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Evaluation of *Mycobacterium tuberculosis* clinical isolates with discordant rifampicin genotypic and phenotypic susceptibility testing results from Pretoria, South Africa

by

Elelwani Elliot Marubini

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MSc (Medical Microbiology)

Department of Medical Microbiology

Faculty of Health Sciences

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"*Change the spiritual fourth dimensional realm within you through positive affirmation*"

Dr. David Yong-Gi Choo

DECLARATION

I, Elelwani Elliot Marubini, hereby declare that the work on which this dissertation is based, is original and that neither the whole work nor any part of it has been, is being or is to be submitted for another degree at this or any other university or tertiary education institution or examination body.

……

Signature of candidate

……

Date

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1 Daum L, Fourie PB, Peters RPH, Rodriguez J, Worthy S, Khubbar M, Bhattacharyya S, Gradus MS, Mboneni T, **Marubini E**, Helm C, Chambers J, Fischer G. (2016) GeneXpert detection of *Mycobacterium tuberculosis* from sputum collected in molecular transport medium. *The International Journal of Tuberculosis and Lung Disease* 20(8):1118-1124.

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1 **Marubini EE**, Malinga L, Mbelle NM, Onweguea B, Fourie PB, Magazi BT (2015) Whole genome sequencing of low level rifampicin resistant *Mycobacterium tuberculosis* strains reveal mutations outside the hotspot region of *rpoB* gene. Federation of Infectious Diseases Societies of South Africa, Drakensburg, South Africa (Poster).

2 Ibidem. Infectious Disease Day, University of Pretoria, South Africa (Poster)

3 **Marubini EE**, Malinga L, Mbelle NM, Onweguea B, Fourie PB, Magazi BT (2015) Unique mutation patterns of multidrug resistant tuberculosis strains associated with discordant results identified in Tshwane, Pretoria. Faculty Day, University of Pretoria. South Africa. (Poster).

4 BT Magazi, JG Pasipanodya, **E Marubini**, N Mbelle et al. (2014) Heads in the Sand: Rifampicin breakpoints are still too damn high! 54th Interscience Conference on Antimicrobial Agents and Chemotherapy. Washington, DC, USA. (Poster)

LIST OF ABBREVIATIONS AND ACRONYMS

°C	Degrees Celsius
%	Percentage
µm	Micro meter
µl	Microliter
ABC	ATP-binding cassette
ADC	albumin, dextrose, catalase
AG	Arabinogalactan
AIDS	Acquired immune deficiency syndrome
BCG	Bacillus Calmette-Guérin
BP	Base pairs
CD	Cluster of differentiation
CFU	Colony forming units
CLSI	Clinical and Laboratory Standard Institutes
CO_2	Carbon dioxide
DOTS	Directly observed treatment, short-course
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DST	Drug susceptibility testing
EDTA	Ethylenediaminetetraacetic acid
ETH	Ethambutol
E-Test	Epsilometer test
FDC	Fixed-dose combination
GC	Growth control
G+C	Guanine *plus* cytosine
GXP	GeneXpert MTB/Rif TestMPT
gyrA	Gyrase alpha subunit
HIV	Human immunodeficiency virus
H_2O	Water
H_2O_2	Hydrogen peroxide
IFN	Interferon
INH	Isoniazid

IL	Interleukin
IS	Insertion sequences
LAM	Lipoarabinomannan
LJ	Löwenstein-Jensen
LNA	Library Normalization Additives
LNB	Library Normalization Beads
LNS	Library Normalization Storage Buffer
LNW	Library Normalization Wash
LPA	Line probe assay
MA	Mycolic acids
MABA	Microplate alamar blue assay
MATE	Multidrug and toxic compound extrusion
MDR-TB	Multidrug-resistant tuberculosis
MFS	Major facilitator superfamily
MGIT	Mycobacterial growth indicator tube
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
M-PCR	Multiplex polymerase chain reaction
MPT64	MPT64 (Rv1980c), a protein of *Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MTBDR*plus*	*Mycobacterium tuberculosis* Drug Resistance Plus Test
MUT	Mutations
NaOH	Sodium Hydroxide
NALC	*N*-Acetyl-*L*-Cysteine
NGS	Next generation sequencing
NTM	Non-tuberculous mycobacteria
PAS	Para-aminosalicylic acid
PCR	Polymerase chain reaction
PDIM	Phthiocerol dimy-ocerosate
PE	Pro-Glu
PG	Peptidoglycan
pH	Potential of hydrogen
Pks	Polyketide synthase enzymes
PPE	Pro-Pro-Glu

PZA	Pyrazinamide
RR-TB	Rifampicin resistant tuberculosis
RRDR	Rifampicin Resistance Determining Region
RIF	Rifampicin
rpoA	RNA polymerase alpha subunit
rpoB	RNA polymerase beta subunit
rpoC	RNA polymerase C subunit
RPM	Revolutions per minute
RT-PCR	Real-time polymerase chain reaction
RND	Resistance nodulation division
SANTP	South African National Tuberculosis Programme
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulphate
SIRE	Streptomycin, Isoniazid, Rifampicin, Ethambutol
SNP	Single nucleotide polymorphism
SMR	Small multidrug resistance
STR	Streptomycin
TB	Tuberculosis
TE	Tris-EDTA
TNF	Tumour necrosis factor
TLR	Toll-like receptors
T-lymphocyte	Thymys-derived lymphocyte
TNF	Tumor necrosis factor- α
USA	United States of America
UV light	Ultraviolet light
WGS	Whole Genome Sequencing
WHO	World Health Organization
WT	Wild Type
XDR-TB	Extensively drug-resistant tuberculosis
ZN	Ziehl-Neelsen

Evaluation of *Mycobacterium tuberculosis* clinical isolates with discordant rifampicin genotypic and phenotypic susceptibility testing results from Pretoria, South Africa

by

Elelwani Elliot Marubini

SUPERVISOR **Professor PB Fourie**

CO-SUPERVISOR **Dr BT Magazi**

DEPARTMENT **Medical Microbiology, Faculty of Health Sciences, University of Pretoria**

DEGREE **MSc (Medical Microbiology)**

SUMMARY

Background. In recent years, there has been an alarming increase in cases of multidrug-resistant tuberculosis (MDR-TB), and also extensively drug-resistant tuberculosis (XDR-TB). The emergence of these forms of tuberculosis (TB) worldwide has alerted scientists to the need for improved diagnostic tests that would enable rapid identification and decision-making on patient management. Over the past 15 years, in particular, new technologies have been introduced that enabled much improved methods of detection of anti-TB drug susceptibility, notably at the molecular level. One of the unfortunate consequences of these advances, however, is the fact that different drug sensitivity test (DST) assays might not always agree in the description of drug resistance status of isolates. This is also true for the detection of resistance to the key anti-TB drug, rifampicin (RIF). Specifically, phenotypic resistance as detected by culture-based methods, often differs from genotypic resistance detected by

molecular methods. In this study, we describe the degree to which several currently used assays differ in their description of RIF-susceptibility in a sample of *Mycobacterium tuberculosis* (*M. tuberculosis*) isolates with discordant phenotypic and genotypic results. We also searched for mutations on the *rpoB* gene by Sanger sequencing and by next-generation whole-genome sequencing (NGS) in a subset of these isolates, in an attempt to distinguish between mutations that have clinical relevance, and those that do not.

Materials and Methods. A convenient sample of 89 *M. tuberculosis* isolates were selected from routine sputum specimen submissions to the NHLS Tshwane Academic Hospital TB Laboratory at the University of Pretoria. Current commercially available phenotypic and genotypic methods were used to describe RIF-susceptibility in the study sample. Of the 89 isolates, 34 showed discordance in RIF DST results between GeneXpert® MTB/Rif (Cepheid, California, USA) (Xpert resistant) and BACTEC™ MGIT™ 960 (BD, Sparks MD, USA) (MGIT susceptible). Other isolates showed concordance on DST (resistant = 21; susceptible = 31). One isolate had missing MGIT results for RIF and Xpert failed to detect resistance in another isolate, confirmed as resistant by MGIT and Hain MDRTBp*lus* (HainLifescience GmbH, Nehren, Germany) line probe assay (LPA). Whole genome sequencing was performed on 40 randomly selected isolates. In addition, microplate alamar blue assay (MABA) was performed on 77 of the 89 isolates to determine the relationship between RIF minimum inhibitory concentration (MIC) of the isolates and detection of resistance by other assays.

Results. On Sanger sequencing, the most frequent *rpoB* gene mutations which conferred resistance to RIF occurred in codons 531, 516, and 526 (41%, 29% and 11% respectively) and 1 isolate had a novel mutation (S601T) outside the *rpoB* rifampicin resistance determining region (RRDR). The most frequently identified mutations in discordant isolates were (L511P and D516Y), 40% and 40%, respectively. Ten isolates susceptible to RIF on both Xpert and MGIT revealed mutations outside the RRDR, and in *rpoC and* efflux pump genes (Rv 1145, Rv 1146 and Rv0933).

Twelve of 22 (54%) isolates resistant to RIF on MGIT had RIF MICs greater than 1 µg/ml, whereas three had RIF MICs below the critical concentration used by MGIT. Twenty-two of 27 (81%) isolates with discordant results had RIF MICs below 1 µg/ml. Twelve of 19 (63%) patients who failed RIF-based TB therapy had no *rpoB* mutations (based on currently used molecular-tests) in the hotspot region.

Conclusions. Our data support the recent reports of other investigators that the expression of efflux pump mutations in *mmpL 13a* and *pstB* genes, and alterations in *rpoC* and *gyrA* may have a synergy with other mutations in the *rpoB* gene within or outside the hotspot region. These associations have previously been shown to result in low levels of RIF resistance. We conclude that NGS of all isolates that show discrepancies in DST between MGIT and Xpert or LPA be routinely performed in order to better inform treatment regimens for TB suspects.

Chapter 1: INTRODUCTION

1.1 Background

Tuberculosis remains as a significant global health problem. The World Health Organization (WHO) in 2014 reported approximately 9.6 million cases of TB, globally (WHO, 2015). Tuberculosis and human immunodeficiency virus (HIV) disease ranked as the main causes of death worldwide. In 2014, the case rate in South Africa reached 834 cases per 100 000 in the general population. The burden of TB in South Africa is generally fueled by HIV co-infection (WHO, 2015), since HIV infection increases the risk of reactivation of a latent TB and rapid progression of the disease (Lin PL & Flynn JL. 2010).

The emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) has further complicated the management strategies for the disease globally (WHO, 2015). Multidrug-resistant tuberculosis is caused by *M. tuberculosis* strains that are resistant to at least isoniazid (INH) and RIF (Valvatne *et al*, 2009; Bazira *et al*., 2010) and XDR-TB is caused by bacilli that are resistant to both RIF and INH, with additional resistance to a fluoroquinolone (moxifloxacin, gatifloxacin or ofloxacin) and at least one of the three injectable drugs used for MDR-TB treatment (capreomycin, kanamycin, or amikacin) (Barnard *et al*., 2008; Georghiou *et al*., 2012). Drug-resistant-TB is predominantly caused by inadequate treatment regimens, poor drug quality or patient non-compliance, but increasingly also by transmission of already resistant strains (Müller *et al*., 2013).

In order to effectively treat and curb the spread of *M. tuberculosis* infections, early diagnosis of TB and rapid identification of resistance to anti-TB agents are vital (Lemus *et al*., 2004; Aragon *et al*., 2006). Although microscopy is still used widely as a pivotal tool for TB diagnosis, cultivation of *M. tuberculosis* is required for confirmation of diagnosis (Seni *et al*., 2012). The diagnosis of TB and drug resistant-TB in HIV co-infected individuals using traditional methods of microscopy and culture can be challenging since the concentration of bacilli is generally low at the sites of infection (Balcells *et al*., 2012; Lawn & Zumla, 2012).

The MGIT system was introduced in 2008 for susceptibility testing of *M. tuberculosis* and rapid detection of mycobacteria. This assay is considered to be the "gold standard" for phenotypic DST of RIF, and largely accepted to be highly accurate and reliable (Rigouts *et al*, 2013).

The understanding of the origin of resistance to anti-TB drugs facilitated the development of rapid molecular diagnostic methods (Minh *et al*., 2012). Molecular assays which are currently commercially available and used by the South African National TB Programme, with the endorsement of the WHO, include the Genotype MTBDR*plus* assay (HainLifescience GmbH, Nehren, Germany) and Xpert MTB/Rif. These molecular assays possess two primary advantages over the phenotypic identification, that is, a more rapid turn-around time and improved accuracy in identification of drug resistant *M. tuberculosis* strains (Patel *et al*., 2000).

The Genotype MTBDR*plus* assay was approved by WHO for the rapid identification of *M. tuberculosis complex* (MTBC) and detection of resistance to INH and RIF by means of multiplex polymerase chain reaction and reverse hybridization. This assay can be performed directly on clinical specimens or culture positive sediments. The MTBDR*plus* assay detects four common mutations in the *rpoB* gene which are D516V, H526Y, H526D, S531L, and failed wild type (WT) probe binding (missing WT without a corresponding mutation) representing an additional 24 mutations whose prevalence varies per geographic region.

The level of resistance conferred by these less common mutations is also variable. Results of proficiency testing among supranational TB reference laboratories have revealed that certain strains carrying specific *rpoB* mutations yield discordant results when tested using the MGIT critical concentration approach. In other words, these strains are genetically RIF resistant, but are in fact classified as susceptible when the proportion method is used on MGIT (van Deun *et al*, 2013). This is particularly the case for mutations occurring in codons 511Pro, 516Tyr, 533Pro, 572Phe and 526 (Rigouts *et al*, 2013). The occurrence of such mutations in some strains probably explains the reduced specificity of some molecular assays when compared to MGIT, leading to discrepant results between the phenotypic and genotypic tests. The relative frequency and clinical impacts of such strains on patients treated with RIF based regimens, however, remains largely unknown (van Deun *et al*, 2013). The prevalence of discordant results might be under-estimated because large molecular testing based surveys have not been

done and MIC testing of isolates is not routinely practiced (Ho *et al*., 2013; van Deun *et al*, 2013). This is problematic because such strains might be pivotal in the spread of MDR-TB.

Besides the public health concerns, the emergence and spread of *M. tuberculosis* strains with the discordant resistance pattern presents a great challenge to clinicians as it creates misunderstanding regarding interpretation of DST results. Furthermore, it emphasises a gap in knowledge about the prevalence of these strains, their degree of RIF-resistance, and clinical outcomes of patients infected by these strains when RIF containing regimens are used for treatment.

1.2 Aim of the study

The aim of this study was to evaluate *Mycobacterium tuberculosis* clinical isolates with discordant phenotypic and genotypic rifampicin susceptibility test results using Sanger and next generation whole-genome sequencing methods to determine the underlying mutations and relate these to the level of resistance as determined by the minimum inhibitory concentration.

1.3 The objectives of the study

1 To use NGS techniques to characterize *M. tuberculosis* mutations in clinical isolates that have discordant RIF genotypic and the critical concentration based phenotypic susceptibility test results.

2 To determine RIF susceptibility quantitatively using the MGIT 960 system, and also the MABA and Agar Proportion assays to derive the MICs of these clinical isolates.

3 To compare the performance of these three methods in terms of categorical or essential agreement.

4 To relate the observed mutations to the MICs obtained from the clinical isolates included in the study.

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Chapter 2: LITERATURE REVIEW

2.1 Introduction

Despite advances in research, TB remains a deadly disease globally, with an estimated one third of the world`s population harbouring TB as underlying infection (Govender *et al*., 2014). This disease causes approximately 3 million deaths annually (Arnold *et al*., 2007). The high incidence of TB disease throughout the world is highly escalated by HIV infection (Adeiza *et al*., 2014).

Another major concern in the control of TB is the prevalence of MDR-TB and XDR-TB (Georghiou *et al*., 2012; Santos, 2012; Bakula *et al*., 2013), which is caused by several factors such as inadequate treatment, failure to adhere to the treatment and misuse of TB drugs (Campos *et al*., 2003).

The cornerstone of successful management of *M. tuberculosis* infection is early diagnosis and rapid identification of resistance to anti-TB agents (Lemus *et al*., 2004; Aragon *et al*., 2006).

The main methods for the detection of *M. tuberculosis* in the laboratory are microscopy and culture. Although microscopy is less expensive, simple, and still widely used as a vital tool in the diagnosis of TB (Seni *et al*., 2012), cultivation of *M. tuberculosis* remains the gold standard, despite its long turnaround time (Uddin *et al*., 2013).

The newly developed molecular methods can be used to detect specific mutations related with drug resistance in *M. tuberculosis* isolates (Bakula *et al*., 2013). The GeneXpert® MTB/Rif and Genotype MTBDR*plus* assay are two of the molecular methods currently available for detection of TB and MDR-TB. Although these techniques provide rapid detection of TB and drug resistance (Patel *et al*., 2000; Barnard *et al*., 2012), they are limited only to mutations within the hotspot region (comprising 81 bp) of the *rpoB* gene.

Despite these advances, there remains a need to establish knowledge regarding the frequency and significance of *M. tuberculosis* strains which display discordant genotypic and phenotypic susceptibility test results for RIF.

2.2 History of tuberculosis and its drug resistance

Tuberculosis has been a major cause of mortality for approximately 10 000 years of human history (Lott and Baker, 2004). It is speculated that the genus originated more than 150 million years ago (Daniel, 2006). Tuberculosis existed in Egypt as early as 5000 years ago, with skeletal abnormalities caused by TB evident in Egyptian mummies and portrayed in early Egyptian art (Daniel, 2006). Hippocrates assumed the disease was largely inherited, while Aristotle (4th century B.C.) and Galen, greatest of Roman physicians (2nd century A.D), highlighted its communicable nature (Smith, 2003).

In 1882, Hermann Heinrich Robert Koch first isolated the TB bacillus (Lott and Baker, 2004; Daniel, 2006) and indicated that it was the agent responsible for human TB disease (Lott and Baker, 2004).

In the late 19th and the early 20th centuries, Edward Trudeau's work explained the aetiology of TB with a classic experiment, he showed that TB can be induced in rabbits with a purified culture of virulent *M. tuberculosis*, but the environmental conditions in which the animals were kept influenced the progress of the disease. In this study, five rabbits infected with *M. tuberculosis* were kept in a crowded, dark cage with very little food. Within three months, four of these rabbits died of TB, and one became severely ill of the disease (Smith, 2003).

The discovery of streptomycin (STR) by Schatz and Waksman in the 1940s led to the beginning of the antibiotic era and its use to treat TB and this was followed by the introduction of antibiotics such as INH, RIF and pyrazinamide (PZA) (Smith, 2003).

The development of antimicrobial chemotherapy led to a remarkable improvement in the treatment of TB (Lott and Baker, 2004). The effective treatment of TB started in 1940s, with the introduction of STR (Onyebujoh *et al.*, 2005; Mitchison & Davies, 2012), however, drug resistant-TB developed soon after the introduction of this drug in 1944 due to the use of STR as a monotherapy (Onyebujoh *et al*., 2005; Da Silva and Palomino, 2011). But this problem was solved by the use of multidrug therapy with drugs such as INH and para-aminosalicylic (PAS) (Onyebujoh *et al*., 2005). The duration of TB treatment was shortened from 24 months to18 months after INH was introduced in therapy in 1952. When RIF was introduced into the combination in the 1970s, the treatment duration was shortened to 9 months. The inclusion of

PZA in combination drug therapy in 1980s further reduced treatment duration to the current six months (Iseman, 2002).

2.3 The epidemiology of tuberculosis

Despite the introduction of effective treatment, TB continues to result in approximately 1.2 million deaths annually, of which 98% are from developing countries (Arnold, 2007). It is estimated that one-third of the entire world population is infected with *M. tuberculosis* (Ahmad, 2010; Govender *et al*., 2014) and about 5% to 10% of infected individuals are at risk of developing secondary TB disease over time. Factors such as HIV infection, drug resistant *M. tuberculosis* strains, population expansion, poor case detection, poverty, active transmission in overcrowded places (hospitals, prisons) and migration from high incidence countries, are accountable for the recent TB epidemic (Ahmad, 2010). The highest TB burden occurs in Asia 58%, whereas regions of Eastern Mediterranean, European and America had (8%, 3% and 3%) cases respectively. Africa carries approximately 28% of the global burden. South Africa, India, Indonesia, China, Nigeria, Pakistan had the largest number of incident cases in 2014 (WHO, 2015). Co-infection with HIV results in high rates of mortality in South Africa (Kasprowicz *et al*., 2011). **Figure 1** shows the estimated global TB incidence.

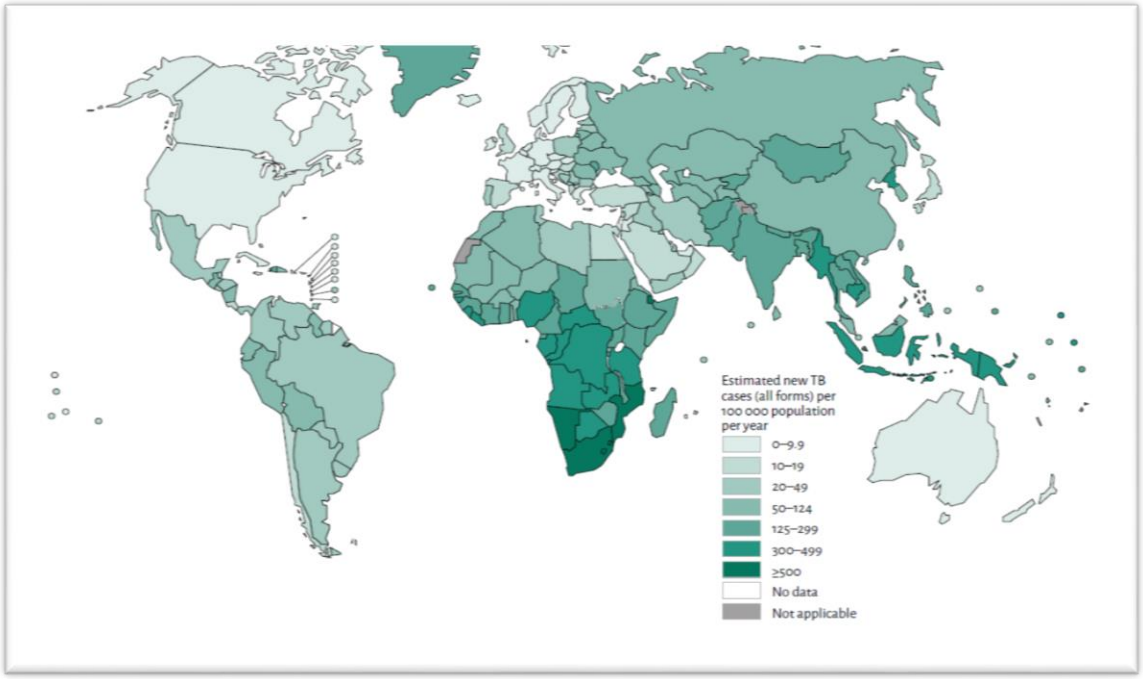


Figure 1: Estimated global tuberculosis incidence, 2014
(Source: WHO, 2015)

2.3.1 Tuberculosis and Human Immunodeficiency Virus co-infection

The HIV pandemic has led to an increase in the incidence of TB. The immuno-suppression caused by HIV infection enables *M. tuberculosis* to proliferate and cause disease, especially the disseminated form characterized by extra-pulmonary manifestations (Campos *et al*., 2003; Kwan & Ernst, 2011; Adeiza *et al*., 2014). As the immune system weakens due to this infection, the susceptibility to TB disease increases by between 20% and 30% (Kwan & Ernst, 2011). Human immunodeficiency virus (HIV) infection escalates the risk of development to active TB in both primary TB infection and the reactivation of latent TB and these alters the natural course of the disease (Kwan & Ernst, 2011; Adeiza *et al*., 2014). Human immunodeficiency virus kills CD4+ T-lymphocytes, resulting in the decline of CD4+ T-lymphocytes counts, promoting rapid progression of TB disease as well as the dissemination of *M. tuberculosis* infection (Adeiza *et al*., 2014).

2.4 Classification of *Mycobacterium tuberculosis*

The genus mycobacterium is divided into two distinct subgroups based on either fast or slow growth rate (Helguera-Repetto *et al*., 2004). Fast growers require at least one week to form colonies on solid growth media (De Groote & Huitt, 2006); in contrast, slow growers require between 10 days and 28 days to form visible colonies on solid media (Cook *et al*., 2009). The population of slow growers include members of the *M. tuberculosis* complex (MTBC) (Arnold, 2007) and *M. leprae*, which are pathogenic to mankind and animals (Helguera-Repetto *et al*., 2004). *Mycobacterium tuberculosis* complex is comprised of eight very closely related mycobacterial species (*M. tuberculosis, M. bovis, M. africanum, M. microti, M. caprae, M. pinnipedii, M. canetti and M. mungi*) (Palomino *et al*., 2007; Reddington *et al*., 2011). The members of MTBC are very similar at the nucleotide level, and this relatedness is the basis of their grouping (Brosch *et al*, 2002). Although these members are related, differences in host tropisms, phenotypes and pathogenicity have been noted (Brosch *et al*, 2002). Most species of the MTBC have been found to cause disease in humans, but the predominant cause of TB in humans worldwide is *M. tuberculosis* (Palomino *et al*., 2007; Reddington *et al*., 2011; Forrellad *et al*., 2013). Furthermore, both *M. africanum* and *M. canetti* have been associated with TB in humans in several regions of Africa. *Mycobacterium bovis* is the causative agent of zoonotic TB in cattle, but can be transmitted to humans and cause disease in man (Forrellad *et al*., 2013).

The other species such as *M. microti*, *M. caprae* and *M. pinnipedii* are pathogenic mainly in animals (Reddington *et al*., 2011; Forrellad *et al.*, 2013).

2.4.1 Scientific classification of *Mycobacterium tuberculosis*

Mycobacteria can exist in soil, dust, rocks, bioaerosols and water, surviving under environmental conditions marked by low nutrient availability, low pH and temperature extremes (De Groote & Huitt, 2006). The genus *Mycobacterium* contains more than 150 acid-fast species, some of which represent parasitic and others free living bacteria (Freidlin, 2013). The genus has several pathogenic species, of which the most prominent are *M. tuberculosis* and *M. leprae*, the causative agents of two of the world's oldest diseases, TB and leprosy (Hett & Rubin, 2008). *M. ulcerans* causes Buruli ulcer, a particularly severe and debilitating disease in mostly West-Africa populations (Garchitorena *et al*., 2015). The genus *Mycobacterium*, together with other acid fast genera which contains mycolic acids in the cell wall, such as *Corynebacterium*, *Nocardia* and *Rhodococcus*, belong to the Order Actinomycetales (Palomino *et al*., 2007; Sakamoto, 2012). Bacteria that belong to this group have a high guanine plus cytosine content (between 61% and 71%) in their DNA (Palomino *et al*., 2007), and a high amount of fatty acid in the cell wall (Palomino *et al*., 2007; Knechel, 2009).

Table 1: The scientific classification of mycobacteria (Palomino *et al*., 2007)

Kingdom	**Bacteria**
Phylum	Actinobacteria
Class	Actinobacteria
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	*Mycobacterium*
Species	*M. tuberculosis* *M. bovis* *M. africanum* *M. ulcerans* *M. microti* *M. canetti* *M. caprae* *M. pinnipedii* *M. mungi*

2.5 Morphological and physiological characteristics of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is non-motile, aerobic, straight or slightly curved rod-shaped gram-positive non-spore forming (Helguera-Repetto *et al*., 2004; Knechel, 2009), acid fast bacterium (Cook *et al*., 2009). The actual size of *M. tuberculosis* is 0.5µm by 3µm and the bacilli have a complex cell wall structure which is essential for their survival (Knechel, 2009). *M. tuberculosis* has a thick, waxy cell wall and highly impermeable outer surface, which give them the ability to persist in a variety of conditions (He & De Buck; 2010; Van der Beken *et al*., 2011).

Tissues with high oxygen partial tension such as lungs (i.e. well aerated upper lobes in particular) promote the growth of the *M. tuberculosis* (Palomino *et al*., 2007). *M. tuberculosis* grow best in the temperature of 37°C and a neutral pH and it`s cultivation in the laboratory is reliant on the atmosphere of 5% to 10% CO_2 (Palomino *et al*., 2007). **Figure 2** shows an electron microscopy image of *M. tuberculosis*.

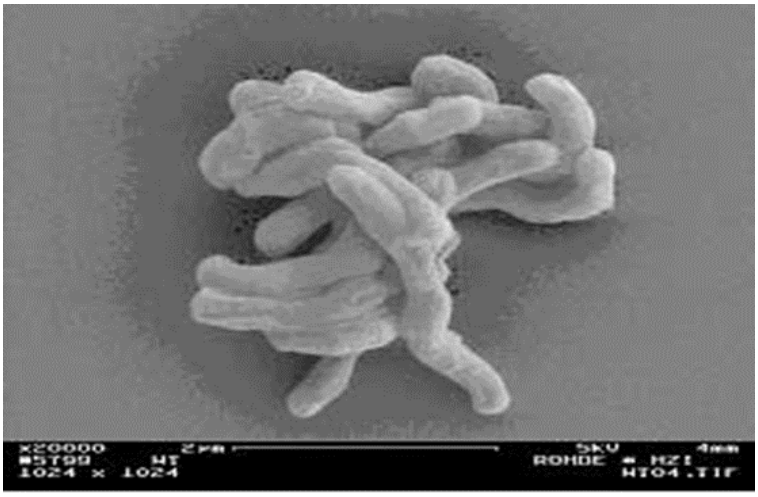


Figure 2: Electron microscopy of *Mycobacterium tuberculosis* growing in culture (Source: Palomino *et al*., 2007)

2.6 The cell-wall composition of *Mycobacterium tuberculosis*

The chemical composition of the mycobacterial cell wall differs slightly from that of other prokaryotes, and it is because of this chemical makeup that its cell wall structure is unique (**Figure 3 -** Hett & Rubin, 2008). This composition is responsible for their resistance to the immune system and antibiotics (Beran *et al*., 2006; Hett & Rubin, 2008). The mycobacterial cell wall is composed of peptidoglycan (PG), arabinogalactan (AG), mycolic acids (MA), lipoarabinomannan (LAM), extractable lipids and proteins (Beran *et al*., 2006). The inner and outer layer of the mycobacterial cell wall surrounds the plasma membrane. The outer part entails both lipids and proteins, these lipids are associated with the cell wall. The outer cell wall connected with the lipid-bound polysaccharides consist of LAM, lipomannan, phthiocerol-containing lipids such as phthiocerol dimycocerosate, dimycolyltrehalose (cord factor), sulfolipids specific to *M. tuberculosis*, and the phosphatidylinositol mannosides (Hett & Rubin, 2008). The covalently linked PG, AG, together with MA forms MA-AG-PG complex. The MA-AG-PG complex makes up the inner layer. The main polysaccharide of the mycobacterial cell wall is the arabinogalactan. This polysaccharide anchors the MA to the PG and it also maintain rigidity of the cell wall (Hett & Rubin, 2008). While, the PG is a polymer of polysaccharide and sugar, that give rigidity to the cell wall, and it also play a role in the regulation of molecular diffusion through the cell (Knechel, 2009; Yao *et al*., 2012). Mycolic acids are found only in the cell wall of the mycolata taxon, which includes *M. tuberculosis*. The MAs are made up of a long chain of α-alkyl, β-hydroxy fatty acids (van der Beken *et al*., 2011). These lipids serve as reserves for carbon and energy and they make up more than half of the dry weight of the mycobacteria (Palomino *et al*., 2007).

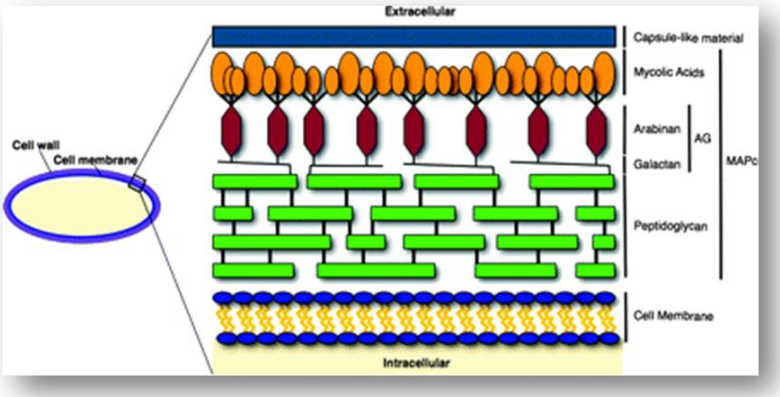
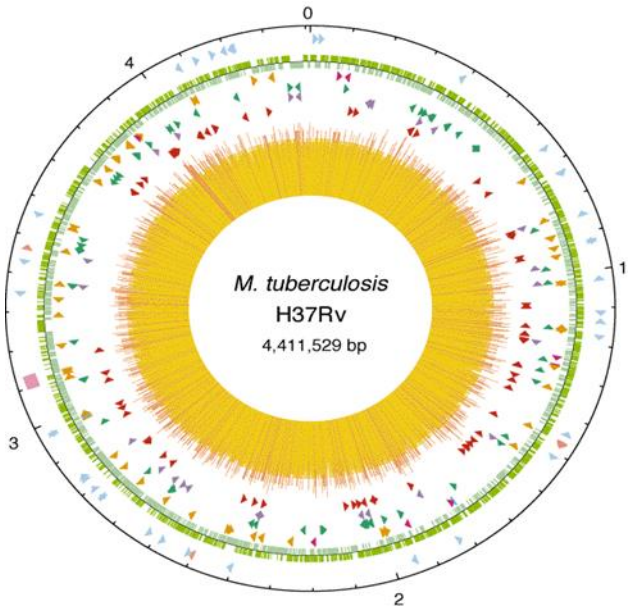


Figure 3: Diagram of the basic components of mycobacterial cell wall

2.7 Genome organization of *Mycobacterium tuberculosis*

In recent years the study of mycobacterial genetics has grown considerably due to the development of molecular methods such as DNA sequencing (Smith, 2003), resulting in the sequencing of the complete *M. tuberculosis* laboratory strain H37Rv genome (Fleischmann *et al*., 2002; Ioerger *et al*., 2010). The H37Rv genome is made up of sequences of 4.4 x10^6 bp in approximately 4000 genes (Fleischmann *et al*., 2002; Smith, 2003). The largest part of the genome contains up to 65.5 % guanine plus cytosine (G+C), which is relatively uniform throughout the genome (Palomino *et al*., 2007). More than 200 of the genes encode enzymes for the metabolism of fatty acids and unrelated Pro-Glu (PE) and Pro-Pro-Glu (PPE) families of acidic, glycine-rich proteins (Smith, 2003). These proteins are assumed to play a vital role in the survival and multiplication of mycobacteria in extreme environments (Palomino *et al*., 2007). The presence and distribution of the insertion sequences in *M. tuberculosis* have been extensively studied (Palomino *et al*., 2007); there are 56 copies of IS elements belonging to the IS3, IS5, IS21, IS30, IS110, IS256 and ISL3 families (Cole, 2002) (**Figure 4**). Only the members of MTBC are known to contain the IS6110 insertion element, which distinguishes them from other mycobacterial species (Coros, 2008). The sequencing of the *M. tuberculosis* H37Rv strain brought important understandings of the biology of the species (Fleischmann *et al*., 2002).



The outer circle shows the scale in Megabytes, with 0 representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, others are pink) and the direct repeat region (pink cube); the second ring inwards shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (centre) represents G + C content, with <65% G + C in yellow, and >65% G + C in red (Cole *et al*., 1998)

Figure 4: Circular map of the chromosome of *Mycobacterium tuberculosis* H37Rv (Source: Cole *et al*., 1998)

2.8 Drug resistant tuberculosis

The current rates of MDR-TB in new and previously treated cases globally are at 3.3% and 20%, respectively. Approximately 480 000 new cases of MDR-TB occurred worldwide in 2014. Of patients with pulmonary TB notified in 2014, an estimated 300 000 had MDR-TB, and nearly half of these patients were in India, China and the Russian Federation. In 2014, approximately 123 000 patients with RIF resistant TB (RR-TB) were reported globally (WHO, 2015). **Figure 5** shows the proportion of new TB cases with MDR-TB.

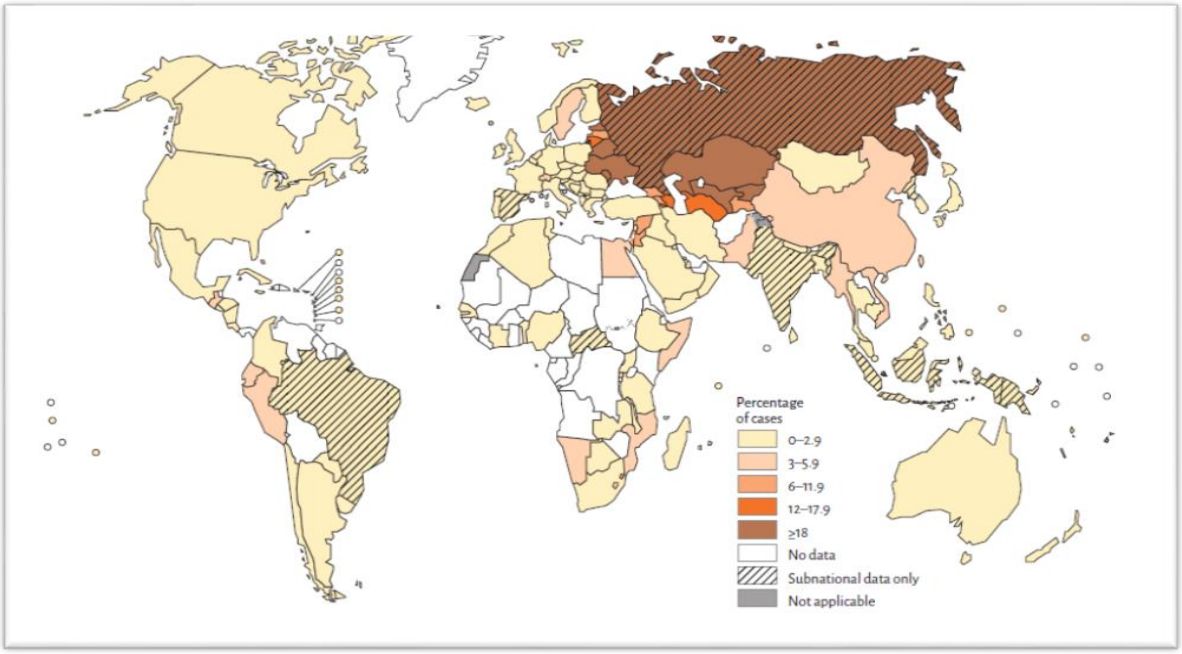


Figure 5: Percentage of new tuberculosis cases with MDR-TB
(Source: WHO, 2015).
(Figure is based on the most recent year for which data have been reported, which varies among countries; data reported before the year 2000 are not shown.)

In contrast, RIF-monoresistance is a rare form of TB disease (Palomino and Martin, 2014). Information on the prevalence of RIF-monoresistance is rare; however, prevalence estimates under 1% for new TB cases have been reported within Europe in 2010, and 3.2% in Zambia (Villegas *et al*., 2016). Other countries like Burundi, Botswana and Brazil also reported case series of RIF-monoresistance (Dramowski *et al*., 2012). Although relatively uncommon, RIF-

monoresistance poses a significant problem to TB control strategies. This form of TB is associated with prolonged treatment duration, high therapeutic cost, and excessive or high failure rates (Dramowski *et al*., 2012). A study done by Mukinda and colleagues (2012) in the Western Cape Province of South Africa reported a rapid increase of RIF-monoresistance, mostly in HIV positive individuals (Mukinda *et al*., 2012). Factors such as variable RIF dosing, impaired absorption, altered metabolism and HIV infection are pivotal in the development of RIF-monoresistant disease in adults (Dramowski *et al*., 2012).

Between 2001 and 2002 the first survey of TB drug resistance done in South Africa showed low estimates (0.9% to 2.6%) of primary MDR-TB (Andrews *et al*., 2007). However, it is estimated that there are approximately 13,000 cases of MDR-TB emerging in South Africa each year with an increasing outbreak of XDR-TB which has a high mortality among HIV infected individuals (Cox *et al*., 2010; Klopper *et al*., 2013). A 2006 outbreak which occurred in rural hospital in KwaZulu-Natal province of South Africa underlined the crisis of drug resistant-TB. In this outbreak 39% (72/185) of culture confirmed patients had MDR-TB, whereas 53 of them had XDR-TB (Abdool-Karim *et al*., 2009)

Drug resistant-TB has further amplified the challenge faced in managing the epidemics of TB (Bernard *et al*., 2008; Cohen *et al*., 2008) and it also frustrates the effectiveness of TB control programmes (Cohen *et al*., 2008). This form of TB seeks urgent necessity for additional control measures, such as new diagnostic methods, better drugs for treatment, and a more effective vaccine (WHO, 2009).

2.8.1 Development of drug resistance in *Mycobacterium tuberculosis*

Drug resistance in bacteria can be due to transmissible elements like transposons and plasmids. Nevertheless, there is still a lack of evidence that acquired genes or plasmids play role in the development of drug resistance in *M. tuberculosis* (Nachega & Chaisson, 2003). In *M. tuberculosis* drug resistant mutants are selected through poor management or non-compliance with treatment (Campos *et al*., 2003).

In the light of this knowledge recent studies have shown that exposure of bacterial cells to sub-lethal concentrations of drugs stimulates cellular mutagenesis leading to increased mutations in other drug resistance genes. This phenomenon might be fundamental in the rapid development of multidrug resistant *M. tuberculosis* strains (Smith *et al*., 2013). Other factors

attributable to drug resistance are suboptimal dosing and malabsorption of antibiotics (Shenoi & Friedland, 2009).

Drug resistance in *M. tuberculosis*, has been shown to be an outcome of multiple mechanisms operating together. It is believed that several mechanisms play a role in the resistance of RIF by *M. tuberculosis* (Sharma *et al*., 2010).

Some of the mechanisms that play a role in conferring RIF resistance in *M. tuberculosis* includes: alterations in the *rpoB* gene, drug efflux pumps (Sharma *et al*., 2010), reduced permeability of the bacterial envelope and compensatory mutations (Poon & Chao, 2005). **Figure 6** shows the mechanisms responsible for drug resistance in *M. tuberculosis*.

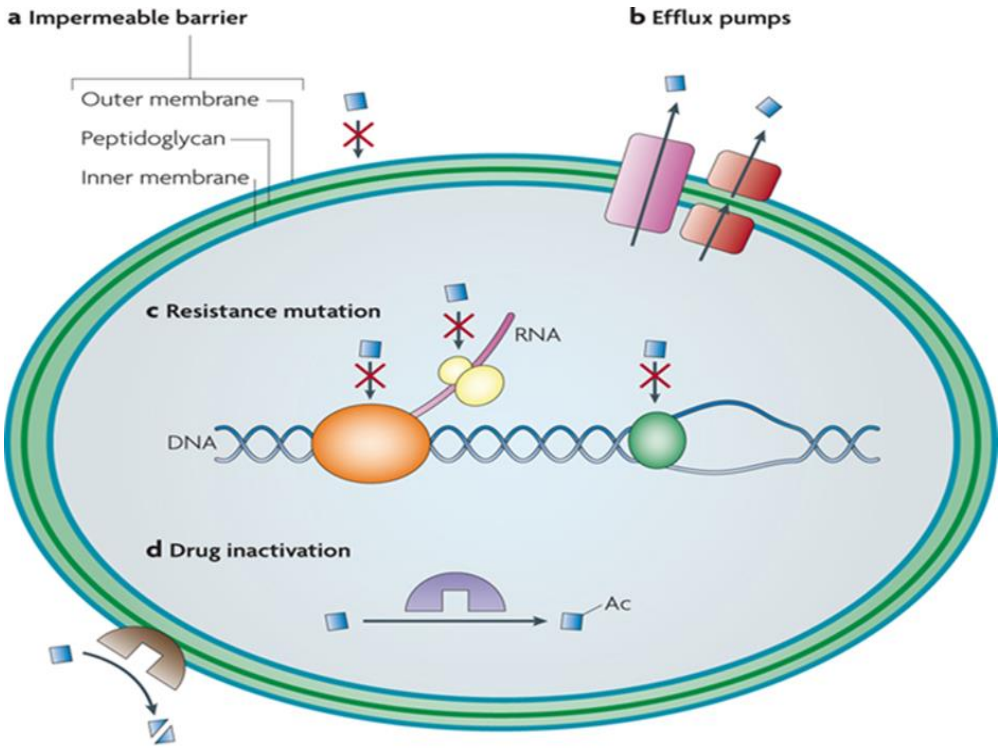


Figure 6: Mechanisms of drug resistance in *M. tuberculosis*
(Source: Allen *et al*., 2010)

2.8.1.1 Mutations in the *rpoB* gene of *M. tuberculosis*

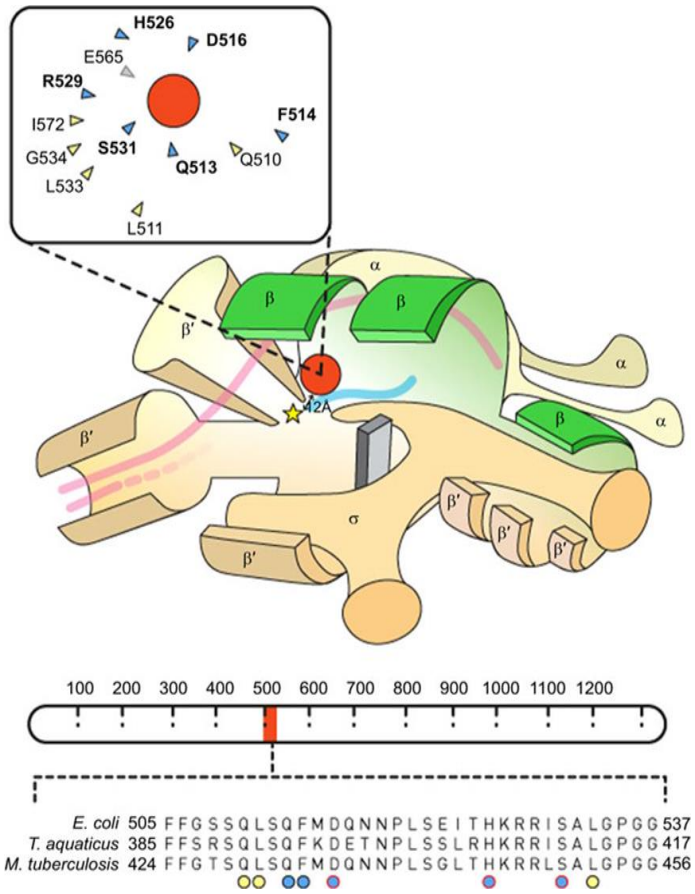


Figure 7: Schematic representation of RNA polymerase structural elements including the RIF resistance determining region
(Source: Sharma *et al.*, 2010)

(Structural annotations have been simplified, and the promoter sequence has been excluded. The *rpoB*-encoded β subunit is highlighted in green. A yellow star represents the RNAP active site and a red circle denotes the RIF molecule which approaches within 12 Å of the active site, inhibiting transcription. Double-stranded DNA is represented by pink lines and, once unwound, only template DNA is shown, with the growing RNA chain coloured in blue. The inset shows a simplified depiction of the RIF binding pocket. Amino acids that form hydrogen bonds with RIF are highlighted in blue and those that form van der Waals interactions are coloured yellow; amino-acid numbering corresponds to that used for *E. coli*. Mutations identified in 11 of the 12 residues that surround the RIF binding pocket have been associated with RIF resistance, albeit at different frequencies (the sole amino acid, E565, which has not been associated with RIF resistance mutations is coloured in grey). A schematic representation of the *rpoB* gene which encodes the β subunit of RNA polymerase is shown below the RNA polymerase cartoon. Amino-acid numbering is shown as dashed demarcations. The RIF resistance determining region is highlighted in blue and the amino-acid sequence of the RIF resistance determining region is magnified below. The alignment contains the amino-acid sequences of *E. coli*, *T. aquaticus* and *M. tuberculosis*. Amino acids that interact directly with RIF are indicated by circles and the colours correspond to the inset diagram. Circles highlighted in red indicate residues that are most frequently observed in RIF resistance isolates.)

Chromosomal (spontaneous, deletion or addition) mutations within the *rpoB* gene are responsible for RIF-resistance in *M. tuberculosis.* It is through these mutations that RNA polymerase continues to function even in the presence of RIF (Sharma S *et al*., 2010). The *M. tuberculosis rpoB* gene encodes the 1,178 amino acid beta subunit for a DNA dependent RNA polymerase enzyme (Daum *et al*., 2012) (**Figure 7**). Additionally, mutations located within the 81 bp region of the *rpoB* gene accounts for approximately 95% of RIF resistant *M. tuberculosis* strains (Viveiros *et al*., 2005; Daum *et al*., 2012; Veluchamy *et al*., 2013). Clearly, classical changes within the *rpoB* gene cannot fully explain resistance to RIF in clinical *M. tuberculosis* isolates (Louw *et al*., 2009). It has been documented that approximately 5% of *M. tuberculosis* isolates resistant to RIF do not have mutations in the hotspot region (81 bp) of the *rpoB* gene (Louw *et al*., 2009). This hypothesis can be validated by the fact that *M. smegmatis* is naturally tolerant to RIF, although no changes in the *rpoB* gene have been noted (Louw *et al*., 2009). This is therefore suggestive of other less-well-defined mechanisms that might play a role in conferring resistance to RIF (Louw *et al*., 2009; Sharma *et al*., 2010). Some of the mechanisms known to cause resistance to RIF include efflux pumping of RIF and the lipid nature of the cell wall together with compensatory mutations

2.8.1.2 Efflux pump-mediated drug resistance in *Mycobacterium tuberculosis*

Several studies suggest that expression of the efflux pumps promotes resistance of *M. tuberculosis* to antibiotics (Louw *et al*., 2009). It has been shown recently that the expression of the efflux pumps causes the reduction of intracellular drug concentrations, and thus aiding antibiotic resistance, since the compounds is halted from reaching the intended targets (Jin *et al*., 2010).

It has been shown that clinical isolates of MDR-TB over-expresses several efflux systems when exposed to antibiotics (Fonseca *et al*., 2015). Resistance to antibiotics caused by efflux pumps, occur due to either increased expression of the efflux pump protein or the enzyme contains an amino acid substitution that makes the protein more instrumental at export (Piddock *et al*., 2006). The decrease in the intracellular concentrations of drugs resulting from heightened activity of efflux mechanisms is responsible for low level of resistance (Fonseca *et al*., 2015)

Most of these efflux pumps seem to operate together in supporting drug resistance (Balganesh *et al*., 2012). These pumps also work in coordination with the cell wall permeability in order to stimulate drug resistance.

Efflux pump systems are categorized into five different families based on their energy sources and structural characteristics namely: the ATP-binding cassette (ABC) superfamily; the major facilitator superfamily (MFS); the multidrug and toxic compound extrusion (MATE) family; the small multidrug resistance (SMR) family; and the resistance nodulation division (RND) family (Sun *et al*., 2014).

2.8.1.3 The lipid metabolism-mediated resistance in *Mycobacterium tuberculosis*

A huge number of lipophilic molecules, ranging from common fatty acid to glycolipids and very long chains of mycolic acid (MA) have been shown to be existing in *M. tuberculosis* (Swanepoel & Loots, 2014). The genome of *M. tuberculosis* comprises of several genes which encode enzymes for the metabolism of fatty acids (Smith, 2003; Swanepoel & Loots. 2014). The primary role of lipids in *M. tuberculosis* is to provide energy required for replication and growth. However, many lipids are responsible for the formation of mycobacterial cell wall, which adds to the virulence of the organism (Swanepoel & Loots, 2014).

