (Berrada et al., 2016).

The study in Canada showed the RIF MIC of ≥100 µg/ml with RIF MIC was found to be 5 µg/ml against strains harboring S531L mutation (Jamieson et al., 2014). This suggests that in the case of this mutation, an increase of the RIF dose might not influence treatment outcomes of the patient. Therefore, other rifamycin derivatives can be considered to replace RIF. The S531W mutation was also associated with highly RIF resistant (MIC of ≥8 µg/ml), which is in line with studies done in countries like USA, Turkey and Canada (Jamieson et al., 2014, Cavusoglu et al., 2004, Berrada et al., 2016).

More than 94% of strains with H526D, H526R and H526Y had high RIF MIC of ≥8 µg/ml and for RFB resistance conferred different levels of resistance ranging from 0.25-16 µg/ml. All RIF-resistant isolates with D516V mutations had low RFB MIC of ≤0.5 µg/ml with the exception of one isolate with RFB MIC of 1 µg/ml. The D516Y mutation was also associated with high levels of RIF resistance but low RFB MIC of ≤0.5 µg/ml. Therefore, RFB is highly recommended for MDR-TB cases with D516Y mutation based on our findings.

The MycoTB plate method is simple and faster alternative for DST of First-Line Drugs (FLDs) and Second-Line Drugs (SLDs). Unlike other conventional DST methods for *M. tuberculosis*, which only test one or two critical concentrations of the antimycobacterial drug, the MycoTB plate provides the minimum inhibitory concentration (MIC) of 12 drugs in one run (Abuali et al., 2012).

The MycoTB plate method is not performed routinely anywhere in the world and clinical studies are required to evaluate the impact of MICs on the treatment outcomes. This will help in shortening TAT for both FLDs and SLDs, this will guide the clinician to select the treatment regimen that is suitable for a particular patient.

Although MycoTB plate method is not a well-established method TB DST, there was overall good categorical agreement when compared with MGIT, and very high sensitivity (94%) and specificity (92%). The high sensitivity and specificity of MycoTB show the

accuracy of the assay in terms of identifying resistant strains as resistant and susceptible strains as susceptible.

The APM for determining *M. tuberculosis* susceptibility is a qualitative method which only gives resistant and susceptible results only and also requires 21 days. The literature review also shows a good agreement when comparing MycoTB and solid agar method (gold standard) for DST.

The MycoTB showed to be very rapid DST methods when compared with two methods (Solid agar proportion and MGIT) which are currently endorsed for susceptibility testing by WHO (Abuali et al., 2012, Lee et al., 2014). In the present study, when comparing MycoTB with MGIT for the antimycobacterial drug-RFB, exhibited kappa co-efficients that was almost perfect (0.85) (Table: 3.2).

CHAPTER 6

CONCLUSION

Our findings in the present study indicated that 26% of in vitro RIF resistant strains are RFB susceptible while only 2% were RFP susceptible. This suggests that 26% of MDR/XDR-TB cases could potentially benefit from RFB to improve the sterilizing effects of the treatment regimen. The study supports the growing data which shows that RIF/resistant-RFB/susceptible MTB strains do exist. Although there are very few clinical studies done to support the use of RFB in RIF resistant isolates, it is currently assumed that RIF-resistant/RFB-susceptible MTB isolates may be treatable with RFB. The study also showed that the use of RFP in RIF resistant MTB cases might not have any positive impact on MDR/XDR-TB cases, because of 98% cross resistance between RIF and RFP.

The study also showed the association between particular RRDR *rpo*B mutations and RIF and RFB cross and differential resistance. The use of GenoType MTBDR*plus* can be beneficial for rapid detection of most common *rpo*B mutations such as S531L, H526Y and H526D which is mostly associated with RIF and RFB cross resistant. Furthermore, strains with S531L, H526Y and H526D mutations were found to confer high levels of RIF resistance. The GenoType MTBDR*plus* assay has a probe that targets the D516V mutation and this mutation was found to be associated with RIF and RFB differential resistance isolates.

Therefore, the use of GenoType MTBDR*plus* Assay will not only benefit in identifying MTBC and RIF resistance but will also help in differentiating rifamycin cross and differential resistance. The Sensititre MycoTB shows that it can provide MICs for first and second-line drugs in clinically relevant time frame. This can help in giving TB patients a personalized rather than empirical treatment and will help in reducing chances of acquired resistance due to inappropriate medication. Incorporation of Sensititre MycoTB into daily routine might as well reduce spread of DR-TB and possible eradicate the disease.

LIMITATIONS OF THE STUDY

- There are no established MIC breakpoints value for Sensititre MycoTB plate, in this study we used WHO recommended clinical breakpoints for MGIT Drug Susceptibility Testing (DST) of RFB and RIF.
- The Genotype MTBDR assay Version 2.0 assay only probe the RRDR of the *rpo*B gene and there are other mutations outside this region which are known to confer RIF resistance.

FUTURE RESEARCH

- More studies are needed to investigate the effectiveness of RFB in RFB susceptible MDR/XDR-TB cases.
- Rifalazil (RFL) is also rifamycin but was not tested in this study. The effectiveness of this drug against RIF resistant MTB need to be investigated as literature review shows that it is very potent against RIF resistant MTB.
- Incorporation of rifamycins derivatives in the MYCO TB plate for determination of MIC could be beneficial.

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APPENDICES

Appendix A: Ethics clearance

R14/49 Mr Richard M. Mojapelo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150754

NAME: (Principal Investigator)	Mr Richard M. Mojapelo
DEPARTMENT:	Clinical Microbiology and Infectious Disease National Institute of Communicable Disease
PROJECT TITLE:	Cross-Resistance Among Rifamycins in Mycobacterium Tubercolusis Clinical Isolates
DATE CONSIDERED:	31 July 2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr AW Dreyer
APPROVED BY:	Professor A Woodiwiss, Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	17/08/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature

Date 21/08/2015

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES