Wild-type minimal inhibitory concentration distributions of second-line drugs in *Mycobacterium tuberculosis* complex clinical isolates in relation to recommended critical concentrations in Limpopo Province, South Africa.

by

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DEDICATION

It is a great honour to dedicate this project to my parents Mr. **Steven Nkoalana Seloma** and Mrs. **Margaret Raesibe Seloma**. My lovely sisters Ditiro, Mahlogonolo, Manku thank you for being pillars of strength. My brothers Edward and Nape you two are living examples of what faith is.

BATHOKWA

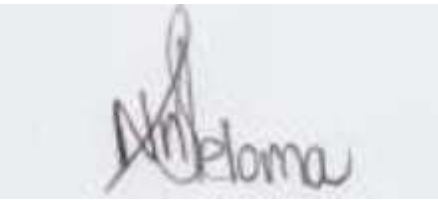
Ke Bathokwa ba Mmaseboko seboko sa go ja Dipudi, Ke Bathokwa ba Mmaseboko ba re Seboko ga se bokwe baneng, ke batho ba bo Phukubje ya lla ga re je re tšhaba go nkga melomo.

Ke ba bo Seulaule Matlakala o reng o beša ka dimeku dikgong di le gona ba bo Matletleretše wa dipekwa wa bo thakga sa masogana motho wa go kgona go itia kati seotseng, ba bo nkgagaripe wa mogoma ke fedile ke lema ka bja maabane bogale a o legoleng la Mma moaga ka ditlhaka, ke batho ba bo Mmamasweng ga ken a mokootlo ga ken a thari ya go belega maswena.

Re ditlogogolo tša Dinoko batho ba bo Mphiri Moja a lla, ba bowago Mahlatji thaba nkgokolo thaba ya Dinoko le di Diphukubje. Ba bo Mošidi Mogabotši letlapa mogatša morula a thaba batho ba bowago Sahlokwe Madikoti Kanana Thabe Phaswa ka morago ga merula e sebentini.

DECLARATION

I **Ngwanamohuba Mologadi Seloma,** hereby declare that the dissertation submitted to the University of Limpopo for Masters' degree in Medical Sciences has not previously been submitted for degree purposes at this or any other University. This is my work in design and execution and all sources contained herein have been duly acknowledged.



12/09/2016

SIGN**.....** DATE**.....**

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ABSTRACT

Background

The reference phenotypic methods for *Mycobacterium tuberculosis* drug susceptibility testing are qualitative and based on drug critical concentrations. Limitations include lack of standardization and variations in laboratory preparation of drug stock solutions. The recommended critical concentrations are determined by consensus and experience rather than scientific data. Consequently incorrect and inadequate susceptibility breakpoints are used and patients receive ineffective antimicrobial therapy. The determination of wild-type minimal inhibitory concentration distribution is an important tool used by European Committee for antimicrobial susceptibility Testing (EUCAST) to establish clinical breakpoints in Europe. This could be applicable in South Africa.

Aim

To determine wild-type minimal inhibitory concentration distributions of first and second-line drugs against *Mycobacterium tuberculosis* complex clinical isolates and compare these with the recommended critical concentration in Limpopo province.

Methods

A sample of 101 *Mycobacterium tuberculosis* complex positive cultures were collected from National Health Laboratory Services in Polokwane (Limpopo province) and sub-cultured on BACTEC MGIT 960 system. The isolates were inoculated on MYCOTB MIC plates to determine the wild-type MIC distributions of first and second-line drugs. The data were compared with currently recommended critical concentrations. DNA was extracted and amplified by PCR. Genotypic drug susceptibility testing was performed using GenoType MTBDR*plus* version 2.0 and GenoType MTBDR*sl* version 2.0 for the first- and second-line drugs, respectively. Genotyping of clinical isolates was performed to determine *M. tuberculosis* strain families using spoligotyping.

Results

Wild-type MIC distributions range reported in this study are as follows rifampin (≤ 0.12 - 0.5 µg/µg/ml), isoniazid (≤ 0.3 - 2.00 µg/ml), rifabutin (≤ 0.12 - 0.25 µg/ml), ethionamide (≤ 0.12 - 5 µg/ml), ethambutol (≤ 0.5 - 2 µg/ml), streptomycin (≤ 0.25 - 0.5 µg/ml), para-aminosalicylic (≤ 0.5 - 4.0 µg/ml), cycloserine (≤ 2 -16 µg/ml), amikacin (≤ 0.12 - 0.5 µg/ml), kanamycin (≤ 0.6 -2.5 µg/ml), moxifloxacin (≤ 0.6 - 0.5 µg/ml), ofloxacin (≤ 0.25 - 1 µg/ml).

GenoType MTBDR*plus* detected (n= 68, 67%) rifampin resistance (MUT 3=26, MUT 2=18, MUT 2B=8) on the *rpoB* gene. Isoniazid resistant (n=20, 19.8%) was detected *katG* MUT (n=20, 19.8%) on *katG* gene (S315T1).

Genotypic resistance to second-line drugs determined by GenoType MTBR*sl* detected no mutations in (n= 98, 97%) isolates on *gyrA, gyrB rrs* and *eis* gene and (n=3, 2.9%) isolates non *mycobacterium tuberculosis* complex were detected.

The frequency and percentage of *Mycobacterium tuberculosis* family strain were identified in (n= 81, 80%) of the clinical isolates which matched 18 pre-existing shared types. The results showed high genotype diversity with the Beijing strain (n= 30, 29.7%) and T family (n= 19, 18.8%) dominating. Twenty isolates (19.8%) had no shared types thus reported as orphan.

Conclusion

The findings obtained in this study suggest wild-type Minimal Inhibitory Concentration distributions may be considered when setting clinical breakpoints. Discordant results were observed between phenotypic and genotypic DST for rifampin, isoniazid, streptomycin, rifabutin and ethambutol, suggesting that breakpoint concentrations for some drugs are set too high while others are too low. The *Mycobacterium tuberculosis* clinical isolates displayed diverse family strain with Beijing and T strain predominate

breakpoints for first-line and second-line drugs used in *Mycobacterium tuberculosis* treatments.

Poster Presentations

Poster presented at faculty of Health science first annual research day on Second-line drug susceptibility breakpoints for *Mycobacterium tuberculosis* using MYCOTB MIC plate. University of Limpopo Tiro hall 16th to 17th September 2014.

Poster presented at National Health Laboratory Service Pathology Research and Development Congress (PathReD) on Determination of families strains of *Mycobacterium tuberculosis* circulating in Limpopo Province, South Africa. Emperors Palace 14th April-16th April 2015.

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ABBREVIATIONS

AMK	Amikacin
CC	Critical Concentration
CAS	Central Asian
CM	Capreomycin
DNA	Deoxyribonucleic Acid
DMD	Dorlands Medical Dictionary
DR	Direct Repeat
DST	Drug Susceptibility Testing
DVR	Direct Variable Repeat
EAIS	East African-Indian Clade
ECOFF	Epidemiological Cut-off Value
ECL	Enhanced Chemiluminescence
EUCAST	European Committee of Antimicrobial Susceptibility Testing
GC	Growth Control
H	Haarlem
INH	Isoniazid
KM	Kanamycin
KZN	KwaZulu Natal
LAM	Latin-American-Mediterranean
MANU2	Manu-Clade
MDR-TB	Multidrug-resistant Tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimal Inhibitory Concentration
MIRU	Mycobacterial Interspersed Repetitive Units
ml	Milliliter
MREC	Medunsa Research Ethics Committee
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
NCCLS	National Committee for Clinical Laboratory Standards

NHLS	National Health Laboratory Services
OFX	Ofloxacin
PAS	Para-aminosalicylic acid
PANTA	Polymyxin Amphotericin Nalidixic Acid Trimethoprim Azlocillin
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
PK	Pharmacokinetics
PZA	Pyrazinamide
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampin
RRDR	Rifampin Resistance Determining Region
S	S-Clade
SDS	Sodium dodecylsulphate
SIR	Susceptible Intermediate Resistant
SIT	Shared International Type
SLD	Second-line drugs
Spoligotyping	Spacer oligonucleotide typing
SPSS	Statistical Package for Social Sciences
STR	Streptomycin
T	T-Clade
T2-T3	T-Clade
TB	Tuberculosis
U	U-Clade
VNTR	Variable Number Tandem Repeat
WHO	World Health Organization
X1	X-Clade
X3	X-Clade
XDR-TB	Extensively drug-resistant TB

TERMINOLOGY

Antibiotic - a substance with sufficient antimicrobial activity that can be used in the treatment of infectious diseases Dorlands Medical Dictionary (DMD, 2010). In this study the term antibiotic or drug is used to define a substance with antimicrobial activity against tuberculosis and multi-drug resistance.

Critical concentration - the lowest concentration of a drug that will inhibit 95% of wild-type strains of *M. tuberculosis* that have never been exposed to drugs, while at the same time not inhibiting clinical strains of *M. tuberculosis* that are considered resistant (NCCLS, 2003; WHO, 2008). In this study critical concentration was used to define the lowest concentration of drug that inhibited at least 95% of wild-type strains of MTB isolates.

Genes - sequence of nucleotides that code for specific proteins (DMD, 2010; www.biology.online.org). In this study the term gene is associated with a change in nucleotide(s) that confers resistance to a particular drug.

Intermediate micro-organism - a micro-organism is defined as intermediate by a level of antimicrobial agent activity associated with uncertain therapeutic effect (EUCAST, 2012). In this study intermediate is associated with uncertain therapeutic effect of a drug to a bacterial isolates.

Minimal inhibitory concentration - the lowest concentration of the antimicrobial agent that inhibits a given bacterial isolates from multiplying and producing any visible growth in the test system (Kahlmeter *et al*., 2006). In this study minimal inhibitory concentration is associated with the lowest concentration that inhibited tuberculosis clinical isolates from multiplying in the MYCOTB sensititre plate.

Missense mutation - a point mutation in which a single nucleotide change results in a codon that codes for a different amino acid (Finken *et al*., 1993). In this study the term missense mutation is associated with a change in a single nucleotide. When encountered it confers resistance to the anti-tuberculosis drugs.

Mutations - heritable changes in genotype that occur spontaneously or are induced by chemical or physical treatment (DMD, 2010; www.biology.online.org). In this study the term mutation refers to the alteration of genetic make-up resulting in development of drug resistance.

Non-wild type micro-organism - a micro-organism is defined as non-wild type for a species by the presence of an acquired or mutational resistance mechanism to the drug

in question (EUCAST, 2012). In this study non wild-type is in an organism with a mutation that confers it resistant to a particular drug.

Objectivity - an investigation or observation where the researcher task is to uncover phenomena without affecting the outcome of the research (www.aqr.org.uk/glossary). In this study objectivity was achieved by reporting the laboratory results without any manipulation of the outcome.

Point mutation - a mutation affecting one or few nucleotides in a gene sequence (Jones-Lopez *et al*., 2011). In this study, the term point mutation refers to a mutation that affects a single nucleotide in the gene sequence.

Primary drug resistance - the presence of resistance to one or more anti-tuberculosis drugs in a patient who has never been treated for tuberculosis before (Espinal *et al*., 2001).

In this study the term primary drug resistance defines the development of drug resistance in *M. tuberculosis* patients.

Resistant micro-organism - a micro-organism is defined as resistant by a level of antimicrobial activity associated with a high likelihood of therapeutic failure European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2012). In this study, the term resistant refers to therapeutic failure of a antimicrobial activity in micro-organism.

Reliability - how accurate will the findings of a test be if repeated in an identical research setting but performed by a different person at a different location. (www.aqr.org.uk/glossary). In this study reliability was achieved by following standard operating procedures for chemical reagents and the manufacturer instructions on equipment operation.

Second-line drugs - treatment used for multidrug-resistant tuberculosis (WHO, 2008). In this study second-line drugs is used to define treatment used for multidrug resistant tuberculosis.

SpolDB4 - is an international spoligotyping database that defines *M. tuberculosis* complex genetic polymorphism, isolates sharing identical spoligotype pattern are defined according to SIT (Spoligotype International Type) isolates with single spoligotype patterns are defined as orphan (Brudey *et al*., 2006). In this study the term SpolDB4 was used to define genetic families with specific signature for various *M. tuberculosis* complex.

Spoligotyping - a polymerase chain reaction (PCR) based method for genotyping strains of the *M. tuberculosis* complex (Driscoll, 2009). In this study the term

spoligotyping is a molecular technique used for genotyping of *M. tuberculosis* complex strains.

Susceptible micro-organism - a micro-organism is defined as susceptible by a level of antimicrobial activity associated with a high likelihood of therapeutic success (EUCAST, 2012). In this study susceptible defines high levels of antimicrobial activity in an organism.

Wild-type - The typical form of an organism, strain, gene or characteristic as it occurs in nature, as distinguished from mutant forms that may result from selective breeding (DMD, 2010). In this study the term wild-type defines an organism in its natural form, with no mutation.

Validity - refers to how well a scientific test actually measures what it is set out to do. (www.aqr.org.uk/glossary) In this study the MYCOTB MIC plate results were considered valid if there was adequate growth in drug free control wells in position H11 and H12. GenoType MTBDR*plus* version 2.0 and GenoType MTBDR*sl* version 2.0 kit results were valid when the amplification control (AC) band developed on the test strip.

CHAPTER ONE

1.1 INTRODUCTION

The increasing rate of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) creates a high demand for accurate and reproducible antimicrobial drug susceptible testing in defining appropriate and effective treatment regimens (Ängeby *et al*., 2012; WHO, 2015).

Drug susceptibility testing in *Mycobacterium tuberculosis* (MTB) is currently based on measuring susceptibility against a critical concentration (CC) defined as the lowest concentration of a drug that will inhibit 95% of a wild-type strains of MTB that have never been exposed to drugs, while not inhibiting clinical strains of MTB that are considered resistant (Schön *et al*., 2009; Ängeby *et al*., 2012). This definition makes the remaining 5% of probably susceptible strains, resistant (Schön *et al*., 2009; Ängeby *et al*., 2012). The recommended critical concentrations used for many second-line drugs are not standardized but determined by consensus and experience rather than scientific facts (WHO, 2008). Consequently, incorrect and inadequate susceptibility breakpoints are used, which leads to inaccurate and unreliable drug susceptibility testing results given to clinicians and thus patients receive ineffective antimicrobial therapy (WHO, 2008).

The determination of wild-type minimal inhibitory concentration (MIC) distribution is an important tool used by European Committee for Antimicrobial Susceptibility Testing (EUCAST) to establish clinical breakpoints in Europe (Kahlmeter *et al*., 2006; Ängeby *et al*., 2010). This could be applicable in South Africa. The clinical breakpoints are established according to the susceptible, intermediate, resistant (SIR) system (Kahlmeter *et al*., 2006; Schön *et al*., 2009). The (SIR) system is mainly useful in wild-type strains that deviate between susceptible and resistant but don't have mechanisms of antibiotic resistance (Schön *et al*., 2009; Silley, 2012). The highest MIC termed the epidemiological cut-off value (ECOFF) can be used to determine low level resistance and non-susceptibility to drugs in situations where there is a gap between the ECOFF and clinical susceptible breakpoint (Schön *et al*., 2009; Werngren *et al*., 2012). The wild-

type MIC will be useful in the management of tuberculosis because it directly determines the accurate breakpoint of clinical isolates.

A bacterial isolate is categorized as non-wild type for a species by applying the appropriate cut-off value in a defined phenotypic test system (EUCAST, 2012). Monitoring low-level resistance can be used for early detection of drug resistance and the discovery of genetic resistance mechanisms that may be included in methods like Genotype MTBDR*plus and* MTBDR*sl* assays (Diacon *et al*., 2007; Ruslami *et al*., 2007; Ling *et al*., 2008).

Mycobacterium tuberculosis can introduce changes in genes and generate different genotypes in different locales, and introduce variability in genes associated with drug effect (Ford *et al*., 2011). This is supported by a study in South Africa where the authors reported difference in the MIC of ofloxacin in clinical isolates from Cape Town and Durban to ofloxacin. Their report clearly shows that, geographical location or environment peculiarities affect the MIC distributions (Williamson *et al*., 2012; Pasipanodya *et al*., 2012).

1.2 Problem Statement

There is a void in the evaluation of CC for second-line drugs against wild-type MIC distributions in clinical isolates of MTB. Studies have reported the discordance between mutated gene sequences, susceptibility testing on Bactec 460 and MIC determination on middlebrook 7H10 agar. The discordant results were mainly observed in strains with MICs close to ECOFFs. There is therefore the need to determine and document the MICs of different locations in South Africa. In the present study Trek Sensititre MYCOTB MIC plate, configured for determination of MIC of first and second-line anti-tuberculosis drugs was used to determine wild-type MIC distributions of first and second line drugs in *M. tuberculosis* complex clinical isolates. It is important to determine wild-type MIC distributions of first and second-line drugs in order to establish reasonable clinical breakpoints for *M. tuberculosis* complex clinical isolates which will be useful in providing guidelines on appropriate dosing and effective regimen for MDR-TB treatment for patients in Limpopo province.

1.3 **PURPOSE OF THE STUDY**

1.3.1 Aim

To determine wild-type minimal inhibitory concentration distributions of first-line and second-line drugs against *Mycobacterium tuberculosis* complex clinical isolates and to compare the values with recommended critical concentrations in Limpopo province.

1.3.2 Objectives

- To determine susceptibility of clinical isolates to first-line drugs (rifampin, isoniazid) and second-line drugs (kanamycin, ofloxacin) using BACTEC MGIT 960.
- To determine wild-type MIC distributions of second-line drugs against *M. tuberculosis* complex clinical isolates using MYCOTB Sensititre MIC plates (Trek Diagnostic, Cleveland, USA).
- To compare data with the currently recommended critical concentrations.
- To analyse the mutations with MIC of drugs that are not comparable with currently used critical concentrations using GenoType MTBDR*plus* assay version 2.0 and GenoType MTBDR*sl* version 2.0 (Hain Lifescience).
- To determine the distribution of strain families among the *M. tuberculosis* complex isolates using spoligotyping.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Tuberculosis chemotherapy

Mycobacterium tuberculosis infections remain a problem worldwide. First-line antituberculosis drugs such as rifampin, isoniazid, ethambutol, pyrazinamide and streptomycin are used as short-course treatment regimen for treatment of drug-sensitive TB (WHO, 2014). However these drugs are ineffective for treatment of multidrug-resistant tuberculosis (MDR-TB) defined as resistant to at least rifampin and isoniazid (WHO, 2006, 2014). The spread of MDR-TB and emergence of XDR-TB create high demand on antimicrobial susceptibility testing of *M. tuberculosis* to produce results that are accurate, clinically meaningful and technically reproducible (Ängeby *et al*., 2012).

2.2 First-line anti-tuberculosis drugs

The current standard chemotherapeutic regimen for treating new pulmonary TB patients consists of a multidrug combination of the first-line anti-TB drugs isoniazid, rifampin, pyrazinamide and ethambutol administered for an intensive, initial period of two months (WHO, 2010; Veluchamy *et al*., 2013). A continuation phase of treatment for an additional four months consists of administration of isoniazid and rifampin. In countries reporting high levels of isoniazid resistance occurring in new TB patients or lacking isoniazid drug susceptibility results, ethambutol is administered throughout the four-month continuation phase of treatment (WHO, 2010, 2014; Veluchamy *et al*., 2013). Upon availability of drug susceptibility profiles and confirmed sensitivity of isoniazid, administration of ethambutol may be omitted in the continuation phase (WHO, 2014; Veluchamy *et al*., 2013).

2.3 Drug Resistance and tuberculosis

Drug resistance develops from random chromosomal mutations, which occur spontaneously at low rates in wild-type strains of MTB (Zhang and Yew, 2009). The rate of natural resistance varies for specific agents: 1 in 0.5×10^4 bacilli for ethambutol, 1 in 3.5×10^6 bacilli for isoniazid, 1 in 3.8×10^6 for streptomycin and 1 in 3.1×10^8 bacilli for rifampin (Jones-Lopez *et al*., 2011). Drug resistance in *M. tuberculosis* can either be intrinsic or acquired. Intrinsic drug resistance is attributed to highly hydrophobic mycolic acids in the cell wall that give the bacteria low permeability for many compounds (Smith *et al*., 2013).

Acquired drug resistance refers to drug resistance in patients with prior history of TB treatment and it is often caused by inappropriate therapy, poor patient adherence to treatment and poor absorption or bioavailability of drugs (Smith *et al*., 2013). Acquired resistance occurs when previously susceptible bacteria become resistant to a particular antibiotic either by mutation or exchange of genetic material with other related species (Smith *et al*., 2013; Kempker *et al*., 2015).

Primary drug resistance refers to the presence of resistance to one or more anti-tuberculosis drugs in a patient who has never undergone treatment for TB. Primary drug resistance may also result from the spread of drug resistance organisms by an infected source to other individuals (Jones-Lopez *et al*., 2011).

2.3.1 First-line drugs mechanism of resistance

Drug-resistance in *M. tuberculosis* has been characterized by a number of mutations in genes that are involved in drug targets. Mutations in *rpoB* and *katG* gene of *M. tuberculosis* have been shown to be responsible for resistance to rifampin and isoniazid respectively (de Freitas *et al*., 2014). Drug resistance in *M. tuberculosis* bacteria arises mainly through the acquisition of mutation in the chromosomal sequence that encodes changes that, block the activity of a drug (mutation in *rpoB*) and prevent binding of rifampin to RNA polymerase and causes inhibition of transcription (Johnson *et al*., 2006; de Freitas *et al*., 2014).

Isoniazid enters *M. tuberculosis* as a prodrug and is activated by catalase-peroxidase, encoded by *katG* gene. Therefore mutation in *katG* leads to loss of the ability of catalase to activate the prodrug (isoniazid) to its active form (Samad *et al*., 2014; Veluchamy *et al*., 2013). Activated INH inhibits NADH dependent Enoyl-ACP reductase which is encoded by *inhA* by interfering with the synthesis of essential mycolic acids (Da Silva et *al*., 2011). Mutations in *inhA* increase the amounts of *inha* protein which interferes with the activity of isoniazid by binding sufficient isoniazid to reduce its effective concentration in the bacterium to below an inhibitory level (Torres *et al*., 2015). The mutations associated with resistance to many of the anti-tuberculosis drugs have been identified, though much work remains to be done to identify the molecular basis of resistance of *M. tuberculosis* strains (Johnson *et al*., 2006).

Resistance to INH has been identified in mutations found in several other genes including *ahpC*, *kasA* and *ndH* (Santos, 2012; Samad *et al*., 2014). In INH resistant isolates missense mutations or small deletions or insertions in the *katG* have been found to prevent activation of the drug leading to resistance (Santos, 2012; Samad *et al*., 2014). Deletions in the *katG* lead to strains with high level resistance to INH however point mutations are more common than deletions (Samad *et al*., 2014). At times, the genes have amino acids substitutions in the *katG* gene codon 315 (Santos, 2012; Samad *et al*., 2014). The S315T mutations are common in strains showing isoniazid resistance and are associated with high level isoniazid resistance with MIC value of ≥ 16 mg/ml (Silva *et al*., 2003; Santos, 2012; Samad *et al*., 2014). The most common amino acid substitution in the *katG* gene is AGC (Ser)-ACC (Thr) and it results in an INH product that is deficient in forming the INH-NAD adduct related to INH antimicrobial activity (Da Silva *et al*., 2011).

Rifampin is a lipophilic ansamycin considered a first-line anti TB drug introduced since 1971 (Zhang and Yew, 2009; Santos, 2012). Mutations in the RRDR are found to reduce the affinity of RNA polymerase to the binding of rifampin (Johansen *et al*., 2003). Point mutations on the *rpoB* at codons 513, 526 and 531 are associated with high levels of rifampin resistance with MIC value of ≥ 128 mg/ml (Bobadila-del-Valle *et al*., 2001). Mutations at codon 511,516, 518 and 522 are associated with low level rifampin

resistance (Bobadila-del-Valle *et al*., 2001). Rifampin resistance is caused by mutations in the *rpoB* gene which encode for beta subunit of RNA polymerase within the RRDR region, for this reason rifampin resistance detection has been identified as a surrogate molecular marker for multidrug-resistance tuberculosis (Kocagoz *et al*., 2005; Yang *et al*., 1998; de Freitas *et al*., 2014).

Ethambutol is a bacteriostatic used as part of intensive phase of TB treatment together with isoniazid, rifampin and pyrazinamide (WHO, 2009). The drug mechanism of action is to target the multiplying bacilli by interfering with biosynthesis of cell wall arabinogalactan (Sreenvatson *et al*., 1997; Bakula *et al*., 2013). Point mutation in the *embCAB* operon 306 is associated with alterations in the *embB* gene which lead to ethambutol resistance.

Streptomycin was the first antibiotic used for tuberculosis treatment derived from soil microorganism actinobacteria *Streptomyces griseus* (Okamoto *et al*., 2007; Da Silva *et al*., 2011). Streptomycin is an aminocyclitol glycoside and its mode of action is to inhibit protein translation by binding to the 30S ribosomal subunits. Genetic basis of resistance to streptomycin is due to mutations in *rpsL* and *rrs* (Okamoto *et al*., 2007; Da Silva *et al*., 2011). Low-level streptomycin resistance has been reported in *gidB* gene, encoding a conserved 7- methylguanosine methyltrasferase for the 16S rRNA (Spies *et al*., 2008; Da Silva *et al*., 2011).

Para-aminosalicylic acid (PAS) was first used with isoniazid and streptomycin as first-line antituberculosis drugs. Currently streptomycin is used for MDR tuberculosis treatment. The drug mechanism of action has not been completely defined (Da Silva *et al*., 2011; Zheng *et al*., 2013). Para-aminosalicylic acid mode of action is suggested to be inhibition of folic acid biosynthesis and iron uptake (Zheng *et al*., 2013; Zhao *et al*., 2014). Clinical isolates with resistance to PAS were identified to have mutations in the *thyA* gene that encodes thymidylate synthase A (Zheng *et al*., 2013; Palomino and Martin *et al*., 2014).

2.4 Molecular genetic assay for identification of *M. tuberculosis* complex

The GenoType MTBDR*plus* version 2.0 is a qualitative *in vitro* used for identification of the *M. tuberculosis* complex and resistance to rifampin and isoniazid from clinical specimens and cultivated samples (Hain lifescience, 2014). A study done in South Africa assessed the MTBDR*plus* assay (Version 2.0) and found that it has sensitivity for detection of 99% for rifampin and 94% for Isoniazid (Barnard *et al*., 2008). The GenoType MTBDR*plus* assay version 2.0 has more features including increased detection of INH resistance due to the inclusion of probes for mutations that confer low resistance found in the promoter region *inhA* compared to MTBDR*plus* version 1.0 Detection of RIF resistance is also increased due to additional wild-type probes for the *rpoB* gene included that cover extra codons from 505-510 (Miotto *et al*., 2008).

Rifampin resistance regions on the *rpoB* gene

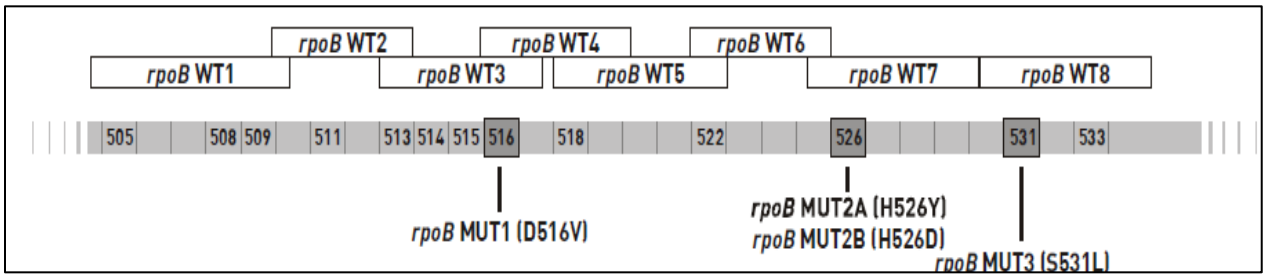


Figure 2.1: Rifampin resistance region of the *rpoB* gene and common resistance-mediating mutations

wildtype probes: *rpoB* WT1- 8, *rpoB* mutation probes MUT1, MUT2A, MUT2B, MUT3, the number (505-533) specify the position of amino acids (codons) analyzed. GenoType MTBDR*plus* assay version 2.0 (Hain Lifescience, 2014).

Mutation in the *rpoB* gene inhibits transcription by targeting the β-subunit of RNA polymerase decreasing affinity for rifampin and resulting in the development of resistance (Palomino and Martin, 2014). Table 2.1 displays mutations on the *rpoB* gene and corresponding wild-type according to (Telenti *et al.,* 1993; Hain Lifescience, 2014).

Table 2.1: Mutations in *rpoB* gene and corresponding failing wild-type bands

Failing Wild type band(s)	Codons Analysed	Developing Mutation band	Mutation
rpoB WT1	505-509		F505L
			T508A
			S509T
rpoB WT2	510-513		E510H
			L511P*
rpoB WT2/WT3	510-517		Q513L*
			Q513P
			Del514-516
rpoB WT3/WT4	513-519	*rpoB* MUT1	D516V
			D516Y
			del515
rpoB WT4/WT5	516-522		Del518*
			N518I
rpoB WT5/WT6	518-525		S522L
			S522Q
rpoB wt7	526-529	*rpoB* MUT2A	H526Y
		rpoB MUT2B	H526D
			H526R
			H526P*
			H526Q*
			H526N
			H526L
			H526S
			H526C
rpoB WT8	530-533	*rpoB* MUT3	S531L
			S531Q*
			S531W
			L533P

* These rare mutations have only been detected theoretically (*in silico*) (Hain lifescience, 2014).

2.5 Second-line antituberculosis drugs

When MDR-TB infections arise second-line drugs are implemented for treatment. These consist of six classes namely Aminoglycoside (amikacin, kanamycin), Polypeptides (capreomycin, viomycin and enviomycin), Fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin), Thioamides (ethionamide and prothionamide) Cycloserine and Terizidone (Gandhi *et al*., 2006, Li *et al*., 2014).

2.5.1 Fluoroquinolones mechanism of action and resistance

The fluoroquinolones (moxifloxacin, levofloxacin, ofloxacin and ciprofloxacin), are bactericidal antibiotics with excellent activity against *M. tuberculosis* and are currently used as second-line drugs in TB treatment (Li *et al*., 2014). New generation fluoroquinolones (moxofloxacin, gatifloxacin) are under clinical evaluation as first-line antibiotics with the goal of shortening the duration of treatment for drug sensitive TB. Fluoroquinolones mode of action is to exert powerful antibacterial activity by trapping gyrase and topoisomerase IV on DNA as ternary complexes thereby blocking the movement of replication forks and transcription complexes (Li *et al*., 2014).

Fluoroquinolone mechanisms of resistance in MTB are most commonly associated with mutations in the conserved quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB* involved in the interaction between the drug and DNA gyrase

2.5.2 Aminoglycosides mechanism of action and resistance

The aminoglycosides (amikacin, kanamycin) and the cyclic polypeptide capreomycin are important injectables drugs in the treatment of MDR-TB. Both the classes of antibiotics exert their activity at protein translation level, through binding of the 30S ribosomal subunit, which affects polypeptide synthesis and ultimately resulting in inhibition of translation (Gonzalo *et al*., 2015). Resistance to aminoglycosides in *M. tuberculosis* usually develops by mutation of ribosome target binding sites. Studies on

cross-resistance to these drugs in MTB isolates have been observed in both clinical isolates and laboratory-generated mutants (Maus *et al*., 2005; Jugheli *et al*., 2009; Via *et al*., 2010; Engström *et al*., 2011).

Resistance to at least fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin) and least one injectables drug or the aminoglycosides (amikacin, kanamycin) is required for a strain to be classified as XDR-TB (CDC, 2013). High level resistance to the aminoglycosides amikacin and kanamycin has been shown to be associated with mutations in the 16S rRNA gene *rrs* at positions 1401, 1402 and 1484 in *M. tuberculosis* (Brossier *et al.,* 2010). Low-level resistance to Kanamycin has been associated with mutations in the promoter region of the *eis* gene that encode aminoglycoside acetyltransferase (Zaunbrecher *et al*., 2009; Palomino and Martin, 2014). Resistance to cyclic peptide capreomycin has been associated with mutations in *tlyA* (Maus *et al*., 2005).

2.5.3 Thioamides mechanism of action and resistance

Thioamide (ethionamide) is a synthetic compound structurally related to isoniazid. It is a prodrug, requiring activation by monooxygenase *ethA*. Similar to isoniazid, ethionamide inhibits mycolic acid synthesis by binding the ACP reductase *InhA*. Approximately three-quarters of *M. tuberculosis* isolates with high-level ethionamide resistance have mutation in *ethA* or *inhA* genes (Carette *et al*., 2012; Palomino and Martin, 2014).

Table 2.2: Summary of mechanisms of resistance in *M. tuberculosis* to first and second-line anti-TB drugs

Drug	Mechanism of action	Common genes involved in resistance	Gene product	References
Isoniazid	Inhibits mycolic acid biosynthesis	*Kat G*	Catalase-peroxidase	Timmins *et al.,* 2004; Zhang and Yew, 2009
		InhA	Enoyl ACP reductance	
Rifampin	Inhibits RNA synthesis	*rpoB*	β subunit of RNA polymerase	Mitchison 1985; Johansen *et al.,* 2003; Kocagoz *et al.,* 2005; Zhang and Yew, 2009
Ethambutol	Inhibits arabinogalactan synthesis	*EmbB*	Arabinosyl transferase	Sreenvatson *et al*., 1997; Bakula *et al*., 2013
Streptomycin	Inhibits protein synthesis	*rpsaL*	S12 ribosomal protein	Ramaswamy *et al*., 1998; Okamoto *et al*., 2007; Spies *et al*., 2008
		Rrs	16s Rrna	
Pyrazinamide	Interferes with pH balance	*PncA*	Nicotinamidase/Pyrazinamidase	Mitchison 1985; Scorpio *et al*., 1997; Zhang and Yew, 2009
Fluoroquinolones	Inhibits DNA gyrase	*GyrA*	DNA gyrase subunit A	Ginsburg *et al.,* 2003; Palomino and Martin, 2013
		GyrB	DNA gyrase subunit B	
Kanamycin/amikacin	Inhibit protein synthesis	*Rrs*	16s Rrna	Maus *et al*., 2005; Jugheli *et al*., 2009; Engström *et al*., 2011
Capreomycin	Inhibit protein synthesis	*ThyA*	rRNAse methyltransferase	Maus *et al*., 2005; Brossier *et al*., 2011
Ethionamide	Inhibits mycolic acid biosynthesis	*etaA/ethA*	Flavin monoxygenase	Brossier *et al*., 2011; Carette *et al*., 2012
Para-aminosalicylic acid	Possibly inhibits foilc and iron metabolism	*ThyA*	Thymidylate synthase	Zheng *et al*., 2013; Zhao *et al*., 2014

2.6 Second-line drugs: mechanisms of resistance

The increasing rates of resistance to kanamycin, amikacin and cyclic peptide are concerns for MDR-TB therapy (WHO, 2010). Mutations in the 3' part of the 16S rRNA gene *rrs* at position 1401, 1402 and 1484 have been associated with resistance to each drug (Palomino and Martin, 2014). Mutations at 1401 and 1484 were found to cause high-level resistance to kanamycin, amikacin and capreomycin, whereas 1402 causes resistance to only capreomycin and kanamycin (Maus *et al.,* 2005; Palomino and Martin, 2014). The *tlyA* gene, which codes a putative rRNA methyltransferase is reported to confer resistance to capreomycin (Maus *et al*., 2005, Palomino and Martin, 2014).

In a study done by Engström *et al.,* (2011) mutations in the gene *rrs* and *tlyA* were correlated with capreomycin resistance in clinical isolates and *in vitro* selected mutants of *M. tuberculosis.* Cross-resistance between kanamycin, amikacin and capreomycin were also investigated. The *in vitro* selected capreomycin resistant mutants had genotypes that were different from the capreomycin resistant clinical isolates. The majority of capreomycin resistant mutants had frameshift or missense mutations in *tlyA* and were susceptible to amikacin and kanamycin (Engström *et al., 2*011*).*

In contrast most clinical isolates with 1401 mutation in *rrs* were resistant to all the other three drugs. The study also observed that the mutation at position 1401 in the *rrs* does not lead to viomycin resistance even though capreomycin and viomycin belong to the same group of antibiotics and bind at same 16S rRNA (Engström *et al*., 2011). This suggests that viomycin might still be an option for XDR-TB patients infected with capreomycin resistant strains of *M. tuberculosis* harboring a 1401 mutation in *rrs* (Stanley *et al*., 2010; Jureen *et al*., 2010). Conflicting results regarding cross-resistance between second-line drugs are most likely due to inappropriately set critical concentration (Engström *et al*., 2011).

In the study on molecular analysis of cross-resistance among the aminoglycoside antibiotics in *M. tuberculosis* isolates. The finding described isolates with high level of

kanamycin resistance that were also resistant to capreomycin which were consistent with *rrs* mutants (Maus *et al*., 2005).

2.7 The need for drug susceptibility testing

The emergence of MDR-TB and XDR-TB makes it necessary to ensure that drug susceptibility testing of *M. tuberculosis* produce rapid and accurate results both for therapy guidance and surveillance of drug resistance (WHO, 2008). Drug susceptibility testing of MTB in diagnostic laboratory classifies isolates as either drug resistant or drug susceptible based on the ability of isolates to grow in the presence of single critical concentration of the tested drug (Böttger, 2011). World Health Organization has declared that the current definition of "critical concentration" defining resistance is often very close to minimum inhibitory concentration (MIC) required to achieve anti-mycobacterial activity, increasing the probability of misclassification of susceptibility or resistance and leading to poor reproducibility of DST results (WHO, 2008; WHO, 2009). Studies have reported significant knowledge concerning molecular mechanism of mycobacterial drug resistance (Böttger, 2011). Drug resistance in *M. tuberculosis* is reported to be heterogeneous and to involve low level, moderate level and high level drug resistance phenotypes (Böttger, 2011; Mpagama *et al*., 2013).

2.7.1 Modern principles of drug susceptibility testing

Antimicrobial susceptibility testing breakpoints are best determined by specialists in clinical trial science and consider pharmacokinetic and pharmacodynamics data. Two such committees composed of these specialists are the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standard Institute (CLSI) of United States of America.

For some bacterial isolates the use of wild-type MIC distribution has become a necessary and widely used tool in the determination of clinical breakpoints. A bacterial isolate is defined as a wild-type if it has no acquired and mutational resistance mechanisms against certain drug (the isolate will have a normal Gaussian MIC distribution when testing MIC for many isolate). Isolates which have MIC outside normal

Gaussian MIC distribution or wild-type distribution will be suspected as having resistance mechanisms (Kahlmeter *et al*., 2006; EUCAST, 2012).

The highest MIC within the wild-type MIC distribution has been labeled the epidemiological cut-off (ECOFF). The ECOFF is used together with pharmacokinetic and pharmacodynamic data to classify a given isolate's wild-type MIC distribution as susceptible (S), intermediate (I), resistant (R) under what is known as SIR system (EUCAST, 2012). The critical concentration is an epidemiological parameter that distinguishes a microorganism as a wild-type strains or non wild-type strains (Schön *et al*., 2009; Böttger, 2011).

2.7.2 Minimal inhibitory concentration distributions

In a study done by Ängeby *et al*. (2010) wild-type MIC distributions of four fluoroquinolones active against *M. tuberculosis* in relation to current critical concentrations used for drug susceptibility testing and PK/PD data on 90 consecutive clinical isolates and 24 drug-resistant strains were described. The wild-type MICs of ciprofloxacin, ofloxacin, moxifloxacin and levofloxacin ranged from 0.125 to 1.0, 0.25 to 1, 0.0032 to 0.5 and 0.125 to 0.5 mg/L, respectively. The MICs for 90 consecutive clinical isolates formed a Gaussian shape distribution as illustrated in figure 2 (Ängeby *et al*., 2010).

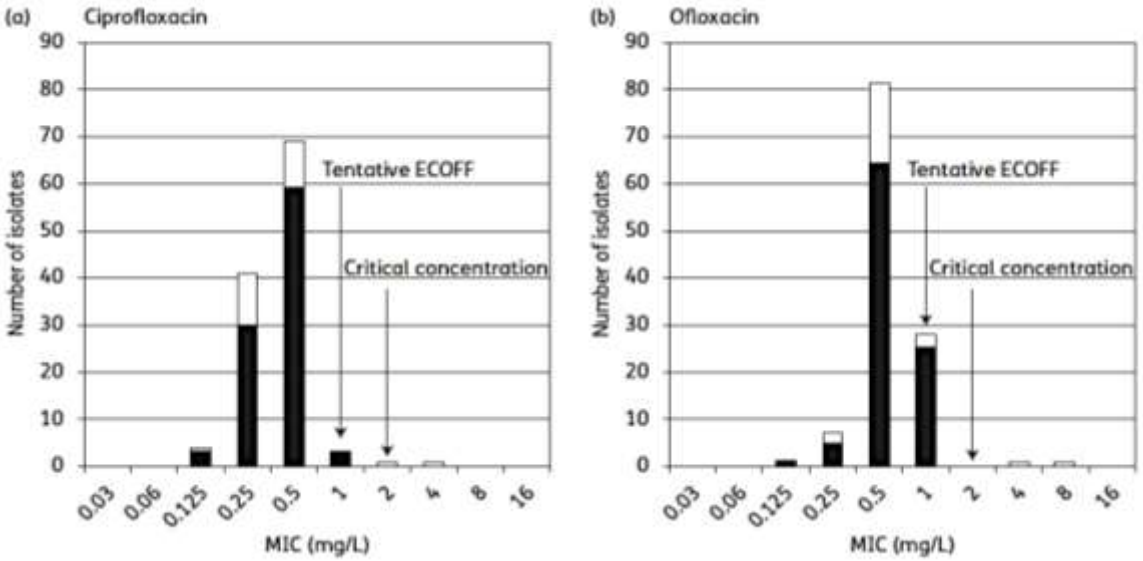


Figure 2.2: MIC distribution of fluoroquinolones for *M. tuberculosis (*Ängeby *et al*., 2010)

The ECOFFs were (S) susceptible ≤ 1.0 mg/L for ciprofloxacin and ofloxacin and S≤ 0.5 mg/L for levofloxacin and moxifloxacin. The wild-type strains were all classified as susceptible when applying the WHO recommended critical concentrations (S ≤ 2.0) for ciprofloxacin, ofloxacin and levofloxacin. Among the 24 drug-resistant strains 2 had MICs higher than the ECOFF, when WHO'S critical concentration of (S ≤ 2.0 mg/L) was applied (WHO, 2008). The two strains were classified as resistant to ofloxacin in agreement to WHO's results of External Quality Assement (EQA) panel, but one strain was doubtfully classified as susceptible to ciprofloxacin and levofloxacin most likely due to artifact of the current critical concentration (Ängeby *et al*., 2010).

The same study proposed susceptibility of ≤ 1.0 mg/L as the tentative epidemiological cut-off (ECOFF) for ofloxacin and ciprofloxacin, and susceptibility ≤ 0.5 mg/L for levofloxacin and moxifloxacin. The study suggests that their ECOFFs would be more appropriate if used as clinical breakpoints on middlebrooks agar rather than the current critical concentration of S ≤ 2.0 for ciprofloxacin, ofloxacin and levofloxacin and S ≤ 0.5 for moxifloxacin if other investigators confirm their findings (Ängeby *et al*., 2010).

In another study Jureen *et al.,* (2010) evaluated wild-type MIC distributions for (injectables) aminoglycoside and cyclic polypeptide antibiotics used for treatment of *M. tuberculosis* infections, 90 consecutive strains and 21 drug-resistant strains MICs

distributions on middlebrook 7H10 medium for amikacin, kanamycin, streptomycin, capreomycin and viomycin were established.

Five out of seven amikacin and kanamycin-resistant isolates were classified as susceptible to capreomycin according to current critical concentration (10 mg/L) but were non-wild type according to the ECOFF (4 mg/L) suggesting that critical concentration for capromycin is too high. Resistant isolates may thus be reported as susceptible. The study suggests that the clinical breakpoint or the critical concentration be reduced to 4 mg/L (Jureen *et al*., 2010).

In another study, the wild-type MIC distributions of ethionamide ranged from 0.5 to 2 mg/L, suggesting that 2 mg/L to be the ECOFF (Schön *et al*., 2011). This is in agreement with a study by Kam *et al.,* (2010) where an ethionamide MIC ranging from 2-3 mg/L was validated against clinical outcome. For cycloserine the wild-type MIC distributions were from 8 to 32 mg/L, suggesting that 32 mg/L to be ECOFF, which may serve as a tentative clinical breakpoint for cycloserine on middlebrook 7H10 agar where there is currently no recommended clinical breakpoint (Schön *et al*., 2011).

2.7.3 Trek Sensititre® *Mycobacterium tuberculosis* MIC plate (MYCOTB)

The conventional phenotypic drug susceptibility testing (DST) agar proportion method (APM) has been used as a gold standard method for *M. tuberculosis* susceptibility testing for rifampin and isoniazid. The diagnostic accuracy, reproducibility of DST for other first-line drugs and second-line drugs are inadequate (WHO, 2008).

Phenotypic methods for DST are based on one or two breakpoints tests and limitations includes variations in laboratories preparation of drug stock solutions and variations in solid agar media used (WHO, 2008; Kim, 2005). The agar proportion method for drug susceptibility testing is labor intensive and requires two to three weeks of incubation before results are available (WHO, 2008). The global increase of *M. tuberculosis* resistance prompts a simple, rapid, accurate quantitative susceptibility method for testing *M. tuberculosis* strains (Sullivan *et al*., 2009).

The Sensititre MYCOTB plate is a 96-well microtiter plate, which contains lyophilized micro-dilution antibiotics, with concentrations prepared and quality controlled by the manufacturer. The plate has 12 first-line and second-line drugs configured for determination of minimal inhibitory concentrations (MIC) of *M. tuberculosis* at seven dilutions per drug (Sullivan *et al*., 2009). The MYCOTB plate is simple to prepare and it can be read manually using mirror box or using a computerized-system Vizion with the SWIN software platform. Results are available as soon as 7 to 10 days (Abuali *et al*., 2012; Sullivan *et al*., 2009).

The MYCOTB MIC plate is a quantitative rapid and efficient method that allows simultaneous drug susceptibility testing of first-line and second line drugs. The MYCOTB MIC plate has shown that wild-type MIC distributions of *M. tuberculosis* clinical isolates to anti-tuberculosis drugs can be considered when setting clinical breakpoints (Schön *et al*., 2009; Ängeby *et al*., 2010).

Sensitire MYCOTB plate has been evaluated for susceptibility testing of *M. tuberculosis* complex against first and second line drugs (Hall *et al*., 2012). The study indicated that MYCOTB MIC plate DST method is equivalent to the gold standard agar proportion method (Hall *et al*., 2012). In this study the MYCOTB plate results were read using semi-automated Vizion technology by two independent reader and qualified laboratory technician. The MYCOTB results were considered susceptible when the MIC breakpoint utilized was equivalent to or lower than agar proportion method (APM) critical concentration and resistant when the MIC breakpoint was higher than the APM critical concentration.

Table 2.3: Schematic representations of MYCOTB MIC plate

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

Abbrevations:

OFL, ofloxacin; MXF, moxifloxacin; RIF, rifampin; AMI, amikacin; STR, streptomycin; RFB, rifabutin; PAS, para-aminosalicylic; ETH, ethionamide; CYC, cycloserine; INH, isoniazid; KAN, kanamycin; EMB, ethambutol; POS, antibiotic- free positive control wells.

2.8 Genotyping of *M. tuberculosis* complex

Genotyping of *M. tuberculosis* isolates is an important tool used to investigate transmission dynamics, circulating strains, natural history of tuberculosis and to identify dominant strains associated with outbreak and drug resistance (Bazira *et al*. 2011). Molecular typing methods commonly used are spoligotyping, IS6110 restriction fragment length polymorphism (RFLP) typing and mycobacterial interspersed repetitive unit typing (MIRU-VNTR) which are PCR based techniques (van Embden *et al*., 1993; Kamerbeek *et al*., 1997; Supply *et al*., 2000).

2.8.1 Spoligotyping

Spoligotyping is a genotyping tool used to provide information on the structure of the direct repeat (DR) region in individual *M. tuberculosis* strains (Streicher *et al*., 2007). Spoligotyping is based on various tandem repeats, are the mostly used methods after IS6110 based fingerprinting (Sharma *et al*., 2008). Spoligotyping technique is fast, precise and exploits the DNA polymorphism in a direct repeat (DR) region of MTB complex strains (Hove *et al*., 2012). *M. tuberculosis* strains vary in the number of DRs, and in the presence or absence of particular spacers (Hove *et al*., 2012). There are different genetic elements that contribute to DNA polymorphism in MTB. The chromosomal locus, contain a large number of 36-base pair (bp) direct repeats interspersed with unique spacer sequences varying in size from 35 to 43 bp, which is the target of spoligotyping (spacer oligonucleotide typing) technique (Kamerbeek *et al*., 1997). Spoligotyping aids in detecting the presence or absence of spacers of a known sequence (Kamerbeek *et al*., 1997). The presence or absence of 43 spacers in the direct repeat region of isolates of MTB is detected by amplifying DRs with primers, one biontinylated. The amplified products are then reverse hybridized to spacer sequence oligonucleotide probes immobilized on a Biodyne C membrane (Groenen *et al*., 1993; Kamerbeek *et al*., 1997). Detection of hybridization signals is made by incubating the membrane in streptavidin peroxidase which binds to biotinylated PCR products and enhances chemiluminescence. The peroxidase present on the streptavidine catalyzes a reaction which results in the emission of light detected by autoradiography of the membrane (Groenen *et al*., 1993; Kamerbeek *et al*., 1997).

2.8.2 Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) analysis with Insertion Sequence (*IS*6110) probe has been considered as the gold standard method for genotyping of *M. tuberculosis* strains (van Soolingen *et al*., 2001; Jiao *et al*., 2008). The RFLP method is laborious, time consuming and because the method lacks PCR amplification steps it requires large numbers of DNA copies (Christianson *et al*., 2010). Similar to most gel based methods, RFLP is susceptible to interpretive errors (van Sooligen *et al*., 2001). This method cannot be used for typing strains with copies fewer than six IS6110 insertions (Soini *et al*., 2001; Christianson *et al*., 2010).

2.8.3 Mycobacterial Interpersed Repetitive Unit Variable Number Tandem Repeat (MIRU-VNTR) Typing.

Mycobacterial Interpersed Repetitive Unit Variable Number Tandem Repeat (MIRU-VNTR) is a well described molecular typing method used for analyzing the diversity and population structure of MTB strain (Supply *et al*., 2000). The advantage MIRU-VNTR has is that it provides better digitation of the generated strain profiles and inter-laboratory comparison as compared to the other techniques like spoligotyping and *IS110*-RFLP. The MIRU-VNTR typing method that is currently used in laboratories worldwide has been standardized (Supply *et al*., 2006). The MIRU-VNTR typing method measures polymorphisms in the entire genome of MTB and it can be used on strains that have low IS6110 copy numbers. Due to its lower discriminatory power with regard to genotyping of the Beijing family strain authors have indicated that it should be coupled with an additional genotyping method such as spoligotyping (Supply *et al*., 2001; Warren *et al*., 2002; Scott *et al*., 2005; Iwamoto *et al*., 2007; Valcheva *et al*., 2008)

2.8.4 Distribution of *M. tuberculosis* family strain

The *M. tuberculosis* Beijing genotype, which was first revealed in 1995, is one of the most successful clades in the present worldwide tuberculosis epidemic (Parwati *et al*.,

2010). The Beijing genotype is a lineage of *M. tuberculosis* that has a worldwide distribution and highly endemic in certain geographical areas and it's also found predominant in South Africa (Viegas *et al.,* 2013). Evolution of *M. tuberculosis* strains has been analyzed by spoligotyping, which can cluster strains into families (i.e., Beijing, T, and Haarlem) based on the deletion patterns of the Direct Repeat (DR) locus (Yokoyama *et al*., 2012).

According to the 4th international spoligotyping database (SpolDB4) strains from the Beijing evolutionary lineage were found to be present in the largest number of countries (Hanekom *et al*., 2011). Beijing and Beijing-like strains represent about 50% of the strains in Far East Asia and 13% of isolates globally (Brudey *et al*., 2006).

In Europe, the Haarlem (H) lineage represents about 25% of the isolates. In South America, about 50% of the strains belong to the Latin American Mediterranean (LAM) family. Three major genotypic families (Haarlem, LAM and T) are the most frequent in Africa, Europe and South America. Outside Europe, the Haarlem strains were mainly found in Central America and Caribbean (about 25%), suggesting a link of Haarlem to the post- Columbus European colonization (Brudey *et al*., 2006). Beijing, Haarlem and African strains responsible for major epidemics are 'modern' types (Bazira *et al*., 2011) The distribution of genotypes amongst MTB strains in the South African provinces suggest spoligotype patterns corresponding to Beijing, LAM4, S, T1, X1 or other genotypes (Streicher *et al*., 2012).

In the Western Cape of South Africa strains of the Beijing genotype are thought to have been introduced following the sea trade routes from East Asia to Europe approximately 400 years ago (Hanekom *et al*., 2011).

Although, MDR and XDR tuberculosis are known to be associated with Beijing strains, in KwaZulu-Natal data suggest that the non-Beijing strain are driving the epidemic (Gandhi *et al*., 2014). The strains that resulted in the outbreak in KZN were classified as F15/LAM4/KZN (Pillay and Sturm, 2007). According to Pillay and Sturm (2007) the F15/LAM4/KZN is found consistently in KZN and is responsible for a significant proportion of transmissible MDR-TB.

Four prominent lineages identified in the Western Cape were Beijing, Haarlem, LAM and X1 are associated with drug resistant *M. tuberculosis* strains (Johnson *et al*., 2010).

A molecular epidemiologic study has been reported in the Eastern Cape, showed that 50% of rifampin-resistant TB isolates (including MDR TB isolates) belonged to the Beijing genotype and that “atypical” Beijing strains were significantly overrepresented (Klopper *et al*., 2013). The identification of the atypical Beijing cluster 86 strain originally identified in the Eastern Cape suggests migration of patients to the Western Cape as this strain was only rarely found in MDR-TB cases in that province (Hanekom *et al*., 2007). Mlambo *et al.* (2008) described a report on the strain diversity of MDR-TB sample in Johannesburg. The spoligotype pattern showed diversity in the predominant strain families and the shared type within certain families. The three strains that are prevalent in Africa (H, LAM, T) were observed in the study and is consistent with other reports from South Africa (Streicher *et al*., 2004; Mlambo *et al*., 2008; Stavrum *et al*., 2009). The study also showed that the EAI1-SOM (SIT 48, 806) family is predominant in Johannesburg.

CHAPTER THREE

3. METHODOLOGY

3.1. Study Area

The research was conducted at the National Institute of Communicable Diseases (NICD), Johannesburg and National Health Laboratory Services (NHLS) Polokwane. Existing archived *M. tuberculosis* complex positive cultures were collected from NHLS in Polokwane hospital, which is a referral laboratory for regional TB hospital and clinics in Limpopo province. The NHLS provides laboratory and related public health services to more than 80% of the population through a national network of laboratories.

3.2. Study design

The study was experimental and wild-type MIC distributions of second-line drugs against *M. tuberculosis* complex was determined on culture isolates and data was compared with recommended CC. GenoType MTBDR*plus* assay was used to detect mutation associated with rifampin and isoniazid. GenoType MTBDR*sl* assay was used to detect mutation associated with second-line drugs. *Mycobacterium tuberculosis* complex isolates were genotyped by spoligotyping to determine strain distributions.

3.3. Study Population

The study targeted *M. tuberculosis* complex positive cultured isolates from patients admitted in wards and out patients in all the regional hospitals and clinics in Limpopo Province whose sputum samples were sent to NHLS at Polokwane hospital. The National Health Laboratory Service receives sputum specimens for culture and drug susceptibility testing (DST) with rifampin and isoniazid using BACTEC MGIT 960 and GenoType MTBDR*plus* line probe assay.

According to a report issued by NHLS and NICD Surveillance in 2011, Limpopo Province had 1931 TB patients and 151 patients with drug resistant TB (NHLS and NICD, 2011). The sample size was determined and calculated based on this report.

A minimum of 109 positive cultured isolates were used from the pool of 437 collected *M. tuberculosis* complex positive cultured isolates.

3.4. Sampling

3.4.1 Study Specimen

Archived *M. tuberculosis* complex, positive cultures were collected from mycobacteria growth indicator tubes (MGIT) 960. The MGIT Samples were collected for a period of six months after approved by Medunsa Research Ethics Committee approval and NHLS sample collection approval.

3.4.2 Sampling Procedure

Cultured isolates were collected by convenience sampling as the samples were not readily available and NHLS keeps the sample for two months only. The cultured isolates are given to the researcher who has been granted approval to collect and conduct TB research at NHLS in Polokwane upon removal of patient's identities.

3.5. Sample Size

To determine the sample size a formula by Morgan was used (Morgan and Krejae, 1970).

$S = X^2 NP (1-P)/ d^2 (N – 1) + X^2 P (1 – P)$.

S = required sample size

X^2 = the table value of chi-square for the 1 degree of freedom at the desired confidence level (3.841).

N = the total number of drug resistance TB patient in Limpopo province reported in 2011 by NHLS and NICD which is 151 (NHLS and NICD, 2011).

P = the population proportion (assumed to be 0.50 since this would provide the maximum sample size).

d = the degree of accuracy expressed as a proportion (0.05)

$S = (3.841) (151) (0.5) (1-0.5)/ (0.05)^2 (151-1) + (3.841) (0.5) (1- 0.5)$

Minimum sample size = 109

3.6. Data Collection

Standard tuberculosis treatments, drug susceptibility test results and critical concentrations used on first-line (rifampin and isoniazid) and second-line drugs (kanamycin and ofloxacin) were collected from NHLS Braamfontein and analyzed after phenotypic methods were performed.

3.7. Laboratory Procedure:

Sputum samples at NHLS were decontaminated with NaOH-NALC (Appendix 1).

Sputum samples were inoculated on Lowenstein-Jensen medium (two tubes per sample) and incubated at 37º C for three weeks to preserve clinical isolates. Lowenstein-Jensen media inhibit growth of other bacteria and limits growth to mycobacteria species (Appendix 2).

Drug-susceptibility testing on BACTEC MGIT 960

The susceptibility of all clinical isolates to first-line drugs rifampin and isoniazid were performed at National Health Laboratory Service (NHLS) in Polokwane hospital Limpopo Province, using BACTEC MGIT 960 methods. The standard operating protocol for DST was followed (Appendix 2). The location and districts of healthcare facilities were recorded to determine the genotypes of *M. tuberculosis* complex clinical isolates.

Sub-culture on BACTEC MGIT 960

Positive clinical isolates (n = 437) were collected and sub-cultured using BACTEC MGIT 960 system. Each BACTEC MGIT vial tube contained 7.0 ml of middlebrook 7H9 liquid media that produces faster growth and recovery of mycobacteria for 7 to 14 days for detection of positive isolates growth. Growth supplement Oleic acid albumin citrate (OADC) 0.8 ml was added aseptically to all tubes and contamination was reduced by addition of antimicrobial PANTA before 500 µl of mixed culture specimen was inoculated in biological safety cabinet in level 3 laboratory (Appendix 2). The MGIT tubes were incubated at 35º C in a MGIT 960 automated system until

there was a green flag light, for positive growth detection next to the tube. A hundred nine positive growth tubes were then used for MIC determination in biological safety level 3 laboratory.

MIC determination using TREK sensititre *Mycobacterium tuberculosis* MIC plate

Trek Diagnostic Sensititre *M. tuberculosis* MIC plate (MYCOTB) was used for drug susceptibility testing. The MYCOTB MIC plate contained first and second-line drugs with a minimum of 7 dilutions per drug. The MIC of an antimicrobial agent was the lowest (minimal) concentration of the antimicrobial agents that inhibits a given bacterial isolate from multiplying and producing visible growth in the test system.

The concentration was determined in the laboratory by incubating 2 ml of bacteria with specified dilutions of the antimicrobial agent to a turbidity equivalent to 0.5 McFarland standards in 11 ml saline Tween solution containing glass beads and vortexed for two minutes. The inoculated saline tween solutions were left for 20 minutes at room temperature inside biological safety cabinet. One ml of the suspension was inoculated to a Middlebrook 7H9 broth (11 ml) containing oleic acid albumin-dextrose catalase. One hundred µl of the 7H9 broth was inoculated to MYCOTB Sensititre MIC plate wells using an automated inoculator (Appendix 3).

The MYCOTB plate were incubated at 37º C in a 5% CO_2 incubator and read after 14 days with semi-automated plate reader (Vizion system) by two independent readers. The growth control wells in position H11 and H12 were checked for adequate growth before evaluating other drugs. The first well with no visible growth was considered to be minimal inhibitory concentration for the 12 drugs.

Genotypic drug susceptibility testing

Genotypic drug susceptibility testing, using GenoType MTBDR*plus* and MTBDR*sl* line probe assay version 2.0 were performed in four separate room molecular laboratory in accordance with WHO recommendations (WHO, 2008).

DNA extraction

DNA was extracted from 109 positive cultured isolates using Genolyse assay for extraction of genomic bacterial DNA from patient specimens and clinical isolates according to the manufacturer's instructions (Hain Lifescience, 2014). One ml of Liquid culture isolates was transferred into a labeled 1.5 ml screw cap tube vortexed for one minute. The pellet bacteria was centrifuged for 15 minutes at 10 000 xg in a standard table top centrifuge.

The supernatant was discarded and resuspend in 100 µl Lysis Buffer (A-LYS). The sample was incubated for 5 minutes at 95^{o}C on a heating block. One hundred µl of Neutralizing Buffer (A-NB) was added to lysate and vortexed for 5 seconds. Centrifuged for 5 minutes at 13 000 xg, 50 µl containing DNA was transferred to a new 1.5 ml screw cap tube and stored at - 20º C until used for amplification and further use with GenoType MTBDR*plus* and GenoType MTBDR*sl* assay version 2.0 (Appendix 4). DNA extraction was performed in a biological safety cabinet in a biological safety level 3 laboratory.

DNA amplification by PCR

Polymerase chain reaction (PCR) is a biochemical process that uses primers to amplify DNA using the enzyme Taq polymerase. GenoType MTBDR*plus* version 2.0 line-probe assay contained Amplification mixes A and B (AM-A and AM-B) which includes reagents needed for DNA amplification such as Taq polymerase, primers optimized for test. The master mix was prepared in a separate room "Master Mix room" free of contaminating DNA. The number of samples was 109 plus one control sample. The master mix containing AM-A and AM-B was mixed into a new screw cap tube labelled master mix according to the laboratory amplification master mix volume chart. Forty five samples were done per session until required samples was reached. Following the chart 460 µl of AM-A was mixed with 1610 µl of AM-B making total to be 2070 µl. Forty five µl of prepared master mix was aliquoted into each labelled PCR tube. The negative control tube was aliquoted with 5 µl of water. In a separate room "Amplification room" 5 µl of DNA was added to the

corresponding PCR tube. The prepared tubes were placed onto the Thermo cycler and the “culture-program” for amplification was selected. Amplicons were then used for hybridization.

Reverse hybridisation procedure includes

Denaturation: separation of amplicons in single-stranded DNA and then binding of labelled amplicons to the membrane strip coated with probes

Hybridisation is the binding of single stranded DNA to the specific probes on the membrane strip

Stringent wash removes unbound DNA probes conjugate reaction binds enzyme to the conjugated protein

Substrate reaction does enzymatic conversion of dye

The strips included in the GenoType MTBDR*plus* ver 2.0 line-probe assay were placed on clean sheet of paper and labelled in “Hybridization room”. Hybridisation Buffer (HYB) and stringent wash (STR) were placed at room temperature to be freed of precipitates. Conjugate (CON) and substrate (SUB) were diluted according to table provided. Twenty (20) µl of Denaturation (DEN) solution was added to the corner of trough tray. Twenty (20) µl of amplicons was added to (DEN) solution and carefully mixed by pipetting up and down. The trough tray was incubated at room temperature to get single stranded DNA. The trough tray was placed into GT-Blot 48 machine. Reverse hybridisation performed for 30 minutes at 45ºc after the GT-Blot machine aspirated Hybridisation buffer (HYB). The labelled strips were placed (Face Side up) in the tray and were completely covered with the reagents in tray wells. Stringent wash (STR) was aspirated for 15 minutes at 45ºC to wash unbound DNA probes. Rinse solution was added for 1 minute. Conjugate was aspirated for 30 minutes at 35ºC. The streptavidin conjugated alkaline phosphates attaches to the biotinylated primer attached to the probe. This was rinsed twice for 1 minute at 35ºC to remove unbound conjugate. Substrate was aspirated for 8 minutes at 35ºC for development of colour. Distilled water was aspirated to stop colour reactions.

The strips were removed and placed on a clean sheet and interpreted with the aid of an evaluation sheet provided with the assay.

Mycobacterium tuberculosis clinical isolates genotypes

Spoligotyping was used to determine *M. tuberculosis* clinical isolates genotypes in accordance with stardardised protocol described by Kamerbeek *et al*. (1997) and Brudey *et al.* (2006) (Ocimum Biosolutions kit). Spoligotyping assay is based on mycobacterial DNA amplification by PCR and hybridisation of the biotin-labeled amplicon to the nylon membrane immobilized oligonucleotides that represents 43 spacers of known sequence. The presence of spacer was visualized on X-ray film as black squares after incubation with streptavidin-peroxidase and chemiluminescent ECL detection.

The *H37Rv* and *Mycobacterium bovis BCG* strains were used as positive controls and molecular biology grade water was used as negative control. The genotyping results were entered in a binary format on excel spread sheet and compared with world-spoligotype database (SpolDB4) of the institute Pasteur Guadeloupe. Based on spoligotype pattern previously described isolates were allocated to family (clade) strains.

3.8. Data Analysis

Statistical Package for Social Sciences (SPPS) Version 23 was used with the assistance of a biostatistician to analyze the data. Percentage of isolates (95% confidence interval) susceptible and resistant to the first-line drugs and second-line drugs was determined. Minimal inhibitory concentration (MIC) to each drug was determined on susceptible strains and resistant strains. The mean MIC (+/-SD) to each drug was calculated. The mean difference was significant at a P-value of ≤ 0.05. Mutational analysis was performed on first-line drugs and chi-square test was used for cross tabulation of MIC and mutations on genes targeted by the drugs.

3 RELIABILITY, VALIDITY AND OBJECTIVITY

Phenotypic and genotypic tests were performed according to the manufacturer's instructions. All laboratory methods employed have been evaluated and standardised to ensure reliability, validity and objectivity. Trek sensititre MYCOTB plates are incorporated with growth control wells in positions H11 and H12. The *M. tuberculosis H37Rv* and *M. bovis BCG* strains were included as control for spoligotyping assay. Purity checks for contamination for each sample were done on blood agar plate. The molecular technique GenoType MTBDR*plus* and MTBDR*sl* version 2.0 have been standardised and validated to ensure reliability, validity and objectivity. GenoType MTBDR*plus* line-probe assay and genoType MTBDR*sl* version 2.0 contains conjugate control strip, amplification control strip and locus control zones for targeted genes to ensure correct performance of the test.

4 BIAS

- The researcher was blinded to results of DST and CC from data collected at NHLS Track Care until phenotypic methods of the study were completed.
- Under internal validity, technical biases were avoided by having two independent readers and a qualified microbiologist read the Drug susceptibility testing MYCOTB MIC plate.
- To avoid bias arising from contamination, purity checks were done on blood agar for each sample.
- To avoid sampling bias, 437 samples were collected randomly at NHLS dating June 2012 to July 2013, as drug resistant tuberculosis isolates are not readily available in the Limpopo province.

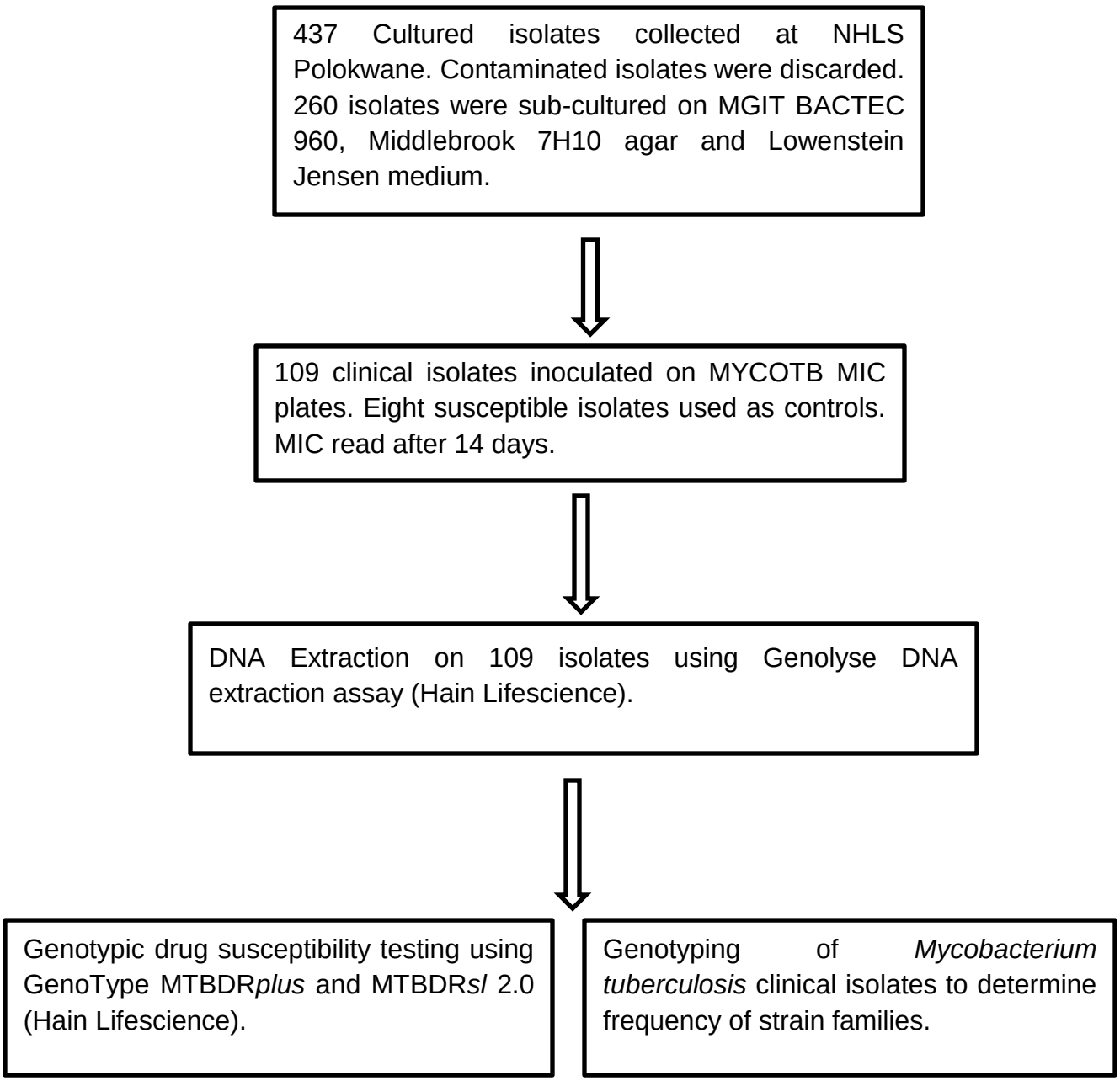
5 ETHICAL CONSIDERATION

The Research study was approved by Medunsa Research Ethics Committee (MREC) for ethical clearance. The ethical clearance certificate was submitted to The Department of Health and NHLS academic research committee for sample collections approval after patient identifiers were removed.

6 SIGNIFICANCE OF THE STUDY

The study will contribute towards efficient TB control, by reporting accurate drug susceptibility testing and the use of wild-type MIC distributions to establish reliable clinical breakpoints for first-line and second-line drugs used for treatment of TB to the Department of Health in the Limpopo Province. The findings of this study will enhance guidelines for therapeutic decision making process by providing dosing guidance and effective tuberculosis treatment regimen for patients in Limpopo Province to the Department of Health. Patients will receive appropriate treatments which will break after chain of transmission and avoid the spread of resistant strains in Limpopo Province.

8 Methodological flow chart



CHAPTER FOUR

RESULTS, INTERPRETATION OF FINDINGS

RESULTS INTERPRETATION

The minimal sample size of 109 sub-cultured *M. tuberculosis* clinical isolates was included in the study. Eight isolates were invalid on MTBDR*plus* assay for genotypic DST were removed from the study. The 101 sample size included 7 isolates susceptible to rifampin and isoniazid, 18 isoniazid mono-resistant isolates, 66 rifampin mono-resistant isolates and 10 (MDR) rifampin and isoniazid resistant isolates. The sample size was categorized into four groups: susceptible to rifampin and isoniazid, INH monoresistant, RIF monoresistant, MDR with resistance to RIF and INH.

The MYCOTB plates were read after 14 days by two independent readers using Vizion semi-automated plate reader. The results were entered on Microsoft excel spread sheet 2010 and the 12 drugs minimal inhibitory concentration (MIC) distributions represented on bar charts figure 4.1 to 4.12. Comparative analysis of drugs MIC and genotypic drug susceptibility results were done on an excel spread sheet. An isolate was considered susceptible on MYCOTB plates when the drug MIC was below or equivalent to agar proportion method and wild-type. An isolates was considered resistant on MYCOTB plates when drug MIC was above and higher than agar proportion methods and non wild-type. Analysis of variance of clinical isolates exhibiting susceptibility and resistance against first and second-line drug was performed using SPSS version 23. $P \leq 0.05$ was considered statistically significance.

Genotypic drug susceptibility testing

GenoType MTBDR*plus* assay version 2.0 was used to detect genomic mutations of *M. tuberculosis* strains conferring resistance to rifampin on *rpoB* gene and resistance to isoniazid on the *katG* gene and *inhA* promoter region. The assay is based on DNA strip technology. Mycobacterial DNA was amplified using PCR. Membrane test strips were coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single-stranded amplicons bonded to the probes by reverse

Hybridization performed automatically using GT-Blot 48 laboratory equipment (Hain Lifescience) The assay was performed according to manufacturer's instructions (Appendix 5) (Hain Lifescience Germany).

The Genotype MDRTBplus assay test strip had five control bands the conjugate control (CC), amplification control (AC), *rpoB*, *katG* and *inhA* locus control. The AC band developed when the test was performed correctly and mistakes during extraction and amplification were excluded. The CC band developed to show efficiency of conjugate binding and substrate reaction. The TUB band developed when the tested isolates belonged to *M. tuberculosis* complex. Only band whose colour intensity matched the AC bands were considered.

The results strips were evaluated and interpreted using evaluation sheet provided in the assay kit. Susceptibility to rifampin was reported when all wild-type (WT1- WT8) probes on the *rpoB* gene stained positive and no mutation probe band was detected. Rifampin resistance was reported when an absence in the wild-type probe (ΔWT1-WT8) or presence of mutation probe band was observed. Susceptibility to isoniazid was reported when wild-type probe of the *katG* gene stained positive and no mutation probe band was detected. The two wild-type probe on the *inhA* gene was evaluated for low level isoniazid resistance.

GenoType MTBDR*sl* assay version 2.0 was used to detect genomic mutations of *M. tuberculosis* strains conferring resistance to fluoroquinolones (ofloxacin, Moxifloxacin) aminoglycosides (kanamycin, amikacin) and low-level kanamycin resistance. The GenoType MTBDR*sl* assay principle is also based on DNA strip technology using reverse hybridization and an enzymatic color reaction performed automatically on GT-Blot 48 device (Hain Lifescience). The tested strip had six control band CC, AC, *gyrA, gyrB, rrs, eis* locus control band and *M. tuberculosis* complex identification band (TUB). The *gyrA* and *gyrB* gene were evaluated for detection of resistance to fluoroquinolones. The *rrs* gene was evaluated for detection of resistance to kanamycin and amikacin, low-level kanamycin resistance was evaluated on the *eis* gene. Similar to GenoType MTBDR*plus* susceptibility to a drug was reported when wild-type probe corresponding to each specific gene stained positive. Resistance to a drug was reported when

absence of wild-type probe was detected and presence of mutation probe was observed.

Spoligotyping results

Spoligotyping results were entered into Microsoft Excel 2010 in octal and binary formats representing black and white squares on the X-ray membrane film and compared with world spoligotyping SITVIT2 database (SpolDB4) of Institut Pasteur de Guadeloupe (Brudey, 2006) available online http:// www.pasteur-guadeloupe.fr.8081/SITVIT-Demo. The database provides information on *M. tuberculosis* spoligotype international type (SIT) patterns. Isolates was assingened genetic family when identical spoligotyping pattern was defined in the database. Isolates that did not match any spoligotype pattern in SITVIT2 database were named orphan. Statistical analysis was performed using SPSS version 23 to calculate frequency distribution and percentages of genotypes shown on table 4.10 and figure 4.16 at 95% confidence intervals and P value of ≤ 0.05 was considered significant.

Wild-type MIC distributions in *M. tuberculosis* clinical isolates

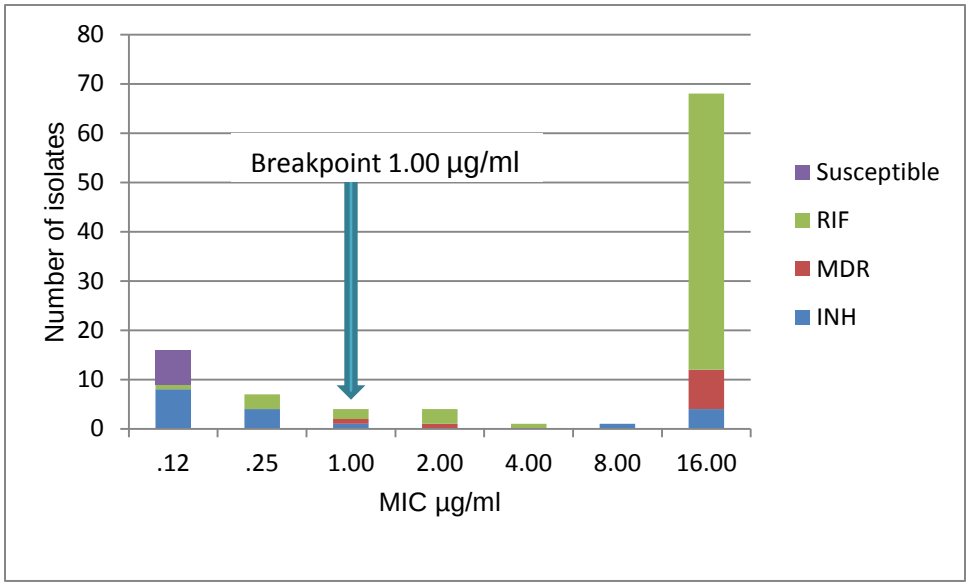


Fig 4.1: Rifampin MIC distributions for wild-type *M. tuberculosis* isolates.

Isolates categorized as susceptible to rifampin and isoniazid (n = 7) had MIC values below the recommended CC of ≤ 1 µg/ml. For isolates categorized as rifampin mono-resistant, eight had MIC distributions within the CC and fifty eight isolates exceeded the CC. Thirteen isolates (INH mono-resistant) had rifampin MIC values within the CC, while for five isolates the MIC values were greater than the CC. The majority of rifampin MIC values for MDR isolates (n = 9) exceeded the rifampin CC, while one MDR isolates, had MIC values were below the CC. Wild-type rifampin MIC distributions range in figure 4.1 is (≤ 0.12 to 0.5 µg/ml).

Number of isolates represents 101 sample size on the y-axis, Breakpoint represents the current critical concentration for rifampin. The seven dilutions minimal inhibitory concentration is represented on x-axis in µg/ml. Susceptible represents isolates susceptible to rifampin and isoniazid, Rif represents rifampin mono-resistant isolates, MDR represent isolates resistant to rifampin and isoniazid, INH represents isoniazid mono-resistant isolates.

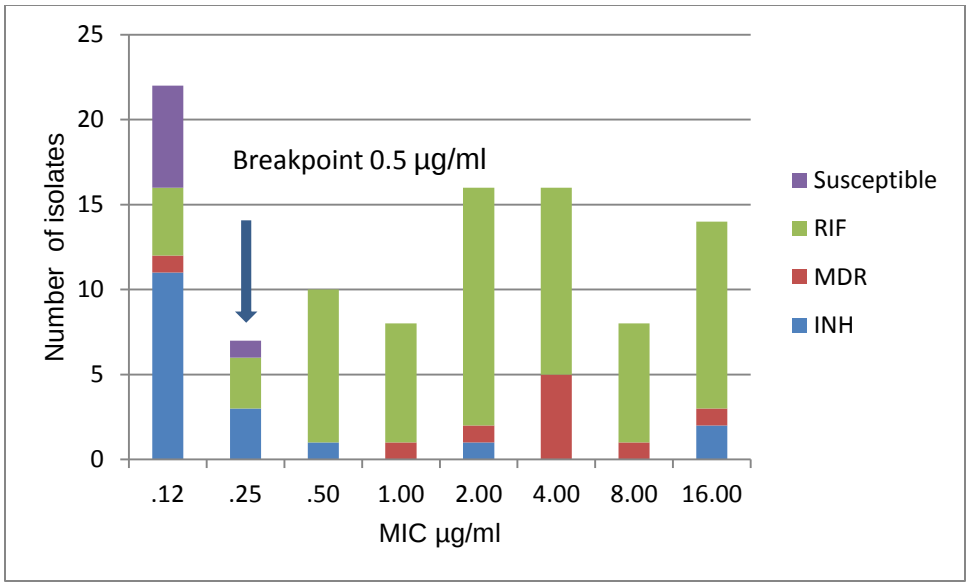


Fig 4.2: Rifabutin MIC distributions for wild-type *M. tuberculosis* isolates.

Susceptible isolates (n = 7) had MIC below the recommended CC of S ≤ 0.5 µg/ml. Isolates categorized as Rif mono-resistant (n = 23) had MIC below the recommended CC and (n = 43) had MIC above the CC. Isolates categorized as MDR (n = 2) had MIC below the recommended CC and (n = 10) had MIC above the CC. Isolates categorized as INH mono-resistant (n = 15) had MIC below the CC and (n = 3) had MIC above the recommended CC. The wild-type MIC distribution for rifabutin in figure 4.2 is (≤ 0.12 - 0.25 µg/ml).

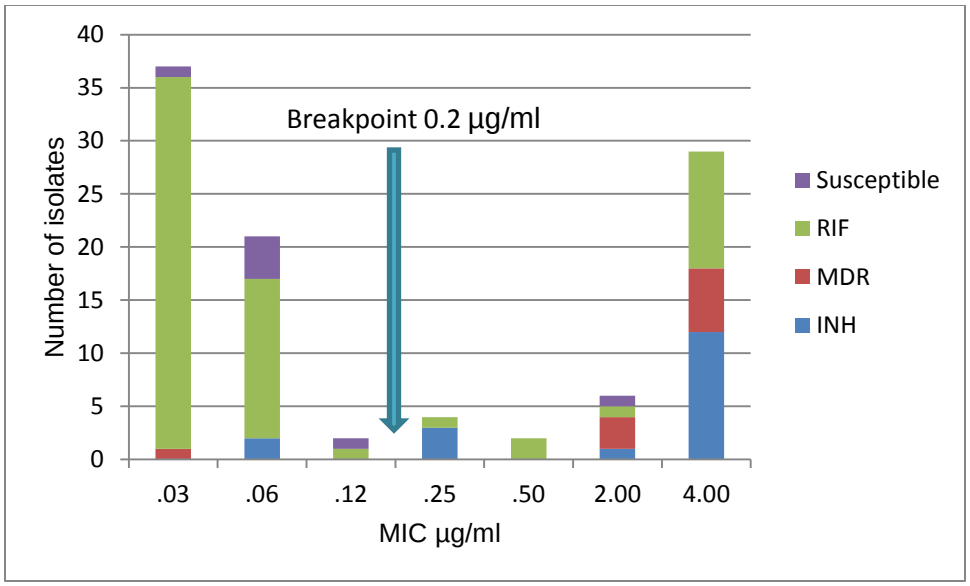


Fig 4.3: Isoniazid MIC distributions for wild-type *M. tuberculosis* isolates.

Isolates categorized as susceptible to isoniazid and rifampin (n = 6) had MIC below the recommended CC of S ≤ 0.2 and (n = 1) had MIC above recommended CC. Isolates categorized as INH mono-resistant (n = 2) had below recommended CC and (n = 15) had MIC above the recommended CC for isoniazid. Isolates catergorized as Rif mono-resistant (n = 51) had MIC below recommended CC for isoniazid and (n = 15) had MIC above the recommended CC. Isolates categorized as MDR (n = 1) had MIC below the recommended CC and (n = 9) had MIC above the CC. Isolates catergorized as INH mono-resistant (n = 2) had MIC below the recommended CC and (n = 16) had MIC above the recommende CC for isoniazid. The wild-type MIC distribution range for isoniazid in figure 4.3 is (≤ 0.3 - 2.00 µg/ml).

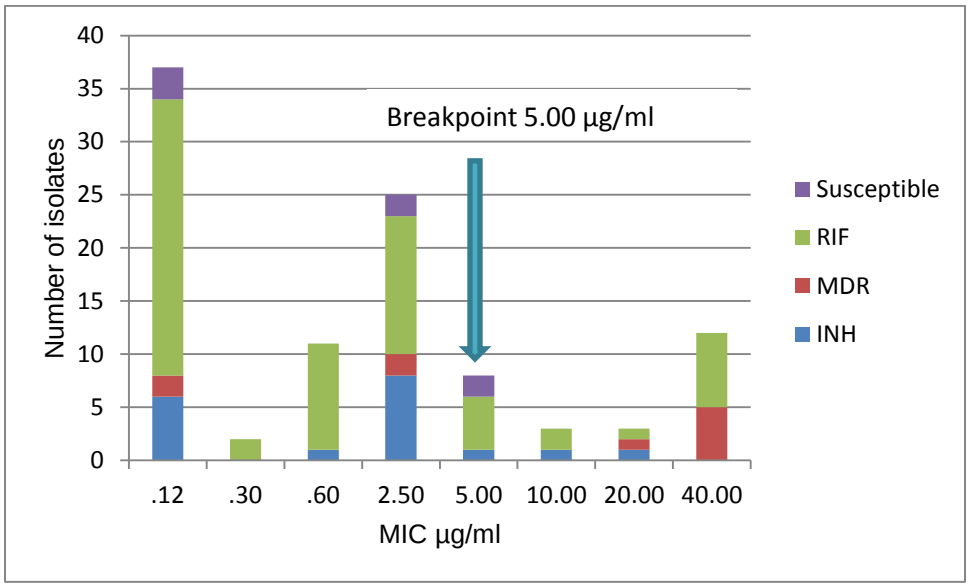


Fig 4.4: Ethionamide MIC distributions for wild-type *M. tuberculosis* isolates.

Susceptible isolates (n = 7) had MIC below the recommended CC of S ≤ 5 µg/ml for ethionamide. Isolates categorized as Rif mono-resistant (n = 56) had MIC below the recommended CC and (n = 10) had MIC above CC. Isolates categorized as MDR (n = 4) had MIC below the recommended CC and (n = 6) had MIC above the recommended CC. Isolates categorized as INH mono-resistant (n = 16) had MIC below the recommended CC and (n = 2) had MIC above the recommended CC. The wild-type MIC distribution for ethionamide range in figure 4.4 is (≤ 0.12 - 5 µg/ml).

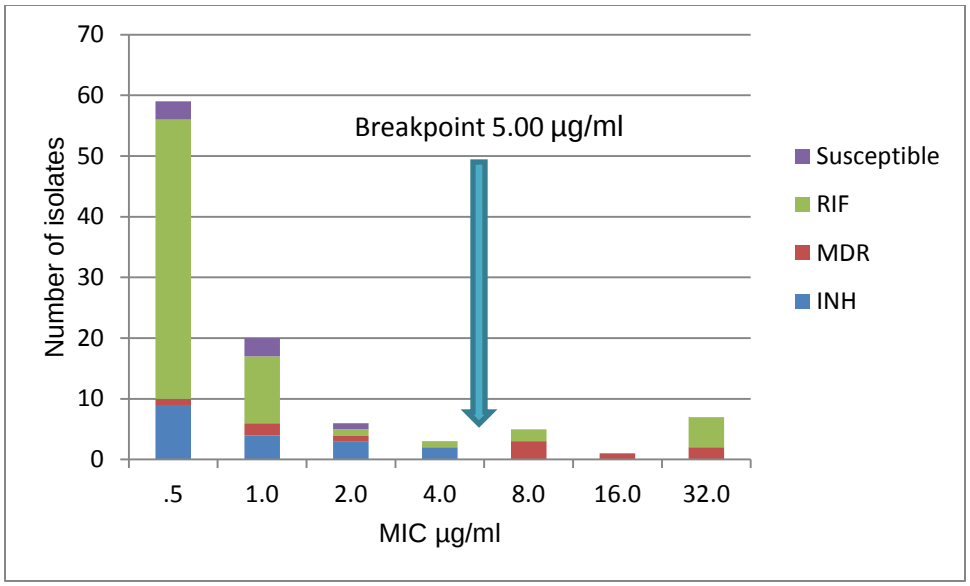


Fig 4.5: Ethambutol MIC distributions for wild-type *M. tuberculosis* isolates.

Susceptible isolates (n = 7) had MIC below the recommended CC. Isolates categorized as Rif mono-resistant (n = 59) had MIC below the recommended CC and (n = 7) had MIC above the recommended the CC. Isolates categorized as MDR (n = 4) had MIC below and (n = 6) had MIC above the recommended CC. Isolates categorized as INH (n = 18) had MIC below the recommended CC. The wild-type MIC distribution for ethambutol range in figure 4.5 is (≤ 0.5 - 2 µg/ml).

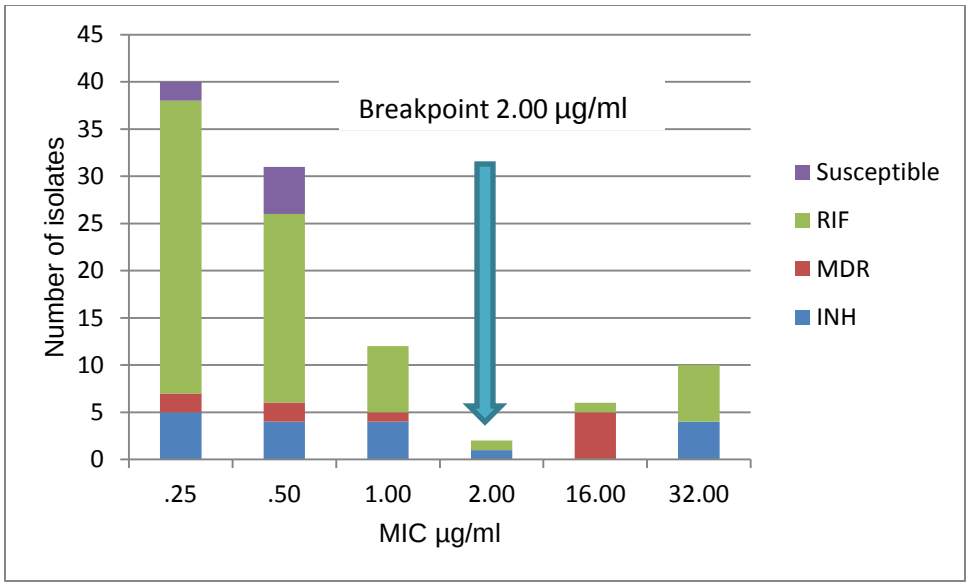


Fig 4.6 Streptomycin MIC distributions for wild-type *M. tuberculosis* isolates.

Susceptible isolates (n = 7) had MIC below the recommended CC of S ≤ 2 µg/ml. Isolates categorized as Rif mono-resistant (n = 58) had MIC below the CC and (n = 8) had MIC above the CC. Isolates categorized as MDR (n = 5) had MIC below the CC and (n = 5) had MIC above the CC. Isolates categorized as INH mono-resistant (n = 14) had MIC above the CC and (n = 4). The wild-type MIC distribution for streptomycin range in figure 4.6 is (≤ 0.25 - 0.5 µg/ml).

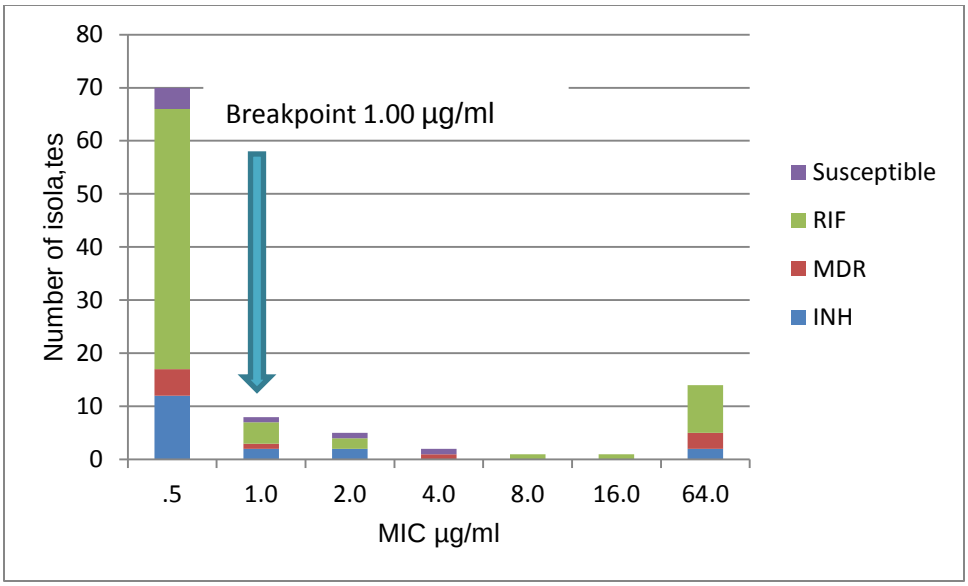


Fig 4.7: Para-aminosalicylic acid MIC distributions for wild-type *M. tuberculosis* isolates.

Susceptible isolates (n = 5) had MIC below the recommended CC of S ≤ 1.00 µg/ml and (n = 2) had MIC above the recommended CC. Rifampin mono-resistant isolates (n = 53) had MIC below the recommended CC and (n = 13) had MIC above the CC. Multidrug-resistant isolates (n = 6) had MIC below the CC and (n = 4) had MIC above the CC. Isoniazid mono-resistant isolates (n = 14) had MIC below the recommended (n = 4) had MIC above the CC. The wild-type MIC distribution range for Para-aminosalicylic acid in figure 4.7 is (≤ 0.5 - 4.0 µg/ml).

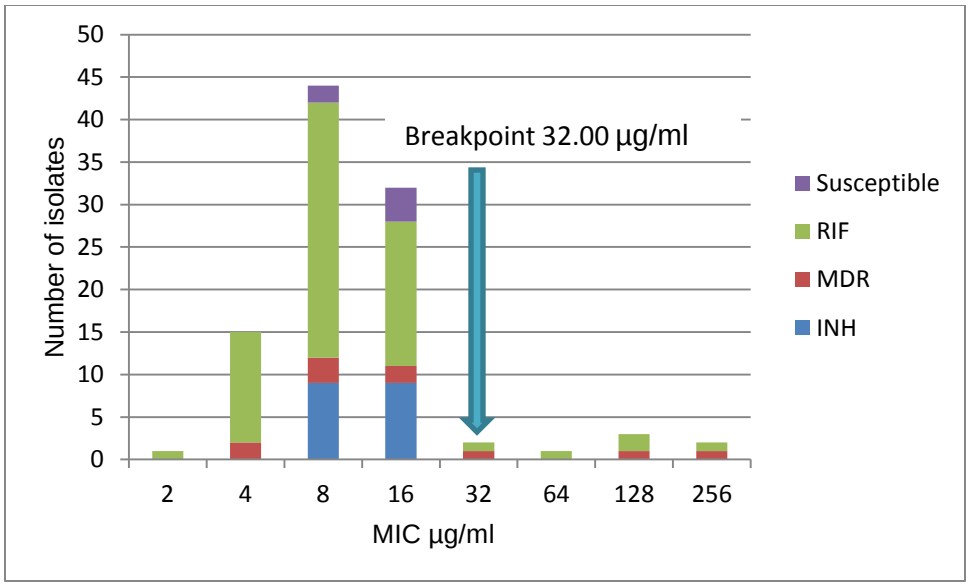


Fig 4.8: Cycloserine MIC distributions for wild-type *M. tuberculosis* isolates.

Susceptible isolates (n = 7) had MIC below the recommended CC of S ≤ 32 µg/ml. Rifampin mono-resistant isolates (n = 62) had MIC below the CC and (n = 4) had MIC above the CC. Multidrug-resistant isolates (n = 8) had MIC below the CC and (n = 2) had MIC below the CC. Isoniazid mono-resistant isolates (n = 18) had MIC below the CC. The wild-type MIC distribution range for cycloserine in figure 4.8 is (≤ 2 - 16 µg/ml).

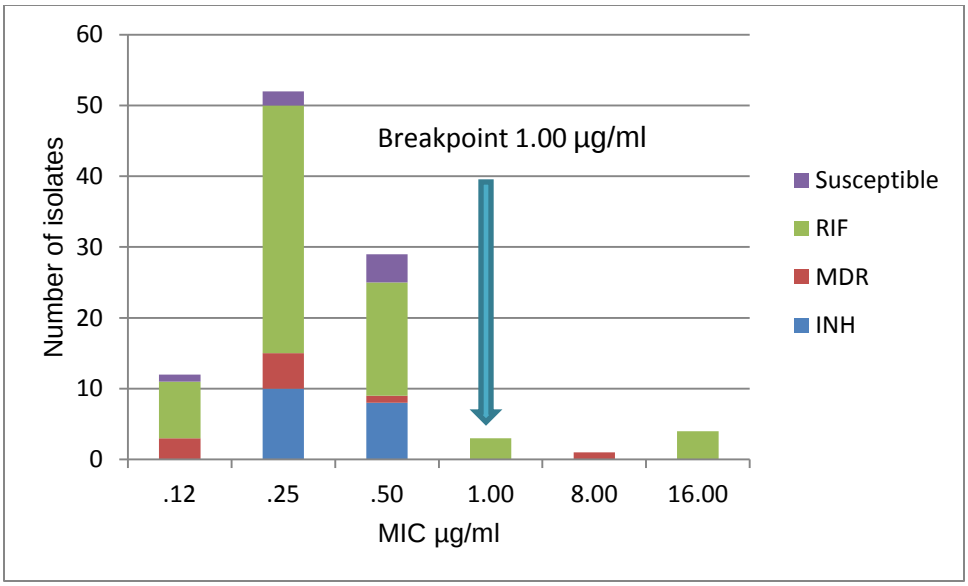


Fig 4.9: Amikacin MIC distributions for wild-type *M. tuberculosis* isolates.

Susceptible isolates (n = 7) had MIC below the recommended CC of S ≤ 1.00 µg/ml. Rifampin mono-resistant isolates (n = 59) had MIC below the recommended CC and (n = 7) had MIC above the CC. Multidrug-resistant isolates (n = 9) had MIC below the CC and (n = 1) had MIC above the CC. Isoniazid mono-resistant (n = 18) had MIC below the CC. The wild-type MIC distribution range for amikacin in figure 4.9 is (≤ 0.12 - 0.5 µg/ml).

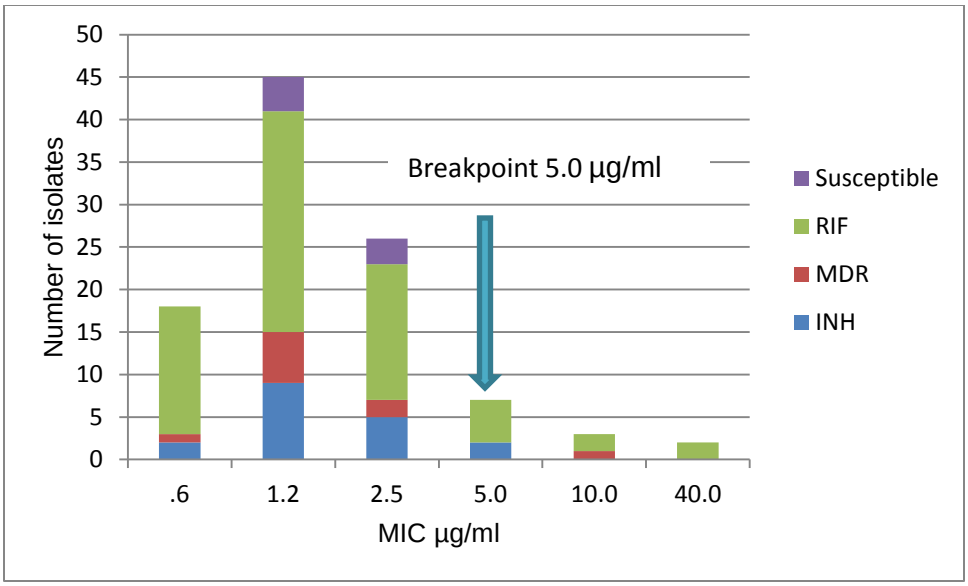


Fig 4.10: kanamycin MIC distributions for wild-type *M. tuberculosis* isolates.

Susceptible isolates (n = 7) had MIC below the recommended CC of S ≤ 5.0 µg/ml for kanamycin. Rifampin mono-resistant isolates (n = 62) had MIC below the CC and (n = 4) had MIC above the CC. Multidrug-resistant isolates (n = 9) had MIC below the CC and (n = 1) had MIC above the CC. Isoniazid mono-resistant isolates (n = 18) had MIC below the CC. The wild-type MIC distribution range for kanamycin in figure 4.10 is (≤ 0.6 - 2.5 µg/ml).

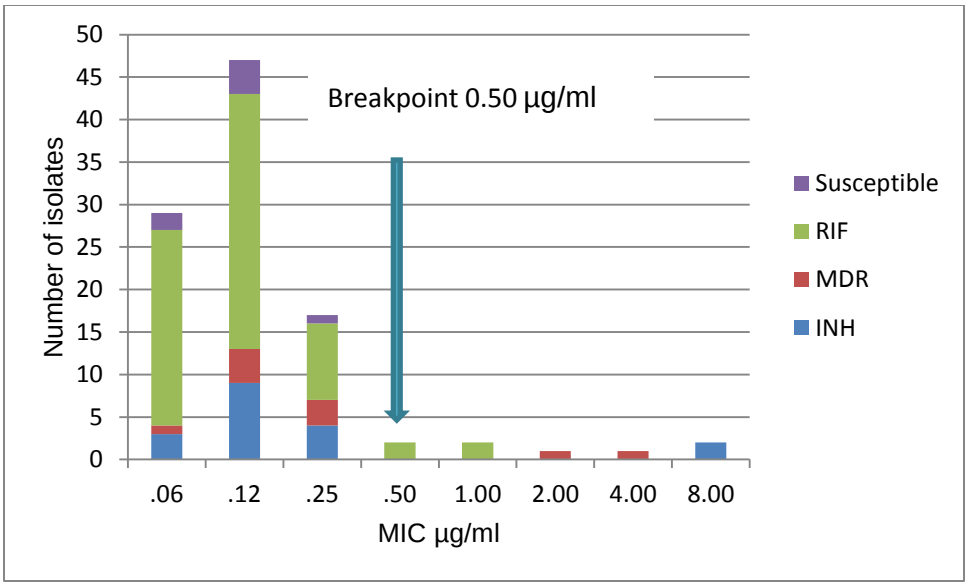


Fig 4.11: Moxifloxacin MIC distributions for wild-type *M. tuberculosis* isolates.

Susceptible isolates (n = 7) had MIC below the recommended CC of S ≤ 0.5 µg/ml. Rifampin mono-resistant isolates (n = 64) had MIC below the CC and (n = 2) had MIC above the CC. Multidrug-resistant isolates (n = 8) had MIC below the CC and (n = 2) had MIC above the CC. Isoniazid mono-resistant isolates (n = 16) had MIC below the CC and (n = 4) had MIC below the CC. The wild-type MIC distribution range for Moxifloxacin in figure 4.11 is (≤ 0.6 - 0.5 µg/ml).

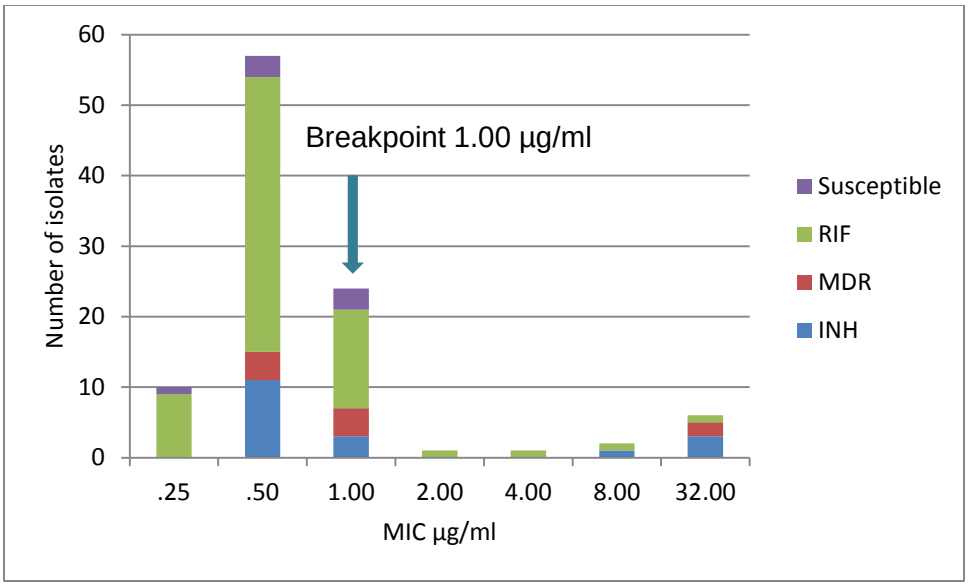


Fig 4.12: Ofloxacin MIC distributions for wild-type *M. tuberculosis* isolates.

Susceptible isolates (n = 7) had MIC below the recommended CC of S ≤ 1.00 µg/ml for ofloxacin. Rifampin mono-resistant isolates (n = 62) had MIC below the recommended CC and (n = 2) had MIC above the recommended CC. Multidrug-resistant isolates (n = 8) had MIC below the CC and (n = 2) had MIC above the recommended CC. Isoniazid mono-resistant isolates (n = 14) had MIC below the CC and (n = 4) had MIC above the CC. The wild-type MIC distribution range for ofloxacin in figure 4.12 is (≤ 0.25 - 1 µg/ml).

Table 4.1: Frequency of mutations conferring resistance to rifampin

rpoB gene mutation pattern	Frequency	Percent
Δ*rpoB* WT2	3	3.0
Δ*rpoB* WT3, Δ*rpoB* WT4	3	3.0
Δ*rpoB* WT3, Δ*rpoB* WT4, *rpoB* MUT1	1	1.0
Δ*rpoB* WT4, Δ*rpoB* WT5	1	1.0
Δ*rpoB* WT5, *rpoB* WT6	1	1.0
Δ*rpoB* WT7, *rpoB* MUT2A	18	17.8
Δ*rpoB* WT7, *rpoB* MUT2B	8	7.9
Δ*rpoB* WT8	2	2.0
Δr*poB* WT8, *rpoB* MUT3	26	25.7
-	3	3.0
rpoB WT, *rpoB* MUT2B, *rpoB* MUT3	1	1.0
rpoB WT, *rpoB* MUT3	4	4.0
WT	30	29.7
Total	101	100.0

Frequency and percentages of genotypic drug susceptibility testing detected by Genotype MTBDR*plus* line probe assay version 2.0 on the *rpoB* gene. The Δ - represent an absent wild-type band. *rpoB* (MUT 1, 2A, 2B, 3) represent corresponding mutation probe band. The - represents non *M. tuberculosis* complex isolates.

Table 4.2: Frequency of mutations conferring resistance to isoniazid

katG mutation pattern	Frequency	Percent
Δ*katG* WT, *katG* MUT1	20	19.8
-	3	3.0
WT	78	77.2
Total	101	100.0

Frequency and percentages of genotypic drug susceptibility testing detected by GenoType MTBDR*plus* line probe assay version 2.0 on the *katG* gene. The Δ represents an absent wild-type band. The - represents Non *M. tuberculosis* complex isolates and k*atG* (MUT 1) represents corresponding mutation probe band.

Table 4.3: Frequency of mutations conferring resistance to isoniazid

inhA mutation pattern	Frequency	Percent
Δ*inhA* WT1	1	1.0
Δ*inhA* WT2, *InhA* MUT3B	1	1.0
-	3	3.0
inhA WT1, *InhA* MUT1	1	1.0
WT	95	94.1
Total	101	100.0

Frequency and percentages of genotypic drug susceptibility testing detected by GenoType MTBDR*plus* line probe assay version 2.0 on the *inhA* gene. The Δ represents an absent wild-type band. The - represents Non *M. tuberculosis* complex isolates and *inhA* (MUT 1, 3B) represents corresponding mutation probe band.

Table 4.4: Frequency of mutations conferring resistance to rifampin and isoniazid

MDR mutation pattern	Frequency	Percent
Δ*rpoB* WT4, Δ*rpoB* WT5, Δ*katG* WT, *katG* MUT1,	1	1.0
Δ*rpoB* WT8, Δ*katG* WT, *katG* MUT1	2	2.0
Δ*rpoB* WT8, *rpoB* MUT3, Δ*katG* WT, *katG* MUT1	2	2.0
Δ*rpoB* WT8, *rpoB* MUT3, Δ*katG* WT, *katG* MUT1, Δ*InhA* WT2, *InhA* MUT3B	1	1.0
-	3	3.0
rpoB WT, *rpoB* MUT3, Δ*katG* WT, *katG* MUT1	1	1.0
WT	91	90.1
Total	101	100

Frequency and percentages of genotypic drug susceptibility testing detected by GenoType MTBDR*plus* line probe assay version 2.0 on the *rpoB* and *katG* gene. The Δ represents an absent wild-type band. The - represents Non *M. tuberculosis* complex isolates and (MUT: 1, 3, 3B) represents corresponding mutations probe band.

Rifampin MIC and mutational analysis of the *rpoB* gene

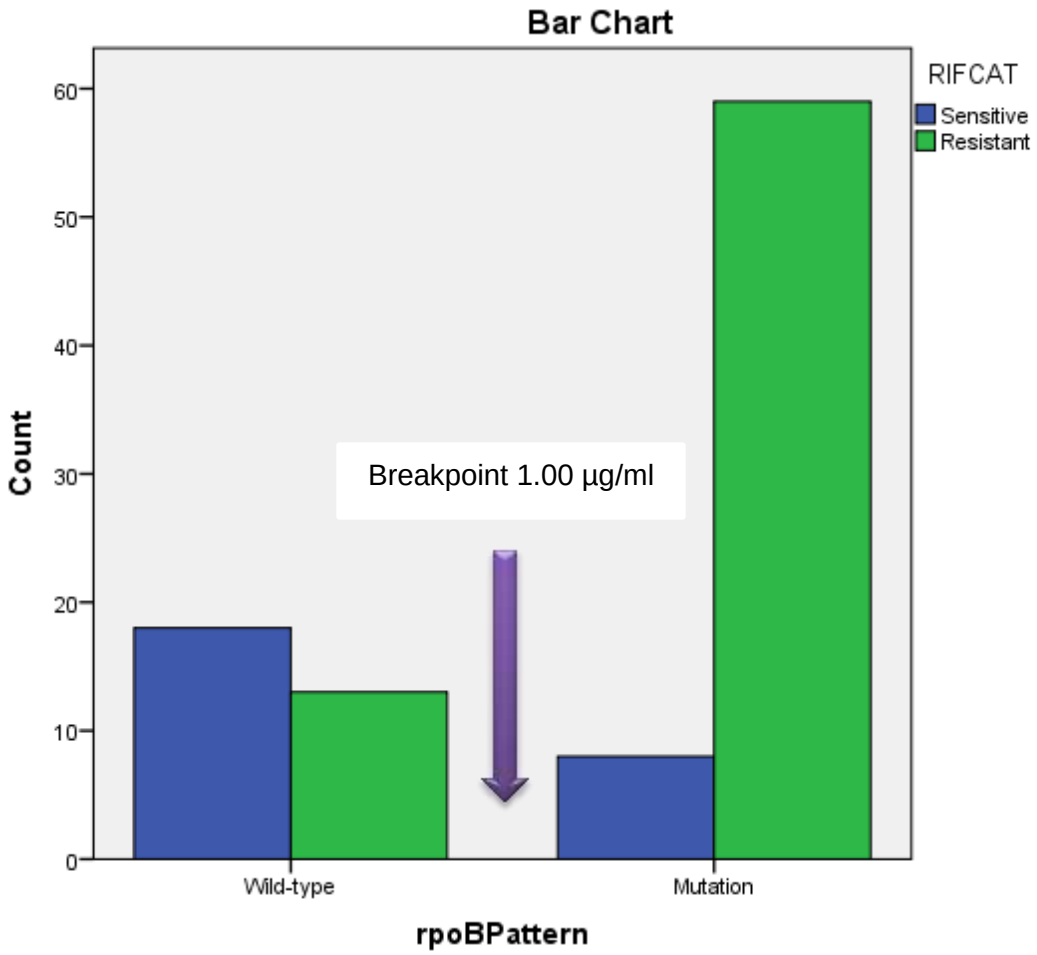


Figure 4.13: Cross tabulation of MIC and Genotypic drug susceptibility testing on the *rpoB* gene

Minimal inhibitory distribution of rifampin for 101 *M. tuberculosis* clinical isolates compared with genotypic drug susceptibility testing result on the *rpoB* gene. Blue colour represents susceptible isolates. Green colour represents resistant isolates. Arrow represents current recommended breakpoint of 1.00 µg/ml for rifampin.

Isoniazid MIC and mutational analysis of *katG* gene

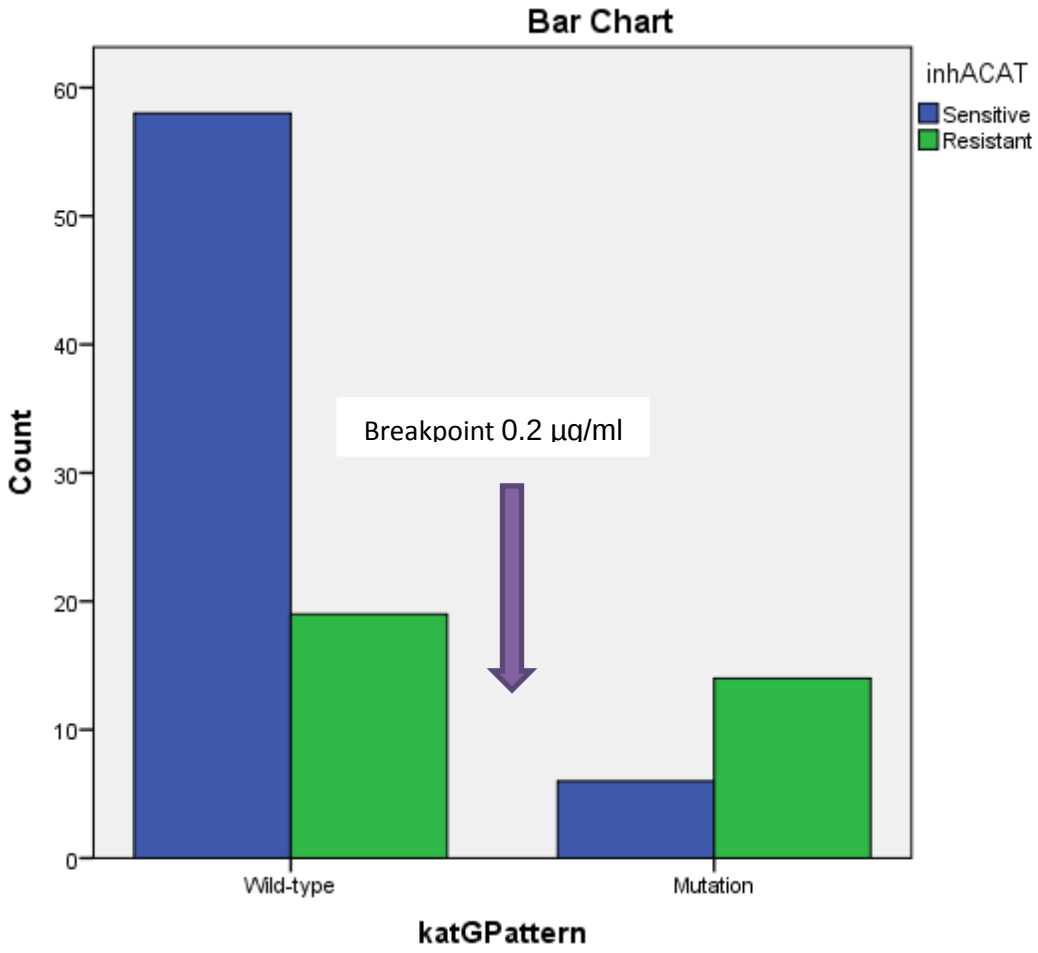


Figure 4.14: Cross tabulation of MIC and Genotype mutational analysis of the *katG* gene.

Minimal inhibitory distribution of isoniazid for 101 *M. tuberculosis* clinical isolates compared with genotypic drug susceptibility results on the *katG* gene. Blue colour represents susceptible isolates, green colour represents resistant isolates and arrow represents current recommended breakpoint of 0.2 µg/ml for isoniazid.

Isoniazid MIC and mutational analysis of *inhA* gene

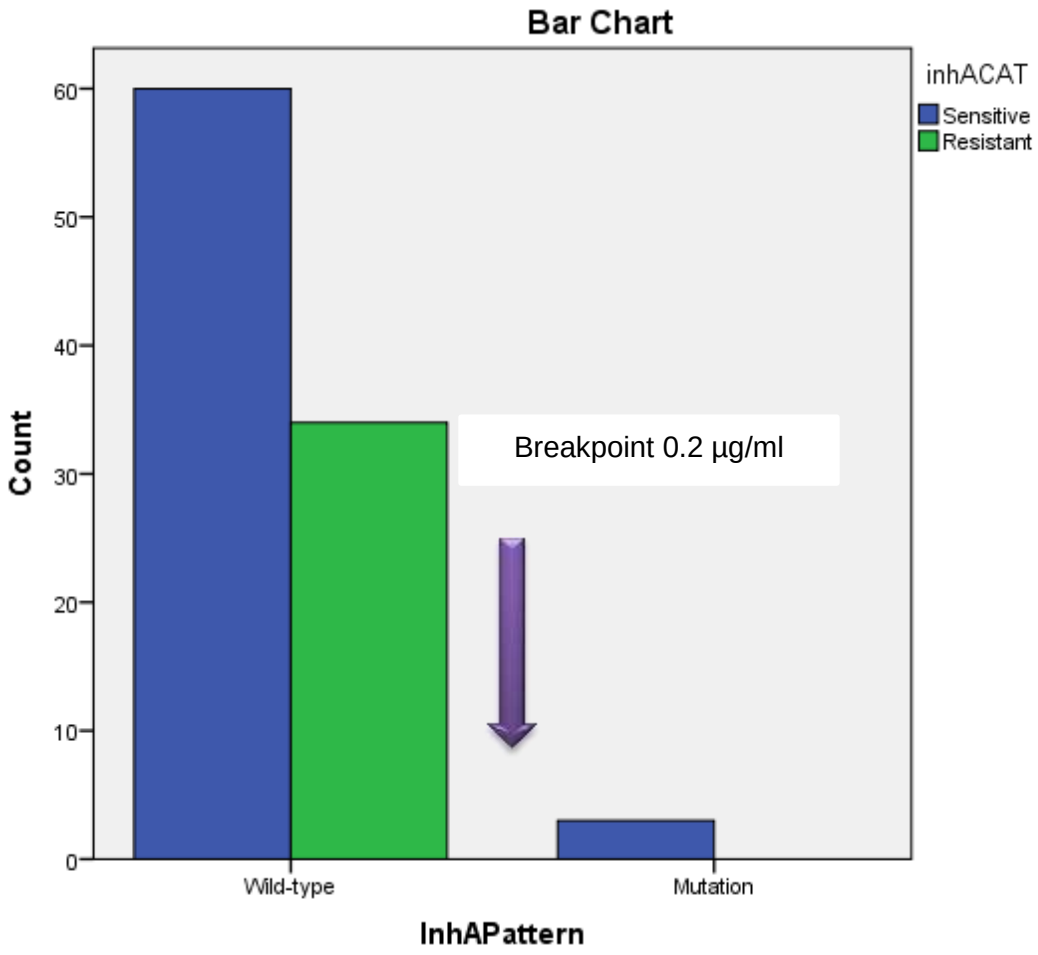


Figure 4.15: Cross tabulation of MIC and Genotype mutational analysis of the *InhA* gene.

Minimal inhibitory distribution of isoniazid for 101 *M. tuberculosis* clinical isolates compared with genotypic drug susceptibility results on the *inhA* gene. Blue colour represents susceptible isolates, green colour represents resistant isolates and arrow represents current recommended breakpoint of 0.2 µg/ml for isoniazid.

Table 4.5: Frequency of mutations conferring resistance to fluoroquinolones (ofloxacin and moxifloxacin)

gyrA gene mutation pattern	Frequency	Percent
WT1 - WT3	98	97.0
-	3	3.0
Total	101	100.0

Frequency and percentage of genotypic drug susceptibility testing detected by GenoType MTBDR*sl* line probe assay version 2.0 on the *gyrA* gene. The *gyrA* locus control stained positive and *gyrA* wild-type band (1, 2 and 3) were detected on 98 isolates.

Table 4.6: Frequency of mutations conferring resistance to fluoroquinolones (ofloxacin and moxifloxacin)

gyrB gene mutation pattern	Frequency	Percent
WT	98	97.0
-	3	3.0
Total	101	100.0

Frequency and percentage of genotypic drug susceptibility testing detected by GenoType MTBDR*sl* line probe assay version 2.0 on the *gyrB* gene. The *gyrB* locus control stained positive and *gyrB* wild-type band was detected on 98 isolates.

Table 4.7: Frequency of mutations conferring resistance aminoglycoside (kanamycin and amikacin)

rrs gene mutation pattern	Frequency	Percent
WT1 - WT2	98	97.0
-	3	3.0
Total	101	100.0

Frequency and percentage of genotypic drug susceptibility testing detected by GenoType MTBDR*sl* line probe assay version 2.0 on the *rr*s gene. The *rrs* locus control stained positive and *rrs* wild-type band was detected on 98 isolates.

Table 4.8: Frequency of mutations conferring resistance low-level kanamycin resistance

eis gene mutation pattern	Frequency	Percent
WT1 - WT3	98	97.0
-	3	3.0
Total	101	100.0

Frequency and percentage of genotypic drug susceptibility testing detected by Genotype MTBDR*sl* line probe assay version 2.0 on the *eis* gene. The *eis* locus control stained positive and *eis* wild-type (1, 2 and 3) band was detected on 98 isolates.

Table 4.9: Genotyping of *Mycobacterium tuberculosis* clinical isolates

Clade	Clade abbreviation	Frequency	Percent
Beijing		30	29.7
Beijing-Like		1	1.0
EAI1-SOM		6	5.9
EAI5		2	2.0
H1		2	2.0
LAM1		1	1.0
LAM11-ZWE		1	1.0
LAM3		1	1.0
LAM3 AND S		1	1.0
LAM4		1	1.0
LAM9		2	2.0
MANU2		4	4.0
Orphan		20	19.8
S		3	3.0
T1		19	18.8
T2-T3		2	2.0
U		3	3.0
X1		1	1.0
X3		1	1.0
Total		101	100.0

Genotype of 101 clinical isolates showed diverse family strains, 81 spoligotype patterns were identified and evaluated for shared spoligotype pattern. The shared spoligotype patterns among the 81 strains matched with 18 international types (SITs) in the updated SpolDB4. Twenty isolates did not match pre-existing shared type in the SpolDB4.

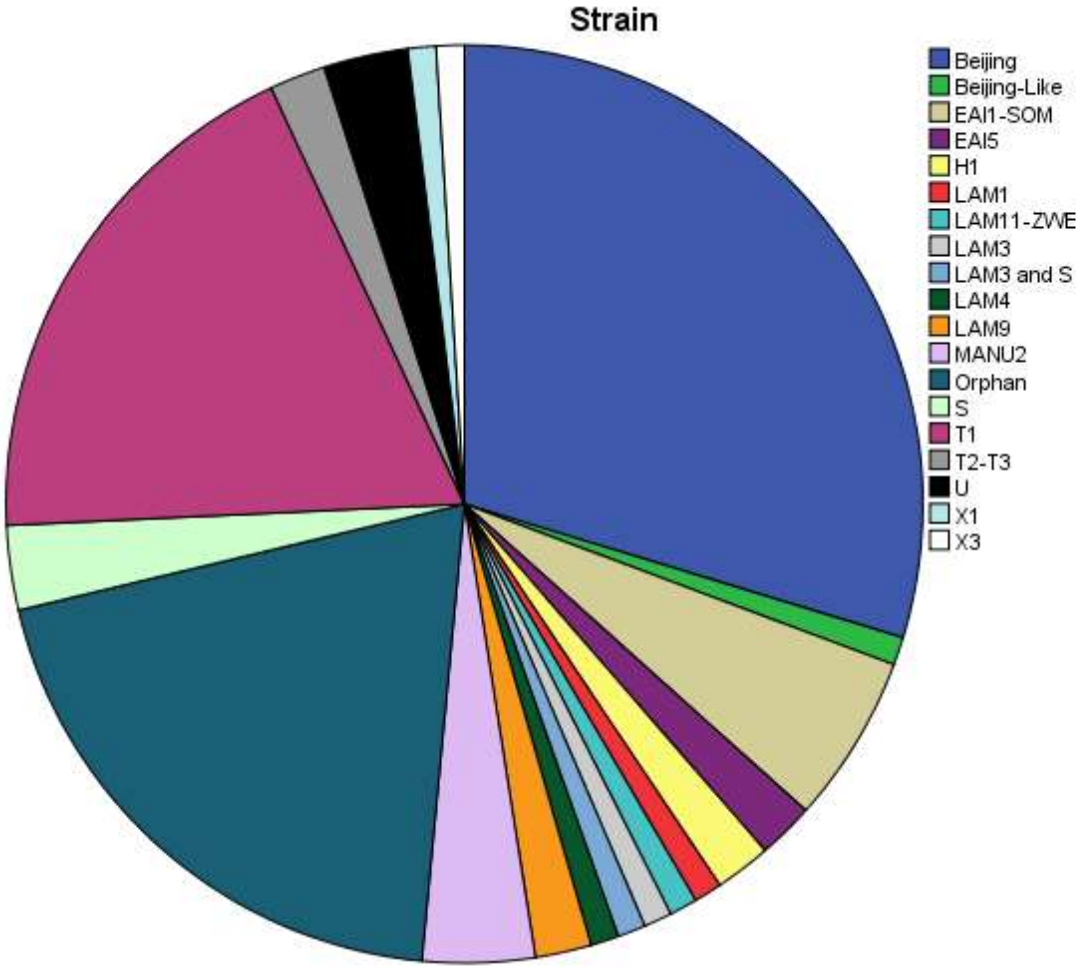


Fig 4.16: Strain distribution on pie chart

The predominant strains in this study were Beijing and T family. The Beijing strain family are known to have a worldwide distribution and it has high endemnicity in certain geographical areas.

4.2 DISCUSSION

Mycobacterium tuberculosis infected people are treated with combination therapy of first-, second-, and third-line drugs. The results for treatment of TB, MDR-TB and XDR-TB are not satisfactory and data are limited. The challenges due to drug-resistant tuberculosis call for urgent need to provide standardized guidelines of drug susceptibility testing against anti TB drugs. In an effort to compare the current critical concentrations in DST for MTB the present study determined wild-type MIC distributions of rifampin, Isoniazid, Ethionamide, Amikacin, Cycloserine, Ethambutol, Kanamycin, Moxifloxacin, Ofloxacin, Para-aminosalicylic acid, Rifabutin, and Streptomycin. A quantitative method (Trek Sensititre® MYCOTB MIC plate) that allowed simultaneous drug susceptibility testing of first-line and second-line drugs was used.

The study focused on *M. tuberculosis* complex clinical isolates resistant to isoniazid or rifampin, the key determinant for first-line drugs treatment failure. Susceptible clinical isolates were used to determine wild-type MIC distributions. The data were compared with the currently recommended critical concentration used for treatment of tuberculosis and multidrug tuberculosis in the Limpopo Province.

The wild-type MIC distribution range for rifampin in this study is (≤ 0.12 - 0.5 µg/ml) (Figure 4.1). The range separates wild-type isolates and those harboring rifampin resistance mutations on *rpoB* gene. The recommended critical concentration of 1.0 mg/ml currently used in the laboratory for rifampin would have categorized (n = 6) isolates as phenotypically susceptible. However, the genotypic results in the (n = 6) isolates showed absence of *rpoB* wild-type 2 band and *rpoB* wild-type 2 with mutation 3 detection. Studies have reported high level rifampin resistance to be associated with mutations at codons 531 and 526 in the *rpoB* gene with MIC value > 16 µg/ml and with low level rifampin resistance in mutations at codon 516 and 522 (Jamieson *et al*., 2014; Heysell *et al*., 2015).

The results correspond with a previous study by Schön *et al*. (2009) who reported results of consecutive susceptible clinical isolates with wild-type MIC distributions of (≤ 0.03 - 0.5 µg/ml). Ocheretina *et al*. (2014) reported rifampin MIC value of (0.0063 - 0.5 µg/ml).

For rifabutin the present study reports a wild-type MIC distribution range of (≤ 0.12 - 0.25 µg/ml) figure 4.2. Mutational analysis on the *rpoB* gene demonstrated isolates below 0.25 to be wild-type. Previous studies have reported that the critical concentration of 1.0 mg/ml is set too high and observed even lower MIC range of rifabutin (≤ 0.008 - 0.06 µg/ml). Ängeby *et al*. (2010) also supported by rifabutin MIC reported by Mpagama *et al*. (2013) of ≤ 0.12 µg/ml. The MIC of ≤ 0.12 µg/ml found in this study showed an excellent agreement for phenotypic and genotypic results for rifabutin in (n= 60) isolates. Rifampin resistant isolates (n= 48) were also found to be resistant to rifabutin. The findings correspond with the results by Schön *et al*. (2013) reporting rifampin resistant and rifabutin susceptible *M. tuberculosis* isolates that harbor mutations on the *rpoB* gene as susceptible due to the breakpoint artefact of 0.5 µg/ml being too high.

Wild-type MIC distribution for isoniazid in figure 4.3 is (≤ 0.3 - 2.0 µg/ml). The reported distribution range is higher than the currently recommended CC for isoniazid on solid agar. Isolates susceptible to isoniazid (n= 20) in this study had MIC at 2.0 µg/ml. A study conducted in South Africa reported that low level resistant isolates with mutations on the C- 15T *inhA* gene were treatable with higher concentration of isoniazid (Schaaf *et al*., 2007) in agreement with recent reports by Kambli *et al*., (2015).

Ethionamide wild-type MIC distribution demonstrated on figure 4.4 is (≤ 0.12 - 5 µg/ml). Ethionamide resistance is reported to be conferred by mutation in one of the major genes (*ethA, ethR* and *inhA* promoter region). All clinical isolates that were categorized as isoniazid resistant had MIC of 10µg/ml and 20 µg/ml on the ethionamide MIC distribution with mutations in the i*nhA* gene. The finding of the current study is in agreement with the recommended critical concentration of 5 mg/L on solid agar. Schön *et al*. (2010) reported wild-type MIC for ethionamide of (0.5 - 2 mg/L) however the agreement between middlebrook 7H10 and BACTEC 460 was poor. The use of

intermediate classification for strain with MIC level of 4 mg/L was suggested to be able to increase reproducibility.

Ethambutol wild-type MIC distribution demonstrated on figure 4.5 is (≤ 0.5 - 2 µgml). The result is compatible with MIC data of (0.5 - 2mg/L) reported by Ängeby *et al*. (2010) and that obtained by Kam *et al*. (2010) where ethambutol range was reported to be (2 - 3 mg/L).

Streptomycin wild-type MIC distribution demonstrated on figure 4.6 is (≤ 0.25 - 0.5 µg/ml). Isolates that exceeded the recommended CC of 2 mg/ml for streptomycin showed no mutation on the *rrs* gene. The *rpsL* gene is not included in the genoType MTBDR*sl* line probe assay. The MIC for streptomycin is consistent with the currently recommended CC and wild-type MIC of 2 mg/L reported in a study conducted by Jureen *et al*. (2010).

Para-aminosalicylic acid MIC distribution demonstrated on figure 4.7 is (≤ 0.5 - 4.0 µg/ml). The t*hyA* and *folC* gene were not analyzed for mutations that confer resistant to PAS as they are not included in GenoType MTBDR*sl* line probe assay. Mpagama *et al*. (2013) reported susceptible MIC of 0.5 and borderline susceptible range of (1.0 to 8.0 µg/ml).

Cycloserine MIC distribution demonstrated on figure 4.8 is (≤ 2 -16 µg/ml). Majority of the isolates had MIC below the recommended CC of 32 mg/L for cycloserine and only six isolates exceeded the CC. Mutations that confer resistance to cycloserine on the *alrA* gene were not evaluated in this study. The result obtained is in agreement with recommended CC of 32 mg/L. Similar MIC range of (8-32 mg/L) was reported by Schön *et al*. (2011).

Amikacin wild-type MIC distribution demonstrated on figure 4.9 is (≤ 0.12- 0.5). Eight isolates exceeded the recommended CC and genotypic analysis on the *rrs* gene detected no mutations. A study on second-line DST reported MIC range of (≤ 0.12 - 0.25 µg/ml) for isolates susceptible to amikacin and borderline susceptible (≤ 0.5 - 1.0 µg/ml) (Mpagama *et al*., 2013).

Kanamycin wild-type MIC distribution demonstrated on figure 4.10 is (≤ 0.6 - 2.5 µg/ml). Five isolates had MIC that exceeded the recommended CC and no mutations were detected on the *rrs* and *eis* gene. Kam *et al*. (2010) reported MIC distribution of (3.0 - 5.0 mg/L.

Moxifloxacin wild-type MIC distribution demonstrated on figure 4.11 is (≤ 0.06 - 0.25 µg/ml). Six isolates had MIC above the recommended CC of 0.5 mg/L and no mutations were detected on the *gyrA* and *gyrB* gene. Similar finding are reported in Trollip *et al.* (2014) study on MODS assay that defined Moxifloxacin breakpoints to be 0.5 µg/ml. The data corresponds with currently recommended CC for Moxifloxacin.

Ofloxacin wild-type MIC distribution demonstrated in figure 4.12 is (≤ 0.25 - 1.00 µg/ml). Ten isolates had MIC that exceeded CC and no mutations were detected on the *gyrA* and *gyrB* gene. The result is consisted with the recommended CC of 1.0 mg/L. The data correspond with MIC distribution of 1.0 mg/L result obtained in Kam *et al*. (2010) and Ängeby *et al*. (2010).

Genotypic drug susceptibility testing

Hundred and nine clinical isolates were analyzed for mutations that confer resistance to rifampin and isoniazid in *M. tuberculosis* using GenoType MTBDR*plus* assay version 2.0. Table 4.2 shows frequency of mutations conferring resistance to rifampin. The assay detected 101 isolates of which 3 were non tuberculosis complex, 30 were wild-type and 68 isolates were resistant to rifampin. Rifampin resistant was reported when an absence of wild-type (∆WT) band and corresponding mutation probe was observed. Missing *rpoB* wild-type 8 and corresponding *rpoB* MUT3 was detected in 26 isolates. Missing *rpoB* wild-type 7 and corresponding *rpoB* MUT2A was detected in 18 isolates. Missing *rpoB* wild-type 7 and corresponding *rpoB* MUT2B was observed in 8 isolates. Isolates with missing wild-type and no apparent mutation probe were also considered resistant to rifampin. Three isolates had absent *rpoB* wild-type 2 band, 4 isolates had absent *rpoB* wild-type 8 band and 3 isolates had absent wild-type 4 and wild-type 5. Heteroresistance was identified in 5 isolates with a wild-type band and mutation probe. Studies have reported that point mutations on the *rpoB* MUT3 at codon 513, 526 and

531 to be associated with high levels of rifampin resistance with MIC value of ≥ 128 mg/ml (Bobadilla-del-Valle *et al*., 2001) and mutations *rpoB* MUT1 at codon 511, 516, 518 and 522 are associated with low level rifampin resistance (Bobadilla-del-Valle *et al*., 2001).

Table 4.3 shows frequency of mutations conferring resistance to isoniazid, 78 isolates were wild-type for isoniazid and 20 isolates had missing wild-type band on *katG* gene and corresponding *katG* MUT1 mutation probe. Table 4.5 shows frequency of mutation on the *inhA* gene. One isolate had missing wild-type band with no corresponding mutation probe. Missing *katG* wild-type gene band and corresponding inhA MUT1 was observed in one isolate. Missing *katG* wild-type band and corresponding *inhA* MUT3B was detected in one isolate.

The highest concentration for isoniazid was 4 µg/ml on the MYCOTB MIC plate used in this study. Majority of the isoniazid resistant isolates had MIC at 4 µg/ml n = 29. One isolate had all wild-type bands present but a mutation band appeared on codon S531T. The DST method used in this study observed more isoniazid resistant isolates than the genotypic method. The discrepancies between the two methods in this study can be

associated with factor that mutations occurred at alternative genes that were not detected by GenoType MTBDR*plus* line probe assay such as *ahpC*, *KasA* and *ndh* which explains the reason the study observed more isolates with INH resistance on the phenotypic method and absence of mutations in the *katG* gene (Ferro *et al*., 2013). Table 4.5 shows frequency of isolates that had mutations on the *rpoB* and *katG* gene, 7 isolates were detected and MIC for rifampin was ≥ 16 µg/ml.

Figure 4.13 demonstrate MIC of 101 *M. tuberculosis* clinical isolates compared with genotypic drug susceptibility testing on the *rpoB* gene. Among the isolates 58 had MIC ≥ 1.0 µg/ml and presence of mutation in the *rpoB* gene and 5 isolates had MIC ≥ 1.00 µg/ml and absence of mutations on *rpoB* gene. Nineteen isolates had MIC of ≤ 1.00 µg/ml with no mutation detected and 15 isolates had MIC of ≤ 1.00 µg/ml with mutation detected on the *rpoB* gene. Böttger, (2011) reported various level of phenotypic drug resistance in *M. tuberculosis* to be associated with specific mutations within 81-bp

Rifampin resistance-determining region (RRDR) on the *rpoB* gene. High level rifampin resistant isolates were reported to present mutations at position S531L, H526Y and S531W and low level resistance isolates are associated with mutation on H526L (Jamieson *et al*., 2014).

A variety of other mutations associated with low-level RIF-resistant often reported as susceptible by automated growth-based methods are F505L, L511P, S522L and D516V (Rigouts *et al*., 2013; van Deun *et al*., 2013). Fifty eight isolates had MIC ≤ 0.2 µg/ml and absence of mutation on the *katG* gene and 18 isolates had MIC ≤ 0.2 µg/ml and presence of mutations on *KatG* gene. Fifteen isolates had MIC ≥ 0.2 µg/ml and mutation on *katG* gene while 5 isolates had MIC ≥ 0.2 µg/ml absence of mutation on *katG* and *inhA* gene. The most frequent mutations associated with high level isoniazid resistance are reported to be *katG* MUT1 codon (S315T1) and *KatG* MUT2 codon S315T2 on the *katG* gene (Ferro *et al*., 2013). Low-level isoniazid resistance are reported in *inhA* MUT1 codon C-15T, *inhA* MUT2 codon A-16G, *inhA* MUT3A codon T-8C and *inhA* MUT3B codon T-8A on GenoType MTBDR*plus* Hain, lifescience.

GenoType MTBDRs*l* line probe assay was used to detect resistance to fluoroquinolones (ofloxacin, Moxifloxacin), aminoglycosides (kanamycin, amikacin) and low-level kanamycin resistance. The test identified 98 clinical isolates as wild-type for the second-line drugs. The wild-type (WT) gene probes stained positive and corresponding mutant probe produced no staining on the *gyrA, gryB, rrs* and *eis* gene. Three isolates were identified as Non *Mycobacterium tuberculosis* complex and eight isolates were excluded due to the colour intensity of band being lesser than that of the Amplification control zone.

Additionally the study determined the frequency and distribution of *M. tuberculosis* strain families. Genotyping of 101 clinical isolates showed diverse family strains, 81 spoligotype patterns were identified and evaluated for shared spoligotype pattern. The shared spoligotype patterns among the 81 strains matched with 18 international types (SITs) in the updated SpolDB4 demonstrated on table 4.9. Twenty isolates (19.8%) that did not match pre-existing shared type were reported as orphans. The diverse strain families that were grouped by SpolDB4 database belonged to Beijing family (n = 30,

29.7%), T1 family (n = 19, 18.8%), U family (n = 3, 3.0%), LAM3 family (n = 1, 1.1%), H1 family (n = 2, 2.0%), X1 family (n = 1, 1.0%), MANU2 family (n = 4, 4.0%), EAI5 family (n = 2, 2.0%), S family (n = 3, 3.0%), EA11-SOM (N = 6, 5.9%), X3 (n = 1, 1.0), T2-T3 (n = 2, 2.0%), LAM and S (n = 1, 1.0%), LAM-ZWE (n = 1, 1.0%), LAM1 (n = 1, 1.0%), T2-T3 (n = 2, 2.0%), Beijing-like (n= 1, 1.0%), LAM 9 (n = 2, 2.0%). Studies have identified three major strain families in Africa Haarlem, Lam, T (Brudey *et al*., 2006; Victor *et al*., 2004; Streicher *et al*., 2004). In this study the Beijing and T family strains were predominate, displayed on figure 4.16. The EA11-SOM, S and X strain observed in this study are geographically distributed in South Africa, Mozambique, Malawi. A study on genetic diversity of extensively drug-resistant tuberculosis in South Africa reported EA11-SOM strain family to be dominant in isolates from Johannesburg (Mlambo *et al*., 2008).

CHAPTER FIVE

CONCLUSIONS

The findings of this study suggest that wild-type MIC distributions may be used to re-evaluate current recommended critical concentrations and define breakpoints for first-line and second-line drugs used for treatment of *M. tuberculosis* in Limpopo Province. Effective TB treatment will prevent the emergence of drug resistance in the Limpopo Province. Our findings support previous reports that certain mutations in the *rpoB* gene codon (S531L and H526Y) and *katG* gene codon (S315T) confer high level resistance to rifampin and isoniazid propelling drug resistant tuberculosis. The study provides preliminary information on diversity of circulating *M. tuberculosis* strain complex and drug resistance pattern.

RECOMMENDATIONS

To increase drug susceptibility testing accuracy, reproducibility and clinical utility the susceptible, intermediate and resistant classification may be considered to report low-level, moderate level and high level and correlate phenotypic (MIC) and genotypic (genetic profile) for tuberculosis diagnostic results. More studies should be conducted to gain detailed knowledge of distribution of mutations linked to drug resistance and their association with MIC in different provinces of South Africa. This will promote a better understanding of the current molecular test and information on drug susceptibility testing to optimize treatment of infections.

CHAPTER SIX

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11 APPENDICES

APPENDIX 1: Decontamination of Mycobacterium sputum samples with NaOH-NALC (Peres *et al*., 2009).

The sodium hydroxide digested debris, normal flora and other infectious organisms in the sputum. Mycobacterium has a high quality of mycolic acid which can resist entry of sodium hydroxide.

NaOH- Nalc decontamination solution

NaOH- Na citrate stock solution (4% NaOH/ 2.9% Na Citrate)

Procedure:

- 0.1 g of N-acetyl-L-cysteine was dissolved in every 20 ml of decontamination solution required.
- 2 ml of sputum sample was placed into a 15 ml centrifuge tube. 2 ml of NaOH-Nacl solution was then added.
- The tube was tightly closed and vortexed for 20 seconds then allowed to stands for 15 minutes.
- 14 ml of phosphate buffer was added into the tube to neutralize alkali decontamination process was halted.
- The tube was centrifuged at 3000 xg for 15 minutes, the supernatant was carefully decanted and pellet retained.

APPENDIX 2: Subculture on BACTEC™ MGIT™ 960™ (Siddiqi SH; Rusch-Gerdes S, 2006)

Principles:

1. MGIT medium

The MGIT (Mycobacterium Growth Indicator Tube) consists of liquid broth medium that is known to produce better recovery and faster growth of mycobacteria. The MGIT tube contains 7.0 ml of modified Middlebrook 7H9 broth base. This medium is sterilized by autoclaving. Enrichment media MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) or MGIT 960 Growth Supplement, was added to make the medium. This

Growth Supplement is essential for growth of many mycobacteria, belonging to *M. tuberculosis* complex. MGIT PANTA was added to suppress contamination.

2. Principle of detection and drug susceptibility testing

Each vial tube contains Middlebrook 7H9 liquid media, an oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenonthroline ruthenium chloride pent hydrate, embedded in silicone at the bottom of the tube. As the bacterial grew within the tube, the free oxygen was utilized and is replaced with carbon dioxide. With depletion of free oxygen, the fluorochrome was no longer inhibited, resulting in fluorescence within the MGIT tube when visualized under UV light. The intensity of fluorescence is directly proportional to the extent of oxygen depletion.

MGIT tubes may be incubated at 37ºC and read manually under a UV light or entered into a MGIT 960 instrument where they are incubated and monitored for increasing fluorescence every 60 minutes. Growth of bacteria as well as mycobacteria increases the fluorescence. In case of *M. tuberculosis*, at the time of positivity, there are approximately 10^5-10^6 colony-forming units (CFU) per ml of medium. The instrument declares a tube negative if it remains negative for six weeks (42 days). The detection of growth can also be visually observed by the presence of a non-homogeneous light turbidity or small granular/flaky appearance in the medium. Growth of some NTM (most commonly rapid growers) results in light turbidity, while contaminating bacteria generally produce heavy turbidity. Drug susceptibility testing can be performed based on the same principle. Two MGIT tubes are inoculated with the test culture. A known concentration of a test drug is added to one of the MGIT tubes, and growth is compared with the MGIT tube without the drug (growth control). If the test drug is active against the isolated mycobacteria, it will inhibit the growth and thus there will be suppression of fluorescence, while the growth control will grow

Uninhibited and will have increasing fluorescence. Growth is monitored by the BACTEC 960 instrument which automatically interprets results as susceptible or resistant.

Materials and Reagents

MGIT medium 7 ml tube

MGIT growth supplement or OADC enrichment

MGIT Panta
Micropipette
Sterile pastuer pipette
Filtered pipette

b. Inoculation of MGIT medium

MGIT tubes were labelled with specimen number.

- The cap was unscrewed and aseptically added 0.8 ml of MGIT growth supplement/PANTA to each MGIT tube. Use of an adjustable pipette is recommended.
- A sterile pipette was used to add a well mixed processed specimen up to 0.5 ml mark to the appropriately labelled MGIT tube.
- The tube was recapped tightly and mixed by inverting the tube several times.
- Tubes and caps were wiped with a mycobactericidal disinfectant and left inoculated tubes at room temperature for 30 minutes.
- The work was done under the biologic safety cabinet for the specimen inoculation.

c. Inoculation of additional media, Lowenstein Jensen

Additional solid medium was used for maximum recovery of mycobacteria.

Three drops of specimen was added on Lowenstein-Jensen bottles.

Incubated at 37ºC temperature.

Incubation

All inoculated MGIT (7ml) tubes were entered in the BACTEC MGIT 960 instrument after scanning each tube. The cap was kept tightly closed during the incubation to maintain the oxygen gradient in the medium. The instrument was checked that it maintains 37ºC ± 1ºC temperature. Since the optimum temperature for growth of *M. tuberculosis* is 37ºC.

Length of incubation: MGIT tubes were incubated for 14 days and checked if the instrument flags them positive. After a maximum of six weeks, the instrument flags the

tubes negative that had no growth. The MGIT tube that flagged positive were included as study samples.

F. Detection of positive growth

The instrument signals a tube positive for growth when a green indicator light shows on the exact location of the positive tube in the drawer of the instrument. The tube were removed and scanned outside the instrument. The tube was observed visually for mycobacterial growth which appears granular, while contaminating bacterial growth appears very turbid. Growth, especially of the *M. tuberculosis* complex, settles at the bottom of the tube. Information about the time-to-detection of positive growth was retrieved from the print out of the unloaded positives report.

APPENDIX 3: Inoculation on LJ medium (Haung *et al*., 2005)

Inoculation of additional media, Lowenstein Jensen
Additional solid medium was used for maximum recovery of mycobacteria.
Three drops of specimen was added on Lowenstein-Jensen bottles.
Incubated at 37ºC temperature.

APPENDIX 4: MIC determination using MYCOTB Sensititre Plates (Hall *et al*., 2012)

Materials and Reagents

MYCOTB MIC plate

Middlebrook 7H9 broth

Saline tween with glass beads

Automated inoculator

Vortex

Stop watch

Pasteur pipette

Paper towel

Tuberculocidal disinfectant

35-37ºC incubator with 5-10% CO_2

Vizion system, computerized MYCOTB plate reader

MIC determination using MYCOTB Sensititre Plates

- Several colonies were selected from MGIT tube using a pasteur pipette and inoculated into a test tube containing saline-Tween and glass beads (TREK Diagnostics).
- After being vortexed for two minutes, the inoculum was allowed to settle for 15 minutes and adjusted to a 0.5 McFarland standard equivalent using a nephelometer.
- One hundred microliters of the inoculum was transferred to 11 ml of Middlebrook 7H9 broth containing oleic acid-albumin-dextrose-catalase (TREK Diagnostics) and vortexed for 20 seconds.
- One hundred microliters was transferred to the MYCOTB plate wells containing the lyophilized antibiotics.
- MYCOTB plate was covered with plastic seals provided by the manufacturer, and the entire outer surface of the plate was disinfected with a tuberculocidal agent.
- Plates was incubated at 37°C in 5 to 10% CO2 and examined on the bench top without opening at 7, 10, 14, and 21 days using both a manual read aided by a mirrored viewer and the semi-automated plate reader (Vizion system).
- The first well with no visible growth was determined to be minimal inhibitory concentration.

APPENDIX 5: DNA Extraction (Hain Lifescience, 2014)

Materials and Reagents

Vortex

Stop watch

Pasteur pipette

Micro-pipettes

Filtered pipette tips

Digital block heater

Screw cap tubes

1% freshly prepared sodium hypochlorite solutions

Paper towel

MGIT Bactec positive clinical isolates

GenoLyse DNA extraction kit

Procedure

- Transfer 1ml of Liquid culture sputum-specimen preparation into a 1.5 ml screw cap tube.
- Centrifuge for 15 minutes at 10 000 xg in a standard table top centrifuge.
- Discard supernant and resuspend in 100 µl lysis buffer (A-LYS).
- Incubate sample for 5 minutes at 95ºC on heating block.
- Add 100 µl neutralizing buffer (A-NB) to lysate and vortex sample for 5 sec.
- Spin down for 5 minutes at full speed and directly use 5-10 µl of the supernatant for PCR.

In case the DNA solution is to be stored for an extended time, transfer supernant to a new tube.

APPENDIX 6: Genotypic drug susceptibility testing.

Materials and reagent

Paper towel

Micro pipette 10, 20, 200 and 1000 µl

GenoType MTBDR*plus* assay kit version 2.0

Filtered pipette tip

Tweezer

GT Blot 48 machine

Gel ink pen

1 % freshly prepared sodium hypochlorite solutions measuring cylinder

GenoType®MTBDR*plus* assay

Principle:

The GenoType®MTBDR*plus* assay is based on DNA STRIP technology. It uses DNA amplification followed by reverse hybridization to detect the presence of *M. tuberculosis* DNA and the most common genetic mutations conferring resistance to RIF and INH. Membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single-stranded amplicons bind to the probes (Hybridization).

Procedure:

- When using a hybridization instrument from HainLifescience, refer to the document "Overview equipment programs" available on www.hain-lifescience.com for the name of the hybridization protocol to be used. The following protocol describes the manual hybridization using a twincubator.
- Switch on the twincubator and prewarm solutions HYB and STR (of the GenotypeMTBDR*plus* Kit) to 37-45^{0}C before use. The reagents must be free from precipitates. Warm the remaining reagents with exception of CON-C and SUB-C to room temperature. Using a suitable tube, dilute conjugate concentrate (CON-C) and substrate Concentrate (SUB-C) with the respective buffer in the amounts needed.
- Dispense 20µl of Denaturation solution (DEN) in a corner of each of the wells used. Add to the solution20µl of amplified sample, pipette up and down to kix well and incubate at room temperature for 5 minutes.

- Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the coloured marker.
- Carefully add to each well 1 ml of prewarmed HYB, gently shake the tray until the solution has a homogenous color.
- Place strip in each well and place tray onto a twincubator and incubate for 30 minutes at 45^0C.
- Completely aspirate hybridization buffer and add STR to each strip and incubate for 15 minutes at 45^0C in the twincubator.
- Completely remove stringent wash solution (STR) and wash each strip once with 1 ml of RIN (rinse solution) for 1 minute in the twincubator (pour out RIN after incubation).
- Add 1 ml of diluted conjugate to each strip and incubate for 30 minutes on twincubator.
- Remove solution and wash each strip twice for 1 minute with 1 ml of RIN and once for 1 minute with 1 ml of distilled water on twincubator (pour out solution each time).
- Add 1 ml of diluted substrate to each strip and incubate protected from light without shaking.
- Stop reaction as soon as bands are clearly visible by briefly rinsing twice with distilled water.
- Using tweezers remove strips from the tray and dry them between 2 layers of absorbent paper.

Omission of a wild type band or the appearance of a mutant band in the resistance-determining region of a gene indicates the existence of a resistant strain.

6.2 GenoType®MTBDR*sl* assay

Materials and reagent

Paper towel

Micro pipette 10, 20, 200 and 1000 µl

Genotype MTBDRsl assay kit version 2.0

Filtered pipette tip

Tweezer

GT Blot 48 machine

Gel ink pen

1 % freshly prepared sodium hypochlorite solutions measuring cylinder

Principle:

GenoType MTBDR*sl* is based on the DNA-strip technology: Mycobacterial DNA is extracted from the specimen, specifically amplified via PCR and detected on a membrane strip using reverse hybridization and an enzymatic color reaction. The test system thus permits the simultaneous detection of MTB complex as well as relevant mutations that are associated with resistance to fluoroquinolones, aminoglycosides/ cyclic peptides and ethambutol. The test can be performed starting from pulmonary patient specimens or cultivated samples.

Procedure:

- In this study The MTBDR*sl* assay was performed on extracted DNA samples .
- The MTBDR*sl* assay was performed according to the instructions provided by the manufacture (Hain Life Science GmbH, Nehren, Germany).
- Amplification mixture contained 35 µl of the primer nucleotide mix, 5 µl of 10 x polymerase incubation buffer, 5 µl of 25 Mm $MgCl_2$, 1 µl of AmpliTaq polymerase (5 µl; AppliedBiosystems), and 5 µl of the supernatant of the cell lysate, for a final volume of 50 µl.
- The amplification protocol consists of 5 min of denaturation at 95°C, followed by 10 cycles comprising 30 s at 95°C and 2 min at 58°C, an additional 20 cycles comprising 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C, and then a final extension at 70°C for 8 min.

- Hybridization and detection was performed using GT Blot48 (Hain Life science GmbH, Nehren, Germany). The hybridization procedure includes the following steps: chemical denaturation of the amplification products at room temperature for 5 min, hybridization of the single-stranded biotin-labeled amplicons to membrane-bound probes at 45°C for 30 min, stringent washes, addition of a streptavidin–alkaline phosphatase (AP) conjugate at room temperature for 30 min, and an AP staining reaction to detect colorimetric bands.

The GenoType MTBDR*sl* DNA strip is coated with 22 probes. Briefly resistance to fluroquinolones will be based on the use of 3 wild-type probes covering GyrA codons 85 to 97. The presence of the most frequently observed mutations are confirmed by positive hybridization with 6 mutant probes (A90V, S91P, D94A, D94G and D94H). For aminoglycoside and cyclic peptide resistance, two wild-type probes cover nucleotides 1401G and 1402 and 1484, and two mutant probes specifically detect the A1401G and G1484T exchages. For ethambutol resistance, one wild-type probe covers codon 306 and the presence of the most frequently observed mutations, M306V and M3061, is confirmed by positive hybridization with two probes. The amplication reactions and the GenoType MTBDR*sl* will be performed as recommended by the manufacturer, using 5 µl ot the extracted DNA.

APPENDIX 7: Spoligotyping (Brudey *et al*., 2006)

Principle

This test is based on DNA polymorphism present at one particular chromosomal locus, the "Direct Repeat" (DR) region, which is uniquely present in *Mycobacterium tuberculosis* complex bacteria. This locus was first described by (Brudey *et al*., 2006) who sequenced this region in *Mycobacterium bovis* BCG, the strain used worldwide to vaccinate against tuberculosis. The DR region in *Mycobacterium bovis* BCG consists of directly repeated sequences of 36 base pairs, which are interspersed by non-repetitive DNA spacers, each 35 to 41 base pairs in length. The number of copies of the DR

sequence in *Mycobacterium bovis* BCG was determined to be 49. In other M. tuberculosis complex strains the number of DR elements was found to vary significantly. The vast majority of the *Mycobacterium tuberculosis* strains contain one or more IS6110 elements in the DR region

Procedure

In vitro amplification of spacer DNA by PCR

1. Chromosomal DNA of *Mycobacterium tuberculosis* strain H37RV and *Mycobacterium bovis* BCG P3 were used as positive controls and water as a negative control

2. Reaction mixture was prepared as follows:

- 2 µl template DNA
- 3 µl primer DRa (0.2µmol/µl)
- 3 µl primer DRb (0.2µmol/µl)
- 20 µl 2×Taq PCR Master mix
- 12 µl MQ water (to a final volume of 40 µl)

3. A drop of mineral oil was placed into the tubes to prevent evaporation of the PCR mix

4. The tubes were placed in a PCR apparatus for amplification

5. The following temperature cycling was performed:

- 3 min 94ºC 1 cycle
- 1 min 94ºC
- 1 min 55ºC
- 30 sec 72ºC 25 cycles

- 7 min 72ºC 1 cycle
- ∞ 4ºC

Hybridization with PCR product and detection

Purpose

Hybridization of the biotin-labelled PCR products to the immobilized spacer-oligos that represent spacers of known sequence. The presence of the spacers was visualized on the film as black spacers after incubation with streptavidin-peroxidase and ECL-detection

All buffers were pre warmed before use. The following buffers were prepared from concentrated stocks, using demineralized water for dilution

- 2×SSPE/0.1% SDS, 42ºC
- 2×SSPE/0.5% SDS, 60ºC
- 2×SSPE/0.5% SDS, 42ºC
- 2×SSPE, room temperature

Twenty (20) µl of the PCR products was added to 150 µl of 2×SPPE/0.1% SDS

The diluted PCR product was heat-denatured for 10 min at 100ºC and cooled on ice immediately.

The membrane was washed for 5 min at 42ºC in 250 ml 2×SPPE/0.1% SDS

The membrane and a support cushion were placed into the miniblotter, in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides

Residual fluids were removed from the slots of the miniblotter by aspiration using sterile pipette

The slots were filled with diluted PCR product (air bubbles were avoided) and hybridized for 60 min at 60ºC on a horizontal surface (no shaking), contamination of neighboring slots was avoided

The samples were removed from the miniblotter by aspiration and the membrane was taken from the miniblotter using forceps

The membrane was washed twice in 250 ml 2×sspe/0.5% SDS for 5 min at 60ºC

The membrane was placed in a rolling bottle and allowed to cool down to prevent inactivation of the peroxidase in the next step

Five (5) µl streptavidin-peroxidase conjugate was mixed with 14 ml of 2×SSPE/0.5% SDS and the membrane was incubated in this solution for 60 min at 4ºC in the boiling bottle

The membrane was washed twice in 250 ml of 2×SSPE/0.5%SDS for 10 min at 42ºC

The membrane was rinsed twice with 250 ml of 2×SSPE for 5 min at room temperature

For chemiluminiscent detection of hybridizing DNA, the membrane was incubated for 1 min in 16 ml ECL detection liquid

The membrane was covered with a transparent plastic sheet or Saran-wrap and a light sensitive film was exposed to the membrane for 20 min

If the signal is too weak or too strong it can be used again directly to expose another film for a shorter or longer period

Regeneration of the membrane

The hybridized PCR product is dissociated from the membrane in order to regenerate the membrane for the next hybridization. A membrane can be regenerated for at least 10 times

The membrane was washed twice by incubation in 1% SDS at 80ºC for 30 min

The membrane was washed in 20mM EDTA pH 8, for 15 min at room temperature

The membrane was stored at 4ºC until use (sealed in plastic or wrapped in Saran-wrap, to avoid dehydration of the membrane)

UNIVERSITY OF LIMPOPO
Medunsa Campus

MEDUNSA RESEARCH & ETHICS COMMITTEE

CLEARANCE CERTIFICATE

MEETING:	01/2014
PROJECT NUMBER:	MREC/HS/43/2014: PG
PROJECT:	
Title:	Wild-type minimal inhibitory concentration distributions of second-line drugs in Mycobacterium tuberculosis clinical isolates in relation to recommended critical concentrations in Limpopo province, South Africa
Researcher:	Miss NM Seloma
Supervisor:	Mrs NTC Maguga-Phasha
Co-supervisor:	Dr EF Makgatho
Department:	Medical Sciences, Public Health & Health Promotion
School:	Health Sciences
Degree:	MSc Medical Science

DECISION OF THE COMMITTEE:

MREC approved the project

DATE: 04 February 2014

UNIVERSITY OF LIMPOPO
Medunsa Campus
2014 -02- 04
MEDUNSA RESEARCH ETHICS COMMITTEE
MREC CHAIRPERSON

PROF GA OGUNBANJO
CHAIRPERSON MREC

The Medunsa Research Ethics Committee (MREC) for Health Research is registered with the US Department of Health and Human Services as an International Organisation (**IORG0004319**), as an Institutional Review Board (**IRB00005122**), and functions under a Federal Wide Assurance (**FWA00009419**)
Expiry date: 11 October 2015

Note:
i) Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee.
ii) The budget for the research will be considered separately from the protocol.
PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH

Enquiries: Latif Shamila Ref:4/2/2

Seloma NM
University of Limpopo
Sovenga
0727

Greetings,

Re: Wild-type minimal inhibitory concentration distributions of second-line drugs in *Mycobacterium tuberculosis* clinical isolates in relation to recommended critical concentrations in Limpopo Province, South Africa.

1. The above matter refers.
2. Permission to conduct the above mentioned study is hereby granted.
3. Kindly be informed that:-
   - Further arrangement should be made with the targeted institutions.
   - In the course of your study there should be no action that disrupts the services.
   - After completion of the study, a copy should be submitted to the Department to serve as a resource.
   - The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.

Your cooperation will be highly appreciated.

Head of Department Date 13/05/2014

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15 May 2014

Applicant: Ms NM Seloma
Institution: University of Limpopo
Faculty: Health Sciences
Department: Medical Sciences, Public Health & Health Promotion
Address: Private Bag X1106, Sovenga, 0727
Contact Number: 015 268 3280

Re: Approval to conduct a study at the National Health Laboratory Service (NHLS) - Polokwane

Your application to undertake a research project titled "**Wild-type minimal inhibitory concentration distributions of second-line drugs in *Mycobacterium tuberculosis* clinical isolates in relation to recommended critical concentrations in Limpopo Province, South Africa**" has been reviewed. This letter serves to advise that the application has been approved.

Please note that the approval is granted on your compliance with the NHLS conditions of service and that the study can only be undertaken provided that the following conditions have been met.

- Full Ethics clearance have been obtained from an approved local Ethics Committee
- Processes are discussed with the laboratory manager and/or the pathologist and are agreed upon
- Confidentiality is maintained at participant and institutional level and there is no disclosure of personal information or confidential information as described by the NHLS policy.
- A final report of the research study and any published paper resulting from this study are submitted and addresses to the NHLS Academic Affairs and Research office and the NHLS has been acknowledged appropriately.

Please note that this letter constitutes approval by the NHLS Academic Affairs and Research. Once all requirements have been met, please contact the Laboratory Manager: Lucas Kgoetsane at 015 2971099 who will provide approval and communicate with the relevant people.

Yours sincerely,

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