The chemical composition of the mycobacterial cell wall is quite unique (Beran *et al*., 2006; Hett & Rubin, 2008). The combination of high MA content with a variety of other intercalated lipids is known to contribute to the low permeability of mycobacterial cells (Jin *et al*., 2010). The low permeability of the cell-wall accounts for the low susceptibility of this organism to immune system actions (Beran *et al*., 2006; Hett & Rubin, 2008) and to anti-TB agents from a variety of antibiotic classes (Beran *et al*., 2006; Hett & Rubin, 2008; Jin *et al*., 2010). In a study comparing the lipid metabolomes of *M. tuberculosis* to further characterize RIF-resistance more fully, two genetically distinct *rpoB* mutants of *M. tuberculosis* strains (S552L and S531L) were tested against *M. tuberculosis* wild type strain (du Preez & Loots, 2014). The study showed that the *M. tuberculosis rpoB* mutant strain was accompanied by an altered fatty acid metabolism. These findings support the hypothesis that lipids may acquire structural changes which may give rise to resistance in *M. tuberculosis*.

Genes which encode 9 polyketide synthase enzymes (*pks*) were also recognized in the *M. tuberculosis* genome. Polyketides are multi-domain proteins responsible for the production of complex lipids and a variety of metabolites connected with the mycobacterial cell envelope (Swanepoel & Loots, 2014). Remarkably, the *pks* gene complex is located directly upstream of *mas*, and both their respective products, phthiocerol and mycocerosic acid, are used to produce another necessary cell wall component, phthiocerol dimy-ocerosate (PDIM), which is also strongly associated with mycobacterial virulence. Metabolomics together with a proteomics approach was instrumental in demonstrating a significant upregulation of the polyketide synthase genes, *ppsA-ppsE*, and *drrA* (Rv2936), responsible for the transport of PDIM over the mycobacterial cell envelope in Beijing and Haarlem *rpoB* mutant *M. tuberculosis* strains (Swanepoel & Loots. 2014).

2.8.1.4 The role of compensatory mutations in *Mycobacterium tuberculosis*

The concept of compensatory evolution is less understood, yet very significant (Poon & Chao, 2005). Compensatory mutations can be defined as either mutations that reduce the fitness related with drug resistance mutations (Maisnier-Patin & Andersson, 2004; de Vos *et al*., 2013) or to regain fitness due to the epistatic influence of mutations in other related genes (Poon & Chao, 2005) (**Figure 8**). Notably, the acquisition of a compensatory mutation does not automatically occur after the development of a resistance-associated mutation. Mutations may be present that influence the development of drug resistance through compensating for the structural consequence the target mutation might have, or by acting synergistically to increase resistance. A study done by Gagneux (2009) measured the growth rate of RIF-resistant *M. tuberculosis* mutants in relation to drug susceptible strains. The investigations employed spontaneous RIF-resistant mutants selected in *vitro*, and strains obtained from TB patients who developed drug resistance. The findings showed that the competitive fitness was dependent on the nature of the *rpoB* gene mutations and the genotype of the strain. The inference from these findings is that the absence or presence of compensatory mutations might contribute to the inferred fitness of cost. The concept has been further elaborated upon by Muller and colleagues (Muller *et al*., 2013).

A large database of RIF-susceptible and RIF-resistant isolates was used to validate the findings in regard to the role of compensatory mutations (Koch *et al*., 2013). The study revealed that a significant proportion of RIF- resistant strains harboured mutations in *rpoA* or *rpoC* genes;

however, same alterations were not identified in the susceptible isolates. In addition to this observation, a high occurrence of *rpoC* gene mutations was reported in closely related strains from South Africa, hinting a link between the propagation of RIF-resistance and the presence of mutations in the *rpoC* gene (Koch *et al*., 2013). These compensatory changes occurring in the *rpoA-rpoC* interaction region of *rpoC* gene (amino acid positions 356 to 756) are believed to be responsible for increased in *vitro* fitness (de Vos *et al*., 2013; Fonseca *et al*., 2015). The mutations occur in the strains that are resistant to RIF due to the nucleotide alterations in the *rpoB* gene, in the mechanisms thought to be influenced by continued exposure to antibiotics (Fonseca *et al*., 2015). **Figure 8** shows epistasis-mediated drug resistance in *M. tuberculosis.*

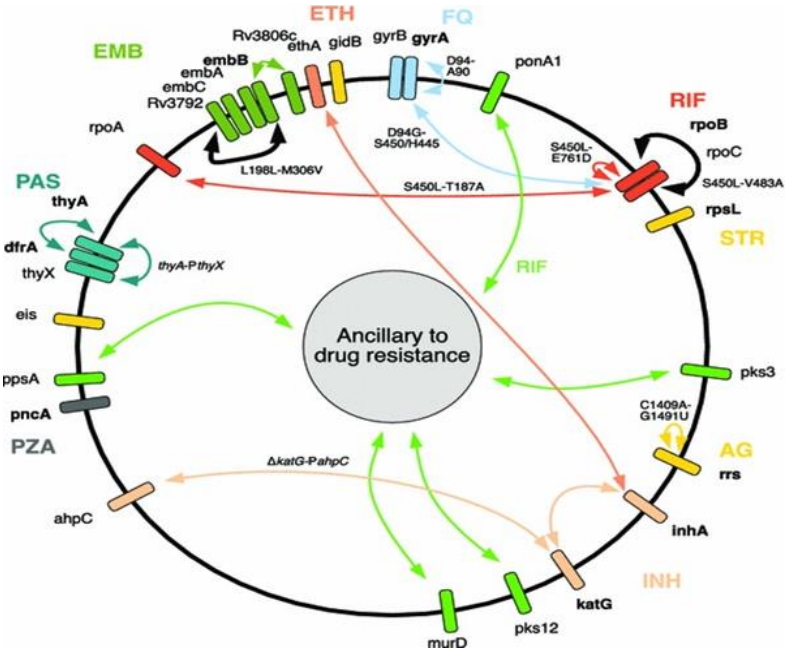


Figure 8: Schematic representation of epistasis-mediated drug resistance in *Mycobacterium tuberculosis*
(Source: Trauner *et al*., 2014)

(A web of epistasis mediates drug resistance in *M. tuberculosis*. Key genes in *M. tuberculosis* drug resistance have been plotted, taking into account their approximate position in the genome. Genes in bold are known to be directly involved in antibiotic resistance. Lines denote putative epistatic interactions; connecting genes involved in the physiology of a drug as well as more broad/indirect mechanisms referred to as 'ancillary to drug resistance'. This categorization is meant to include factors mediating complex aspects of cell physiology, such as cell permeability and mutation-induced physiological changes. Bold lines connecting *rpoB* to *rpoC* and *embB* to Rv3972 refer to in vitro validated compensatory mechanisms.)

2.9 Mode of transmission of tuberculosis

Tuberculosis is a communicable disease which is caused by small airborne sputum particles carrying *M. tuberculosis*, called droplet nuclei, generated by the coughing and sneezing of individuals with pulmonary TB. The droplet nuclei, which are particles of 1 μm to 5 μm in diameter causes infection when inhaled (Mathema *et al*., 2006, Ahmad, 2010). These droplet nuclei can remain suspended in the air for several minutes to hours after release (Knechel, 2009; Ahmad, 2010). Several factors such as closeness of contact, bacillary load inhaled, exposure of the bacilli to UV light and the immune status of the host influence the transmission and the development of TB disease (Knechel, 2009; Ahmad, 2010). Approximately 30% of people exposed to *M. tuberculosis* become infected, but only 5% to 10% of those infected develop primary TB infection in the absence of HIV or other immunosuppressive conditions (Lin & Flynn, 2010).

2.10 Pathogenesis of tuberculosis infection

The genome of *M. tuberculosis* consists of genes which codes for several virulence factors. These virulence factors play a crucial in the attachment and invasion of the bacilli. Another important character of the virulence factors is that they are able to suppress immune response (Prozorov *et al*., 2014).

Infection begins when the bacteria enter the alveoli and are phagocytized by alveolar macrophages and local dendritic cells (Smith, 2003; Lin & Flynn, 2011). Mycobacterial adhesins attach to the alveolar macrophages in the lungs (Govender *et al*., 2014; Prozorov *et al*., 2014). The binding of adhesins to the host cells activates the immune response, which is important in the defence of the infection (Govender *et al*., 2014).

Subsequent to the adherence of bacilli to the host cells, the bacteria phagocytosed by the macrophages are transferred to the phagosomes. The condition in the phagosomes is unfavourable to the mycobacteria species, but they have established survival strategies (Forrellad *et al*., 2013). Some of those mechanisms for survival are the inhibition of phagosome progression (Forrellad *et al*., 2013; Prozorov *et al*., 2014), the prevention of the induction of apoptosis, and increased resistance to host toxic compounds (Forrellad *et al*., 2013). Additionally, phospholipase enables the bacteria to survive within the macrophages

(Ahmad, 2010). On the other hand, *katG* protein A catalase hydrolyses the H_2O_2 and organic peroxides generated by phagocytes (Smith, 2003; Forrellad *et al.*, 2013) and *AhpC* enzyme, a hydroperoxide reductase which detoxify organic hydroperoxides in the macrophages (Smith, 2003). All these factors contribute to the survival of bacilli within the macrophages. Genes such as *nuoG, katG, sodA/secA2, pkn E and Rv3654cl/RV3655C* are responsible for anti-apoptosis activity of *M. tuberculosis* (Forrellad *et al*., 2013).

The disease progression is stopped when most of the bacilli are killed in the granulomas (Ahmad, 2010); however, some of the bacteria can escape the killing by the immune response, resulting in survival and persistence of the bacilli in a dormant state in the host (Ahmad, 2010). The release of *M. tuberculosis* from the lysosomes to the cytosol outside ruptured macrophages promotes the survival of some bacilli and upon release infects new cells (Prozorov *et al*., 2014).

2.11 Host immune response to tuberculosis infection

The immune response plays a significant role in the control of *M. tuberculosis* infection and in most patients the immune system successfully contains the infection. This is supported by the fact that approximately 90% of people infected with *M. tuberculosis* do not develop the disease (Palomino *et al*., 2007).

Infection begins when the bacteria enter the alveoli and are phagocytized by alveolar macrophages and local dendritic cells (Smith, 2003; Lin & Flynn, 2010). Subsequently, the bacteria together with the antigen engulfed by dendritic cells are taken from the distal airways to the draining regional lymph nodes and this stimulates T-cell response (Lin & Flynn, 2010). Activated macrophages, along with lymphocytes, travel to the primary site of infection resulting in the formation of granulomas, which contain the bacilli (Raja, 2004; Ahmad, 2010; Lin & Flynn, 2010).

Mycobacterium tuberculosis resides in the phagosomes and its antigens are exposed to major histo-compatibility complex) (MHC) class II molecules (Kaufmann, 2001). Antigenic peptides in the cytosolic compartment are processed by MHC class I and when CD8 T-cells recognise this molecule, differentiate into effector cells, and produce cytolytic molecules and cytokines that kill both host cells and intracellular bacilli (Prezzemolo *et al*., 2014). On the other hand, MHC class II process mycobacterial antigen and the derived antigenic peptide is identified by

$CD4^+$ T-cells (Prezzemolo *et al.*, 2014). The $CD4^+$ T-cells produce cytokines (van Crevel *et al*., 2002; Raja, 2004; Lin & Flynn, 2010), interferon gamma (IFN- γ), and are also essential for optimal function of the $CD8^+$ T-cells (Lin & Flynn, 2010).

Interferon gamma is the most vital cytokine that stimulates the macrophages to produce reactive oxygen and nitrogen species (Sakamoto, 2012). The ligation of TLR2 and TLR4 fuels the macrophages to produce cytokines such as tumor necrosis factor- α (TNF-α), IL-1β, IL-6 and IL-12. Tumor necrosis factor- α controls the formation of granulomas (Sakamoto, 2012), induces macrophage activation (van Crevel *et al*., 2002), and is associated with fever and wasting in progressive TB disease (Sakamoto, 2012). The inflammation seen in the lung of TB infected patients is caused by the activity of both the chemokines and cytokines (Garlanda *et al*., 2007).

The transfer of bacilli from the primary site of infection causes *M. tuberculosis* to disseminate to other organs, which can lead to miliary or meningeal TB disease. Human immunodeficiency virus-infection depletes the number of $CD4^+$ T-cells and $CD8^+$ T-cells, and consequently promotes the persistence of bacilli in the blood (Krishnan *et al*., 2010).

2.12 Clinical manifestations of tuberculosis infections

The various stages of TB seen during pathogenesis include latent, primary, primary progressive, and extra-pulmonary disease. Individuals with latent TB infection show signs of the disease but they cannot spread the bacilli (Knechel, 2009). On the other hand, primary TB in adults is often asymptomatic and is difficult to diagnose (Palomino *et al*., 2007). But, in some individuals TB manifests radiologically as parenchymal disease, lymphadenopathy and pleural effusion (De Backer *et al*., 2006).

Primary progressive disease develops only in 5% to 10 % of latently infected individuals. This form of disease result from the activation of the latent primary infection, but in some patients this form of TB develops due to the continuation of primary disease (De Backer *et al*., 2006). Primary progressive disease is accompanied by progressive fatigue, cough, malaise, weight loss, and a low-grade fever followed by chills and night sweats (Knechel, 2009).

Individuals infected with HIV/AIDS are more likely to develop extra-pulmonary TB disease (Gray & Cohn, 2013), caused by haematogenous spread of the bacilli during primary infection

(Knechel, 2009). Signs and symptoms for extra-pulmonary TB disease depend on the organ infected (Palomino *et al*., 2007). Most frequently, extra-pulmonary TB disease manifests as lymphatic, pleural, bone/joint (particularly the thoracic spine), soft tissue, central nervous system, or pericardium disease (Sterling *et al*., 2010).

2.13 Treatment of tuberculosis

Antibiotic monotherapy in the treatment of TB commonly results in the development of drug resistant strains (Chiang *et al*., 2010). Therefore, combination chemotherapy is the basis for the effective treatment of TB (Laurenzi *et al*., 2007). Current TB therapy takes a period of six months, and is based on four first-line antibiotics (INH, RIF, PZA and EMB) given together for a period of two months, followed by administration of INH and RIF for the subsequent four months under direct observation by a healthcare worker (Chopra *et al*., 2012). The ultimate goal of TB therapy is to attain cure, to prevent death and relapse, to render patients non-infectious rapidly, and to prevent the development of drug resistant strains. The anti-TB drug combination that includes INH, RIF, PZA and EMB ensures that actively metabolizing bacilli in the cavities are destroyed, including actively replicating bacilli in the acidic and anoxic closed lesions in the lung (Onyebujoh *et al*., 2007).

Several factors, including poor adherence of patient to treatment, may promote the development of drug tolerant strains of *M. tuberculosis* (Wolff & Nguyen, 2012). The problem with drug resistant-TB, is that treatment with second line drugs are often associated with severe toxic side effects and high cost (Wolff & Nguyen, 2012). A combination therapy of a fluoroquinolone (moxifloxacin, gatifloxacin or levofloxacin), injectable aminoglycoside (amikacin or kanamycin), and any first line drug to which the isolate is susceptible, as well as the addition of drugs such as cycloserine or terizidone, and ethionamide, is generally used to treat MDR-TB. The intensive phase with injectable drugs takes eight months, whereas continuation phase last for 12 months to 18 months (Calligaro *et al*., 2014). Patients with XDR-TB infection are treated with drugs that are less effective and much more toxic than those used in the management of MDR-TB. At least five drugs to which isolates are susceptible are included in the therapy, and first-line drugs with good susceptibility results should be chosen over second-line agents (Prasad, 2012).

2.14 Action of anti-tuberculosis drugs and the molecular mechanisms of resistance

Isoniazid is the most effective drug in the treatment of TB disease. It is an inactive drug that undergoes conversion by the enzyme catalase-peroxidase into isonicotinic acid. Isonicotinic acid prevents the biosynthesis of MA, which is the most important part of the mycobacterial cell wall (Palomino *et al*., 2007). The molecular mechanisms which are responsible for INH resistance are quite complex, as they have been associated with a variety of mutations which affect one or several genes involved in MA biosynthesis (Johnson *et al*., 2006; Alcaidea & Coll, 2011). Genes which are associated with INH resistance are *katG, inhA, and ahpC*; however, a more frequent mutation in INH resistant isolates is located in codon 315 of *katG* genes. Mutations in the *katG* genes are responsible for between 60% and 70% of INH-resistant strains (Aragon *et al*., 2006; Johnson *et al*., 2006).

Pyrazinamide is a key first-line drug, capable of killing a population of latent bacilli living in acidic environment (Da Silva & Palomino, 2011). The inclusion of PZA in the drug combination shortened the therapy of TB from nine to six months (Zhang *et al*., 2003). Pyrazinamide is a pro-drug that needs to be converted to pyrazinoic acid by the enzyme pyrazinamidase (Mitchison & Davies, 2012). Pyrazinamidase is a product of the *pncA* gene (Da Silva & Palomino, 2011; Mitchison & Davies, 2012). Pyrazinamide resistance in *M. tuberculosis* is caused by mutations in *pncA* gene (Da Silva & Palomino, 2011). It has been reported that between 72% and 95% of clinical isolates of *M. tuberculosis* resistant to PZA carry *pncA* mutations, which are spread across all parts of the gene (Santos, 2012).

Ethambutol is an important TB drug for preventing the development of drug resistance (Santos, 2012). Ethambutol is a bacteriostatic, inhibiting the growth of multiplying bacilli (Da Silva & Palomino, 2011; Santos, 2012) and preventing growth in non-replicating bacteria (Santos, 2012). Ethambutol acts on the cell wall by interfering with arabinosyltransferases encoded by the *embCAB* operon, consisting of three homologous genes designated as *embA*, *embB* and *embC* (Bakula *et al*., 2013). Resistance to ETH is caused by the mutations in *embCAB* operon, specifically the *embB* gene (Santos, 2012). The most common mutation which confers resistance to ETH occurs in codon 306 of *embB* gene (Da Silva & Palomino, 2011).

Streptomycin is an aminocyclitol glycoside drug used as an alternative first line anti-TB drug (Santos, 2012). Streptomycin inhibits protein synthesis by binding to 30S ribosomal subunit

and 16S rRNA (Ahmad & Mokaddas, 2009; Santos. 2012). Resistance to STR in *M. tuberculosis* is conferred in the *rrs* or *rpsL* gene (Da Silva & Palomino, 2011).

Rifampicin is known to interfere with transcription in bacteria by binding to the β-subunit of RNA polymerase (the product of the *rpoB* gene) (Lee *et al*., 2005; Comas *et al* 2012; Veluchamy *et al*., 2013). The *M. tuberculosis rpoB* gene encodes the 1,178 amino acid beta subunit for a DNA dependent RNA polymerase enzyme (Daum *et al*., 2012). Furthermore, most mutations responsible for RIF-resistance occur are located within the hot-spot 81 bp region of the *rpoB* gene (Viveiros *et al*., 2005; Daum *et al*., 2012; Veluchamy *et al*., 2013). The rate of genetic mutations leading to RIF-resistant mutants in strains not previously exposed to anti-TB drugs varies from a frequency of 10^{-7} to 10^{-8} (Spindola deMiranda *et al*., 2001). Evidently, the frequency of codon alteration within the *rpoB* gene of RIF-resistant *M. tuberculosis* isolates varies across different geographical regions (Horng *et al*., 2015). It has been reported that the most common mutations in the 81 bp region occur in codons 526 and 531, and account for 62.5% to 81.1% of RIF-resistant strains, respectively (Horng *et al*., 2015). Nevertheless, the degree of resistance to RIF differs with the nature of the mutations (Horng *et al*., 2015). Amino acid changes in codons 526 and 531 causes high level of resistance than alterations in codons 511, 516, 518, and 522 (Horng *et al*., 2015). Table 2 shows the mechanisms of action and genetic markers of first-line anti-TB drugs. Although most changes in codons occur in the *rpoB* gene, rare RIF resistant mutations have been reported, such as those at codons 176 (Val176Phe), 381 (Ala381Val), 490 (Gln490His), 500 (Ala500Val), 502 (Ile502Val), 505 (Phe505Ser), 538 (Leu538Pro), 146 (Val146Phe), and 572 (Ile572Phe). However, information regarding their clinical significance is lacking (Horng *et al*., 2015).

Table 2: Mechanisms of action and genetic markers of first-line anti-tuberculosis drugs

TB drug	Mode of action	Gene(s)	Prominent codons	References
RIF	Inhibit transcription	*rpoB*	511, 516, 518, 522, 526 & 531	(Horng *et al*., 2015) (Veluchamy *et al*., 2013)
INH	Inhibit mycolic acid synthesis	*katG* *inhA* *oxyR, ahpC, furA, ndh*	315 C15T, -24, -16, -8, -7	(Palomino *et al*., 2007).
PZA	Blocks trans-translation	*pncA* mutations		(Da Silva & Palomino, 2011)
ETH	Inhibit cell wall arabinogalactan synthesis	embCAB	*embB*-codon 306	(Santos, 2012)
STR	Protein translation disruption	*rpsL* *rrs*		(Da Silva & Palomino, 2011)

2.15 Laboratory diagnosis of active tuberculosis

Diagnosis of TB is conventionally done by detecting the presence of acid fast bacilli under the microscope, cultivation of *M. tuberculosis*, identification of MTBC, followed by DST testing (Parsons *et al*., 2011). Molecular techniques, such as PCR, can also be useful in the identification of MTBC (Singh & Kashyap, 2012).

2.15.1 Microscopic examination of mycobacteria

Microscopic evaluation of the stained sputum smear is the cornerstone for pulmonary TB diagnosis in developing countries (Desikan, 2013; Uddin *et al*., 2013; Weldu *et al*., 2013; Dezemon *et al*., 201). This technique is less costly, rapid, easy to perform (Desikan, 2013; Uddin *et al*., 2013), and it can also detect the most infective patients **(**Desikan, 2013). The requirement of 5000 to 10 000 bacilli per millilitre to allow the detection of the bacteria in smears, and time-intensive manual processing are some of the limitations of this technique (Dezemon *et al*., 2014).

The sensitivity of smear microscopy has been reported to range between 20% and 80% (Parsons *et al*., 2011). In the case of extra-pulmonary specimens, the sensitivity of smear microscopy has been shown to be 51% (Vadwai *et al*., 2011). Furthermore, in HIV co-infected individuals, cavitary lung disease is less common and as a result patients present with fewer organisms in the lungs, which makes microscopic examination of sputum highly insensitive (Parsons *et al*., 2011; Kaforou *et al*., 2013). A study done by Padmapriyadarsini and colleagues (2011) showed that the sensitivity of microscopy in examination of TB sputum smear in HIV infected TB patients ranged from 43% to 51%.

Ziehl-Neelsen (ZN) along with fluorochrome techniques are the two commonly used procedures for staining of acid fast bacilli (Dezemon *et al*., 2014). Ziehl-Neelsen staining is the most commonly used technique, but it has a low sensitivity when compared to fluorescence microscopy, which is 10% more sensitive (Parsons *et al*., 2011; Dezemon *et al*., 2014). The examination of smears at lower magnifications with fluorescence microscopy reduces the turn-around time with this technique (Parsons *et al*., 2011).

2.15.2 In vitro growth of *Mycobacterium tuberculosis*

Culturing of *M. tuberculosis* on media is still regarded as the gold standard because it is highly specific (Achkar *et al.*, 2011). Cultivation of the bacilli is required for conducting follow-on procedures such as DST and genotyping. The sensitivity of culture has been estimated to be approximately 500 times more than that of microscopy (Huggett *et al*., 2003; Parsons *et al*., 2011).

Mycobacteria can be grown either on solid or in liquid media. On solid media, bacterial growth can be observed after six to nine weeks, whereas liquid culture media yield results within 10 to 21 days (Huggett *et al*., 2003). The Löwenstein-Jensen (LJ) culture method is the most commonly used for growing tubercle bacilli on solid media. The most outstanding limitation of LJ media is the long waiting time of at least six weeks for growth to become visible (Rodrigues *et al*., 2009; Chihota *et al*., 2010). Middlebrook 7H9 broth is the most commonly used liquid growth medium. This growth method results in shorter turnaround times for diagnosis of TB by culture, but is generally more expensive than other methods, given the need for supplementation to allow for DST (Parsons *et al*., 2011).

When traditional methods of culture are used, the diagnosis of TB and drug resistance strains of *M. tuberculosis* in HIV co-infected individuals can be challenging because the number of bacilli is usually low in the tissues at the sites of infection (Balcells *et al*., 2012; Lawn & Zumla, 2012). Besides the low sensitivity of culture in this context, there is also the difficulty of obtaining clinical specimens from deep-seated organs (Lawn & Zumla, 2012). As a result, the sensitivity of culture for the detection of extra-pulmonary TB is only about 53% (Vadwai *et al*., 2011).

2.16 Identification of *Mycobacterium tuberculosis* from culture

Antigen detection assays differentiate MTBC species from non-tuberculous mycobacteria (NTM) by lateral flow. Antigen MPT64 assay (Standard Diagnostics, Seoul, South Korea) is one of the antigen detection tests used to differentiate between MTBC species and NTM. Members of MTBC secrete MPB64 protein (Machado *et al*., 2014), whereas the NTM do not release this protein. The principle of this technique is based on the detection of MPB64 protein using monoclonal antibodies. This assay is easy to perform, and it provides a rapid identification of MTBC in liquid culture (Machado *et al*., 2014). The sensitivity and specificity

of the SD AgMPT64 kit has been reported to be 97% and 100%, respectively (Kumar *et al*., 2011).

Biochemical tests target the metabolic compounds produced by organisms. Several biochemical tests for mycobacteria are available currently. P-nitro benzoic acid assay is an example of the biochemical methods which identifies mycobacterial species. P-nitro benzoic acid inhibits the growth of MTBC members whereas other mycobacteria show slight or no inhibition when tested (Rodrigues *et al*., 2009). This test is simple, rapid, well adapted for the MGIT 960 system, and is highly accurate (Sharma *et al*., 2010). A study done by Rodrigues and colleagues (2009) has shown that this assay has a sensitivity and specificity of 97.6% and 100%, respectively.

2.17 Drug susceptibility testing for *Mycobacterium tuberculosis*

The emergence of MDR-TB demands the performance of DST. Antimicrobial testing of *M. tuberculosis* is done phenotypically by observing the growth in the presence anti-TB drugs, and genotypically by detecting mutations in the genes related to drug action (genotypic DST) (Kim, 2005).

