Antimicrobial Susceptibility Testing and Sequencing of *Mycobacterium tuberculosis* Clinical Isolates

by

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Dedication

This work is dedicated to my late father Sweethern and my grandmother Simbisai who sadly passed away during my PhD studies

Without your support, this would not have been possible.
Thank you.

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree. Where information from other sources and collaboration was used, it has been indicated with references and acknowledgement

Signature: .....

Date: .....

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LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
AMI	Amikacin
APM	Agar proportion method
ADM	Agar dilution method
ART	Antiretroviral therapy
BCG	Bacille Calmette-Guerrin
CFU	Colony forming units
CYC	Cycloserine
^{14}C	Radioactive Carbon 14
CO_2	Carbon Dioxide
CLSI	Clinical and Laboratory Standards Institute
DOTS	Directly-observed, short course treatment strategy
DST	Drug susceptibility testing
DNA	Deoxyribonucleic acid
EMB	Ethabutol
EMB	Ethambutol
ETH	Ethionamide
Etest	Epsilometer test
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FQ	Fluoroquinolones
HIV	Human immune-deficiency virus
IPT	Isoniazid preventive therapy
INH	Isoniaizid
IGRA	Interferon-gamma release assay
KAN	Kanamycin
LPAs	Line Probe Assays
LTBIs	Latent TB infection
MBC	Minimum bactericidal concentration
MDR-TB	Multidrug-resistant TB
MODS	Microscopic Observation Drug Susceptibility
MIC	Minimal Inhibitory Concentration
MIGT	*Mycobacterium* Growth Indicator Tube
MTBC	Mycobacterium tuberculosis complex
MOTT	Mycobacteria other than *mycobacterium tuberculosis*
MXF	Moxifloxacin

NTM	Non-tuberculosis mycobacteria
NTA	Nitrate reductase assay
NX	Nextera
NICD	National Institute of Communicable Diseases
OADC	Oleic acid-albumin dextrose-catalase
OFX	Ofloxacin
PCR	Polymerase chain reaction
PPD	Purified protein derivative
PAS	Para-aminosalicyclic acid
PZA	Pyrazinamide
PD	pharmacodynamics
PK	Pharmacokinetics
RIF	Rifampicin
rRNA	ribosomal ribonucleic acid
RFB	Rifabutin
RRDR	Rifampicin resistance drug-resistance
SM	Streptomycin
TB	Tuberculosis
TLA	Thin-layer agar
WHO	World Health Organization
WGS	Whole genome sequencing
XDR-TB	Extensively drug-resistant TB

LIST OF ARTICLES IN PREPARATION FOR SUBMISSION AND CONFERENCE CONTRIBUTIONS

PUBLICATIONS

1 **Rukasha I**, **Said HM, Omar SV, Koornhof H, Dreyer AW, Musekiwa A, Moultrie H, Hoosen AW, Kaplan G, Fallows D and Ismail N** (2016) Correlation of *rpo*B mutations with minimal inhibitory concentration of Rifampin and Rifabutin in *Mycobacterium tuberculosis* in an HIV/AIDS endemic setting, South Africa. *Frontiers in Microbiology* **7***:*1-7 doi:10. 3389/fmcbd.2016..0197

2 **Said HM, Kushner N, Omar SV, Dreyer AW, Koornhoof H, Erasmus L, Gardee Y, Rukasha I, Shashkina E, Beylis N, Kaplan G, Fallows D and Ismail NA (2016).** A novel molecular strategy for surveillance of multidrug resistant tuberculosis in high burden settings. A novel molecular strategy for surveillance of multidrug resistant tuberculosis in high burden settings. PLOS one 11(1): p e0146106 doi: 10.13371/journal.pone.0146106

3 **Birkhead M, Naicker S, Blasich P, Rukasha I, Thomas J, Sriruttan C, Abrahams s, Mavuso GS and Govender NP (2018)** Cryptococcus neoformans: Diagnostic dilemmas, electron microscopy and capsular variants doi 10.3390/tropical med 40100001

4 **Wake RM, Britz E, Sriruttan C, Rukasha 1, Omar T, Spencer DC, Nel JS, Mashamaite S, Adelekan A, Chiller TM, Jarvis JN, Harris TS and Govender NP** (2017) High cryptococcal antigen titers in blood are predictive of subclinical cryptococcal meningitis among human immunodeficiency virus-infected patients. *Clinical Infectious Diseases* 66(5) 686-692.doi.10.1093/cid/cix872

5 **Dikmans AC, Rukasha I and Hoosen AA** (2014) Trichomoniasis in women attending an antiretroviral clinic in South Africa. *International Journal of Infectious Disease* 21:422

6 **Rukasha I, Said H and Ishmael N** (2014) Evaluation of the Sensititre MYCOTB MIC plate for susceptibility testing of *Mycobacterium tuberculosis* against the Etest and agar proportion methods. *International Journal of Infectious Diseases* 21:61

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8 **Rukasha I, Said HM, Omar SV, Joseph L, Koornhof HJ, Dreyer AW, Musekiwa AA, ,Hoosen AA, Fallows DA and Ismail NA** (2019) Evaluation of the Sensititre ® MYCOTB MIC plate method against agar dilution MIC method for susceptibility testing of *Mycobacterium tuberculosis.* To be submitted to the *Plos One* Journal

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1 **Said HM, Omar SV, Fallows D. Rukasha I, Ismail NA** (2017) Resistance to second line anti-TB drugs in South Africa: Association between MIC and genetic resistance determinants. FIDDSSA Conference November 2017 (Poster Presentation)

2 **Rukasha I, Said HM, Omar SV, Fallows D, Ismail NA (2015)**46th Union World Conference on Lung Health Cape Town, South Africa (International Union Against Tuberculosis and Lung Disease, The Union); 2-6 December 2015 (Poster Award).

3. **Rukasha I, Said HM, Omar SV, Fallows D, Ismail NA (2014)** The 16th International Congress on Infectious Diseases, April 2-5, April 2014, Cape Town South Africa (Oral presentation).

4. **Rukasha I, Hoosen AA and Kock MM** (2010) Prevalence of *Trichomonas vaginalis* in HIV positive women. Faculty Day, Faculty of Health Sciences, University of Pretoria on 29 to 30 August 2011 (Poster presentation)

5. **Kock MM, Rukasha I and Hoosen AA** (2011) Detection of *Trichomonas vaginalis* in HIV positive women attending Tshwane District Hospital, Pretoria, South Africa. International Society for Sexually Transmitted Diseases Research Conference, Québec, Canada on 10 to 13 July 2011 (Poster presentation)

6. **Rukasha I, Ehlers MM and Kock MM** (2012) Genetic relatedness of *Trichomonas vaginalis* isolates obtained from Tshwane District Hospital, South Africa. South African Society of Biochemistry and Molecular Biology Federation of African Societies of Biochemistry and Molecular Biology, Champagne Sports Resort, Drakensberg, KwaZulu-Natal, South Africa from 29 January to 01 February 2011 (Poster presentation)

7. **Rukasha I, Ehlers MM and Kock MM** (2012) Genetic relatedness of *Trichomonas vaginalis* isolates obtained from Tshwane District Hospital, South Africa Faculty Day, Faculty of Health Sciences, University of Pretoria on 24 to 25 August 2012 (Oral and Poster presentation)

Antimicrobial Susceptibility Testing and Whole Genome Sequencing of *Mycobacterium tuberculosis* Clinical Isolates

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SUMMARY

With the global rise in drug resistant tuberculosis (DR-TB), drug susceptibility testing (DST) is key to ending the disease. Universal access to a prompt and comprehensive DST is therefore a major component towards the End TB strategy. Currently, diagnosis of DR-TB still relies mainly on conventional DST, which distinguish resistant and susceptible strains based on critical concentration (CC). However, studies have shown that *M. tuberculosis* is not binary but diverse involving low, moderate and high levels of drug resistance. The CC could also change over time with more exposure to anti-TB drugs and for many of the anti-TB drugs the CC is near the wild type minimum inhibitory concentration (MIC). Consequently, phenotypic DST based on CC testing, may provide inaccurate results, possibly leading to suboptimal treatment regimens. This necessitates to continually revise and evaluate CCs. Thus, validation of quantitative methods determining MIC instead of CCs are needed to enable formulation of optimal regimens. In addition, determination of MIC facilitates monitoring trends of drugs resistance. Methods based on CC cannot detect subtle MIC changes until the mode shifts to the next category. The geometric mean MIC is a more sensitive marker for changes in MIC distributions. Shift in MIC population distributions may have important implications for treatment.

While introduction of rapid molecular techniques has improved DR-TB case detection, a comprehensive catalogue of genetic markers of clinically relevant mutations does not exist and mutations associated with resistance to newer and repurposed drugs are yet to be identified. Literature suggests that different genetic polymorphisms are associated with distinct phenotypic resistance levels. However, the impact of those mutations on the MIC remains to be investigated. Previous studies on genetic association for drug resistance in *M. tuberculosis* mainly relied on phenotypes defined by DST performed at a single CC. MICs are more appropriate to assess the biological effects of genomic variation in understanding the mechanism of resistance. Thus, correlating specific mutations conferring drug resistance with specific MICs for given drug classes are essential to predict levels of resistance which can be used to guide clinical decision-making.

This study aimed i) To validate the Sensititre MTCOTB broth microdilution (MYCOTB) method for first and second-line anti-TB drugs ii) To determine the association between specific *rpo*B mutations and the MIC of rifampin (RIF) and rifabutin (RFB) among clinical MDR-TB isolates and iii) To determine the association between different genetic polymorphisms and resistance at an MIC level and iv) To evaluate the trend of anti-TB MIC for *M. tuberculosis* clinical isolates over a three year period in order to observe a MIC creep, if any and investigate the role of mutations in MIC changes over time.

The MYCOTB broth microdilution method was validated against the agar dilution method (ADM). Strains showing discordant results between the two methods after repeat testing, were resolved using the next generation sequencing. For this purpose, a collection of MDR-TB strains from a cross-sectional MDR-TB study were used. The MYCOTB plate is based on 12 lyophilized anti-TB drugs including first and second-line drugs. For ADM, 11-welled plates of Middlebrook 7H11 medium was used representing all drugs on MYCOTB except for CYC. The categorical, essential as well as sensitivity and specificity of MYCOTB were determined in comparison with ADM. The MYCOTB plate showed good overall performance with categorical agreement ranging from 88% to 98% for the drugs tested. The sensitivity of the plate ranged from 60-100%, with exception of para-aminosalicylic acid (PAS), which had 11%, while specificity ranged from 94% to 100%. Whole genome sequencing resolved 70% of the isolates in favour of the MYCOTB plate.

Rifampicin resistance is often associated with the presence of mutations in the 81-bp RIF resistance determining region (RRDR) of the *rpo*B gene, but the effect of these *rpo*B mutations on RFB resistance is less well understood. Some *rpo*B mutations, detectable by rapid molecular diagnostics, confer resistance to RIF but not rifabutin (RFB), suggesting RFB may be effective for treatment of *M. tuberculosis* with these mutations. The current study investigated the association between *rpo*B mutations and MIC of RIF and RFB as well as the prevalence of RFB susceptible isolates among RIF resistant strains. MICs for first- and second-line drugs were determined using the MYCOTB method and the RRDR region of the *rpo*B gene was sequenced. Cross-resistance between RIF and RFB was found in 73% of the isolates. Mutations S531L, H526D and H526Y were associated with both RIF and RFB (p=0.0001), while, D516V and L533P mutations were found in RIF-resistant but RFB susceptible isolates (p=0.001). A total of 27% isolates were resistant to RIF but retained susceptibility to RFB.

To determine the association of genetic polymorphism and resistance at MIC level, MICs for first- and second-line drugs were linked to the corresponding genetic mutations. The MICs were determined using the MYCOTB method and relevant genes were sequenced. The Kruskall Wallis static was used to determine the association between MICs and the different mutations. The *kat*G mutations S315T, S315G and double peak S315T were significantly associated with high INH resistance (MIC: 2 to 4ug/ml; p=00001). However, *kat*G mutations were not significantly associated with ETH MICs (p=0.832). The *inh*A mutations C-15T, T-8A and G-17T were significantly associated with high resistance to both INH (0.5 to 4 ug/ml, p=0.013) and ETH resistance (10 to 40 ug/ml, p=0.001). For MXF and OFX, *gyr*A mutations at codon 90 and 91 were associated with lower MIC compared to isolates at codon 94. Isolates with *gyr*A mutation at the codon 90 had lower MIC (OFX: 4 to 8 ug/ml; p=0.0001: MXF 1 ug/ml; p=0.0001) compared to isolates at codon 94 (OFX: 8 to 32 ug/ml; p=0.000, MXF: 1 to 8ug/ml; p=0.0001). The mutations A1401G, C492T, C492T_A1401G and A514C_A1401G were associated with high MICs for KAN (10 to 40ug/ml, P=0.0001) and AMI (16ug/ml, p=0.0001). Cross-resistance between AMI and KAN was found in 85% of resistant isolates. The *emb*B mutation was not significantly associated with MIC ranges in this study.

To investigate the MIC trend and possible MIC creep, the distributions of individual MICs were plotted against time to evaluate changes over the study period. Additionally, the MIC_{50}, MIC_{90} and MIC range, modal MIC, geometric mean and median MIC were determined over the three-year period. The MIC trends over the three years and the significance of changes was assessed using paired T-test. A P-Value <0.05 was considered as significant. The study showed MIC creep for three drugs RFB (p=0.0001), MXF (p=0.0001) and OFX (p=0.0001), whereas EMB (p=0.0067) and INH (p=0.0218) showed decrease in MIC over time. All the other drugs; RIF, PAS, SM, KAN, CYC, AMI, and ETH showed stable MICs over three years.

The study showed the MYCOTB assay is a good alternative to conventional DST methods; relatively rapid and provides quantitative data on susceptibility to first- and second-line drugs, thus facilitating therapeutic decision-making and therapeutic drug monitoring to optimize regimen efficacy. The 96-well microplate format without the need for equipment will allow its use in resource-limited settings. The study showed that up to 27% of MDR-TB patients may benefit from a treatment regime that include RFB as a substitute for RIF resistance. Different drug resistance mutations were associated with different MIC ranges; *kat*G, *inh*A *gyr*A and *rrs* mutation were associated with high MICs of their respective drugs while mutations such as *rps*L (for KAN and AMI) and *emb*B were not significantly associated with MIC ranges. This information can help in guiding clinical decision-making. The study further showed a general increase in the proportion of resistant strains over the study period for 9 of the 11 drugs tested, with evidence of creep for three drugs (MOX, OFX and RFB). Thus the monitoring of MIC changes of drugs is important to prevent gradual loss of drug efficacy.

CHAPTER 1

1.1 BACKGROUND AND RATIONALE OF THE STUDY

Drug resistance is a serious problem in South Africa. Currently, South Africa treats the third-highest number of drug resistant tuberculosis (DR-TB) patients globally, after India and Russia (WHO, 2018). Treatment outcomes are poor among DR-TB patients in South Africa, with a success rate of approximately 50% nationally and globally. A virtually untreatable strain of TB was reported from Eastern Cape in 2013, accounting for approximately 90% of multidrug resistant (MDR) and extensively-drug resistant (XDR) cases (Klopper et al., 2013, Ismail et al., 2018).

Effective management of M/XDR-TB relies upon the rapid diagnosis and treatment of resistant infections. While implementation of new diagnostics has improved DR-TB case detection, the diagnosis of drug resistance still relies largely on conventional drug susceptibility testing (DST) methods which classify *M. tuberculosis* isolates as either drug resistant or drug susceptible on the basis of determination of critical concentrations (CCs) (Richter et al., 2009, Böttger, 2011). Drug-resistance for the *M. tuberculosis* involves low level, moderate level and high level drug resistance phenotype (Richter et al., 2009, Böttger, 2011). The current CCs have limited evidence base and are largely based on consensus, not from clinical or pharmacokinetics/pharmacodynamics studies. Many of the CC defining resistance is often very close to the minimum inhibitory concentration (MIC) required to achieve anti-mycobacterial activity, increasing the probability of misclassification of susceptibility or resistance. In addition, *M. tuberculosis* always seems to be adapting and evolving with more exposure to anti-TB drugs and thus there is a need to continually revise and evaluate CCs. Quantitative methods demining minimum inhibitory concentration (MIC) instead of CCs are needed to reflect and accommodate the biological complexity of drug-resistance. The introduction of the Sensititre MYCOTB microdilution method, for MIC testing of first and second-line drugs is a major improvement in the current standard for detecting drug resistance. The current study is the first study to

evaluate the assay with agar dilution method. The assay has been previously evaluated only with CC based DST methods.

Determination of MIC also facilitates monitoring trends of drugs resistance. Despite the problem of drug resistance and poor cure rates of DR-TB in South Africa, the changes or shifts in MIC levels has not been monitored. This is mainly due to lack of MIC testing methods for TB. The categorical classification of TB as sensitive or resistant cannot detect subtle MIC changes until the mode shifts to the next category. The geometric mean MIC is a more sensitive marker and can accurately reflect changes in MIC distributions when compared with conventional methos (Kim, 2005). Thus, a gradual and unnoticed increase in MIC levels may have occurred over time, a phenomenon known as MIC "creep" or "drift". Hence, in this study we evaluated the trend of anti-TB MIC for *M. tuberculosis* clinical isolates over a three years period to observe MIC creep, if any and investigate the role of mutations in MIC changes over time.

Diagnosis of TB has entered an era of molecular detection that provides faster and more cost-effective methods to diagnose TB and drug resistance. However, the impact of different drug resistance mutations on the MIC remains to be investigated. Studies have shown that different genetic mutations affect phenotypic resistance in different ways with MIC levels strongly correlating with the position and nature of the encoded amino-acid substitution (Sirgel et al., 2013, Jamieson et al., 2014, Lee et al., 2014, Rukasha et al., 2016). The level of resistance, reflected by the MIC, is important in guiding therapeutic decision-making for clinicians treating patients, in order to determine whether to increase the dosage or change the regimen. In addition, this knowledge could improve our molecular prediction of levels of drug resistance for clinical and diagnostic use, as considerable gaps remain in prediction of resistance to many of anti-TB drugs. Correlating specific mutations conferring drug resistance with specific MICs for given drug classes are essential to predict the level of resistance. Therefore, in the current study we compared MICs of first and second line drugs to drug resistance mutations determined by sequencing.

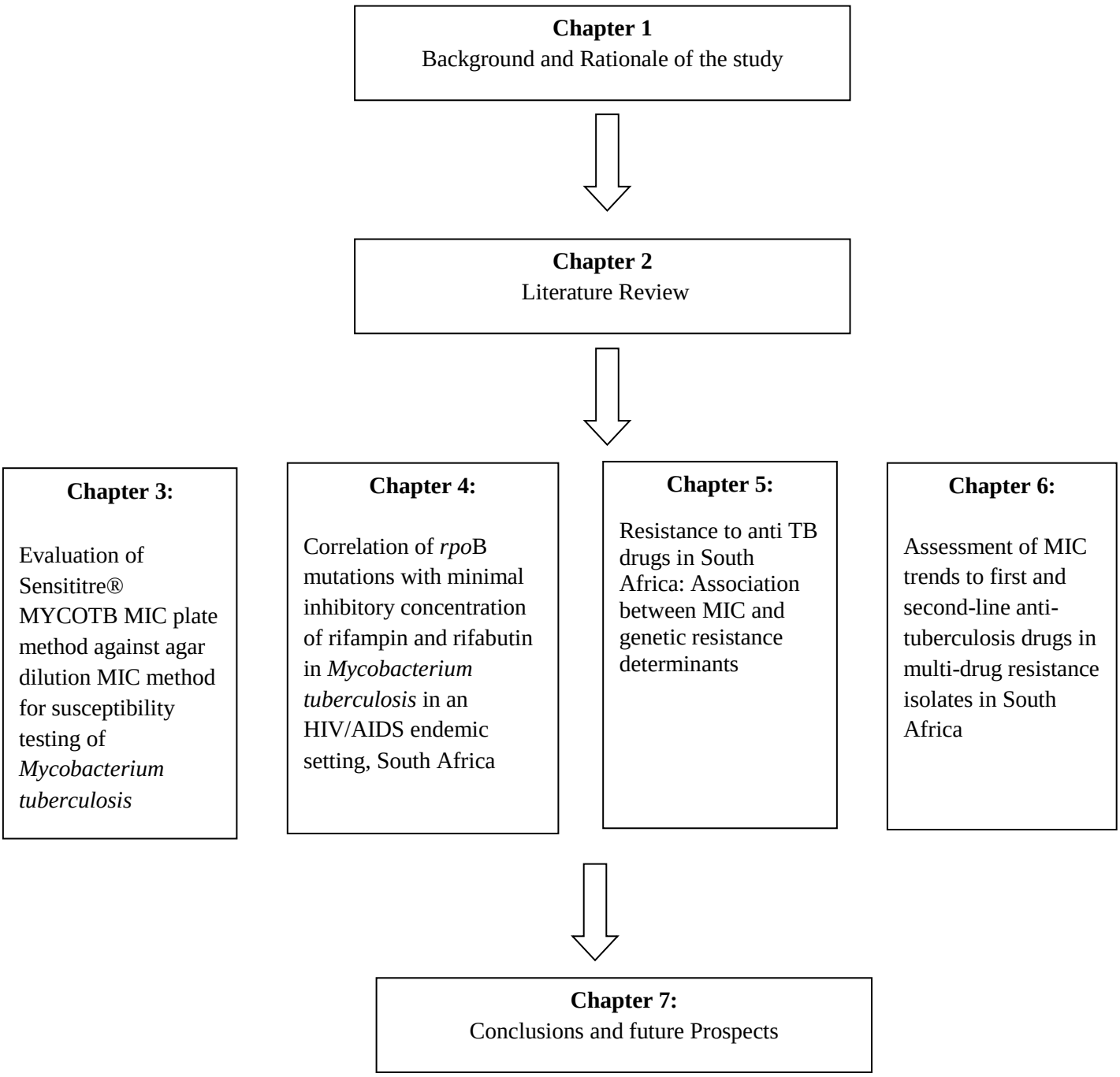
The main aims of the study include

i. To validate the Sensititre MTCOTB broth microdillution method (MYCOTB) for first and second-line anti-TB drugs against the agar dilution method (ADM)
ii. To determine the association between specific *rpo*B mutations and the MIC of RIF and RFB among clinical MDR-TB isolates
iii. To determine the association between different genetic polymorphisms and resistance at an MIC level as different resistance levels are reported to be associated with distinct genetic polymorphisms and
iv. To evaluate the trend of anti-TB MIC for *M. tuberculosis* clinical isolates over a three years period and to observe MIC creep, if any and investigate the role of mutations in MIC changes over time

Study objectives

i. To collect *M. tuberculosis* isolates with known spectrum of resistance profiles
ii. To compare the turnaround time, ease of set up of the MYCOTB plate method against ADM
iii. To determine *M. tuberculosis* MICs categorical and essential, modified essential agreements by comparing the MYCOTB plate method against ADM
iv. To determine the performance indices (specificity, sensitivity and positive predictive value) of the MYCOTB plate method against ADM
v. To resolve any discordant results between the two methods by whole genome sequencing (WGS)
vi. To determine genetic mutations for first and second-line drugs using the Sanger sequencing technique
vii. To determine the association of different genetic resistance mutations and MIC of *M. tuberculosis* isolates for first and second-line anti-TB drugs
viii. To determine MIC trends and MIC creep if any for MDR-TB isolates over three years period and evaluate the impact of mutation over time

Thesis Layout



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CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

Tuberculosis (TB) is a leading cause of deaths from a single contagious infectious disease (WHO, 2018). *Mycobacterium tuberculosis* (*M. tuberculosis*) is spread mainly through the air, when an infectious person coughs, sneezes, talks or spits saliva droplets containing the tubercle bacilli (Banuls et al., 2015). Tuberculosis has been known to, mankind since ancient times and history suggests that genus *Mycobacterium* originated more than 150 million years ago (Barberis et al., 2017).

Mycobacterium is a genus of Actinobacteria, and family *Mycobacteriacae* (Iseman, 2000, Ryan and Ray, 2004). Generally, mycobacteria is classified as *M. tuberculosis* complex which are slow growing and cause tuberculosis: *M. tuberculosis*, *M. bovis*, *M, africanus,*, *M. microti* and *M. leprae* (Cassidy et al., 2009, Winthrop et al., 2010, Johnson and Odell, 2014, Kendall and Winthrop, 2013). The other group include the nontuberculous mycobacteria (NTM) also known as Mycobacteria other than tuberculosis (MOTT) which are opportunistic environmental mycobacteria capable of causing the other disease resembling TB : *M. avium, M. kansasii and M. abscessus* (Crow et al., 1957, Ryu et al., 2016, Sharma et al., 2018). *Mycobacterium tuberculosis* are aerobic, non-motile, non-sporulating, non-encapsulated, weakly gram-positive, acid-fast bacillus (Barry et al., 1998, Pfyffer et al., 2002, Sakamoto, 2012, Jilani and Siddiqui, 2018). The size of the genome is 4 million base pairs, with around 4 000 genes (Cole et al., 1998, Cole, 2002).

Upon inhalation, *M. tuberculosis* bacteria travel to the lungs and end up in the alveoli. In some persons the bacilli are cleared but in immunocompromised people it can grow leading to TB manifestations (Martineau et al., 2007, Vankayalapati and Barnes, 2009, Getahun et al., 2015, Korb et al., 2016). *Mycobacterium tuberculosis* symptoms are gradual in onset and are dependent on age, immune status and co-existing diseases (Knechel, 2009, Falzon et al., 2011, D'Ambrosio et al., 2015). In patients with drug-susceptible TB a global standard first-line TB treatment is a short-course regimen which include INH, RIF, PZA, EMB and SM (Zumla et al., 2013, Alqahtani

and Asaad, 2014). However, in resistant TB, the WHO in 2018 showed preference of oral drugs over injectable drugs and included new and repurposed drugs.

At present the only available vaccine is Bacille Calmette-Guerrin (BCG) made from an attenuated strain of *M. bovis* (Zumla et al., 2015, WHOc, 2018). There are two commercially available methods for diagnosis of latent tuberculosis namely, the Mantoux test also known as tuberculin skin test or an interferon-gamma release assay (Mazurek et al., 2010, McNerney et al., 2012). In terms of performance none of the tests is preferred tests and preference depends on population being investigated (Dumm et al., 2015). Diagnosis of active TB infection is by microscopy which can be enhanced with staining methods, Ziehl-Neelsen and Kinyoun or by using the fluorescent microcopy. The gold standard for diagnosis of TB can be made by culturing on solid media (egg-based or agar-based solid media) or liquid media (Mycobacteria Growth Indicator Tube [MGIT] and VersaTREK (Ängeby et al., 2003, Woods et al., 2007, Moore and Shah, 2011).

Nucleic acid amplification tests (NAAT) offer several advantages which include faster turnaround times and the opportunity of omitting culture (Boehme et al., 2011, Alqahtani and Asaad, 2014, Sharma et al., 2016a, Sharma et al., 2016b). There are number of commercially available molecular methods which include the line probe assay (Hain Lifescience , Nehren, Germany) and Xpert® MTB/RIF (Cepheid, Sunnyvale, CA) (Richter et al., 2009, Blakemore et al., 2010). Line-probe assays include the GenoType® MTBDR*plus*, INNO-LiPA® Rif TB and GenoType® MTBDR*sl*. In 2010 WHO endorsed the real-time based PCR, Xpert® MTB/RIF, Cepheid for the direct identification of *M. tuberculosis* complex bacteria with simultaneous detection of RIF resistance from specimens (WHO, 2011). The Xpert® MTB/RIF has been modified to the Xpert MTB/RIF Ultra assay, which offer better sensitivity and specificity (Chakravorty et al., 2017, Dorman et al., 2018, WHO.XpertMRT, 2017). The Xpert MTB/RIF Ultra assay has sensitivity and specificity of 92% and 99% respectively while Xpert® MTB/RIF assay had 87% and 82.9% (Chakravorty et al., 2017, Dorman et al., 2018, WHO.XpertMRT, 2017).

The gold standard NAAT tests is the DNA sequencing based approaches which provide the highest level of information. Sequencing has generally first-generation which generally refers to "sanger sequencing" while, next generation is generally used to refer to any of the high-throughput

methods which were developed after Sanger sequencing (Abdelaal et al., 2009, Tyler et al., 2016). However, NAAT assays cannot detect silent mutations that do not necessarily translate to mutations.

Susceptibility testing of *Mycobacterium* is categorized into resistance or sensitive without considering whether its high level or low-level resistance. Which is in contrast to drug susceptibility testing used for most other bacteria, which uses minimum inhibitory concentration (MICs) to accommodate for moderate level or low-level resistance. A number of MIC based method have been proposed which include the Etest (bioMerieux). The Etest employs thin plastic strips that are impregnated with a dried antibiotic concentration gradient and are marked on the upper surface with a concentration scale. Preparation of MICs in solid media (7H10 medium or Lowenstein-Jensen medium) and liquid media (7H11 medium) involves preparation of serial dilutions of anti-TB drugs in the respective media (Das et al., 2003, Schaaf et al., 2007, Springer et al., 2008, Schönfeld et al., 2012). However, these methods are laborious and expensive. To overcome the limitations of the previous methods; micro-dilution method for determination of MICs have been introduced in recent years. Use of MICs have been shown to accommodate for the varied *M. tuberculosis* susceptibility variability (Sirgel et al., 2013, Jamieson et al., 2014) as 13 opposed to conventional methods based on binary categorization. However, MICs are still phenotypic methods and thus have long turn aroud time whereas molecular tests have faster turn around time. Correlating specific mutations with MICs can provide results faster. Conferring drug resistance with specific MICs for a given drug class is essential before successful implementation of these technologies.

2.2 HISTORY OF *MYCOBACTERIUM TUBERCULOSIS*

Tuberculosis has been known to mankind since ancient times. The exact dates when TB started are unknown but scientific work investigating the evolutionary origin of *Mycobacterium tuberculosis* (*M. tuberculosis)* complex has concluded that the most recent common ancestor of the complex dates back 40, 000 years ago, corresponding to the period subsequent to the expansion of Homo sapiens out of Africa (Daniel, 1998, Daniel, 2006, Barberis et al., 2017).

Researchers discovered the disease in human bones from the Neolithic era, in a settlement in the eastern Mediterranean (Hershkovitz et al., 2008). Signs of the disease have also been found in the spinal cords of Egyptian mummies constituting "Pott's disease", dated between 3000 and 2400 BC (Zink et al., 2001, Zink et al., 2003, Daniel, 2006). It is believed that humans first acquired *M. tuberculosis* in Africa about 500 years ago and that TB spread to other humans through to domesticated animals in Africa, such as goats and cows and along various trade routes (Zink et al., 2001, Zink et al., 2003, Daniel, 2006). Seals and sea lions that bred on African beaches are believed to have acquired the disease and carried it across the Atlantic to South America (Gibbons, 2001). Hunters along seas and oceans would have been the first humans to contract the disease in America (Gibbons, 2001, Frith, 2014).

In the 19th century the concept of keeping TB patients isolated in sanatoriums was started (Davis, 1996). Infectious persons were isolated from society and treated with rest and improved nutrition (Daniel, 20059). When all this was happening, scientists had conflicting ideas of the etiology of TB: In Northern Europe scientists felt TB was generally a hereditary disease and in the Southern Europe it was considered an infectious disease (Daniel, 2006). Tuberculosis has been known by different names throughout history such as consumption (because of severe weight loss), phthisis pulmonaris, scrofula, Pott's disease and the white plague (because of the extreme pallor seen among those infected) (Daniel, 1997, Daniel, 2006). The face of TB was unveiled by Robert Koch between 1843 to 1910 (Daniel, 1997, Daniel, 2005). Robert Koch a Prussian physician in 1882 revealed for the first time that the causal agent of the disease was "*M. tuberculosis*" or "Koch's bacillus" (Daniel, 1997, Daniel, 2005). Koch made the Nobel prize winning presentation to the tenth International Medical Conference in Berlin in 1882 when he presented evidence that he isolated tubercle bacilli that could be transmitted between animals and that it was the single cause of TB (Daniel, 2005, Daniel, 2006). Today the outlook and the course of TB in patients changed dramatically with the introduction of chemotherapy (Daniel, 2006). The discovery of the bacteriostatic drug para-aminosalicylic acid (PAS) in 1943 and streptomycin in 1944 followed by the highly bactericidal drugs isoniazid and the rifamycins in 1952 and 1957 respectively signaled a new era (Ahmad et al., 2011, Nasibullah et al., 2015). Sanatoria were closed and effective public health measures became possible (Daniel, 2006, Daniel, 2011, Barberis et al., 2017).

2.3 CLASSIFICATION OF *M. TUBERCULOSIS*

Mycobacterium is a genus of Actinobacteria, and family Mycobacteriacae (Iseman, 2000, Ryan and Ray, 2004). The name of the species termed "*M. tuberculosis*" is derived from the Greek words for fungus (myces) and small rod (bakterion) alluding to the way mycobacteria have been observed to grow in a mold-like fashion on the surface of liquids when cultured (Iseman, 2000, Ryan and Ray, 2004). Members of the *Mycobacterium* genus occur widely in natural ecosystems as free living organisms, while some which include *M. tuberculosis* and *M. leprae*, are obligate parasites (Iseman, 2000, Ryan and Ray, 2004). Mycobacteria can be divided into two main groups namely the slow and rapid growers.

The members of the *M. tuberculosis* complex (MTBC) are slow growing and include *M. tuberculosis, M africanum, M microti, M canetti, M caprae* and *M. pinnipedi,* (Kubica et al., 2003, Cvetnic et al., 2007, Kiers et al., 2008). All MTBC members can cause TB in humans and other primates, but can also cause disease in other animals (Ryan and Ray, 2004, Malone and Gordon, 2017, Romha et al., 2018). *Mycobacterium tuberculosis and M. africanum* are primarily human pathogens (Iseman, 2000, Ryan and Ray, 2004) while, *M. microti* mainly causes disease in rodents, although a few cases have been reported in humans as well (Iseman, 2000, Ryan and Ray, 2004). *Mycobacterium bovis* and *M. caprae* cause disease in both humans (zoonotic TB) and animals (cattle, goats, elephants, dear, seals cats etc.) although most infections are in animals (Iseman, 2000, Kubica et al., 2003, Kumar et al., 2005, Cvetnic et al., 2007, Kiers et al., 2008). Whereas, *Mycobacterium pinnipedi* (seals and sea lions) (Cousins et al., 2003).

Non-tuberculous mycobacteria (NTM) or mycobacteria other than *Mycobacterium tuberculosis* (MOTT) are opportunistic environmental mycobacteria capable of causing other diseases resembling tuberculosis, including pulmonary disease, lymphadenitis, skin disease or disseminated disease (Cassidy et al., 2009, Winthrop et al., 2010, Johnson and Odell, 2014, Kendall and Winthrop, 2013, Das et al., 2018). The MOTT or NTM group includes the *M. avium* complex (MAC) species which include *M. avium, M. indicus pranii*, *M. colombiens,* and *M. avium- silvaticum.* Other *Mycobacterium* species include *M. gordonae M. kansansii*, *M. simiae*, *M. terrae,* the intermediate pace growing *M. intermedium* and the rapidly growing *M. fortuitum,*

M. parafortuitum, *M. chelonae* and *M. vaccae* (Cassidy et al., 2009, Winthrop et al., 2010, Kendall and Winthrop, 2013, Johnson and Odell, 2014, Sharma et al., 2018).

2.4 CHARACTERISTICS AND MORPHOLOGY OF *M. TUBERCULOSIS*

Mycobacterium tuberculosis are aerobic, non-motile, non-sporulating, non-encapsulated, weakly gram-positive, acid-fast bacilli (Barry et al., 1998, Pfyffer et al., 2002, Sakamoto, 2012, Jilani and Siddiqui, 2018). Microscopically the bacilli are present in clumps and appear as straight or slightly curved rods. The bacteria are 1 to 4 µm in length and 0.3 to 0.6 µm wide (Barry et al., 1998, Pfyffer et al., 2002, Sakamoto, 2012). *Mycobacterium tuberculosis* is a slow-growing bacterium, characterized by a 12 to 24 hour division rate and prolonged culture period with visible growth seen from 3 to 8 weeks on solid media (Barry et al., 1998, Pfyffer et al., 2002, Sakamoto, 2012). This is much slower than 1 hour division time for most bacterial pathogens (Pfyffer et al., 2002, Sakamoto, 2012, Harries et al., 2004). The *M. tuberculosis* organisms are facultative intracellular bacteria that multiply within phagocytic cells, particularly macrophages and monocytes and on culture tend to grow in parallel groups, producing colonies characteristically exhibiting serpentine cording on microscopy (Barry et al., 1998, Pfyffer et al., 2002, Sakamoto, 2012).

2.4.1 Structure of the cell envelope

The cell envelope of *M. tuberculosis* comprises an inner plasma membrane which is homologous to plasma membranes of other bacteria and a cell wall core (Riley, 2006, Meena, 2010, Sakamoto, 2012, Daffé, 2015). The cell wall core is built of three macromolecules covalently linked together forming peptidoglycan which contains meso-diaminopimelic acid and N-glycosylated muramic acid residues, arabinogalactan and mycolic acids (Riley, 2006, Meena, 2010, Sakamoto, 2012, Daffé, 2015). Surrounding this core is a capsule-like outer structure of non-covalently linked glycans, lipids and proteins (Daffé, 2015, Minnikin et al., 2015) Beneath the cell wall there are layers of arabino-galactan and peptidoglycan that lie just above the plasma membrane (Riley, 2006, Minnikin et al., 2015). The waxy cell wall confers many of the unique advantages to *Mycobacterium tuberculosis* which include acid-fastness, extreme hydrophobicity. In addition the cell wall offers resistance to desiccation, acidity or alkalinity, chemical disinfectants as well as many antibiotics (Daffé and Draper, 1997, Sakamoto, 2012, Daffé, 2015). Phosphatidyl-inositolmannosides are the main plasma membrane components and form the lipid anchor of

lipoarabinomannan and lipomannan, which belong to the upper segment of the cell wall together with free lipids and proteins (Riley, 2006, Meena, 2010, Sakamoto, 2012).

2.4.2 Genomics of *M. tuberculosis*

The genome of *M. tuberculosis* was well studied, generally using the strain *M. tuberculosis* $H_{37}R_V$ and published in 1998 (Camus et al., 2002). The size of genome is 4 million base pairs, with around 4 000 genes (Cole et al., 1998, Cole, 2002). The Guanine (G) + Cytosine (C) content is characteristically high, about 65% (Cole et al., 1998, Cole, 2002). This represents the second-largest bacterial genome sequence currently known (after that of *Escherichia coli*) (Cole, 2002). The genome is rich in repetitive DNA, particularly insertion sequences and in new multi-gene families and duplicated housekeeping genes (Cole et al., 1998, Aranaz et al., 1999). The G + C content is relatively constant throughout the genome indicating that horizontal transfer of genes is probably absent (Cole et al., 1998, Aranaz et al., 1999). Concerning transcriptional regulation, *M. tuberculosis* codifies for 13 putative sigma factors and more than 100 regulatory proteins (Cole et al., 1998, Aranaz et al., 1999). The presence of a single ribosomal ribonucleic acid (rRNA) operon contrary to most eubacteria that have more than one operon, has been suggested as a factor contributing to the slow growth of *M. tuberculosis* (Brosch et al., 2002). Genes that code for enzymes involved in lipid metabolism constitute a very important part of the bacterial genome (Cole, 2002) In contrast to other microorganisms, a very large po rtion of *M. tuberculosis* genes (approximately 6% or 250 genes of the genome) encode enzymes that are involved in lipogenesis and lipolysis (Cole, 2002). The different species of the *M. tuberculosis* complex show a 95-100% DNA relatedness based on studies of DNA homology, and the nucleotide sequence of the 16S rRNA gene is exactly the same for all species (Brosch et al., 2002, Cole, 2002). As a result of this, some scientists suggest that they should be grouped as a single species while others argue that they should be grouped as varieties or subspecies of *M. tuberculosis* (Aranaz et al., 1999, Borrell et al., 2018). Plasmids in *M. tuberculosis* are important in transferring virulence because genes on the plasmids are more easily transferred than genes located on the chromosome (Aranaz et al., 1999, Cole, 2002). One such 18kb plasmid in the *M. tuberculosis* $H_{37}R_V$ strain was proven to conduct gene transfers (Aranaz et al., 1999, Cole, 2002).

2.5 TRANSMISSION OF *M. TUBERCULOSIS*

Tuberculosis is mainly transmitted through inhalation of aerosol droplets with diameter of 1-5µm containing tubercle bacilli directly expectorated from an individual with active pulmonary disease (Sia and Wieland, 2011, Acuña-Villaorduña et al., 2016, Yates et al., 2016, Churchyard et al., 2017, Tostmann et al., 2008). The infectious dose for a person is reported to be between 1 and 200 bacillli, however, as a single aerosol droplet can contain any number from 1 to 400 bacilli (Tostmann et al., 2008, Sia and Wieland, 2011, Acuña-Villaorduña et al., 2016). The tubercle bacillus can bind directly to mannose receptors on macrophages via the cell-wall associated mannosylated glycolipid lipoarabinomannan or directly via certain complement receptors to Fc receptors (Tostmann et al., 2008, Sia and Wieland, 2011, Churchyard et al., 2017). The highest risk of transmission occurs among patients with cavitary pulmonary disease or patients with positive acid-fast bacilli smears, although people with negative smears but positive cultures may still transmit the disease (Tostmann et al., 2008, Yates et al., 2016). Transmission of TB is more likely to occur in schools, public transport settings, workplaces, healthcare facilities, mines and prison (Escombe et al., 2010, Andrews et al., 2013, Yates et al., 2016).

2.6 THE PATHOGENESIS AND IMMUNOLOGICAL RESPONSE TO *M. TUBERCULOSIS*

Upon inhalation, *M. tuberculosis* bacteria travel to the lungs and end up in the alveoli, where they are recognized in an immune-competent host as foreign and are rapidly attacked by the body's macrophages and phagocytosed by alveolar macrophages (Martineau et al., 2007, Vankayalapati and Barnes, 2009, Getahun et al., 2015, Korb et al., 2016). Macrophages engulf the bacteria and dissemble them or halt progression of infection as part of the process of a body's defense mechanisms, in combating disease (Martineau et al., 2007, Vankayalapati and Barnes, 2009, Getahun et al., 2015). In some persons the bacilli are cleared, whereas in others infection is established (Modlin and Bloom, 2013, Gibson et al., 2018). The infected areas gradually transform into a granuloma, comprising predominantly a wall of macrophages intended to contain the infection (Martineau et al., 2007, Vankayalapati and Barnes, 2009). In susceptible individuals, this allows the *M. tuberculosis* bacilli to continue growing and overwhelm the phagocytic cells it

has infected until they die (process of necrosis) (Vankayalapati and Barnes, 2009, Sakamoto, 2012). In some cases the necrotized lesions may heal with some amount of scarring and calcification (Vankayalapati and Barnes, 2009, Sakamoto, 2012).

The *M. tuberculosis* bacteria escape the host immune–mediated clearance mechanisms using multiple strategies. *Mycobacterium tuberculosis* has the ability to remain dormant within the host cells for years, at the same time retaining the potential to be activated (McKinney et al., 2000, Wayne and Sohaskey, 2001, Korb et al., 2016). The dormancy or latency of *M. tuberculosis* allows the bacterium to escape the activated immune system of the host (McKinney et al., 2000, Wayne and Sohaskey, 2001, Meena, 2010). *Mycobacterium tuberculosis* have antioxidants which not only provide direct protection against host-generated oxidants but also suppress early oxidant-mediated immunological responses of the host needed for efficient antigen presentation, including the activation and apoptosis of macrophages (Meena, 2010, Sharma et al., 2012). To neutralize the effects of antioxidants, *M. tuberculosis* has the ability to detoxify oxygen radicals, using at least three mechanisms: i) the oxidative burst may be counteracted by production of catalase and superoxide dismutase enzymes; ii) compounds including glycolipids, sulfatides and lipoarabinomannose down regulate the oxidative cytotoxic mechanism (Chan et al., 1991, Chatterjee et al., 1992, Meena, 2010, Korb et al., 2016) and iii) macrophage uptake via complement receptors may bypass the activation of respiratory burst (Chan et al., 1991, Chatterjee et al., 1992, Meena, 2010, Korb et al., 2016). The high lipid concentration in the organism's cell wall offers considerable protection to *M. tuberculosis* which may involve three mechanisms: i) the thick cell wall causes impermeability and resistance to entry of antimicrobial agents; ii) the unique cell wall protects the bacilli from acidic and alkaline compounds in both the intracellular and extra-cellular environment and iii) the cell wall offers resistance to osmotic lysis via complement deposition and attack by lysozyme (Chan et al., 1991, Chatterjee et al., 1992, Meena, 2010). The inhibition of growth and killing of intracellular pathogens within the host cell of the mononuclear phagocyte lineage are considered to be dependent on phago-lysosome fusion however, *M. tuberculosis* bud out from the fused phago-lysosomes into vacuoles that fail to fuse to the secondary lysosome and thus escape lysosomal killing (Hackam et al., 1998, Meena, 2010, Korb et al., 2016).

2.7 CLINICAL MANIFESTATIONS OF PULMONARY AND EXTRA-PULMONARY TB

The clinical manifestations of TB occur progressively in stages which include latency, primary progressive disease and extra-pulmonary disease and are dependent on age, immune status and co-existing diseases (Knechel, 2009). Persons with latent TB have no signs or symptoms of the disease, do not feel sick and are not infectious (Guyot-Revol et al., 2006, Campion et al., 2015). Approximately one-third of the world's population is asymptomatically infected with *M. tuberculosis* (Rajagopalan, 2016). The *M. tuberculosis* organisms are enclosed and inactive (Guyot-Revol et al., 2006). However, viable bacilli can persist in the necrotic material for years or even a lifetime if the immune system is not compromised (Jensen et al., 2005). Active TB develops in only 5% to 10% of persons exposed to *M. tuberculosis* (Zumla et al., 2013, Rajagopalan, 2016). Co-infection with HIV/AIDS is the most notable cause for the progression to active disease although other factors such as diabetes mellitus, sepsis, renal failure, old age, malnutrition, smoking, chemotherapy and immune-suppression associated with organ transplantation can trigger reactivation of latent TB (Zumla et al., 2013). There are two main types of clinical manifestations of tuberculosis (TB) which include pulmonary and extra pulmonary TB (Singh, 2018).

Manifestations of TB often include progressive coughing lasting more than three weeks, chest pain with breathing or coughing, fatigue, malaise, weight loss, and low grade fever accompanied by chills and night sweets (Paton et al., 2004, Knechel, 2009). Wasting, a classic feature of TB is due to the lack of appetite and involves the loss of both fat and lean tissue (Ddungu et al., 2006). A cough develops in most patients which eventually advance to a productive cough of purulent sputum (Knechel, 2009). The sputum may also be streaked with blood (hemoptysis) due to destruction of a vessels located in the wall of the cavity, or the formation of an aspergilloma in an old cavity (Knechel, 2009).

Extra-pulmonary disease occurs in more than 20% of immune-competent patients, and the risk of extra-pulmonary TB increases with immune-suppression with HIV positive patients more than 50% have (Knechel, 2009, Lam et al., 2016). Miliary TB progresses rapidly and can be difficult to

diagnose because of its systemic and nonspecific signs and symptoms such as fever, weight loss and weakness (Knechel, 2009). The most serious location of extra-pulmonary TB is the central nervous system, where the infection may result in almost always fatal tubercular meningitis or space occupying tuberculomas (Frieden *et al*., 2003; Knechel, 2009, Cochicho et al., 2016). Other sites involving extra-pulmonary TB include bones, joints, pleura, the lymphatic and genitourinary systems, pericardial TB gastrointestinal TB and showing symptoms such as difficulty in swallowing (Knechel, 2009, Lam et al., 2016, Tuli, 2016).

2.8 TREATMENT OF *MYCOBACTERIUM TUBERCULOSIS*

Treatment of TB is not only aimed to cure the disease but also to interrupt the transmission and prevent relapse (Falzon et al., 2011, D'Ambrosio et al., 2015). In patients with drug-susceptible TB a global standard first-line TB treatment is a short-course regimen and is used in most high burden countries on drug-sensitive TB which include INH, RIF, PZA, EMB and SM (Zumla et al., 2013, Alqahtani and Asaad, 2014). Each treatment regimen for pulmonary TB caused by susceptible organisms has an initial 2 months intensive phase with INH, PZA, EMB and RIF, followed by a continuation phase with INH and RIF for 4 to 6 months (Zumla et al., 2015, Alqahtani and Asaad, 2014). The current standard four-drug treatment regimen of first-line drugs achieves cure rates of more than 90% in treatment under oversight of tuberculosis-control programs (Zumla et al., 2015, WHOb, 2017, Gilpin et al., 2018). Risk factors for relapse include cavitation, extensive disease, non- adherence to treatment, immunosuppression, and a sputum culture that remains positive at 8 weeks (Zumla et al., 2013, Alqahtani and Asaad, 2014). If any of these risk factors is present, therapy may be extended up to 9 months (Zumla et al., 2013, Alqahtani and Asaad, 2014, WHOb, 2017, Gilpin et al., 2018). Streptomycin can be used as an interchangeable drug with EMB in the initial phase treatment in cases when the patient's *M. tuberculosis* isolate has been proved to be sensitive (Blumberg et al., 2003, D'Ambrosio et al., 2015).

2.8.1 Treatment of drug-resistant tuberculosis

The WHO issued a communication for treatment of MDR-TB and RR-TB (WHOa, 2018). The guidelines is determined by i) preference for oral over injectable agent ii) the results of drug-susceptibility testing iii) the reliability of existing DST methods iv) population drug resistance levels v) history of previous use of the medicine in a patients v) drug tolerability vi) and potential drug tolerability. Based on this the WHO guidelines classified available anti-TB drugs into three groups (see Table 2.1) (D'Ambrosio et al., 2015, Zumla et al., 2015). First-line anti-TB drugs (Group A) are medicines to be prioritised, Group B are medicines that have to be added to the priority list in group A, while group C are medicines to be included to complete the regimens and when agents from groups A and B cannot be used.

The WHO currently recommend a two-treatment regimen; the long regimen (which does not contain any injectable drugs) and shorter regimen (contain injectable drug) (WHOb, 2018). The long regimen recommended for MDR-TB and RR-TB is composed of bedaquiline, linezolid, levofloxacin (or moxifloxacin) and cycloserine or clofazimine taken for 18 to 20 months. With some drugs taken for shorter period (WHOb, 2018) The WHO shorter regimen consists of amikacin, moxifloxacin, prothionamide, clofazimine, pyrazinamide, high dose isoniazid and ethambutol taken for nine to 12 months. The regimen is different from previous short regimen since it does not contain kanamycin instead of amikacin. Currently the South African treatment regimen contain bedaquiline, moxifloxacin, ethionamide, clofazimine, high dose isoniazid, ethambutol and pyrazinamide (Ndjeka et al., 2018).

Table 2.1: WHO Grouping of medicines recommended for use in longer MDR-TB regime (WHOb, 2018)

Group	Medicine	Abbreviation
Group A **Include all three medicines** **(Unless they cannot be used)**	Levofloxacin or Moxifloxacin	LFX MXF
	Bedaquiline,	BDQ
	Linezolid	LZD
Group B **Add both medicines** **(Unless they cannot be used)**	Clofazimine	CFZ
	Cycloserine or Terizidone	CS, TRD
	Ethambutol	EMB
Group C **Add to complete the regimen and when medicines from groups A and B cannot be used**	Delamanid	DLM
	Pyrazinamide	PZA
	Imipenem-cilasstatin or Meropenem	IPM-CLN, MPM
	Amikacin or Streptomycin	AM , SM
	Ethionamide or Prothionamide	ETO, PTO
	p-aminosalicyclic acid	PAS

2.9 TREATMENT OF *M. TUBERCULOSIS* IN HIV POSITIVE INDIVIDUALS

Human lmmuno-defiency Virus (HIV) infection is the most important risk factor for the development of active TB (Abdool Karim et al., 2011, Blanc et al., 2011, Havlir et al., 2011). Human lmmuno-defiency Virus destroys CD4 lymphocytes and macrophages cells that play a central role in anti-mycobacterial defenses, leading to an increase in HIV replication and

accelerates progression of HIV infection, with attendant high mortality (Abdool Karim et al., 2011, Blanc et al., 2011, Havlir et al., 2011). Early initiation of antiretroviral therapy (ART) results in a reduction in mortality among patients with TB (Abdool Karim et al., 2011, Blanc et al., 2011, Havlir et al., 2011). Patients who do not receive ART and those with very low numbers of $CD4^+$ cells have a high short-term risk of death (Abdool Karim et al., 2011, Blanc et al., 2011, Havlir et al., 2011). WHO recommends that ART be started within the first 8 weeks after the initiation of TB treatment and that patients with a CD4 cell count of less than 50 per cubic millimeter receive ART early within the first 2 weeks (WHO, 2012). For ART in patients with active TB, regimens with non-nucleoside reverse transcriptase inhibitors are preferred, and efavirenz is the drug of first choice (Narendran and Swaminathan, 2016). Rifabutin is preferred to rifampicin because it is an less potent inducer of cytochrome P3A (Horne et al., 2011, Jo et al., 2013).

2.10 NEW DRUGS

Currently two new drugs have been approved by the WHO, United States Food and Drug Administration and by the European Medicines Agency and the WHO. These two drugs are Bedaquiline also known as TMC207 or Sirturo and delamanid (Diacon et al., 2014).

2.10.1 Bedaquiline

Bedaquiline is the first new anti-TB drug to be introduced into the market in almost 50 years (Andrews, 2016, Pontali et al., 2016, Sullivan and Amor, 2016). Bedaquiline belong to the diarylquinoline class of antibiotics which selectively targets the proton pump of ATP synthesis, leading to inadequate Adeno-Triphosphate synthesis, which is necessary for bacterial metabolism (Diacon et al., 2014, Pontali et al., 2016). The MICs of bedaquiline against *M. tuberculosis* is very low, and its bactericidal activity in the murine model is superior to that of INH and RIF (Diacon et al., 2014, Pontali et al., 2016). A number of studies have shown that a standard 2-month treatment regimen with Bedaquiline yielded high culture conversion rates, rapid sputum culture conversion and low acquired resistance to companion drugs in newly diagnosed MDR-TB cases (Diacon et al., 2014). Based on the available evidence, WHO recommended the use of Bedaquiline to treat

MDR-TB in adults when the following conditions are met, pharmacovigilance is in place, informed consent is ensured and monitoring is possible as safety concerns for this drug remain (Diacon et al., 2014, WHO, 2014). In 2014 the South African government began to use Bedaquiline to treat XDR-TB patients on a strict inclusion and exclusion criteria (Ndjeka, 2014, Ndjeka et al., 2015, Ndjeka et al., 2018). Expanded Access programme in South Africa showed 76% (48 out of 63) and 146/200 (73%) treatment success rate among patients who received Bedaquiline after 6 months (Ndjeka, 2014, Ndjeka et al., 2015). The French compassionate use programme has been similarly reassuring at 6 months with 97% (28/29) participants which had culture conversion with minimal side effects and deaths reported (Guglielmetti et al., 2015, Pym et al., 2015).

2.10.2 Delamanid pretomanid and repurposed drugs

Delamanid and pretomanid belong to the nitro-imidazoles class of antibiotics, presently undergoing phase II and phase III clinical trials (D'Ambrosio et al., 2015). They inhibit the synthesis of mycolic acids, which are components of the cell envelope of *M. tuberculosis* (D'Ambrosio et al., 2015). WHO recommends the use of delamanid when pharmacovigilance is in place and informed consent ensured treatment (Diacon et al., 2014). Pretomanid (previously known as PA-824) has low MIC for *M. tuberculosis* comparable to that of INH and thus was found to be effective in TB treatment (Diacon et al., 2014). In addition, sutezolid SQ109 and benzothiazinones are also under consideration and are in phase II of clinical trials (D'Ambrosio et al., 2015). Repurposed drugs are drugs formerly used to treat other infectious diseases and now they have been repurposed for treatment of TB. Clofazimine has been used to treat leprosy, linezolid and oxazolidone previously used for treatment of Gram-negative bacterial infections while, combinational therapy of amoxicillin/clavulanic acid was used in treatment of bacterial infections (Diacon et al., 2014, WHO, 2018). Clofazmine have now been included in the treatment of MDR and XDR TB regimen.

2.11 CROSS-RESISTANCE BETWEEN ANTI-TB DRUGS

All rifamycins, including rifapentine, rifabutin (RFB) and rifampicin are known to have a high level of cross-resistance (Uzun and Erturan, 2002, Senol et al., 2005, Chikamatsu et al., 2009). Many studies have shown cross-resistance between RFB and RIF, ranging from 54% to 88% (Uzun and Erturan, 2002, Senol et al., 2005, Chikamatsu et al., 2009). Fluoroquinolones have variable cross-resistance: in vitro data shows that *M. tuberculosis* strains remain susceptible to some newer-generation fluoroquinolones when they are resistant to earlier-generation fluoroquinolones (Sirgel et al., 2012, van der Heijden et al., 2013). Aminoglycosysides; kanamycin and amikacin exhibit high level cross-resistance while, viomycin shows high level of cross resistance with its structural analogue capreomycin (peptide) as these two drugs bind at the same site on the *M. tuberculosis* ribosome (Jugheli et al., 2009). Other aminoglycosides and polypeptides show lower degrees of cross-resistance. Protionamide has the same drug target as INH, namely, *inh*A, but is activated from the pro-drug form to the active drug by a different enzyme, *Eth*A, similar to ETH (Witney et al., 2016). As a result, protionamide and ETH show a reported 100% cross-resistance (Witney et al., 2016). In the same way ETH can exhibit cross-resistance with INH if the *inhA* mutation is present (Zhang, 2005, Witney et al., 2016).

2.12 PREVENTION AND CONTROL FOR *M. TUBERCULOSIS* INFECTION

The main reason of preventive therapy is to prevent latent (asymptomatic) infection from progressing to clinical disease as well as to prevent initial infection and to prevent recurrence. (Laupland and Valiquette, 2015). Persons with latent *M. tuberculosis* infection who are at increased risk of active TB require preventive treatment (Campion et al., 2015, Getahun et al., 2015). While chemotherapy is the mainstay prevention method used worldwide, prevention using vaccine or therapy with immune adjuvants are important potential options that have been evaluated in clinical trials in 2014 (Laupland and Valiquette, 2015).

2.12.1 Chemoprophylaxis as a method of prevention of active TB

Usually INH prophylaxis is used in situations which are known or suspected of posing a risk of primary TB infection (e.g HIV positive individuals and people living with TB patients) (Akolo et al., 2010). Isoniazid can be used alone in prophylaxis because the load of tubercle bacilli in a subclinical primary lesion is small in relation to that in reactivation TB (Zumla et al., 2013). Clinical trials have shown that isoniazid preventive therapy (IPT) dramatically reduces the incidence of active TB in individuals who have a positive tuberculin test (Akolo et al., 2010, Laupland and Valiquette, 2015). For the prophylactic use of drugs (chemoprophylaxis) to prevent TB disease, the WHO recommends the drug INH daily for 6 to 9 months and such regimens have efficacies ranging from 60 to 90% (Akolo et al., 2010, Sterling et al., 2016). A 2004 Cochrane review found that IPT reduced the risk of TB by 33% overall and by 64% when targeted to people living with HIV (Akolo et al., 2010, Laupland and Valiquette, 2015). Other studies have shown that three months of weekly rifapentine and isoniazid was more effective and safe for treatment of latent *M. tuberculosis* and had better tolerability and treatment completion than 9 months of IPT (Sterling et al., 2016).

2.12.2 Vaccines

Modeling studies show that without new vaccines TB can never be eliminated by advances in diagnostic and therapeutic strategies alone (Zumla et al., 2013). In addition, the poor cure rates, high case fatality and extraordinary cost of treating patients makes development of vaccine of high importance (Beresford and Sadoff, 2010, WHOc, 2018). A vaccine can have the potential to limit initial infection, progression of disease and reactivation of latent TB (Beresford and Sadoff, 2010). Currently there is only one licensed vaccine against TB which is available worldwide: Bacille Calmette-Guerrin (BCG) (Zumla et al., 2013). This vaccine has been used since 1921 and protects children from the severe forms of TB such as meningeal TB and other disseminated forms of TB in infants and children (Colditz et al., 1995, Trunz et al., 2006, Zumla et al., 2013). The BCG vaccine comprises a live attenuated strain of *M. bovis* that has lost its virulence by passing through 230 in vitro passages over a 13 year period (Liu et al., 2009). This vaccine is widely used in TB endemic countries where newborns are immunized with a single intra-dermal dose soon

after birth (Hesseling et al., 2008). The BCG vaccine has been shown to provide protection against pulmonary TB with an efficacy varying between nil to 80% (Colditz et al., 1994, Behr, 2002, Ottenhoff and Kaufmann, 2012). Therefore, since pulmonary TB is the most prevalent form of disease in both adolescents and adults, BCG is estimated to have little impact in limiting TB spread (Trunz et al., 2006). A safer and more effective vaccine needed that can either boost BCG's initial effects (booster vaccine) or replace BCG (priming vaccine). Currently fourteen candidate vaccines are in clinical trials, and only one vaccine the Chinese candidate, "Vaccae, has entered phase 3 testing (WHOc, 2018). The TB vaccine pipeline incorporates various vaccine platforms including whole cell vaccines, adjuvanted proteins, and vectored subunit vaccines (WHOc, 2018) .