2.17.1 Phenotypic drug susceptibility testing of *Mycobacterium tuberculosis*

Phenotypic DST determines if the organism is resistant or susceptible to an anti-TB drug by evaluating the growth of the organism in the presence of the drug compared with the growth control in conventional culture (Nataraj, 2011). Various phenotypic DST methods have been introduced recently, for example MGIT-based tests, Epsilometer test (E-test) and the MABA (Mei *et al*., 2014). These methods can be performed either on solid or liquid media (Tanoue *et al*., 2002). The down-side of traditional phenotypic methods is that they are characterized by long turn-around times (Narasimooloo & Ross, 2012).

2.17.1.1 Agar proportion method for drug susceptibility testing

Agar proportion method performed on Middlebrook 7H10/11 is recommended as the gold standard for antimicrobial susceptibility testing for MTBC by Clinical and Laboratory Standard Institutes (CLSI) (Ardito *et al*., 2001); however, it has a long turnaround time of about 21 days (Ardito *et al*., 2001). The proportion method allows an exact estimation of the number of

mutants in a bacterial population that is resistant to a specific antibiotic and this proportion is expressed as a percentage. The proportion of resistance is expressed by comparing the number of colony forming units (CFU) that develop on drug containing medium to those growing in drug free medium. A study done by Birinci and colleagues (2002) shown a correlation between proportion method with MGIT and E-test for the first line anti-TB drugs (Birinci *et al*., 2002).

2.17.1.2 Epsilometer test for drug susceptibility testing

The Epsilometer-test (AB BIODISK) method on Middlebrook 7H11, utilizes strips containing gradients of impregnated antibiotics to determine the susceptibility of drugs (Varma *et al*., 2002; Karabulut *et al*., 2014). This test is not labour intensive, has good turnaround times and can be used in a clinical laboratory to give quantitative susceptibility testing results (Sanic *et al*., 2000). Some of the limitations of this assay include the high rate of contamination, difficulty in standardizing the technique, obtaining uniform results, and dealing with the potential hazard arising from the high inoculum needed for this test (Verma *et al*., 2010). This technique has been shown to be equivalent to agar proportion method in determining MIC of RIF in *M. tuberculosis* strains (Sanic *et al*., 2000). An excellent agreement was reported between MGIT and E-test compared with the agar proportion method. Concordance was reported as 98% for INH and 100% for RIF, STR, ETH (Karabulut *et al*., 2014).

2.17.1.3 Microplate alamar blue assay for drug susceptibility testing

Microplate alamar blue assay is one the colorimetric tests which rely on the colorimetric reagents such as redox indicators measuring bacterial viability. Such tests provide a low cost alternative method for the determination of MIC (Chauca *et al*., 2007). With this method, resistance is detected by a change in colour of the indicator after the addition of reagents into inoculated wells of a microplate. The colour change is directly associated to the quantity of viable mycobacteria in the medium. Alamar blue is a soluble redox dye that is stable in culture and is non-toxic. The oxidized dye is blue and non-fluorescent; upon reduction it turns pink and fluorescent. Growth is determined by a visual colour change or by using spectrophotometry (Rampersad, 2012). Microplate alamar blue assay is dependent on the reduction of tetrazolium salt. The most frequently used tetrazolium salts are 3-(4,5-dimethyethiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), sodium 3′- [1-phenylamino)-carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6-nitrobenzene) sulfonic acid hydrate (XTT), and

4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, water-soluble tetrazolium salt (WST-1) (Rampersad. 2012)

The colorimetric methods are highly sensitive and specific in the rapid detection of resistance to RIF and other anti-TB drugs in culture isolates. A review by Bwanga and colleagues (2010), showed a 100% concordance between MGIT and MABA in the detection of RIF resistance (Bwanga *et al*., 2010).

2.16.1.4 BACTEC TB 460 system for drug susceptibility testing

The liquid based radiometric BACTEC TB 460 (BD, Sparks, USA) quantitatively measure the CO_2 released by the metabolism of C-labelled substrate present in the medium (Rodrigues *et al*., 2007). This assay has a short turnaround time of 10 days to 14 days (Rodrigues *et al*., 2009). Although the BACTEC TB 460 method has a short turnaround, it has some disadvantages like labor intensiveness, use of radioisotopes which requires special regulation. Although this technique was found to perform better than LJ in the identification and isolation of *M. tuberculosis* from clinical specimens (Rodrigues *et al*., 2007), the high cost associated with radioactive waste disposal, forced the manufacturer to consider the alternative systems (Rodrigues *et al*., 2009). This system is, consequently, now obsolete and no longer in use, having been replaced by the BACTEC MGIT 960 system.

2.16.1.5 BACTEC MGIT 960 system for drug susceptibility testing

The BACTEC MGIT 960 system was approved by the WHO in 2008 for susceptibility testing of *M. tuberculosis.* The system tests the growth of mycobacteria in the presence of a known concentration (i.e., the critical concentration) of the drug. A growth control without the drug is added to the test system. At the end of the testing protocol the growth in the presence of a drug is compared to growth in the drug free control. This qualitative test is a form of the proportion method in which resistance is established at >1%, meaning if >1% of the total bacterial population tested is resistant to the drug, the organism is considered resistant for clinical purposes.

The BACTEC MGIT 960 system boasts the advantage of reduced turnaround times when compared to culture on solid media, and qualitative susceptibility testing to first line drugs can be done using a 21-day protocol with most results interpretable within 4 days to13 days. It has

been evaluated against the BACTEC TB 460 radiometric susceptibility method with high concordance rates (Scarparo *et al*., 2004; Garrigó *et al*., 2007). Thus, it is now considered the "gold standard" for phenotypic susceptibility testing of RIF, and largely believed to be highly accurate and reliable. Indeed, the MGIT system is highly sensitive in picking up resistance due to the common *rpoB* mutations in codons 513 and 531 (Rigouts *et al*, 2013). While MGIT certainly has more advantages over other culture based methods, high contamination rates remain the main limitation of this assay (Zhao *et al*., 2014). Another drawback is that it fails to detect low level RIF resistance caused by certain specific *rpoB* mutations (so-called disputed mutations), leading to misclassification of RIF susceptibility results (Van Deun *et al*., 2015).

2.17.2 Molecular methods for *Mycobacterium tuberculosis* detection and drug susceptibility testing

Early diagnosis of TB in clinical samples and confirmation of clinical and radiological diagnosis in patients can be done using nucleic acid amplification test (NAAT) procedures (Bicmen *et al*., 2011). Certain of the molecular methods used for the rapid detection of mycobacterial species and RIF-resistance involve nucleic acid probes, and conventional or multiplex real-time PCR assays (Williams *et al*., 2007), DNA sequencing, LPA and DNA microarrays (Morgan *et al*., 2005).

2.17.2.1 Polymerase chain reaction methods

Polymerase chain reaction methods are useful tools for rapid identification of mycobacteria in clinical specimens such as sputum, bronchial lavage, cerebrospinal fluid, and ascitic fluid. Several of the available PCR assay platforms, e.g. multiplex PCR and real-time PCR, have been shown to perform better than microscopy or culture (Singh & Kashya, 2012). A study done by Zakham and colleagues (2012) showed 88.2% specificity and 81.1% sensitivity for the detection of *M. tuberculosis* from clinical specimens by PCR.

Multiplex-PCR assays allow for the rapid detection of two or more target genes in a single reaction step, but are technically demanding compared to the conventional monoplex-PCR assay. Technically, it is difficult to optimise this assay, since conditions such as competition and/or homology between the chosen primers, annealing temperature, and optimum concentration of the primers used, require appropriate standardisation. If these conditions are not properly optimised, the sensitivity of the assay is reduced (Gopinath & Singh, 2009).

The real-time PCR approach provides for rapid detection of MTBC without the need for processing of the amplicons (Surat *et al*., 2014), since it utilizes fluorescent probes for the detection of genes (Grassi *et al*., 2006). Fluorescent probes enable real-time PCR to simultaneous amplify and detect the desired sequences from clinical specimens (Tobler *et al*., 2006). The region targeted for the detection and characterization of MTBC is the specific insertion element known as IS6110 (Surat *et al*., 2014), which is unique to MTBC. IS6110 has become an important diagnostic tool for the detection of MTBC species (Coros *et al*., 2008). A study done by Richardson and colleagues (2009) determined a sensitivity and specificity of greater than 99 % for the detection MTBC, when a syber-green multiplex real-time PCR was used. These findings are in support of those published by Esfahani and colleagues (2012). Other targets such as *16S rDNA, rpoB, recA, sodA, gyrB, dnaJ*, and the *16S-23S rDNA* have been found to be also suitable for the identification of mycobacteria (Esfahani *et al*., 2012). Though real-time PCR has advantages over conventional real-time PCR, it is technical demanding and costly, posing challenges to routine use (Gopinath & Singh, 2009).

2.17.2.2 GeneXpert assay for the detection of mutations in the *rpoB* gene

The GeneXpert MTB/Rif assay is a semi-automated, simplified version of real-time polymerase chain reaction. This assay has proved to be an effective molecular technique for the detection of *M. tuberculosis* and RIF-resistance. GeneXpert uses five different nucleic acid hybridization probes in the same multiplex reaction in order to detect DNA sequences amplified in a hemi-nested real-time PCR assay.

This technique is fast, accurate, and easy to operate. The chances of contamination in this method are reduced since all the steps, except decontamination of sputum, are automated (Blakemore *et al*., 2010; Helb *et al*., 2010). Furthermore, Xpert MTB/Rif provides semi-quantitative measurements based on the number of PCR cycles required for detection of a critical amount of DNA (Cycle Threshold or C_T). Samples obtained from untreated TB patients were used to validate correlation between quantitative C_T readouts from Xpert MTB/Rif with semi (quantitative) results of conventional microbiological tests such as microscopy grade and solid culture CFU counts (Kayigire *et al*., 2013), and sensitivities of 90% to 100% have been shown for the detection of *M. tuberculosis* and RIF^-resistance in smear-positive patients. In smear-negative patients, sensitivity was 72% and specificity 100% (Blakemore *et al*., 2010; Helb *et al*., 2010; Kayigire *et al*., 2013).

The limitations of this technique include the high cost (Evans, 2011) and the fact that it detects only mutations within 81 bp *rpoB* gene (Ioannidis *et al*., 2011), meaning that approximately 5% of RIF-resistance which is not encoded by *rpoB* gene mutations cannot be detected (Blakemore *et al*., 2010).

2.17.2.3 Line probe assay for the detection of *Mycobacterium tuberculosis* mutations

Line probe assays utilize reverse hybridization DNA strip technology (Palomino *et al*., 2007; Ling *et al*., 2008), with specific DNA probes immobilized in parallel lines in a nitrocellulose membrane (Palomino *et al*., 2007). These assays provide a rapid detection of TB, but they fail to identify novel mutations (Potdar & Thakur, 2013). Two LPAs which are currently commercially available for detecting first and second-line drug resistance are the INNO-LIPA Rif.TB (Innogenetics, Ghent, Belgium) and Genotype MTBDR*plus* assays (Ling *et al*., 2008).

INNO-LIPA TB Rif assay detects MTBC and the mutations in the *rpoB* gene that confers resistance to RIF (Morgan *et al*., 2005; Ling *et al*., 2008). This assay entails DNA extraction (from culture isolates or directly from clinical samples) and amplification of the *rpoB* gene hotspot region. Amplicons obtained from the PCR are hybridized on the nitrocellulose strips containing probes (Morgan *et al*., 2005). Some studies have reported a high specificity (approximately 100%) and sensitivity of 96.9% for the detection of the RIF-resistance profile (Viveiros *et al*., 2005). However, this assay has a high probability for false positive results, which makes it less useful for the detection of MTBC (Traore *et al*., 2000).

The Genotype MTBDR*plus* assay involves DNA extraction, amplification with a multiplex-PCR, reverse hybridization, and colorimetric visualization of the strips (Daum *et al*., 2012). The assay can be performed directly on clinical specimens or culture positive sediments. The Genotype MTBDR*plus* assay has demonstrated to possess high sensitivity (95%) and specificity (98%) in the detection of RIF-resistance from smear positive clinical specimens and culture positive sediments (Bazira *et al*., 2010). Nevertheless, sensitivity falls to less than 75% when smear negative clinical specimens are tested directly, thus requiring culture before such specimens are processed (Bazira *et al*., 2010). This cultivation of *M. tuberculosis* negatively affects time to diagnosis in the HIV-TB co-infected patients as most of them have smear negative disease. Cognisant of this, the manufacturers have recently introduced version 2 of the assay with improved sensitivity for smear negative specimens (Barnard *et al*., 2012)

Missing WT and corresponding mutant bands in a sample is interpreted as resistant according to Genotype MTBDR*plus* assay, but there remains uncertainty, since the type of amino acid change is not directly characterized. The absence of WT and mutant bands as an indication of drug resistance using Genotype MTBDR*plus* assay could possibly give a false positive result, if different amino acid residues in known amino acid positions affect resistance or levels of resistance to a particular drug (Daum *et al*., 2012).

2.17.2.4 DNA Sequencing

Polymerase chain reaction-based sequencing assays explain the molecular basis of drug resistance in *M. tuberculosis* and have a specificity of 100%, providing a better insight into drug resistance, and leading to greater confidence in choice of suitable treatment options (Potdar & Thakur, 2013). The major drawbacks of this test are that it is expensive, technically demanding and cumbersome for routine use (García de Viedma, 2003).

The Sanger sequencing method has been a research and commercial standard technology for more than 30 years, because of its technical ease-of-use and reliability of results generated (Dewey *et al*., 2012). But, the recently introduced parallel sequencing technologies, also known as NGS, have brought significant changes in the field of biological research. These sequencers have the ability to produce high-throughput reads of short lengths of random reads at a moderate cost (El-Metwally *et al*., 2013; McElroy *et al*., 2014). Some platforms which belong to these categories include the 454 from Roche, MiSeq and HiSeq from Illumina, SOLiD and Ion Torrent from Life Technologies, RS system from Pacific Bioscience, and Heliscope from Helicos Biosciences (El-Metwally *et al*., 2013).

Whole genome sequencing can be achieved using these platforms, with the additional advantage of differentiating *M. tuberculosis* isolates with much greater resolution (Bryant *et al*., 2013). Whole gene and whole genome sequencing yield valuable information on drug resistance, virulence determinants and genome evolution, and it also has the potential of becoming the ultimate tool for diagnostics (Roetzer *et al*., 2013).

Table 3 shows the advantages and disadvantages of the various methods employed in RIF detection.

Table 3: Phenotypic and molecular methods used to detect RIF resistance

Phenotypic methods for RIF resistance detection			
Method	**Advantages**	**Disadvantages**	**References**
Agar proportion method	- Exact estimation of the number of mutants	- Long turnaround time (21 days)	Ardito *et al*., 2001
MABA	- Highly sensitive and specific - Provides rapid detection of RIF-resistance in culture isolates - MIC determination - Low cost		Bwanga *et al*., 2010
BACTEC MGIT 960 system	- Short turnaround time - High sensitivity to 513 and 531 mutations	- Fails to detect low-level RIF resistance - High contamination rate - Expensive	Garrigó *et al*., 2007 Rigouts *et al*, 2013 Mokaddas *et al*. 2015 Zhao *et al*., 2014
Molecular methods for RIF resistance detection			
Conventional PCR	- Short turnaround time (2-3h) - Very sensitive (81.13%) - Very specific (88.24%)	- Post processing of the amplicons	Singh & Kashyap, 2012 Esfahani *et al*., 2012 Zakham *et al*., 2012 Zakham *et al*., 2012
Real-Time PCR	- Easy to perform - Short turnaround time (3h) - No post processing of the amplicon - High sensitivity 89-100% - High specificity 99-100%	- Expensive	 Surat *et al*., 2014 Esfahani *et al*., 2012 Kocagoz *et al*., 2005 Kocagoz *et al*., 2005
Line probe assay	- Rapid detection of RIF resistance	- Gives false positive results	Potdar & Thakur, 2013; Daum *et al*., 2012
	- Sensitivity of 96.9% for the detection of RIF resistance	- Fails to identify mutations outside *rpoB* hotspot	Traore *et al*., 2000; Potdar & Thakur, 2013
	- Highly specific (100%)	- Low sensitivity (75%) in smear negative clinical specimens - False positive result	Traore *et al*., 2000, Bazira *et al*., 2010 Daum *et al*., 2012
GeneXpert assay	- Rapid detection of RIF-resistance - High sensitivity (100%) to smear positive isolates	- Limited to mutations within 81 bp - Low sensitive (71.7%) to - smear-negative culture-positive isolates	Ioannidis *et al*., 2011 Kayigire *et al*., 2013, Wilson, 2011
		- False-positive RIF-resistance due to silent mutations	Mokaddas *et al*. 2015
	- Highly specific (100%)		Kayigire *et al*., 2013
DNA Sequencing	- Gold standard for DST - Highly specific (100%)	- Cumbersome for routine use - Prone to interpretation errors	García de Viedma, 2003 García de Viedma, 2003

2.18 Prevention and control of tuberculosis infection

The pillars of TB control, such as case finding, treatment and vaccination, are continuously in need of improvement in order to prevent TB (Arbelaez *et al*., 2000; Churchyard *et al*., 2014). For this reason, the WHO recommended the DOTS programme several decades ago (Chopra *et al*., 2012). The four important pillars of DOTS are: identification of smear positive pulmonary TB using sputum microscopy in patients presenting themselves to public clinics, directly observed treatment with short course chemotherapy; guaranteed continuous drug supply; and a case recording system tracking treatment outcomes (Obermeyer *et al*., 2008). Although widely implemented, the impact on curbing the epidemic was not as pronounced as generally hoped for, largely because of the overwhelming demands on service provision that followed in the wake of the escalating epidemic of HIV-TB co-infection.

Bacillus Calmette-Guérin (BCG), derived from an attenuated *M. bovis* strain, is the only vaccine currently available against TB (Brandt *et al*., 2002). Most countries recommend the administration of BCG to children (Franco-Paredes *et al*., 2006) because it provides protection against severe forms of TB in this cohort, more especially the disseminating and meningeal forms (Ottenhoff & Kaufmann, 2012). Its efficacy against severe forms of TB disease ranges from 60% to 80% in children (Arbelaez *et al*., 2000), but it is not more than 50% effective against pulmonary TB in adults and adolescents (Ottenhoff & Kaufmann, 2012). Due to the disadvantages and limitations of the currently available vaccine, it is imperative that effective and safer TB vaccines be developed in order to prevent TB disease (Franco-Paredes *et al*., 2006).

2.19 References

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Chapter 3: CHARACTERIZATION OF *rpoB* MUTATIONS AND MIC DISTRIBUTIONS IN PHENOTYPIC AND GENOTYPIC RIFAMPICIN DISCORDANT *Mycobacterium tuberculosis* ISOLATES

NOTE: Editorial style of the Journal of Diagnostic Microbiology and Infectious Disease was followed in this chapter, which was prepared in the format of a manuscript to be submitted for publication to the chosen journal. However, headings, tables, figures and references follow the numbering in accordance with those of the other chapters.

3.1 Abstract

It has been documented that approximately 5% of *Mycobacterium tuberculosis* isolates resistant to rifampicin (RIF) by phenotypic test methods do not harbour mutations in the hotspot region of the *rpoB* gene. Earlier reports showed that amino acid changes in the efflux pump genes and epistasis between genes are associated with low level of RIF resistance, but little is known about the changes occurring outside the hotspot region of the *rpoB* gene. Widely used genotypic drug susceptibility test methods, such as GeneXpert MTB/Rif or Genotype MTBDR*plus* line-probe assays, would fail to detect RIF resistance due to mutations outside of the *rpoB* hotspot region. In this study, the aim of the study was to characterize, in a collection of 89 isolates from sputum specimens of TB suspects, the novel mutations occurring outside the rifampicin resistance determining region of *rpoB* gene using whole genome sequencing and correlating these mutations with the phenotypic drug susceptibility test results of the same isolates. All isolates were subjected to GeneXpert MTB/Rif, Genotype MDRTB*plus* version 2, and BACTEC 960 MGIT assaying using the standard critical concentration cut-point of 1 µg/ml. Furthermore, Sanger sequencing of the *rpoB* gene was carried out in a randomly selected subset of 40 isolates, and Illumina Miseq whole gene sequencing of the *rpoB, rpoC, rpoA, gyrA* (as an indicator gene for mutations related to multi-drug resistance in *M. tuberculosis*), and efflux pump genes were performed on 17 of these isolates (included 10 discordant, i.e. susceptible by MGIT but no mutations observed by Sanger sequencing of *rpoB*). Illumina MiSeq NGS revealed alterations outside the hotspot region of the *rpoB* gene, with these mutations most frequently occurring in codons 1156 (2%) and 184 (2%). One amino acid change (A1125V, n=3) and 2 single nucleotide changes (C623G, n=4) and G675E (n=1) were

identified in the *rpoC* gene. The most frequently identified mutations in the efflux pump genes were: Rv 1145 (*mmpL 13a* (Leu277fs), and Rv0933 (*pstB/*T61M), 11% and 4%, respectively. Mutations (E21E=21%, S95T=21% and G668D=21%) were the most frequently identified in *gyrA* gene. Furthermore, 9 isolates had mutations in the *rpoC* gene, and these alterations occurred in codons G675E (n=1), C623G (n=4) and A1125V (n=4). Resistance to RIF was mostly due to mutations in codons 531, 526 and 516. The degree of resistance to RIF in strains with mutations in codon 526 of the (81 bp of *rpoB* gene) differed with amino acids involved. The change of histidine to arginine was related to high levels of RIF resistance (≥16 µg/ml), whereas replacement of histidine to leucine was related with moderate levels of RIF resistance (2 µg/ml). Our data support the recent reports of other investigators that the expression of efflux pump mutations in the *mmpL 13a, mmpL 13b and pstB* genes, and alterations in *rpoC* and *gyrA,* may have synergy with other mutations in the *rpoB g*ene within or outside the hotspot region. These associations have been shown to result in low levels of resistance. We conclude that next generation sequencing of all isolates that show discrepancies in drug susceptibility testing between MGIT and GeneXpert or MTBDR*plus* LPA be performed in order to better inform treatment regimens for TB patients.

3.2 Introduction

The development of drug resistant-TB has complicated the eradication of *M. tuberculosis* infection. Although drug-resistant-TB has been a problem since the early days of the introduction of antibiotics, MDR-TB continues to be part of a mounting problem (Comas et al., 2011). Multi-drug resistant tuberculosis is, by definition, characterized by *M. tuberculosis* strains resistant to both INH and RIF, however, resistance to RIF is the key determinant for treatment failure (Van Deun *et al*., 2009). In most settings, particularly where fixed-dose combination (FDC) first-line anti-TB drugs are used, resistance to RIF is strongly associated with resistance to INH. Detection of RIF resistance therefore serves as a reliable (although not complete) proxy for MDR-TB (WHO, 2015).

Rifampicin is a pivotal anti-TB drug used for the treatment of TB infection. Rifampicin is known to interfere with transcription in the bacterium by binding to the β-subunit of RNA polymerase (the product of the *rpoB* gene) (Comas *et al*., 2012; Kumar & Jena. 2014). Approximately 95% of *M. tuberculosis* strains with resistance to RIF contain distinct mutations located within the 81 bp(the 27 codon range 505 to 533) region of the β-subunit of the *rpoB*

gene (Kumar & Jena. 2014; Mboowa *et al*., 2014). These mutations mostly occur in codons 516, 526 and 531 (Valvatne *et al*., 2009). Furthermore, nucleotide changes in codons 526 and 531 causes high level of RIF resistance, whereas mutations in codons 511, 516, 518, and 522 are associated with low level RIF resistance (Horng *et al*., 2015). It has been reported in some parts of the world that disputed *rpoB* mutations are responsible for over 10% of RIF resistance and treatment failure in first line regimens (Van Deun *et al*., 2015). Additionally, little is known about the levels of RIF resistance in clinical isolates with *rpoB* mutations situated outside the hotspot (81 bp) region of the *rpoB* gene (Jamieson *et al*. 2014).

Rifampicin DST by conventional culture methods, together with new rapid molecular methods, is considered a most reliable diagnostic system (Van Deun *et al*., 2009). The MGIT 960 system boasts the advantage of reduced turn-around times (Scarparo *et al*., 2004) and is considered the "gold standard" for phenotypic susceptibility testing of RIF because of its high accuracy and reliability (Ahmad *et al*., 2016). Unfortunately, some *M. tuberculosis* isolates harboring *rpoB* gene mutations will test susceptible for RIF by the critical concentration method, leading to misclassification of results (Williamson *et al*., 2012).

Molecular assays which are currently commercially available and used by the South African National TB Programme, with the endorsement of the WHO, include the Genotype MTBDR*plus* assay (Hain Lifescience GmbH, Nehren, Germany) and GeneXpert MTB/Rif (Cepheid, California, USA). These molecular assays possess two primary advantages over the phenotypic identification, that is, a more rapid turn-around time and improved accuracy in identification of drug resistant *M. tuberculosis* strains (Barnard *et al*., 2012). The limitation of these assays is that they only detect mutations within the 81 bp region of the *rpoB* gene that confers resistance to RIF (Ioannidis *et al*. 2011).

Results of proficiency testing among WHO supranational TB reference laboratories have revealed that certain strains carrying specific *rpoB* mutations yield discordant results when tested using the MGIT critical concentration system. In other words, these strains are genetically RIF resistant, but are in fact classified as susceptible when the proportion method is used on MGIT 960 system (Van Deun *et al*., 2013). This is particularly the case for mutations occurring on codons 511Pro, 516Tyr, 533Pro, and several 526 mutations (Rigouts *et al*., 2013). The occurrence of such mutations in some strains probably explains the reduced specificity of the currently widely used molecular assays, when compared to MGIT testing, with resultant

discrepancy between the phenotypic and the genotypic tests (Van Deun *et al*., 2013). The fact that their prevalence might be under-estimated, because large molecular testing based surveys have not been done and MIC testing of isolates is not routinely practiced (Van Deun *et al*., 2013), is of concern because such strains might be pivotal in the spread of MDR-TB.

Ultimately, however, sequencing of the amplified target DNA has been found to be the most specific, accurate and reliable method to detect resistance to RIF; this technique is becoming less expensive, even though it demands more specialized equipment (Patel *et al*., 2000). Parallel sequencing technologies, such as NGS, have brought significant changes in the field of biological research. These sequencers have the ability to produce high-throughput reads of short lengths of random reads at a moderate cost (El-Metwally *et al*., 2013; McElroy *et al*., 2014). Sequencing techniques yield valuable information on drug resistance and detects all the mutations within specific genes, which is an advantage over the currently available molecular assays in that they are limited to the mutations occurring in the hotspot region of the *rpoB* gene.

In this study we evaluated *M. tuberculosis* clinical isolates with discordant RIF phenotypic and genotyping susceptibility test results using Sanger sequencing and NGS methods to describe the underlying mutations and correlate these mutations with MIC results obtained for each isolate.

3.3 Materials and methods

3.3.1 Collection and selection of *Mycobacterium tuberculosis* isolates

In this study, 89 *M. tuberculosis* clinical isolates with RIF susceptibility test results for BACTEC MGIT 960 (MGIT) and GeneXpert MTB/Rif (Xpert) were sourced from the National Health Laboratory Services (NHLS), Tshwane Academic Division TB Laboratory from January 2014 to September 2015, after routine investigations were performed. As part of routine investigation, clinical specimens were decontaminated using the standard *NALC*-NaOH method (National Department of Health, 2014). Each specimen was inoculated into MGIT tubes for incubation. Isolates were also identified as *M. tuberculosis* using ZN acid-fast staining and confirmed with the BD MGIT TBc identity kit (MPT64 antigen) (Becton Dickinson, Sparks, MD). Following identification, all of the *M. tuberculosis* isolates were tested for susceptibility to RIF using the MGIT SIRE kit (Becton, Dickinson, Sparks, MD)

according to manufacturers' recommendation, with critical drug concentration applied at the recommended 1.0 µg/ml for RIF.

3.3.2 Phenotypic drug susceptibility testing

3.3.2.1 MGIT 960 system for drug susceptibility testing

Phenotypic DST was performed using MGIT for RIF according to the manufacturer's instructions, in all the isolates without prior results. Briefly, the test inoculum was prepared on a positive 7 ml MGIT tube within 5 days after it first became positive on the MGIT instrument. For day 1 or day 2 positives, a MGIT broth suspension was used for preparing the inoculums for susceptibility testing. For day 3, day 4, or day 5 positives, a 1:5 dilution (1 ml of the positive broth diluted in 4 ml of sterile saline) was used to prepare the inoculum for susceptibility testing. Three tubes containing 7 ml of Middlebrook 7H9 were labelled, one as growth control (GC), one as RIF, and one as INH. A 0.8 ml quantity of MGIT SIRE (BD, Sparks, MD, USA) supplement was aseptically added to each tube. Thereafter, 100 µl RIF and INH drugs were added aseptically to the appropriately labelled tubes, using a micropipette. No antibiotics were added to the GC tube. From there, 0.1 ml of the organism suspension was aseptically pipetted into 10 ml of sterile saline to prepare the 1:100 GC suspension, which was mixed thoroughly, before inoculating 0.5 ml of the GC suspension into the tube labelled "GC". Aseptically, 0.5 ml of the organism suspension was pipetted into RIF and INH containing tubes and the tubes recapped and mixed thoroughly. The tubes were then loaded into the MGIT instrument. When the test growth unit was less than 100 when the GC reaches 400 the test was deemed susceptible, conversely for 100 or more the test isolate was deemed resistant.

3.3.3 Determination of minimum inhibitory concentrations using the microplate alamar blue assay

MABA was performed as described by Ocheretina and colleagues (2014), with few modifications. Briefly, a sterile 96-flat bottom well microplate was used to carry out the tests. Rifampicin stock solutions of 800 µg/ml were prepared and stored in -80˚C. The range of concentrations tested was 0.00005 µg/ml to 8 µg/ml. A growth control containing no antibiotic was included in each plate. Three to five days old positive MGIT cultures were used to determine RIF MICs. The MGIT tubes were vortexed vigorously and allowed to stand for 5 to 10 min. From the supernatant, 100 µl of bacterial suspension was inoculated to the wells of a

96-well plates. Perimeter wells on each plate were filled with sterile water to avoid dehydration of the medium during incubation. The plates were sealed and incubated at 37°C under 5% CO_2 for 7 days. After 5 days of incubation, 50 µl of 1:1 alamar blue (Invitrogen, Carlsbad, USA) and 10% Tween 80 solution was added in the GC wells. When a color change was observed, 50 µl of the same solution was added to all the wells. A colour change from blue to pink indicated growth of the bacteria, and the MIC was defined as the lowest drug concentration that prevented a full colour change of the solution from blue to pink. The MIC of a positive control H37Rv was determined as well. The same RIF stock was used for all assays.

3.3.4 Determination of minimum inhibitory concentrations using the agar proportion method

The agar proportion method was carried out in sterile 12 well RIF plates (Media-Mage, Florida Hills, South Africa) and drug concentrations within a range of 0.125 µg/ml to 16 µg/ml were tested. Day 1 positive MGIT cultures were used for inoculation. All the sets of the plates were labelled properly with the isolate number. Mycobacteria growth indicator tubes were vortexed vigorously and allowed to stand for 5 to 10 min. One hundred microliter of the inoculum was added in growth control well and all the drug containing wells. Plates were sealed with parafilm, followed by incubation at 37°C in 5% CO_2. The inoculated media were examined for contamination a week after incubation and DST interpretation was done after 3 weeks to 6 weeks of incubation. A strain was considered susceptible if there were no colonies or considerably less than 1% of growth in drug medium compared with GC media. A strain was considered to be resistant if the number of colonies on drug containing medium exceeded that on the GC.

3.3.5 Extraction of genomic DNA from *Mycobacterium tuberculosis* isolates

Mycobacterium tuberculosis genomic DNA was isolated as described by van Soolingen and colleagues (1991), with some modifications. *M tuberculosis* isolates were grown on LJ medium for 3 to 4 weeks. Colonies were gently scraped off with the inoculation loop and washed down in 1.5 ml of sterile water. The suspension was transferred to 2 ml screw cap tubes. After centrifugation at 3000 X g for 10 min, cells were re-suspended in 450 µl of TE buffer (0.01 M Tris-HCl, 0.001 M EDTA [pH 8.0]). One-hundred µl of lysozyme was added to a final concentration of 10 mg/ml, and the tube incubated for 1 h at 37°C. One-hundred µl of 10% sodium dodecyl sulfate (SDS) and 50 µl of proteinase K (at a 10-mg/ml concentration)

was added, and the mixture incubated for 10 min at 65°C. Two-hundred µl of 5 M sodium chloride and 160 µl of preheated (at 65°C) cetrimide saline solution were added, mixed gently by inversion, and incubated for 30 min at 55°C. The addition of NaCl blocked the binding of cetrimide to DNA. DNA extraction was further carried out applying chloroform-isoamyl alcohol in a ratio of (24:1). This combination resulted in the formation of an aqueous layer, which contained DNA. Subsequently, recovered DNA was washed once with 70% ethanol and the air-dried pellet covered with 100 µl of TE buffer and stored at 4°C overnight to allow the pellet to dissolve.

3.3.6 Molecular drug susceptibility testing

3.3.6.1 GeneXpert MTB/Rif assay for drug susceptibility testing

GeneXpert MTB/Rif (Xpert) is the recommended diagnostic test for the rapid evaluation of TB suspects in South Africa. In this study Xpert was performed only on isolates without prior results from routine testing. To analyse positive culture isolates using Xpert, 1 ml of bacterial suspension from a positive MGIT was mixed with 2 ml of the sample reagent, and 2 ml of the solution transferred to the Xpert cartridge. Cartridges were loaded for processing and allowed to run for 1 h 30 minutes. Results were recorded as RIF resistance detected or RIF resistance not detected.

3.3.6.2 Drug susceptibility testing by Genotype MTBDR*plus*

The Genotype MTBDR*plus* line-probe assay (LPA) was performed on DNA extracted from positive MGIT cultures as per manufacturer's recommendations. Drug susceptibility testing for RIF was done during the routine testing of the specimens. Polymerase chain reaction (PCR) was performed using primers and DNA precursors provided by the manufacturer, and subsequent hybridization performed in an automated device (GTBLOT 20; Hain LifeScience, GmbH) following the manufacturer's recommendations. Hybridized amplicons were colorimetrically detected using streptavidin conjugated with alkaline phosphatase and substrate buffer. Finally, strips containing hybridized amplicons were air dried and fixed on evaluation paper for interpretation of drug resistance patterns of the isolates.

3.3.6.3 Drug susceptibility testing using Sanger sequencing

In 40 isolates randomly selected from the study set of 87, genomic DNA was extracted from the culture isolates as previously described, followed by amplification and sequencing of the *rpoB* gene (resource constraints limited the number of isolates that could be included). Primers used at an annealing temperature of 55ºC with GGCACCGCGCTG as flanking sequences were:

- *rpoB* F CCACCCAGGACGTGGAGGCGATCACAC; and
- *rpoB* R CGTTTCGATGAACCCGAACGGGTTGAC (Ravibalan *et al*., 2014)

Sequences were analysed using DNASTAR® SeqMan® NGen® Software.

3.3.6.4 Next-generation sequencing

From the series of 40 isolates subjected to Sanger sequencing, 17 isolates were successfully sequenced by NGS (23 not successful) in order to explore and describe other mutations in the *rpoB*, *rpoC* and *rpoA* genes. Efflux pump mutations were also screened. Isolates confirmed to be MDR-TB, RIF susceptible or discordant isolates were sequenced. Sequencing was carried out on Illumina Miseq platform (Illumina, San Diego, CA). The Nextera sample preparation kit was used to prepare a DNA quality sample of 1 nanogram, following the manufacturer`s instructions (Illumina, San Diego, CA). Libraries were prepared and run on the Illumina Miseq instrument according to the manufacturer` instructions. Sequence reads were trimmed and aligned to *M. tuberculosis* H37Rv reference genome using CLC Bio software (v. 602) (Aarhus, Denmark).

3.4 Results

3.4.1 Drug susceptibility testing using Genotype MTBDR*plus* line-probe assay, GeneXpert MTB/Rif, and MGIT 960 system

Of the 89 isolates included in the study series, MTBDR*plus* assay showed 81 (91%) as resistant to RIF. Xpert MTB/Rif showed resistance in 57 (64.0%) and MGIT 960 in 22 (24.7%). These proportions differed statistically significantly from one another (Fischer Exact Test $p<0.0001$).

Twenty-one (23.6%) of the 89 isolates were RIF-resistant and 7 (7.9%) RIF-susceptible by all assays (MTBDR*plus* assay, Xpert, MGIT 960). Overall, discordance between assays on RIF susceptibility was seen for 61 (68.5%) of isolates. Except for one isolate, all of the discordant

outcomes (n=60, excluding one invalid result for MGIT 960) related to differing outcomes between MGIT 960 susceptibility determinations and molecular test results. In 59 of the 60 isolates, MGIT 960 showed susceptibility to RIF, with MDRTB*plus* and/or Xpert also detecting resistance. **Table 4** shows the characteristics of isolates collected in this study.

Table 4: Summary characteristics of *Mycobacterium tuberculosis* isolates collected between 2013 and 2015 at Tshwane Academic Hospital, Pretoria

Genotype MTBDR*plus* (RIF[a])	GeneXpert MTB/Rif	MGIT 960 (RIF)	Number of isolates
R[b]	R	S[c]	34
R	R	R	21
R	S	S	24
R	S	R	1
S	S	S	7
R	R	Invalid[d]	1
S	R	S	1
Total isolates			**89**

[a]RIF=rifampicin; [b]R=resistant; [c]S=ssusceptible; [d]Invalid=uninterpretable results

3.4.2 *rpoB* gene sequencing by Sanger system

Because of limited resources, Sanger sequencing of the *rpoB* gene was performed on only 40 of the 89 isolates collected in this study. Isolates were randomly selected and all the sequencing runs were successful.

Sequencing results are shown below for all isolates that were concordantly resistant to RIF on MTBDR*plus*, Xpert, and MGIT 960 (n=17) (**Table 5**), and all isolates that showed RIF resistance on MTBDR*plus* and Xpert but were reported RIF susceptible on MGIT 960 (n=10) (**Table 6**). The remaining 13 isolates that showed RIF resistance only on MDRTB*plus* (n=11), or were susceptible to RIF on all three the assays (n=2), did not show any mutations in *rpoB* by Sanger sequencing, and have been excluded from the tables. Overall, a total of 30 previously known mutations were recognized and one novel mutation (S601T) was identified.

3.4.2.1 Description of *rpoB* gene mutations in phenotypically uniformly confirmed RIF-resistant strains

The most frequent *rpoB* gene mutations described by Sanger sequencing in the 17 isolates that were designated resistant by MTBDR*plus*, Xpert and MGIT 960, occurred in codons 531, 516, and 526 (41%, 29% and 11% respectively). A mutation in codon 533 (L533P) was found in 1 isolate, and a novel mutation (S601T) outside the *rpoB*-RRDR recognized in one more isolate. In one isolate, the mutations Q513P and DEL514-516 were found, and in another, mutations L533P and D516G occurred concurrently, suggesting mixed strains. One strain which was resistant to RIF on all three assays did not show any mutations by Sanger sequencing (**Table 5).**

Table 5: Microbiological characteristics of phenotypically RIF-resistant *Mycobacterium tuberculosis* isolates subjected to *rpoB* gene sequencing (n=17)

GeneXpert MTB/Rif	Genotype MTBDR*plus* (RIF[a])		*rpoB* gene mutations (Sanger sequencing)	MGIT (RIF)	Number of isolates
	No WT band	Interpretation			
R[c]	WT8	R	S531Q & D516G	R	1
R	WT8	R	S531Q	R	1
R	WT8	R	Mixed strains	R	1
R	WT8	R	S531W	R	1
R	WT8	R	S531L	R	1
R	WT 3,4	R	L533P	R	1
R	WT 3,4	R	NO MUT[d]	R	1
R	WT 3,4	R	DEL514-516	R	1
R	WT 3,4	R	S601T	R	1
R	WT 3,4	R	D516Y	R	1
R	WT 3	R	Q531L& DEL514-516	R	1
R	WT 3	R	Q531L	R	1
R	WT 2,3,8	R	DEL	R	1
R	WT 7	R	H526R	R	3
R	WT 7	R	H526L	R	1
R		R	S531L/(D516G)	R	1
Total isolates					**17**

[a]RIF=rifampicin; [b]WT=wild type; [c]R=resistant; [d]NO MUT=no mutation

3.4.2.2 Description of *rpoB* gene mutations in isolates with phenotypic and genotypic discordant RIF susceptibility test results

Four amino acid changes were detected in 5 isolates with discordant RIF-susceptibility test results (MGIT susceptible and Xpert resistant). L511P, D516Y, H526N, or S531Q occurred as single mutations in each of 7 isolates. Two isolates had double mutations (L511P/D516G and Q531P/DEL514-516). A tenth isolate showed presence of multiple (mixed) strains (**Table 6**).

Table 6: Discrepant phenotypic and genotypic results based on *rpoB* testing of the strains

GeneXpert MTB/Rif	Genotype MTBDR*plus* (RIF[a])		*rpoB* gene mutations (Sanger sequencing)	MGIT (RIF)	Number of isolates
	Absent WT	Interpretation			
R	WT 2	R	L511P & D516G	S	1
R	WT 2,3	R	Q531P & DEL514-516	S	1
R	WT 3,4	R	D516Y	S	2
R	WT 4	R	D516Y	S	1
R	WT 6,7,8	R	L511P	S	1
R	WT 7	R	H526N	S	2
R	WT 7	R	Mixed strains	S	1
R	WT 8	R	S531Q	S	1
Total					**10**

[a]R=resistant; [b]S=susceptible; [c]WT=wild type

3.4.3 *rpoB* mutations detected by Genotype MTBDR*plus*

As shown above under 3.4.1, the reporting of RIF-resistance was very significantly higher for the MTBDR*plus* assay than for any of the other assays.

Of concern, however, is the observation that 21 of all RIF-resistant isolates and 31 of RIF-susceptible isolates had inconclusive RIF results on this assay (**Table 7)**, i.e. a missing WT band in the *rpoB* gene not accompanied by a corresponding mutant band, as required, on the Genotype MTBDR*plus* assay **(Figure 9**). Results from this assay need to be treated with caution.

Table 7: RIF inconclusive results on Genotype MTBDR*plus* assay

Genotype MTBDR*plus* assay (RIF)	Genotype MTBDR*plus* assay (RIF)	GeneXpert assay (RIF)	GeneXpert assay (RIF)	No of isolates
Absent WT[a] band	**Mutant band**	**Resistant**	**Susceptible**	
WT 2	Absent	7	1	8
WT 3	Absent	3	0	3
WT 2,3	Absent	1	0	2
WT 4	Absent	5	1	6
WT 3,4	Absent	8	0	8
WT 6,7,8	Absent	1	2	3
WT 7	Absent	12	1	13
WT 8	Absent	19	19	38
Total				**81**

[a]WT-Wild type, [b]RIF-Rifampicin

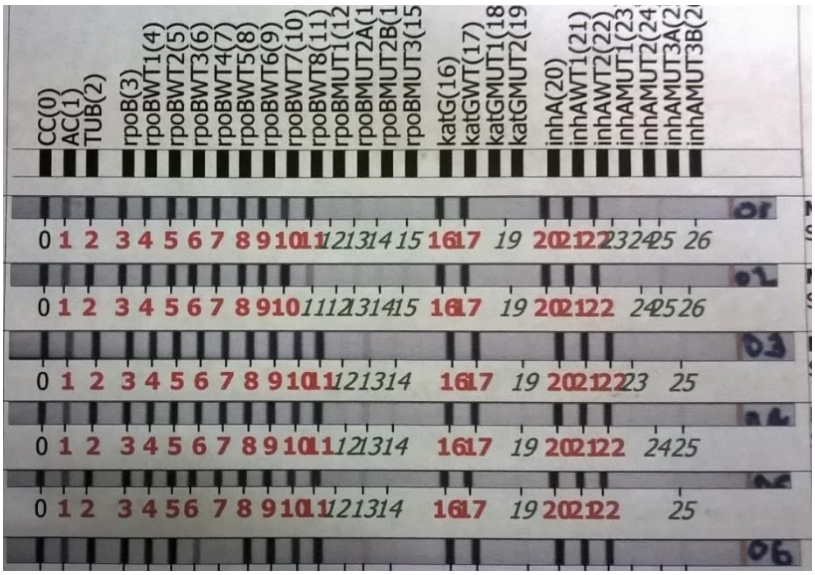


Figure 9: Rifampicin inconclusive results on Genotype MTBDR*plus* assay

3.4.4 Description of *rpoB* mutations by MiSeq next-generation sequencing

Illumina MiSeq NGS was performed on all 40 isolates selected for Sanger sequencing, but unfortunately, only 15 of the runs were interpretable. Repeat runs on the unsuccessful samples were not possible because of funding limitations.

3.4.4.1 Resistance to rifampicin due to *rpoB* mutations

In 4 isolates resistant and 11 susceptible to RIF by MGIT, a total of 23 mutations were identified in the *rpoB* gene (**Table 8**). Most frequently, mutations were identified in codons 516 and 531 (22% and 9% respectively).

Five isolates had multiple mutations within the *rpoB* gene (D516G/L533P/I1187T, S531L/Q1056H, D516Y/A938P, Y1096S/Y1099S/M1106L and Q531L/R908C). Seven novel mutations in the *rpoB* gene were identified (Q1056H, R908C, A938P, Y1096S, Y1099S, I1187T and M1106L), and 2 silent mutations observed outside the hotspot region of the *rpoB* gene (T1156C in 4 isolates and C184T in one isolate). Three RIF-resistant strains had mutations in *rpoC* (C542G, A253V/V333, and C62T) and efflux pump encoding genes (Rv1145/Rv1146 and Rv0933). Mutation S531L was associated with a mutation in the *rpoC* gene. **Table 8** summarises the r*poB*, *rpoC* and efflux pump gene mutations.

Table 8: Description of mutations in *rpoB, rpoC* and efflux pump genes revealed by MiSeq whole genome sequencing

MGIT 960 (RIF[a])	**MiSeq next-generation whole genome sequencing**			**No. of isolates**
Susceptibility status	***rpoB* MUT**	***rpoC* MUT**	**efflux pump MUT**	
Resistant	D516G/L533P/I1187T	C542G		1
	S531L/Q1056H	A253V/V333L	Rv1145/Rv1146	1
	Q531L/R908C	C62T	Rv1145/Rv0933	1
	D516Y			1
Total isolates				**4**
Susceptible	D516Y/A938P	G675E	Rv1146 (resistant GXP)	1
	D516Y	G675E		1
	Y1096S/Y1099S/M1106L	C623G	Rv1145(I358fs)	1
	T1156C	A1125V	Rv1145	3
		C623G		1
	C184T	G675E		1
	T1156C		Rv1145	1
	D516Y			1
		C542G	Rv0933	1
Total				**11**

[a]**RIF-rifampicin,** [b]**MUT-mutation**

3.4.4.2 Resistance to RIF due to mutations outside of the *rpoB* RRDR (81 bp hotspot region), or in the *rpoC*, *gyrA* or efflux pump genes

Ten isolates susceptible to RIF on both Xpert and MGIT revealed mutations outside the RRDR region, *rpoC and* efflux pump genes. One isolate had three mixed amino acid changes (Y1096S/Y1099S/M1106L) located outside the RRDR region. Mutations T876G/G1027C/T1027C were found in 1 isolate. One amino acid change (A1125V, n=3) and 2 single nucleotide changes (C623G, n=4) and G675E (n=1) were identified in the *rpoC* gene.

The identified mutations in efflux pump genes were: Rv1145/*mmpL13a* (Leu277fs), and Rv0933/*pstB* (T61M). Mutations E21E, S95T and G668D were the most frequently identified in *gyrA* gene (**Table 9; Figure 10**).

Table 9: Description of mutations in *rpoB* (outside the 81 bp RRDR), and *rpoC* and efflux pump genes revealed by whole genome sequencing in GeneXpert MTB/Rif and MGIT 960 RIF-susceptible isolates

***rpoB* MUT[a] outside RRDR**	***rpoC* MUT**	***GyrA* MUT**	***pstB*/Rv0933**	***mmpL13a (Rv1145)***	**No of isolates**
C184T	G675E	E21E S95T G668D			1
	C623G	E21E S95T G668D	T61M		2
	C623G	E21E S95T G668D			1
T1156C	A1125V	E21E S95T G668D		Leu277fs	4
Y1096S/Y1099S/M1106L		E21E S95T G668D		Leu277fs	1
	C623G	E21E S95T G668D			1
Total					**10**

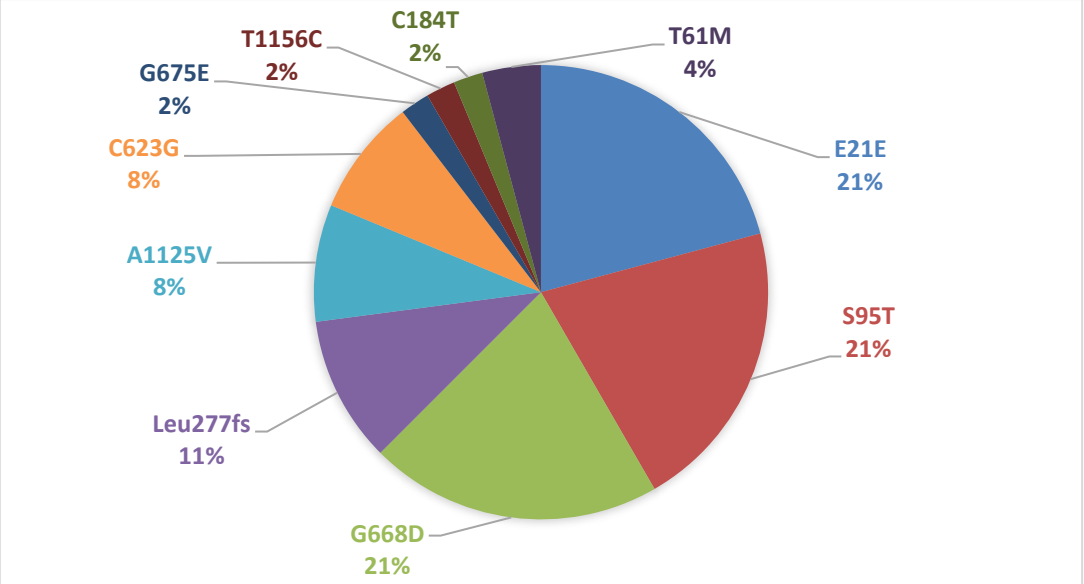


Figure 10: Frequency of mutations occurring in *rpoB* outside the RRDR, *rpoC, GyrA, pstB* and *mmpL13a* as detected by MiSeq NGS in 10 isolates defined as RIF susceptible by Xpert and MGIT 960.

3.4.5 Determination of minimum inhibitory concentrations by microplate alamar blue assay and Agar Proportion Method, and correlation with RIF-resistance by other phenotypic/genotypic assays

In order to further elucidate the microbiological characteristics of the isolates included in this study, and to relate MIC levels to RIF-resistance as determined by the molecular and in vitro assays used, the MABA was performed on 77 of the initial 89 isolates (some of the isolates were not viable at the time of this experiment).

As is evident from **Table 10**, MABA is a reasonable predictor of RIF-resistance and a very good predictor of RIF-susceptibility status as determined by MGIT 960. In 17 isolates resistant to RIF by all three assays used in this study (MTBDRplus, Xpert, MGIT 960), MABA agreement reached only 70.6%. However, in isolates classified as RIF-susceptible by MGIT 960, and depending on whether Xpert RIF-resistance was flagged as positive or negative (irrespective of the MTBDR*plus* status), agreement varied between 92.6% and 100%. In isolates classified as RIF-susceptible by all assays, MABA MICs were consistently below 1 µg/ml, i.e. with 100% agreement to the MGIT 960 results.

Table 10: Agreement between RIF MIC levels determined by MABA and observed RIF-resistance/susceptibility on MGIT 960, with/out concordant results by Xpert or MTBDR*plus* assays

RIF Resistance on LPA/Xpert/MGIT	High MIC (>4 µg/ml): Resistant	Medium MIC (>1.0 – 4.0 µg/ml): Resistant	Low MIC (≤1 µg/ml): Susceptible	Agreement between MABA and MGIT 960
R/R/**R** (n=17)	**11**	**1**	5	12/17 (70.6%)
R/R/**S** (n=27)	0	2	**25**	25/27 (92.6%)
R/S/**S** (n=23)	0	0	**23**	23/23 (100%)
S/S/**S** (n=8)	0	0	**8**	8/8 (100%)
Total samples*	**11**	**3**	**61**	**68/75 (90.7%)**

*Two isolates from the study series of 77 had missing data for one of the variables used in this table, and were excluded from the analysis

The agar proportion method for MIC determination was simultaneously performed to serve as an in vitro comparison to MABA, with results obtained for 48 of the 77 isolates tested by MABA. **Table 11** shows the agreement between the two methods, based on the classification of isolates as per MIC categories high (>4 µg/ml), medium (>1.0 – 4.0 µg/ml) or low (≤1 µg/ml). The two methods were highly correlated (91.7%) on MIC category.

Table 11: Agreement between microplate alamar blue assay and agar proportion method for determining rifampicin minimum inhibitory concentration levels in 48 *Mycobacterium tuberculosis* isolates

MIC category by Agar Proportion Method (APM)	MIC category by Microplate Alamar Blue Assay (MABA)			Total
	High MIC (>4 µg/ml): Resistant	Medium MIC (>1.0 – 4.0 µg/ml): Resistant	Low MIC (≤1 µg/ml): Susceptible	
High	**7**	0	0	
Medium	1	0	2	
Low	0	1	**37**	
Total	8	1	39	**48**
Agreement between MABA and APM: 44/48 = 91.7%				

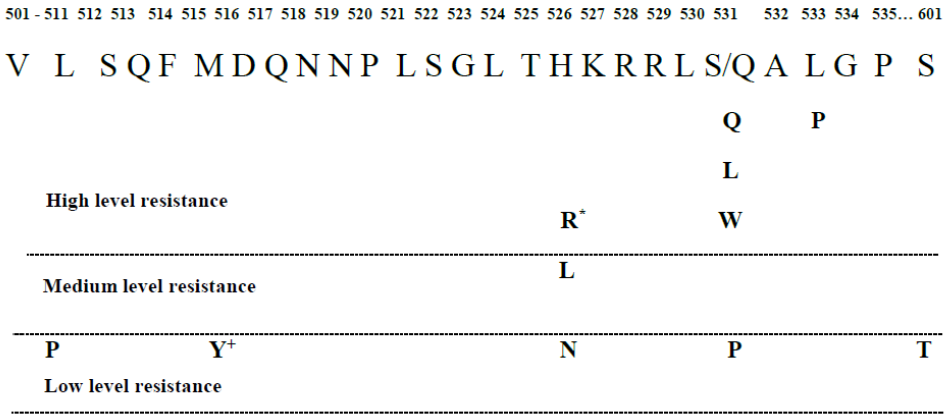
3.4.6 Minimum inhibitory concentrations of rifampicin-resistant/susceptible isolates and corresponding mutations in the *rpoB* gene detected by Sanger sequencing

Isolates with the L511P, H526N, D516Y and Q531P mutations were found to be RIF-susceptible. These isolates showed low level MICs (<1µg/ml). Furthermore, these isolates were characterized as discordant isolates since they were resistant genotypically (Xpert, MTBDR*plus*) but susceptible phenotypically at 1µg/ml RIF on MGIT 960 system. Rifampicin resistant isolates characterized by mutations L533P (n=1), S531L (n=1), S531Q (n=1), H526R (n=1), S531W (n=1) and Q531L (n=1) presented high MIC level (MIC >4 µg/ml) on both MABA and Agar proportion methods. All of these isolates were resistant to RIF on MGIT 960. One RIF- resistant isolate, with a mutation outside the hotspot (81 bp) region of *rpoB* gene had low MIC level and this isolate was found to be resistant to RIF on MGIT 960. One isolate with mutation H526L had moderate MIC level (>1µg/ml to 4 µg/ml) (**Table 12**). Mutations in *rpoB* and their known correlation with RIF-resistance levels are shown in **Figure 11**.

Table 12: Frequency of *rpoB* mutations and minimum inhibitory concentration distribution

***rpoB* MUT[c]**	**MGIT 960 (RIF)**	**RIF[a] MIC[b](µg/ml) on MABA[d]**	**Agar proportion**	**No of isolates with MUT**
L533P	R	>8 µg/ml	>16 µg/ml	1
Q531L	R	>8 µg/ml	>16 µg/ml	1
H526R	R	>8 µg/ml	≥16 µg/ml	1
S531Q	R	>8 µg/ml	≥16 µg/ml	1
S531W	R	>8 µg/ml	≥16 µg/ml	1
S531L	R	>8 µg/ml	≥16 µg/ml	1
H526R	R	>8 µg/ml	-	1
H526L	R	2 µg/ml	-	1
Q531P	R	0.5 µg/ml	0.125 µg/ml	1
S601T	R	0.007 µg/ml	-	1
D516Y	S	1 µg/ml	4 µg/ml	1
D516Y	S	0.5 µg/ml	0.5 µg/ml	1
L511P	S	0.25 µg/ml	0.25 µg/ml	1
D516Y	S	0.12 µg/ml	-	1
H526N	S	0.08 µg/ml	<0.12 µg/m	1

[a]RIF- rifampicin; [b]MIC-minimum inhibitory concentration, [c]MUT-mutation, [d]MABA-microplate alamar blue assay. Mutations are numbered according to *Escherichia coli* nomenclature.



Asterisks show double mutations and a plus sign shows three mutations. Selected MICs ranges were to classify the level of resistance to RIF: high level (MIC>4 µg/ml), medium (>1µg/ml - 4 µg/ml) and low level (MICs (<1µg/ml).

Figure 11: *rpoB* mutations and their correlation to RIF resistance levels

3.4.7 Minimum inhibitory concentrations of rifampicin-resistant/susceptible isolates and corresponding mutations in *rpoB* (outside the 81 bp RRDR), *rpoC*, *rpoA* and efflux pump genes

Three isolates with silent mutations in the *rpoB* gene outside the RRDR (T1156C) also had amino acid changes in the *rpoC* gene (A1125V), in addition to efflux pump mutations Rv1145 (I358fs). Two of these strains had MICs that were 10 fold lower than the critical concentration used by MGIT 960 system, whereas 1 isolate had a MICs 1000 fold lower than the 1 µg/ml critical concentration.

Three mutations located outside the 81 *bp* RRDR of the *rpoB* gene (T876G/G1027C/T1027C) occurred simultaneously in 1 strain, and were associated with a MIC of 0.12 µg/ml in the isolate. One isolate with RIF MIC of 0.06µg/ml showed mutations C623G and Rv0933 on MiSeq sequencing.

The remaining RIF-susceptible isolates, with mutations outside the RRDR (Y1096S/Y1099S/M1106L), or in *rpoC* (C623G) or with Rv1145 mutations, showed low MICs of 0.007µg/ml. One isolate with a *rpoB* mutation outside RRDR (C184T) and *rpoC* mutation (G675E) had a MIC of 0.25 µg/ml (**Table 13**).

Table 13: Mutations in *rpoB* outside the RRDR, *rpoC*, and efflux pump genes, and their corresponding MIC values by MABA and agar proportion methods

rpoB non-RRDR MUT	*rpoC* MUT	Efflux pump MUT	MGIT 960 RIF	MABA	Agar proportion
	C623G	Rv0933 (T223H)	S[e]	0.06µg/ml	0.25 µg/ml
Y1096S/Y1099S/M1106L	C623G	Rv1145 (I358fs)	S	0.007µg/ml	
T876G/G1027C/T1027C			S	0.12 µg/ml	≤0.12µg/ml
T1156C	A1125V	Rv1145 (I358fs)	S	0.007 µg/ml	≤0.12 µg/ml
T1156C	A1125V	Rv1145 (I358fs)	S	0.12µg/ml	0.12µg/ml
	C623G		S	0.04 µg/ml	≤0.12 µg/ml
C184T	G675E		S	0.25 µg/ml	0.2 µg/ml
T1156C	A1125V	Rv1145 (I358fs)	S	0.12 µg/ml	0.12 µg/ml
T1156C		Rv1145 (I358fs)	S	0.003 µg/ml	≤0.12 µg/ml
C542G		Rv0933(T142M)	S	0.039 µg/ml	≤0.12 µg/ml
Total isolates (n=10)					

[a]bp=base pair; [b]MUT=mutation; [c]RIF=rifampicin; [d]MABA=microplate alamar blue assay; [e]S=sensitive.

3.5 Clinical outcomes

Information for 19 treatment compliant patients with MGIT 960 DST indicating susceptibility to RIF, but failing drug therapy, could be matched to isolates reported in this study. Seven of these patients showed *rpoB* gene mutations, and 12 had no known mutations by Sanger sequencing (**Table 14**).

Table 14: GeneXpert test outcomes and corresponding mutations identified in 19 MGIT 960 RIF-susceptible patients failing first-line chemotherapy

Study number	Gender	Age	MGIT 960 Results (RIF*)	*rpoB* gene mutations	Previous TB treatment	Previous HIV treatment	1st Line TB Treatment
E01	Male	49	S	L511P/ (D516G)	No	No	Failed
E05	Female	39	S	NO MUT	No	No	Failed
E06	Male	-	S	NO MUT	No	No	Failed
E07	Female	31	S	S531Q	No	No	Failed
E08	Male	33	S	D516Y	Yes	No	Failed
E10	Male	34	S	D516Y	No	No	Failed
E11	Female	26	S	L511P	MDR	No	Failed
E13	Female	34	S	Mixed strains	MDR	No	Failed
E14	Male	54	S	NO MUT	No	No	Failed
E15	Male	64	S	NO MUT	No	Yes	Failed
E16	Male	28	S	NO MUT	No	No	Failed
E17	Female	37	S	NO MUT	No	No	Failed
E18	Female	47	S	NO MUT	No	No	Failed

E27	Female	41	S	NO MUT	No	No	Failed
E29	Male	1	S	NO MUT	No	No	Failed
E30	Female	23	S	NO MUT	No	No	Failed
E32	Female	-	S	NO MUT	No	Yes	Failed
E34	Male	5	S	NO MUT	No	No	Failed
E38	Female	41	S	H526N	No	No	Failed

*S = susceptible to RIF

Based on **Table 12** and **Table 14**, misclassification of isolates with *rpoB* mutations D516Y, L511P and H526N could have occurred, using MGIT 960 RIF DST as the reference. MICs as determined by MABA or agar proportion method support the MGIT 960 results in **Table 12**. Treatment failures on conventional first-line regimens containing RIF were recorded in the patients infected with strains carrying these mutations.

3.6 Discussion

Molecular methods are increasingly being used for the rapid diagnosis of TB, including RIF-resistant TB, in suspects presenting to health services. The WHO-recommended molecular assays, i.e. MTBDR*plus* and GeneXpert MTB/Rif, together with culture-based testing on the MGIT 960 system, form an integral part of the TB diagnostic algorithm in South Africa. Though these methods provide fast results, the limitations of the assays are that they only report on mutations within the RRDR of the *rpoB* gene. The challenge with the currently available commercial methods is that they fail to determine the level of resistance (Jamieson *et al*., 2014), an important aspect for consideration in designing adequate treatment approaches. Furthermore, these methods have difficulty detecting disputed mutations within the hotspot region of the *rpoB* gene (codons 507 to 533) (Van Deun *et al*., 2015). The MGIT 960 system, being a culture-based DST method, would fail to detect this type of resistance, resulting in misclassification of RIF-resistant strains (Van Deun *et al*., 2015).

3.6.1 Inconclusive results reported by the Genotype MTBDR*plus* assay

The sensitivity of MTBDR*plus* assay for the detection of RIF-resistance is known to vary geographically (Farooqi *et al*., 2012), raising an additional concern as to its appropriate application in diagnostic algorithms. In this study, 81 of the 89 isolates showed a missing WT band in the *rpoB* locus, but a large proportion of these (52/89 or 58.4%) without a corresponding mutation band on the Genotype MTBDR*plus* assay. Results were interpreted

as indicating resistance to RIF, but in fact, were inconclusive. In some cases, the occurrence of a missing WT band can be the result of poor quality of DNA. Furthermore, the absence of WT and mutant bands as an indication of drug resistance using MTBDR*plus* assay could possibly give false positive results if different amino acid residues in known amino acid positions affect resistance or levels of resistance to a particular drug (Daum *et al*. 2012).

3.6.2 Rifampicin minimum inhibitory concentrations and the association with *rpoB* (81 bp RRDR) mutations

Mutations located within the 81 bp RRDR of the *rpoB* gene account for the majority of RIF-resistance to *M. tuberculosis* strains (Veluchamy *et al*., 2013). In this study resistance to RIF was mostly due to mutations in codons 531, 526, and 516, and these results were in agreement with findings from China and other parts of the world (Yue *et al*., 2003). The degree of resistance to RIF in strains with mutations in codon 526 differed with amino acids involved. The change of histidine to arginine was related to high levels of RIF resistance (≥16 µg/ml), whereas replacement of histidine by leucine was related with moderate levels of RIF-resistance (2 µg/ml). Notably the change of histidine to asparagine was characterized by low MICs (0.06 µg/ml).

The low MIC observed for H526N and the moderate MIC associated with mutation H526L are in agreement with the findings from the work done Jamieson and colleagues (Jamieson *et al*., 2014). Mutations S531W, S531L, Q533L and L533P were associated with high levels of resistance to RIF. A study done by Sougakoff and colleagues (2004) also showed high MICs for S531W and S531L, confirmed by similar findings in our study. The S531W mutation is rarely seen in clinical *M. tuberculosis* isolates, which might be due to the reduced fitness of isolates carrying this amino acid change (Mariam *et al*., 2004).

Mutations L511P, D516Y, H526N and Q531P were identified in isolates with discordant RIF susceptibility test results between phenotypic and genotypic methods. In our study 3 of these mutations were characterised by MICs 10 times below the critical concentration (1 µg/ml), except mutation H526N which had MIC of 0.06 µg/ml. Interestingly, amino acids change in L511P and H52N were previously known to cause resistance to RIF, however, some studies have indicated that they can be found in both susceptible and resistant isolates (Jamieson *et al*., 2014). Furthermore, these mutations are termed disputed mutations due to the fact that they

cause low level of RIF resistance in clinical on phenotypic DST, which cannot be picked-up by the MGIT 960 system (Van Deun *et al*., 2015).

In this study, one novel S601T mutation was identified in the *rpoB* gene. This novel mutation was located outside the RRDR of the *rpoB* gene, and for that, it couldn`t be identified by GeneXpert MTB/Rif and MTBDR*plus*, since these assays fail to identify novel mutations occurring outside the hotspot region of the gene (Potdar & Thakur. 2013).

3.6.3 Relationship between the results of genotypic and phenotypic drug susceptibility testing and clinical outcome

As introduced earlier (under 3.5 above), 19 patients with strains susceptible to RIF by MGIT 960 failed TB first-line treatment. One of these patients had a mixed infection. This form of infection is believed to arise due to re-infection resulting in the occurrence of more than one strain of *M. tuberculosis* in the same person. In such case, the competition between strains may influence treatment outcome in co-infected patients, with failure in the event of resistance strains dominating (Millet *et al*., 2013).

Three of the patients were on TB treatment before and one was on ARV treatment. We hypothesized that the 3 patients who were on TB treatment before either were relapse or recurrent cases of TB, that might have emerged due to the regrowth of the same strain of *M. tuberculosis* that caused the previous TB episode (Lin & Flynn. 2010), or resulted from poor adherence to treatment.

HIV infection is also known to increase the progression of TB disease (Lin & Flynn. 2010) and to enhance the development of drug resistant *M. tuberculosis* strains, which in turn lead to drug resistance in *M. tuberculosis*. This mechanism might have been instrumental in the development of treatment failure in 3 patients who were HIV positive. **Table 15** summarises clinical, microbiological and genotypic characterization of patients with *rpoB* gene mutations.

Table 15: Summary of clinical, microbiological and genotypic characterization of patients with *rpoB* gene mutations

Clinical characteristics		**Microbiological and Genotypic features**		**No of Isolates**
Previous TB treatment	**Previous HIV treatment**	**BACTEC™ MGIT™ 960 (RIF)**	***rpoB* gene mutations**	
NO	YES	S	NO MUT	1
YES	NO	S	D516Y	1
YES	NO	S	L511P	1
NO	NO	S	L511P/ (D516G)	1
NO	NO	S	S531Q	1
NO	NO	S	D516Y	1
NO	NO	S	H526N	1
YES	NO	S	Mixed strains	1
NO	NO	S	NO MUT	11
Total Isolates				**19**

[a]S=sensitive; [b]R=resistant

Twelve (63%) of the 19 patients who failed RIF-based TB therapy had no *rpoB* mutations (based on currently used molecular tests) in the hotspot region. Other mechanisms, including efflux pumps, might have contributed to RIF-resistance in these cases. We have reported on 10 isolates with no mutation in the 81 bp RRDR of the *rpoB* gene. Next generation sequencing identified SNPs T1156C, C184T, and C542G as the most likely causes for resistance to RIF. Furthermore, 3 novel amino acid changes (Y1096S/Y1099S/M1106L) occurred in one isolate, with 3 distinct SNPs (T876G/G1027C/T1027C) identified. It is possible that these mutations were responsible for the RIF-resistance observed, but more data would be needed to elucidate the findings.

Other mechanisms that could possibly be associated with drug resistance in these isolates, were detected, corresponding to 3 mutations in the efflux pump genes Rv0993 and Rv1145 (Louw *et al*., 2009). A study done by Li and colleagues (2015) indicated that the expression of the efflux pump (*mmpL 13a, mmpL 13b and pstB*) genes can lead to efflux of RIF from the cell. Moreover, nucleotide alterations within *rpoC* may have a synergy with other mutations in the *rpoB g*ene within or outside the hotspot region and these associations often results in low levels of resistance. In this study, one isolate with compensatory mutations in *rpoC* had a mutation S531L, which is inconsistent with other studies done on similar subjects (Jamieson *et al*.,

2014). Although, compensatory mutations in *rpoC* gene were associated with S531L in our study, seemingly, other strains harbouring the mutations in the *rpoB* gene also presented with *rpoC* compensatory mutations.

Several other factors, including poor adherence of patient to the treatment may lead to treatment failure and relapse (Wolff & Nguyen. 2012). However, the findings of this study pose a challenge to several features of the current diagnostics and treatment practices (Ho *et al*., 2013). It needs to be recognised that disputed *rpoB* gene mutations may have an influence in treatment failure. One aspect to pursue in this context regards the recommended cut-point for the critical concentration used in determining RIF-resistance. Our comparative whole-genome sequencing and MIC data suggest possibly misclassifications of mutation-containing isolates and isolates with no mutations.

Based on hollow-fibre-studies and Monte Carlo simulations, it has been suggested that the critical concentrations for determining RIF-resistance should be lowered from the current level of 1.0 µg/ml to 0.0625 µg/ml (Gumbo *et al*., 2014). It is supposed that a lower RIF critical concentration may be reasonably sensitive at identifying *rpoB* gene mutations that confer lower-level RIF resistance (Williamson *et al*., 2012). Applying a lower MIC breakpoint, e.g. 0.0625 µg/ml, to the isolates used in this study would change the susceptibility status of 32 (55%) of the isolates, which were susceptible on MGIT 960 system at 1 µg/ml, to resistant. In turn, it would increase the specificity of molecular assays for RIF susceptibility. More detailed investigations into this aspect are called for.

3.7 Conclusions

In this study full gene sequencing was used to confirm genotypic and phenotypic results obtained by GeneXpert, MTBDR*plus* assay and MGIT 960 system. Our data support the recent reports of other investigators that the expression of efflux pump mutations in *mmpL 13a* and *pstB* genes, and alterations in *rpoC* and *gyrA* may have a synergy with other mutations in the *rpoB* gene within or outside the hotspot region. These associations have previously been shown to result in low levels of RIF resistance. We conclude that NGS of all isolates that show discrepancies in DST between MGIT and Xpert or LPA be routinely performed in order to better inform treatment regimens for TB suspects.

3.8 Limitations of the study

A major limitation of this study concerns the small number of discordant isolates available for investigation, and in particular, the fact that, because of funding restrictions, not all tests could be performed on all available material. The study could have benefitted from whole genome sequencing of all isolates collected in the study, rather than on subsets randomly sampled from the study isolates. Because of this situation, representativeness of the series of isolates used could be questioned, and extrapolation of the findings to other settings away from Pretoria might not be valid. In this study, resistance resulting from mutations in other genes known to be associated with drug resistance was investigated, but MIC testing of these mutations were not specifically targeted. Finally, the study did not have full clinical information available for patients from whom isolates have been obtained. This is a major shortcoming which could, unfortunately, not be avoided or rectified.

3.9 Ethical considerations

Ethics approval for this study was provided by the University of Pretoria Faculty of Health Sciences MSc Degree Committee, and the Research Ethics Committee, in January 2015. Laboratory numbers, not names were used to identify the specimens, samples and essential clinical information were delinked from any personal identifiers to ensure confidentiality.

3.10 Study design and location

This was a prospective study in which consecutive clinical isolates which met the inclusion criteria were collected from the National Health Laboratory Services, Tshwane Academic Division TB Laboratory (NHLS-TAD). Work started soon after ethics approval was received for the study and continued until the required sample size of 89 samples was met. The study was conducted in the laboratories of the NHLS-TAD, and the South African Medical Research Council (SAMRC) in Pretoria. Sanger sequencing was performed at Inqaba Biotec Laboratories in South Africa, and MiSeq whole genome sequencing conducted at the Baylor Research Institute in Texas, United State if America.

3.11 Statistical methods

The data presented in this study are largely descriptive. Sample size for number of strains included were estimated with the assistance of a qualified biostatistician (Prof. Becker, University of Pretoria). Where comparisons have been made between subsets, the Fischer's Exact Test for statistical significance has been used (95% confidence), specifically to accommodate small numbers.

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Chapter 4: CONCLUDING REMARKS

In this study, we were particularly interested to see whether discordant results from phenotypic and genotypic RIF-susceptibility tests are caused by disputed *rpoB* mutations, other mutations outside the hotspot 81 bp of the *rpoB* gene, or compensatory mutations between genes or efflux pump mutations. We also sought to correlate these mutations with the level of MICs observed and to relate the findings to diagnostic and clinical implication.

Overall, these questions were addressed under three themes:

4.1 Comparison of methods in discordant strains (Theme 1)

Of the 89 isolates included in the study series, the MTBDR*plus* assay showed 81 (91%) as resistant to RIF. GeneXpert MTB/Rif showed resistance in 57 (64.0%) and MGIT in 22 (24.7%). Each of these proportions differed statistically significantly from one another (Fischer Exact Test $p<0.0001$). Furthermore, 52 of the 89 isolates showed a missing WT band in the *rpoB* locus, but without corresponding mutation bands on Genotype MTBDR*plus* assay. Twenty-four of 81 isolates were susceptible to RIF on GeneXpert assay, whereas 57 were resistant to RIF on GeneXpert assay.

These findings uncover the limitations of Genotype MTBDR*plus* assay in connection with false resistant results. Missing WT and corresponding mutant bands in a sample is interpreted as resistant according to Genotype MTBDR*plus* assay. Nevertheless, there remains uncertainty, since the type of amino acid change is not directly characterized. The absence of WT and mutant bands as an indication of drug resistance using Genotype MTBDR*plus* assay could possible give a false positive result if different amino acid residues in known amino acid positions affect resistance or levels of resistance to a particular drug (Daum *et al*., 2012). Another problem associated with these inconclusive results is that they lead to delayed interpretation of results in settings where GeneXpert assay is not available, which frustrates the decision-making in terms of drug administration. Consequently, patients continue to spread the disease in their communities.

4.2 Description of mutations involved in genotypic resistant strains not detected by phenotypic methods and minimum inhibitory concentrations distribution (Theme 2)

To resolve the discrepancy between recommended commercially available molecular assay and culture based phenotypic methods two sequencing methods were used to detect mutations associated with resistance to RIF. These methods showed to be reliable and were able to detect other mutations located outside the 81 bp of *rpoB* gene. In this study, the Illumina Miseq technology was used to sequence whole *M. tuberculosis* genome to characterize the full *rpoB* gene, known to express mutations responsible for resistance to RIF.

Novel mutations in the several genes, such as deletions and SNPs, were revealed in this study. Previously characterised mutations that confer resistance to RIF were reported in this study. Thirteen novel mutations were discovered outside the hotspot region (81 bp RRDR) of the *rpoB* gene: A938P, I1187T, Q1056H, S601T, Y1096S, Y1099S, M1106L, T876G, G1027C, T1027C, T1156C, C184T and C542G. These mutations were associated with MICs below the critical concentration used by MGIT 960 system, and resistance due to these mutations couldn't be detected by currently available methods for DST.

4.3 Whole genome sequencing (Illumina Miseq) for detailed analysis of mutations in *rpoA*, *rpoC* and efflux pump mutations and minimum inhibitory concentration distribution (Theme 3)

It has been documented that approximately 5% of *M. tuberculosis* isolates resistant to RIF do not harbour mutations in the hotspot region of the *rpoB* gene (Louw *et al*., 2009). It is therefore important to consider that other alternative mechanisms such as efflux pump activity, compensatory mutations together with mutations is *rpoA* and *rpoC* may confer resistance to RIF. In this study, Illumina Miseq sequencing was used to characterize the target genes *rpoA* and *rpoC*, and certain efflux pump mutations (*mmpL 13a, mmpL 13b and pstB*). Fourteen mutations were found in the *rpoC* gene (A253V, V333L, C623G, A1125V and G675E). Eight of these mutations were found in isolates sensitive on MGIT and GeneXpert, and 6 mutations were found on isolates GeneXpert resistant and MGIT sensitive. Only one mutation was detected in *rpoA* gene in a single isolate (E400K). It would seem that *rpoA* is not important for investigation in the context of discordant DST results. Nine efflux pump mutations were found: *mmpL 13a* (Rv1145 (I358fs), *mmpL 13b (*Rv1146 and *pstB* (Rv0933-T142M). Three

isolates with silent mutations in the *rpoB* gene outside 81 bp RRDRregion (T1156C n=3) had amino acid changes in the *rpoC* gene (A1125V n=3) together with efflux pump mutations Rv1145 (I358fs). Seven of these mutations were found in isolates sensitive on MGIT and GeneXpert, and 2 mutations were found on GeneXpert resistant and MGIT sensitive isolates. Two of these strains showed MICs which were 10 fold lower than the critical concentration used by MGIT 960 system, whereas 1 of these isolates displayed MICs 100 fold lower than the 1 µg/ml. These findings support the hypothesis that synergetic relationships between genes and efflux pump mutations may cause low level of resistance (Fonseca *et al*., 2015). It is there important that efflux pump inhibitors be developed and considered for use in the treatment of TB.

These findings highlight the deficiencies of the MTBDR*plus* and GeneXpert molecular methods. The MGIT 960 system, however, detects RIF-resistance due to these mutations, leading to discordant results. On the other hand, mutations in these genes can easily be detected by whole genome sequencing of *M. tuberculosis* strains.

4.4 Future studies

Inhibition of RIF efflux pump Rv1145, Rv1146, Rv0933 by efflux pump inhibitors can be useful in reducing the dose administered and increasing the efficacy of RIF against *M. tuberculosis* strains. This study paves the way for the exploration of more potent inhibitors of Rv1145, Rv1146 and Rv0933 which may lead to the combination of anti-TB drug and efflux pump inhibitors.

The regulation of the intracellular concentration of the anti-TB drug by efflux pumps indicates that drug resistance in *M. tuberculosis* may be more complex than generally understood. This study outlined that drug resistance can be associated with mutations in drug target genes and/or with upregulation of efflux mechanisms. However, the clinical importance of the upregulation of efflux activity needs to be explored. We speculate that such mechanisms may have impact on the level of resistance as well. There is, therefore, the need to broaden insight into the efflux mechanisms and their role in drug resistance. Likewise, development of accurate and simple diagnostic methods that could identify and characterize efflux events is essential.

4.4 References

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Appendix A: DETAILED METHODS

A.1 Extraction of genomic DNA from *Mycobacterium tuberculosis* isolates

Mycobacterium tuberculosis genomic DNA was isolated as described by van Soolingen and colleagues (1991), with certain modifications. *Mycobacterium tuberculosis* isolates were grown on LJ media for 3 weeks to 4 weeks. Colonies were gently scraped off by inoculation loop and further washed down in 1.5 ml of sterile water. The suspension was transferred to 2 ml screw-cap tubes, after centrifugation at 3000 rpm for 10 min. Thereafter the concentrated cell suspension was heated at 96°C for 20 min to kill the cells. After centrifugation at 3000 g for 10 min, cells were re-suspended in 450 µl of TE buffer (0.01 M Tris-HCl, 0.001 M EDTA [pH 8.0]). Hundred µl of lysozyme was added to a final concentration of 10 mg/ml, and the tube incubated for 1 h at 37°C. Hundred µl of 10% sodium dodecyl sulfate (SDS) and 50 µl of proteinase K (at a 10-mg/ml concentration) were added, and the mixture was incubated in the ultrasonicator for 10 min at 65°C. Two hundred µl of 5 M sodium chloride and 160 µl was added, mixed gently by inversion, and incubated for 30 min at 55°C. The addition of sodium chloride blocked the binding of Cetrimide to DNA. An equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) was added, and the mixture was vortexed for 10 sec. After centrifugation for 5 min at 5 000 g, 900 µl of aqueous layer was transferred to new falcon tubes, and the re-extraction done with chloroform and isoamyl alcohol (24:1). From there, 800 µl was transferred to 1.5 ml eppendorf tubes. To 800 µl of the aqueous layer, 560 µl isopropanol was added at room temperature, and mixed gently until the DNA precipitated out of the solution. After 30 min at -20°C and centrifugation for 15 min, the pellet was washed once with 70% ethanol and the air-dried pellet was covered with 100 µl of TE buffer and stored at 4°C overnight to allow the pellet to dissolve.

A.2 Sanger sequencing methods

Polymerase chain reaction products were cleaned using ExoSAP protocol as follows: The Exo/SAP master mix was prepared by adding the following reagents to a 0.6ml micro-centrifuge tube:

- Exonuclease I (NEB M0293) 20U/ul-50.0 µl
- Shrimp Alkaline Phosphatase (NEB M0371) 1U/ul-200.0 µl

The following reaction mixture was prepared: 10.0 µl of PCR mixture and 2.5µl Exo/SAP Mix, this reaction was mixed vigorously and incubated at 37°C for 30 min. The reaction was stopped by heating at 95°C for 5 min. Sequencing was then done with the ABI V3.1 Big dye kit. The labelled products were then cleaned with the Zymo Seq clean-up kit. Two hundred and forty µl of Sequencing Binding Buffer was added to a sequencing reaction. The mixture was transferred to a provided Zymo-Spin™ IB-96 Plate (Zymo Research Corp, Irvine, USA) mounted onto a collection plate and the mixture was centrifuged at ≥ 3,000 x g (5,000 x g max.) for 2 min. Three hundred µl of sequencing wash buffer was then added to each well of the plate and centrifuged at ≥ 3,000 x g for 5 min. Fifteen to twenty microliters of water was added directly to the column matrix of the filter plate. The Zymo-Spin™ IB-96 plate was placed on top of the supplied 96-well PCR plate and the assembly was mount onto the collection plate, this was followed by centrifugation at ≥ 3,000 x g for 2 min to elute the DNA. Note: Ultra-pure DNA was loaded into the sequencer. After filter plates were re-generated. The cleaned products were injected on the ABI3500XL analysers (Inqaba Biotech, South Africa) with a 50 cm array, using POP7. The generated sequence reads were analysed using DNASTAR® SeqMan® NGen® Software (Inqaba Biotech, South Africa).

A.3 Whole genome sequencing by Illumina Miseq

Library preparation for the WGS was prepared as follows: Nextera Tagmentation assay was prepared by adding 10 µl of tagmentation DNA buffer, 5 µl of DNA sample (1 ng) and 5 µl amplicon tagmentation mix in a 0.2 PCR tube. The mixture was gently vortexed to mix and the tubes were capped, spinned and placed in an ABI 2400 or 9700 thermocycler (Applied Biosystems, USA). The following programme was used: 55℃ for 5 min and held at 10℃. Once the sample reached 10℃ the mixture was neutralized by adding 5 µl of neutralizing buffer to each sample and vortexed to mix. The tubes were capped, spinned and incubated at room temperature for 5 min. Nextera tagmentation mix was amplified by adding 15 µl of Nextera PCR master mix to each PCR sample tube or added to each sample or row of samples in a PCR plate. A 5 µl of selected index 2 (S5XX) primer(s) (white cap) primers (orange caps), one primer to each sample (or row of samples in a PCR plate) was used. The tubes were then capped and spinned to mix. Polymerase chain reaction was performed on the ABI 2400 or 2700 thermocycler (Applied Biosystems, USA) using the following programme: Miseq Indexes 72℃ for 3 min, 95℃ for 30 seconds, 12 cycles of 95℃ for 10 seconds, 55℃ for 30

seconds, 72℃ for seconds, 72℃ for 5 min, held at 10℃. The index 1 and index 2 primers were recorded on the lab tracking form.

A PCR clean-up procedure was performed by quick spinning of PCR tubes to collect condensation. One set of 1.5 ml low-bind and 1 set of regular micro-centrifuge tubes were labelled for each sample. Fifty µl of each PCR-indexed product was pipette into its corresponding low-bind micro-centrifuge tube. AMPure beads were vortexed for 30 sec to ensure even distribution and 30 µl of suspended AMPure beads was added to each sample. The mix was gently vortexed and all the tubes were incubated for 5 min at room temperature. The tubes were further placed on the magnetic stand until supernatant was clear for 2 min. The pipette was then set to 80 µl to remove and discard the supernatant. The tubes were left on magnetic stand and a fresh 200 µl and 80% ethanol was pipetted to the tubes and tubes were rotated on the magnetic stand, and further incubated for 30 sec. The supernatant was removed and discarded. A hot air blower was used to partially dry samples on a magnetic stand. The tubes were removed from magnetic stand and 52.5 µl of resuspension buffer was pipetted into each tube. The samples were then incubated at room temperature for 2 min. The tubes were again placed on the magnetic stand until supernatant was clear for 2 min. Fifty µl of supernatant for each sample was then transferred to its corresponding regular 1.5 ml micro-centrifuge tube.

Library normalization was performed by transferring 20 µl of PCR cleaned sample to its corresponding 1.5 ml low-bind microcentrifuge tube. Library Normalization Additives 1 (LNA1) was mixed with Library Normalization Beads 1 (LNB1) into a 1.5 ml tube. The mixture was vortexed and spun; mix LNA =1 and LNB1 were used immediately. A combined mixture of 45 µl of LNA1/LNB1 was transferred into each sample. The tubes were then capped and vortexed at 1,800 rpm for 30 min. The tubes were placed on the magnet stand until supernatant was cleared for 20 min. With tubes on the stand, the supernatant was carefully removed and discarded. The tubes were then removed from magnetic stand and the beads were washed with Library Normalization Wash (LNW1) by adding 45 µl of LNW1 to each sample tube and the tubes were capped and were put on the shaker to vortex at 1,800 rpm for 5 min. The tubes were then placed on the magnetic stand until supernatant was cleared for 2 min. The supernatant was removed and discarded. The tubes were removed from magnetic stand and 30 µl of 0.1N NaOH was added to each sample tube. The tubes were capped and vortexed at 1,800 rpm for 5 min. Thirty µl of Library Normalization Storage Buffer 1 (LNS1) was added

to each regular labelled 1.5 ml sample tube. The tubes containing 0.1N NaOH were removed from the vortex when the time expired and quick spun. The 0.1N NaOH sample tubes were placed on the magnetic stand until supernatant was cleared for 2 min. Thirty µl of the supernatant was transferred to the new 1.5 ml micro tubes containing the Library Normalization Storage Buffer 1 (LNS1). Library pooling was performed by diluting 5 µl equal volumes of normalised libraries of all samples with 576 µl hybridization buffer and heat-denatured in preparation for cluster generation and sequencing. The mixture was incubated in a heat block for 2 min at 96℃. After incubation, the tubes were inverted 1 to 2 times to mix and were immediately place in an ice water bath for at least 5 min. A 600 µl of prepared diluted libraries was loaded into the load samples reservoir in the cartridge. The flow cell and cartridge were put into the Miseq machine sequencing.

A.4 Determination of RIF MIC using the Microplate Alamar Blue Assay

A.4.1 Drug stock concentration preparation

Drug stock concentrations were stored in -80°C.

a) RIF Concentration calculations (for samples to be tested at a highest concentration of 8ug/ml):

- 2 mg of RIF was weighed off in a 1.5ml Eppendorf tubes.
- Dissolved into 100 µl of 100% DMSO (Concentration. 20 000 µg/ml – Stock 1).
- This was diluted to 800 µg/ml.
    - C1V1=C2V2.
    - (20 000 µg/ml)(x µl)= (800 µl/ml) (500 µl).
    - X = 20 µl (Thus add 20 µl into 480 µl DMSO/7H9 Media) (Concentration. – 800 µg/ml – Stock 2).
    - Twofold serial dilutions of the drug was done in a sterile 96 well plate for all the concentrations tested.
- 2 µl of stock 2 and dilutions was added into 98ul of 7H9 media in the 96-well plate.
- 100 µl of bacteria was added to all the wells.
    - The final test concentration was 8 µg/ml.

b) RIF concentration calculations – For samples to be tested at a highest concentration of 0.08ug/ml.

- 1.6 mg of RIF was weighed off in 1.5 ml Eppendorf tubes.
- Dissolved in 1 ml of 100% DMSO *plus* 9 ml H_2O (Concentration. 160 µg/ml-Stock 1).
- 1:10 dilution of 160 µg/ml-Stock was made to obtain 16 µg/ml.
- Two fold serial dilution was done in a sterile 96 well plate, and the highest concentration. Inoculated in the well was 8 µg/ml. When 8 µg/ml was used the highest concentration of the drug which came into contact with organisms was 0.08 µg/ml: thus the highest concentration tested was 0.08 µg/ml and lowest concentration was 0.005 µg/ml.
    - C_1=8 µg/ml drug concentration.
    - V_1=2 µg/ml of drug inoculated in each well.
    - V_2=200 µg/ml total volume in each well (2 µg/ml of the drug, 98 µg/ml of the media and 100 µg/ml of the bacterial inoculum). C_2=?.
- Thus: $C_1V_1=C_2V_2$
    - (8 µg/ml)(2 µg/ml)= (C_2)(200 µg/ml)
    - C_2=(8 µg/ml)(2 µg/ml)/(200 µg/ml)
    - C_2=0.08 µg/ml: thus

A.4.2 Preparation of the media and Tween 80

2.34 g of Middlebrook 7H9 Broth base (Sigma-Aldrich, Chemie, GmlbH) was suspended in 450 ml (in sterilized 1 000 ml Duran bottles) of distilled water and 2 ml of glycerol was added. The media was sterilized by autoclaving at 121°C for 10 min. After cooling to 45 °C in the water bath, one vial of Middlebrook (albumin, dextrose and catalase) ADC (Sigma-Aldrich, Chemie, GmbH) growth supplement was added aseptically. The media was stored in -4°C after use. Ten % of Tween 80 was prepared as follows: 10 ml of 100% Tween 80 (Batch #F24619) was measured and diluted in 100 ml sterile water sterile duran bottles containing a magnetic stirrer. The mixture was then autoclaved at 121°C for 10 min. After autoclaving the solution was allowed to cool and stored in -4°C.

A.4.3 Drug testing on Microplate Alamar Blue Assay (MABA)

A sterile 96 well, flat bottom microplates containing RIF at concentrations ranging from 0.00005 µg/ml to 8 µg/ml were used to test each sample. Outer perimeter wells on each plate

were filled with 200 µl of sterile water (with a multichannel pipette) to avoid dehydration of the medium during incubation. A GC without RIF was also included for each isolate. Serial twofold dilutions of the drug was made from stock 2 solution (800 µg/ml) was prepared in a 96-well microtitre plate using 50 µg/ml of dimethyl sulfoxide (DMSO). Two microliters of RIF from stock 2 concentration of 800 µg/ml was inoculated in column 2, rows B to E. Identical serial dilutions were continued through column 11, (B11 to E11). Three to 5 days old positive MGIT cultures were used to determine MICs to RIF in a microplate assay, and the concentration of the bacteria ranged from (0.8×10^5 to 3.3×10^5) CFU/ml. Mycobacterial cultures in MGIT were vortexed vigorously and allowed to stand for 5 to 10 min, from the supernatant, 100 µl of bacterial suspension was inoculated to the wells in rows B to E in columns 2 to 11 (yielding a final volume of 200 µg/ml per well) and wells in column 11 served as drug-free (inoculum-only) controls. The plates were sealed with Parafilim and were incubated at 37°C for 5 days. Fifty microliters of a freshly prepared 1:1 mixture of Alamar Blue (Accumed International, Westlake, Ohio) reagent and 10% Tween 80 was added to wells in B11 to E11, and the plates were reincubated at 37°C for 24 h. If wells B11 to E11 turned pink, the reagent mixture was added to all wells containing the test reagents in the microplate. The microplates were resealed with parafilm and were incubated for an additional 24 h at 37°C, and the colours of all wells were recorded. A blue color in the well was interpreted as no growth, and a pink colour was scored as growth. The MIC was defined as the lowest drug concentration that prevented a colour change from blue to pink.

Table A1. Colorimetric redox indicator assay plate setting up for RIF

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20
B	200 µl H_20	100 µl Sample B2	100 µl Sample B3	100 µl Sample B4	100 µl Sample B5	100 µl Sample B6	100 µl Sample B7	100 µl Sample B8	100 µl Sample B9	100 µl Sample B10	100 µl Positive control	200 µl H_20
C	200 µl H_20	100 µl Sample C2	100 µl Sample C3	100 µl Sample C4	100 µl Sample C5	100 µl Sample C6	100 µl Sample C7	100 µl Sample C8	100 µl Sample C9	100 µl Sample C10	100 µl Positive control	200 µl H_20
D	200 µl H_20	100 µl Sample D3	100 µl Sample D3	100 µl Sample D4	100 µl Sample D5	100 µl Sample D6	100 µl Sample D7	100 µl Sample D8	100 µl Sample D9	100 µl Sample D10	100 µl Positive control	200 µl H_20
E	200 µl H_20	100 µl Sample E3	100 µl Sample E3	100 µl Sample E4	100 µl Sample E5	100 µl Sample E6	100 µl Sample E7	100 µl Sample E8	100 µl Sample E9	100 µl Sample E10	100 µl Positive control	200 µl H_20
F	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20
G	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20
H	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20

Appendix B: DATA AND DETAILED RESULTS

B.1 Determination and interpretation of RIF MIC using the alamar blue assay

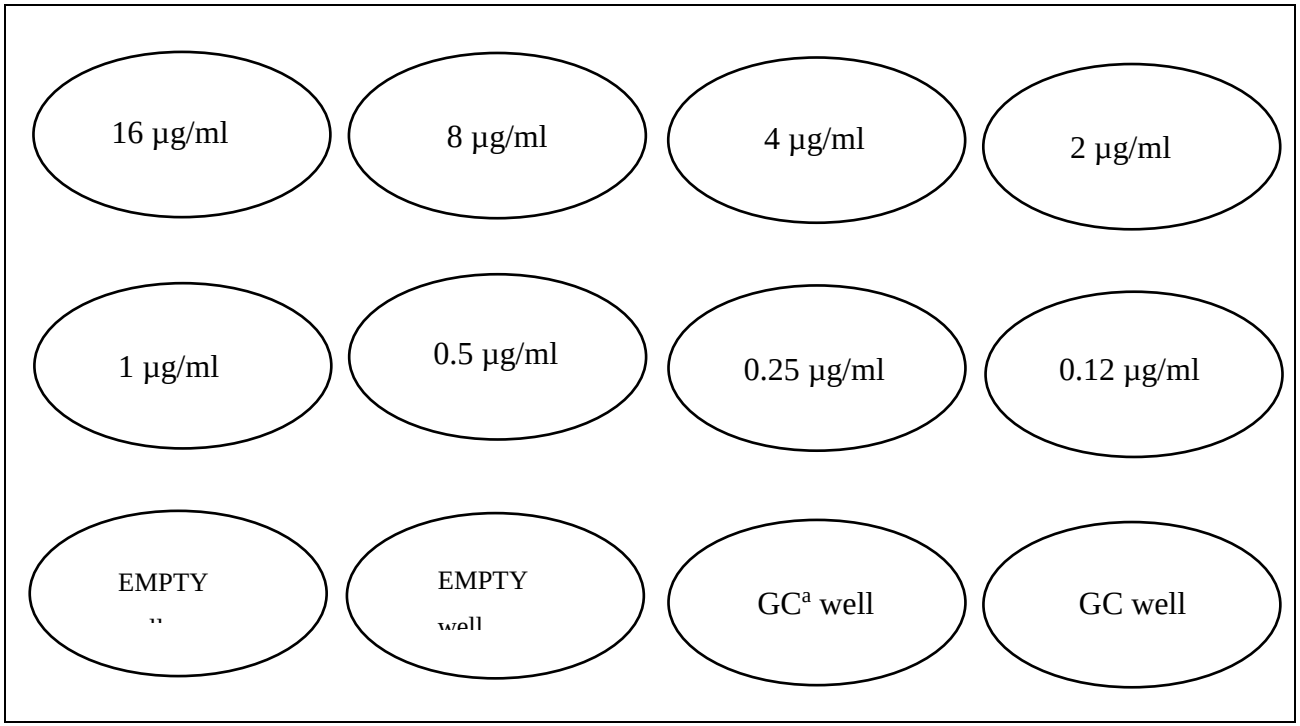
Table B1. Colorimetric redox indicator assay, drug concentration within the test wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 µl H_20	200 µl H_20[a]	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20
B	200 µl H_20	8 µg/ml RIF B2	4 µg/ml RIF B3	2 µg/ml RIF[b] B4	1 µg/ml RIF B5	0.5 µg/ml RIF B6	0.25 µg/ml RIF B7	0.12 µg/ml B8	0.06 µg/ml B9	0.03 µg/ml B10	NO Drug added	200 µl H_20
C	200 µl H_20	8 µg/ml RIF C2	4 µg/ml RIF C3	2 µg/ml RIF C4	1 µg/ml RIF B5C5	0.5 µg/ml RIF C6	0.25 µg/ml C7	0.12 µg/ml C8	0.06 µg/ml C9	0.03 µg/ml C10	NO Drug added	200 µl H_20
D	200 µl H_20	0.016µg/ml RIF D3	0.008 µg/ml RIF D3	0.004 µg/mlRIF D4	0.002 µg/mlRIF D5	0.001 µg/mlRIF D6	0.0005 µg/mlRIF D7	0.00025 µg/mlRIF D8	0.0001 µg/mlRIF D9	0.00006 µg/mlRIF D10	NO Drug added	200 µl H_20
E	200 µl H_20	0.016 µg/ml RIF E3	0.008 µg/mlRIF E3	0.004 µg/mlRIF E4	0.002 µg/mlRIF E5	0.001 µg/mlRIF E6	0.0005 µg/mlRIF E7	0.00025 µg/mlRIF E8	0.0001 µg/mlRIF E9	0.00006 µg/mlRIF E10	NO Drug added	200 µl H_20
F	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20
G	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20
H	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20	200 µl H_20

Abbreviation [a]H_20-water; [b]RIF-rifampicin

Figure B1: Minimum inhibitory concentration: Colour change signifying breakpoint

B.2 Determination and interpretation of RIF MIC using agar proportion kit



Abbreviation: [a]GC–growth control

Figure B2. Agar proportion plate set up for rifampicin

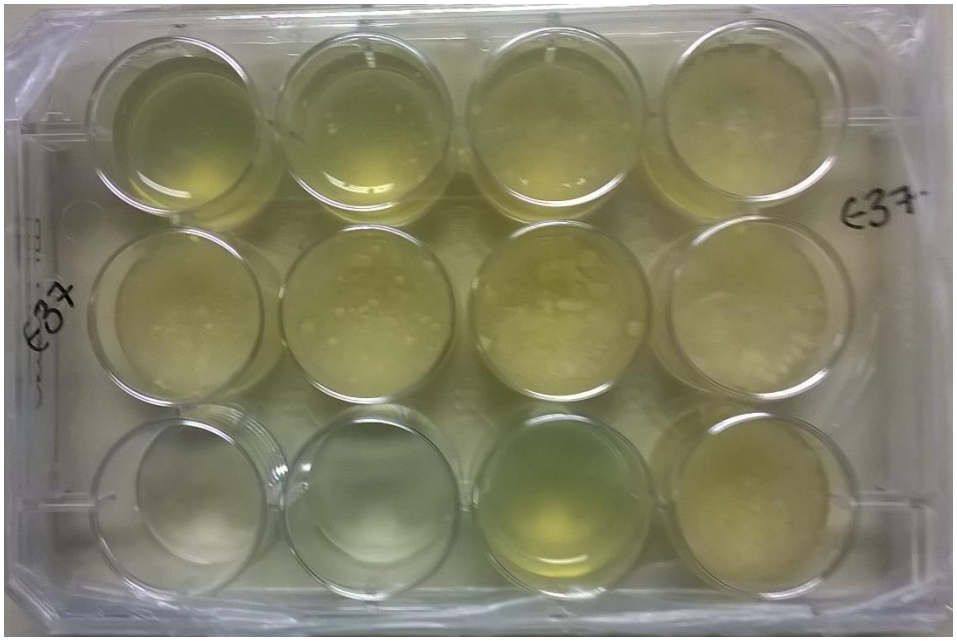


Figure B3: Representative readout for drug susceptibility testing in the 12-well agar microtitre plate

Table B2: Individual drug susceptibility test results for rifampicin

S/no[a]	Absent WT[b] band description	MTBDR*plus* Results(Rif[c])	GXP[d] Results(Rif)	MGIT Results(Rif)	Sanger *rpoB* gene mutations	Miseq WGS[e]			MABA[g] RIF 0.0005-8µg/ml	Agar proportion RIF 0.12-16 µg/ml
						rpoB MUT[e]	*rpoA* MUT	*rpoC* MUT		
E01	WT 2	Resistant	Resistant	Sensitive	L511P/ (D516G)	NO Results	NO Results	NO Results	Not Done	Not Done
E02	WT 8	Resistant	Resistant	Resistant	S531Q/ (D516G)	NO Results	NO Results	NO Results	≥8 µg/ml	Not Done
E03	WT 3,4	Resistant	Resistant	Resistant	L533P/(D516G)	D516G/L533P/I1187T	NO MUT	C542G	≥8 µg/ml	≥16 µg/ml
E04	NO	Resistant	Resistant	Resistant	S531L/(D516G)	S531L/Q1056H	NO MUT	A253V/V333L	8 µg/ml	≥16 µg/ml
E05	WT 8	Resistant	Sensitive	Sensitive	NO MUT	NO MUT	NO MUT	C542G	0.06 µg/ml	≤0.12 µg/ml
E06	WT 8	Resistant	Sensitive	Sensitive	NO MUT	NO Results	NO Results	NO Results	0.007 µg/ml	≤0.12 µg/ml
E07	WT 8	Resistant	Resistant	Sensitive	S531Q	NO Results	NO Results	NO Results	Not Done	Not Done
E08	WT 3,4	Resistant	Resistant	Sensitive	D516Y	D516Y/A938P	NO MUT	G675E	0.5 µg/ml	0.5 µg/ml
E09	WT 6-8	Resistant	Sensitive	Sensitive	NO MUT	NO MUT	NO MUT	C623G	0.06µg/ml	0.25 µg/ml
E10	WT 3,4	Resistant	Resistant	Sensitive	D516Y	D516Y	E400K	G675E	0.12 µg/ml	Not Done
E11	WT 6,7,8	Resistant	Resistant	Sensitive	L511P	NO Results	Not Done	Not Done	0.25 µg/ml	0.25 µg/ml
E12	WT 8	Resistant	Resistant	Resistant	S531Q	NO Results	Not Done	Not Done	≥8 µg/ml	≥16 µg/ml
E13	WT 7	Resistant	Resistant	Sensitive	Mixed strains	NO Results	Not Done	Not Done	≥0.08 µg/ml	Not Done
E14	WT 8	Resistant	Sensitive	Sensitive	NO MUT	Y1096S/Y1099S/M1106L	NO MUT	C623G	0.007 µg/ml	Not Done
E15	WT 8	Resistant	Sensitive	Sensitive	NO MUT	NO Results	NO Results	NO Results	0.06 µg/ml	Not Done
E16	WT 8	Resistant	Sensitive	Sensitive	NO MUT	T876G/G1027C/T1027C	NO MUT	NO MUT	0.12 µg/ml	≤0.12
E17	WT 8	Resistant	Sensitive	Sensitive	NO MUT	T1156C	NO MUT	A1125V	0.03 µg/ml	≤0.12 µg/ml
E18	WT 8	Resistant	Sensitive	Sensitive	NO MUT	T1156C	NO MUT	A1125V	0.12µg/ml	0.12µg/ml
E19	WT 8	Resistant	Resistant	Resistant	Mixed strains	NO Results	NO Results	NO Results	≥0.08 µg/ml	Not Done
E20	WT 3,4	Resistant	Resistant	Resistant	NO MUT	NO Results	NO Results	NO Results	Not Done	Not Done
E21	WT 3,4	Resistant	Resistant	Resistant	DEL/ (DEL514-516)	NO Results	NO Results	NO Results	≥0.08 µg/ml	Not Done
E22	WT 2,3	Resistant	Resistant	Sensitive	Q531P/(DEL514-516)	NO Results	NO Results	NO Results	0.25 µg/ml	0.25 µg/ml
E23	WT 3	Resistant	Resistant	Resistant	Q531L/(DEL514-516)	NO Results	NO Results	NO Results	Not Done	Not Done
E24	WT 2,3,8	Resistant	Resistant	Resistant	DEL	NO Results	NO Results	NO Results	≥8 µg/ml	Not Done

E25	WT 7	Resistant	Resistant	Resistant	H526R	NO Results	NO Results	NO Results	≥8 µg/ml	Not Done
E26	WT 8	Resistant	Resistant	Resistant	S531W	NO Results	NO Results	NO Results	≥8 µg/ml	16 µg/ml
E27	NO	Sensitive	Sensitive	Sensitive	NO MUT	NO MUT	NO MUT	C623G	0.03 µg/ml	≤0.12 µg/ml
E28	WT 3,4	Resistant	Resistant	Resistant	S601T	NO Results	NO Results	NO Results	0.007 µg/ml	Not Done
E29	WT 8	Resistant	Sensitive	Sensitive	NO MUT	C184T	NO MUT	G675E	0.25 µg/ml	≤0.12 µg/ml
E30	WT 8	Resistant	Sensitive	Sensitive	NO MUT	T1156C	NO MUT	A1125V	0.12 µg/ml	≤0.12 µg/ml
E31	WT 7	Resistant	Resistant	Sensitive	H526N	NO Results	NO Results	NO Results	0.06 µg/ml	≤0.12 µg/ml
E32	WT 8	Resistant	Sensitive	Sensitive	NO MUT	NO Results	NO Results	NO Results	0.06 µg/ml	Not Done
E33	WT 8	Resistant	Resistant	Resistant	S531L	NO Results	NO Results	NO Results	Not Done	Not Done
E34	WT 6,7,8	Sensitive	Sensitive	Sensitive	NO MUT	T1156C	NO MUT	NO MUT	0.004 µg/ml	≤0.12 µg/ml
E35	WT 7	Resistant	Resistant	Resistant	H526L	NO Results	NO Results	NO Results	2 µg/ml	Not Done
E36	WT 3	Resistant	Resistant	Resistant	Q531L	Q531L/R908C	NO MUT	C62T	≥8 µg/ml	≥16 µg/ml
E37	WT 3,4	Resistant	Resistant	Resistant	D516Y	D516Y	NO MUT	NO MUT	1 µg/ml	0.5 µg/ml
E38	WT 7	Resistant	Resistant	Sensitive	H526N	NO Results	NO Results	NO Results	Not Done	Not Done
E39	WT 4	Resistant	Resistant	Sensitive	D516Y	D516Y	NO MUT	NO MUT	1 µg/ml	4 µg/ml
E40	WT 7	Resistant	Resistant	Resistant	H526R	NO Results	NO Results	NO Results	≥8 µg/ml	≥16 µg/ml
E41	WT 8	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.25 µg/ml	Not Done
E42	WT 8	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	1 µg/ml	1 µg/ml
E43	WT 8	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	≤0.12 µg/ml
E44	WT 8	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	2 µg/ml	1 µg/ml
E45	NO	Sensitive	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.0 6 µg/ml	Not Done
E46	WT 8	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.12 µg/ml	≤0.12 µg/ml
E47	WT 8	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	Not Done
E48	WT 8	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.12 µg/ml	≤0.12 µg/ml
E49	WT 3	Resistant	Resistant	Resistant	Not Done	Not Done	Not Done	Not Done	≥8 µg/ml	≥16 µg/ml
E50	WT 8	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.03 µg/ml	Not Done
E51	WT 8	Resistant	Resistant	Resistant	Not Done	Not Done	Not Done	Not Done	Not Done	Not Done
E52	WT 8	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	Not Done
E53	WT 8	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.03 µg/ml	0.25 µg/ml

E54	WT 2	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	Not Done
E55	WT 7	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.25 µg/ml	Not Done
E56	WT 2	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	Not Done
E57	WT 4	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.25 µg/ml	0.25 µg/ml
E58	WT 7	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	0.25 µg/ml
E59	WT 8	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.25 µg/ml	0.25 µg/ml
E60	WT 8	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	Not Done
E61	WT 8	Resistant	Sensitive	Resistant	Not Done	Not Done	Not Done	Not Done	0.5 µg/ml	Not Done
E62	WT 8	Resistant	Resistant	Resistant	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	≤0.12 µg/ml
E63	WT 8	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	0.25 µg/ml
E64	WT 8	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.015 µg/ml	≤0.12 µg/ml
E65	WT 8	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	Not Done
E66	WT 4	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	1 µg/ml	0.5 µg/ml
E67	WT 2	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	Not Done	Not Done
E68	WT 7	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.12 µg/ml	Not Done
E69	WT 7	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.03 µg/ml	Not Done
E70	WT 4	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.12 µg/ml	≤0.12 µg/ml
E71	WT 8	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	Not Done	Not Done
E72	WT 2	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.12 µg/ml	0.5 µg/ml
E73	WT 2	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	Not Done	Not Done
E74	WT 4	Resistant	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	Not Done	Not Done
E75	WT 8	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	Not Done	Not Done
E76	WT 7	Resistant	Resistant	NO Results	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	Not Done
E77	NO	Sensitive	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.12 µg/ml	Not Done
E78	WT 4	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.5 µg/ml	0.25 µg/ml
E79	WT 8	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	1 µg/ml	4 µg/ml
E80	WT 7	Resistant	Resistant	Resistant	Not Done	Not Done	Not Done	Not Done	8 µg/ml	2 µg/ml
E81	WT 8	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.5 µg/ml	0.5 µg/ml
E82	NO	Sensitive	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.5 µg/ml	0.5 µg/ml

E83	NO	Sensitive	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	≤0.12 µg/m
E84	WT 3,4	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.5 µg/ml	0.5 µg/ml
E85	NO	Sensitive	Sensitive	Sensitive	Not Done	Not Done	Not Done	Not Done	0.5 µg/ml	NO Results
E86	WT 7	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.5 µg/ml	1 µg/ml
E87	NO	Sensitive	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.06 µg/ml	≤0.12 µg/ml
E88	WT 2	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	4 µg/ml	Not Done
E89	WT 2	Resistant	Resistant	Sensitive	Not Done	Not Done	Not Done	Not Done	0.12 µg/ml	1 µg/ml

Abbreviation [a]S/no-study number, [b]WT–wild type, [c]Rif-rifampicin, [d]GXP-geneXpert, [e]WGS–whole genome sequencing, [f]MUT-mutation, [g]MABA-microtitre plate alamar blue assay

Table B3: Information pathways and efflux pump gene mutations detected by whole genome sequencing

S/no	*RpoA* MUT	*rpoB* MUT[a] outside (81 bp	*rpoC* MUT	*GyrA* MUT	*pstB*/ Rv0933	*mmpL13a* (Rv1145)	*mmpL13b* (Rv1146)
E3		I1187T	C623 G				
E4		Q1056L/T1156C	A253V/V1333L/ C142T/C254A			Leu277fs	G165A
E5			C542G		T61M		
E8		A938P	G675E	E21E			A364C
				S95T			
				G668D			
E9			C623G	E21E	T61M		
				S95T			
				G668D			
E10	E400K		G675E	E21E			
				S95T			
				G668D			
E14		Y1096S	C623G	E21E			
		T1099S	T589C	S95T			
		M1106L	G598C	G668D			
E16		T957G		E21E		Leu277fs	
		G1108C		S95T			
		T1156C		G668D			
E17		T1156C	A1125V			Leu277fs	
E18		T1156C	A1125V	E21E		Leu277fs	
				S95T			
				G668D			
E27			C623G	E21E			
				S95T			
				G668D			
E29		C184T	G675E	E21E			
				S95T			
				G668D			
E30		T1156C	A1125V	E21E		Leu277fs	
				S95T			
				G668D			
E34		T1156C		E21E		Leu277fs	
				S95T			
				G668D			
E36		R908C	C143T	E21E	Thr50fs	Leu277fs	
		T1156C		S95T			
				G668D			

S/no-Study number, bp-base pair

Table B4: Summary of clinical, microbiological and genotypic characterization of patients with *rpoB* gene mutations

S/NO	Gender	Age	MGIT Results(Rif[b])	*rpoB* gene mutations	Previous TB treatment	Previous HIV treatment	1st Line TB Treatment
E01	Male	49	Sensitive	L511P/ (D516G)	no	no	**Failed**
E05	female	39	Sensitive	NO MUT	no	no	Failed
E06	male		Sensitive	NO MUT	no	no	Failed
E07	female	31	Sensitive	S531Q	no	no	Failed
E08	male	33	Sensitive	D516Y	yes	no	Failed
E10	male	34	Sensitive	D516Y	no	no	Failed
E11	female	26	Sensitive	L511P	MDR regimen	no	Failed
E13	female	34	Sensitive	Mixed strains	MDR regimen	no	Failed
E14	male	54	Sensitive	NO MUT	no	no	Failed
E15	male	64	Sensitive	NO MUT	no	yes	Failed
E16	male	28	Sensitive	NO MUT	no	no	Failed
E17	female	37	Sensitive	NO MUT	no	no	Failed
E18	female	47	Sensitive	NO MUT	no	no	Failed
E27	Female	41	Sensitive	NO MUT	no	no	Failed
E29	male	1	Sensitive	NO MUT	no	no	Failed
E30	Female	23	Sensitive	NO MUT	no	none	Failed
E32	female		Sensitive	NO MUT	no	yes	Failed
E34	male	5	Sensitive	NO MUT	no	no	Failed
E38	female	41	Sensitive	H526N	no	no	Failed

Abbreviation: [a]S/NO-study number; [b]Rif-rifampicin;

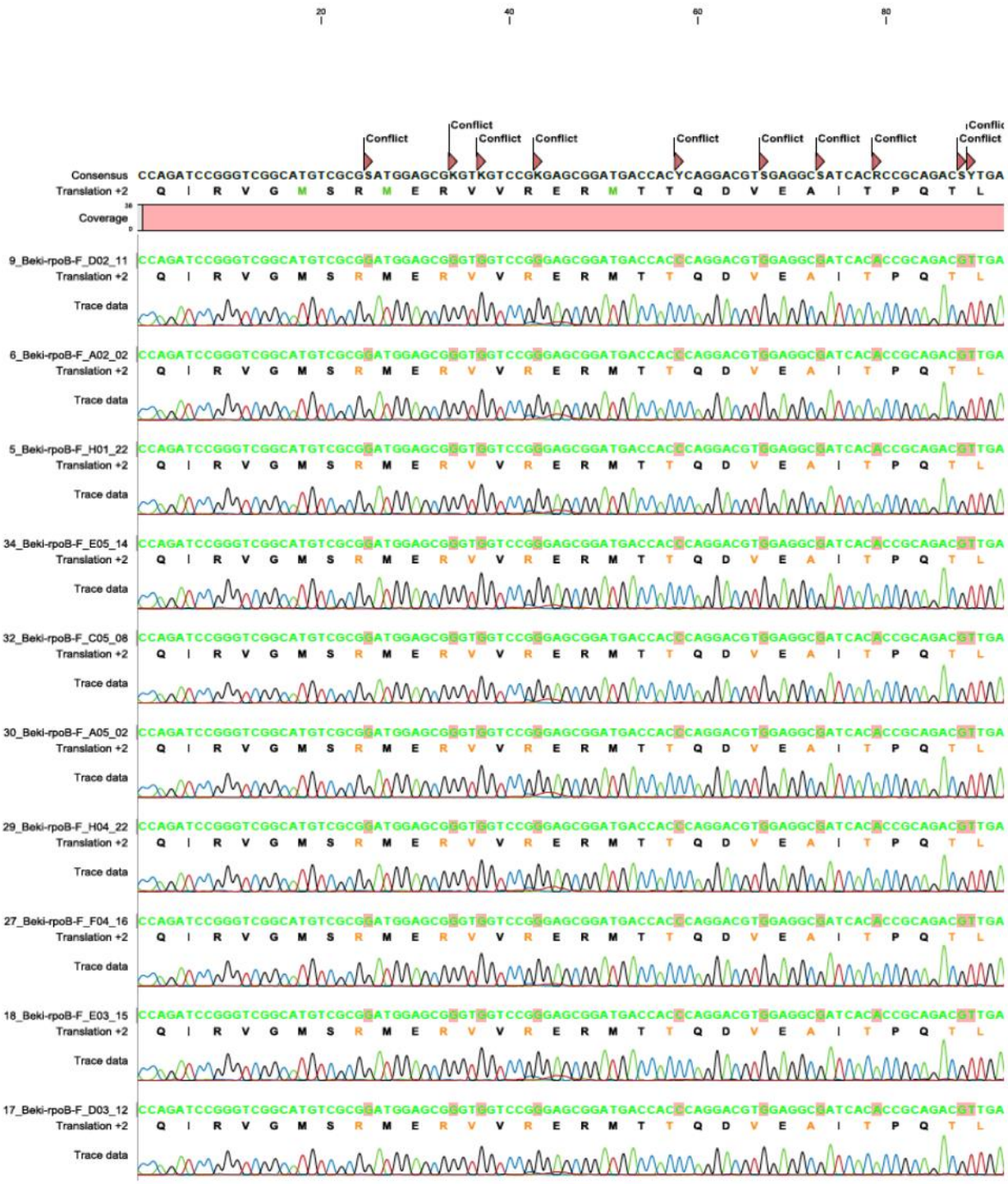


Figure B3: Alignment of reads generated by Sanger sequencing

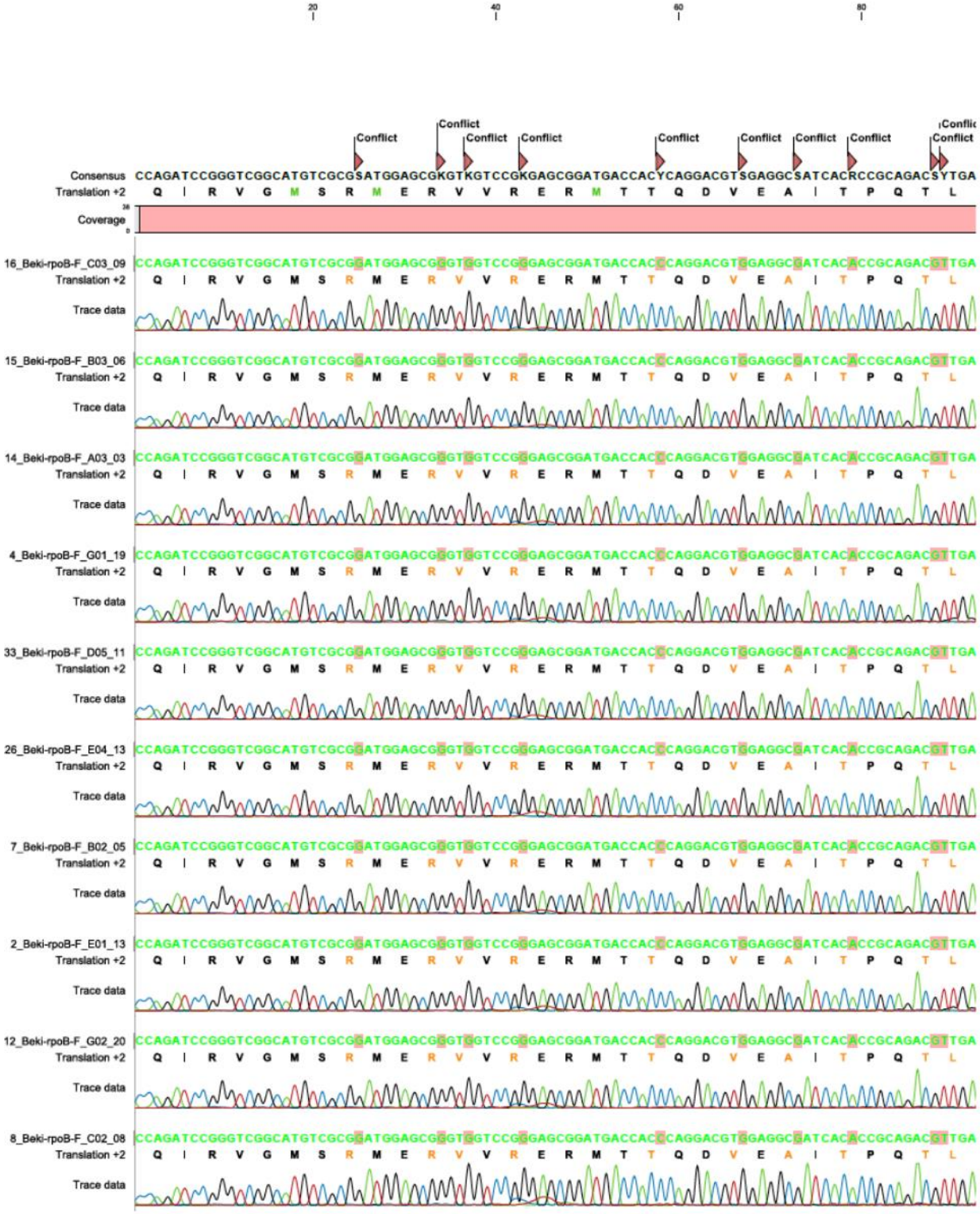


Figure B3: Alignment of reads generated by Sanger sequencing

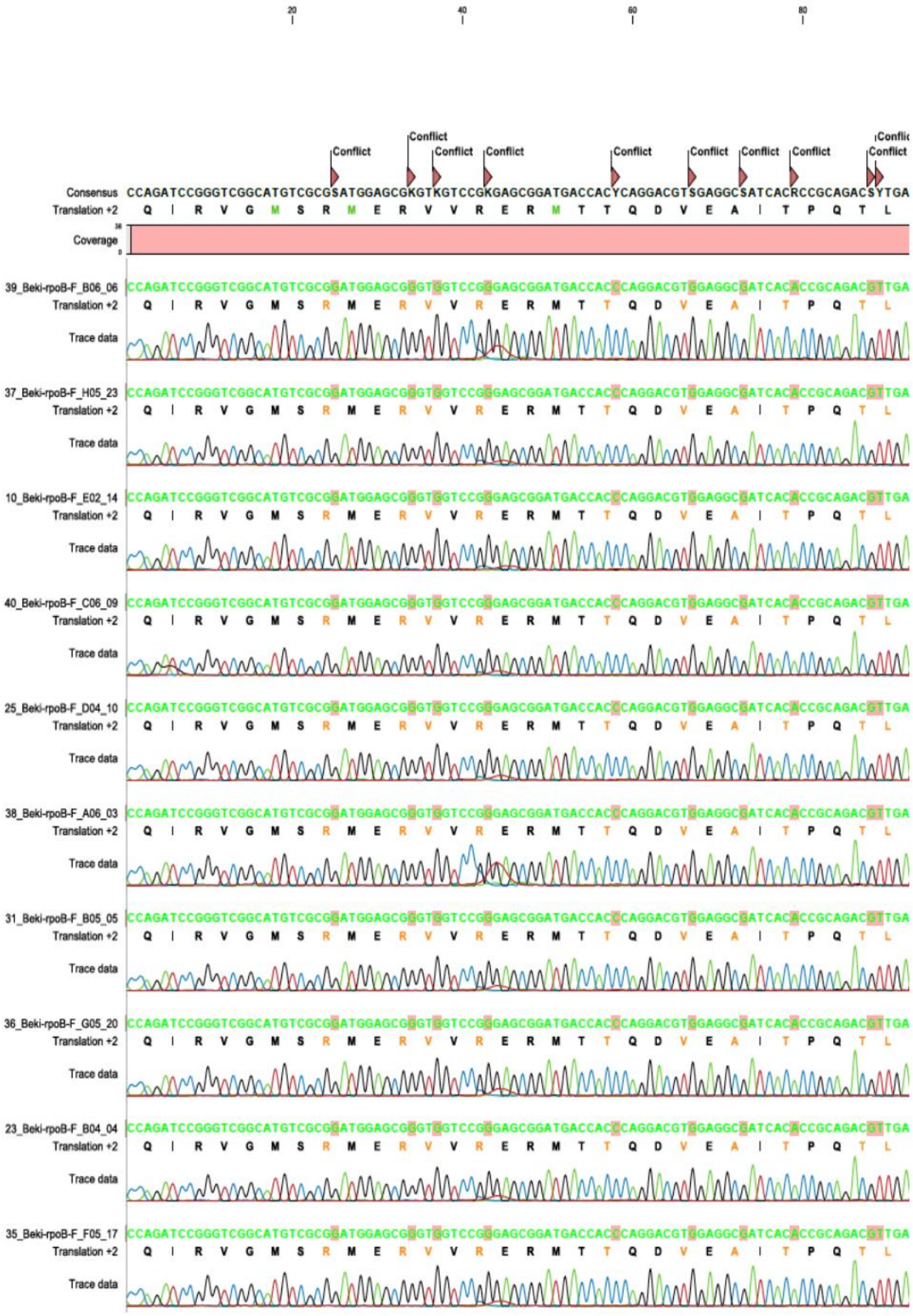


Figure B3: Alignment of reads generated by Sanger sequencing

Appendix C: CONFIRMATION OF COMPETENCY IN TUBERCULOSIS LABORATORY PRACTICE

University of Pretoria

Department of Medical Microbiology

Training log for BSc Honors and Masters Students MARUBINI, E.E.

15010016

Purpose

The purpose of this training is to ensure that University of Pretoria students accessing the Tuberculosis laboratory in the department of Medical Microbiology are competent to do so and safely without endangering themselves or the environment. Training will be provided by competent individuals and verified as such. The training document provided will be used to confirm full competency in the laboratory.

Laboratory safety:

PPE

Principle of the biosafety cabinet

Maintenance of a biosafety cabinet

Working under biosafety cabinet

Disposal of infectious material (waste disposal)

Hand washing in the laboratory

Department of Medical Microbiology
University of Pretoria
NHLS Tshwane Academic Division
2016 -02- 25
PRIVATE BAG X323
ARCADIA 0007
SOUTH AFRICA

Verified.....date.....4/2014.....

Laboratory procedures

Decontamination of sputum samples

Date	Lab number	Name of decontaminant
02/02/2015	MG01216226	NALC - NaOH
02/02/2015	RH00522042	NALC - NaOH
02/02/2015	MG01236293	NALC - NaOH
03/02/2015	MG01233097	NALC - NaOH
03/02/2015	MG01233547	NALC - NaOH
03/02/2015	MG01233547	NALC - NaOH
03/02/2015	RH00522812	NALC - NaOH

Verified.....date.....5/2/15.....

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Microscopy

Auramine staining

Date	Lab number	Auramine staining
02/02/15	MG012/6226	✓
02/02/15	RH0052202	✓
02/02/15	MG 0123 6293	✓
02/02/15	MG01233097	✓
03/02/15	MG01233547	✓
03/02/15	BD00522812	✓
03/02/15	BD RH00522820	✓
03/02/15	MG01237452	✓
03/02/15	MG01236809	✓
03/02/15	MG01238076	✓

Verified.....date 5/2/15

Ziehl Neelson staining

Date	Lab number	Ziehl Neelson staining
04/02/15	MG01240524	✓
04/02/15	MG01240560	✓
04/02/15	MG01240581	✓
04/02/15	MG01240900	✓
05/02/15	RI00053107	✓
05/02/15	MG01237530	✓
05/02/15	MG01242411	✓
05/02/15	MG01242057	✓
05/02/15	BD00526911	✓
05/02/15	RH00523695	✓

Verified.....date 5/2/15

Culture

Principle of the MGIT

Inoculation of the vials

Loading of the vials in the machine

Date	Lab number
02/02/2015	MG01216226
02/02/2015	RH005-22 042
02/02/2015	MG01233097
02/02/2015	MG01236293
03/02/2015	MG012335 47
03/02/2015	DA 005 2 2 812
03/02/2015	RH00522 320
03/02/2015	MG01234452
03/02/2015	MG01236309
03/02/2015	MG01238076

Handling of vials which give a positive flag

Verified..... date 5/2/15

Identification techniques

Using the MGIT machine

MPT64 assay

Verified..... date 5/2/15

Susceptibility testing

Principle of phenotypic drug testing

Using the MGIT machine

Verified..... date 5/2/15

Principles of genotypic drug testing

HAIN Line probe assays

Gene Xpert

Verified..... date 2015/03/9

Department of Medical Microbiology
University of Pretoria
NHLS Tshwane Academic Division
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