2.13 DIAGNOSIS OF LATENT TB INFECTION

The screening and treatment for latent *M. tuberculosis* infection is often recommended for high risk groups including individuals from regions and populations in which TB is endemic (Africans and Asian descendants), persons in whom risk of reactivation disease is high which includes HIV positive patients, patients receiving immune-suppressive drugs therapy, as well as recent contacts with a TB infected person (Horsburgh Jr and Rubin, 2011, Ferrara et al., 2012, Zumla et al., 2013). Latent infection can be diagnosed with either a Mantoux test also known as tuberculin skin test or an interferon-gamma release assay (Mazurek et al., 2010, McNerney et al., 2012).

2.13.1 The Tuberculin skin test (Mantoux test)

Mantoux test first introduced in 1890, is a delayed hypersensitivity test known as the tuberculin skin test where a purified protein derivative (PPD) is employed as the antigen (Franken et al., 2007, McNerney et al., 2012). Purified protein derivative is prepared by attenuating a culture of *M. tuberculosis, M. bovis* and other non-tuberculous mycobacteria (Franken et al., 2007, McNerney et al., 2012). The TST is less expensive however it is subjective to interpretation errors and need several visits to health facility to get results.

2.13.2 Interferon Gamma Release Assays (IGRAs)

Two commercially available blood tests for detecting latent TB infection, the QuantiFERON TB-Gold (QFT-D, Qiagen, GmbH, Germany) and the T-SPOT-TB (Oxfold Immunotec Limited, Abingdon, UK) have been developed (Mazurek et al., 2010, McNerney et al., 2012, Pai et al., 2014). In these tests interferon γ-secretion from T-cells is measured after exposure to the *M. tuberculosis* derived antigens ESAT-6, TB7.7 and CFP-10 that are specific for the *M. tuberculosis* complex (Mazurek et al., 2010, McNerney et al., 2012, Pai et al., 2014). Secretion of INF-γ indicates either latent or active infection, but is not suitable to distinguish the two states of infection (Pai et al., 2014). Because the antigens used are not present in either the BCG vaccine or in most non-tuberculosis mycobacteria (NTM), these tests are more specific than tuberculin-based tests (McNerney et al., 2012). The IGRAs are expensive and also, false negatives are seen in AIDS patients, as well as other conditions such as malnutrition, and in patients on steroid therapy (Franken et al., 2007, McNerney et al., 2012).

2.13.3 Chest X-rays

Chest radiographs are among the most commonly used tools for TB diagnosis (Geng et al., 2005, Ryu, 2015, Curtis, 2016). A posterior-anterior radiograph is used to check for lung abnormalities in people who have signs and symptoms of TB disease in the lungs (Geng et al., 2005, Curtis, 2016). Computer-aided readings of Chest-X-rays have been reported with higher sensitivity and specificity (Melendez et al., 2016). Chest X-rays have advantages of being able to detect early disease of TB (Ryu, 2015, Curtis, 2016). However, chest X-rays may only suggest that TB disease is present, changes alone cannot definitely diagnose TB infection (Ryu, 2015, Curtis, 2016) and cannot distinguish pathological manifestation of pulmonary TB from other pulmonary infections such as nocardiosis which is closely related to TB (Harries et al., 2004). Also, chest X-rays cannot distinguish previously treated TB from active TB, as post-primary TB heals with parenchymal scarring and nodules, thus one cannot determine whether these residual findings are indicative of active disease or past disease (Ryu, 2015).

2.14 DIAGNOSIS OF ACTIVE TB INFECTION

2.14.1 Sputum Microscopy

Sputum smear microscopy is the most commonly used method to diagnose active TB especially in limited resources setting (Steingart et al., 2006). A number of methods are used for acid-fast staining including Ziehl-Neelsen and Kinyoun methods (Marais et al., 2008, Magalhães et al., 2018). Improved visualization of *M. tuberculosis* is conducted using the light emitting diode or the fluorescent microscopy using auramine–O or auramine-rhodamine dyes (Marais et al., 2008, Goel et al., 2018). Smear examination is rapid (results within 2 hours), inexpensive, technically simple and highly specific method for detection of acid-fast bacilli (Urbanczik, 1985, Mfinanga et al., 2008). In some developing countries, microscopic examination of sputum smears is the only tool available for the laboratory diagnosis of TB (Zingue et al., 2018). However, smear microscopy lacks sensitivity, varying between 20-60% as between 5, 000 to 10, 000 bacteria/ml are needed for a positive result (Urbanczik, 1985, Mfinanga et al., 2008). Furthermore, microscopy gives presumptive diagnosis since it cannot discriminate between *M. tuberculosis* and other mycobacteria (Urbanczik, 1985, Mfinanga et al., 2008). The identification of pulmonary TB patients by smear microscopy is regarded as indicating a highly infectious state in the patients (Urbanczik, 1985, Mfinanga et al., 2008). Thus microscopy remains the primary diagnostic tool for TB diagnosis.

2.14.2 Culture

Culture techniques refer to methods used to grow *M. tuberculosis* in a laboratory. Culture of *M. tuberculosis* is often used as the reference or gold standard method for TB diagnosis (Parsons et al., 2011). Culture is also essential for species identification and drug susceptibility testing (Parsons et al., 2011). Traditionally, egg-based or agar-based solid media have been used for the isolation of mycobacteria (Ängeby et al., 2003, Woods et al., 2007, Richter et al., 2009, Forbes et al., 2018). Culture can detect as low as 10 bacterial/ml of sputum and the sensitivity is lower for mucoid/salivary sputum (Acuña-Villaorduña et al., 2017). However, it is laborious and require complex laboratory facilities (biosafety cabinets) for handling of *M. tuberculosis* organisms

(WHO, 2016a, Magalhães et al., 2018). A major drawback of this test is the length of time it takes to obtain the results which can range from 2-8 weeks. This delay may cause patient death and further transmission of the disease (Ängeby et al., 2003, Woods et al., 2007, Richter et al., 2009).

2.15 DIAGNOSIS OF DRUG RESISTANCE IN *M. TUBERCULOSIS*

By

For decades, TB diagnosis has relied almost entirely on smear microscopy, which did not provide any information on drug resistance and most patients were put onto a standardized first-line regimen without any knowledge of drug susceptibility testing (Wells et al., 2013, Alqahtani and Asaad, 2014). However, the increasing awareness of MDR and XDR-TB has drawn greater attention to the need for DST, with a number of methods being continuously developed (Wells et al., 2013, Alqahtani and Asaad, 2014).

2.15.1 Conventional drug susceptibility testing methods

The WHO and the International Union against Tuberculosis and Lung Disease in 1945 agreed on three categories of acceptable methods for susceptibility testing: i) the absolute concentration method, ii) the resistance ratio method and the iii) proportion method. In practice all three principal methods can perform adequately, provided that the technical protocols are followed exactly (Vareldzis et al., 1994, Heifets, 1996).

2.15.1.1 The absolute concentration method

The drug to be tested is incorporated into agar or Lowenstein-Jensen medium at a fixed concentration (and for some drugs at two fixed concentrations), or is used in a broth dilution method based on growth in fixed concentrations of drugs in a liquid medium. The limit of susceptibility is defined as the lowest concentration of the drug that inhibits growth (<20 colonies) (Vareldzis et al., 1994, Heifets, 1996, Drobniewski et al., 2007). Drug concentrations, and particularly inoculum sizes, must be carefully standardized with reference to wild-type cultures. Variation in inoculums size is the major source of error in this method (Vareldzis et al., 1994, Heifets, 1996, Drobniewski et al., 2007).

2.15.1.2 The Resistance ratio method

This is a refinement of the absolute concentration method, in which the minimal inhibitory concentration for a given isolate is compared with the MIC of a known susceptible strain when the test and control isolates are tested on a solid medium containing different drug concentrations (Canetti et al., 1963, Canetti et al., 1969, Heifets, 1996, Drobniewski et al., 2007). The resistance ratio methological approach to DST is based on the MIC for the test isolate divided by the MIC for a standard susceptible strain e.g $H_{37}Rv$, or a recently isolated susceptible wild type strain (Canetti et al., 1963, Canetti et al., 1969, Heifets, 1996, Drobniewski et al., 2007). If the ratio is two or less, or eight or more, the isolate is fully susceptible or highly resistant, respectively. Inoculum size needs to be standardized, but the critical concentration does not need to be determined because of the direct comparison with a susceptible isolate (Canetti et al., 1963, Canetti et al., 1969, Heifets, 1996, Drobniewski et al., 2007).

2.15.1.3 Proportion method

The proportion method compares the degree of growth of test organism in drug containing media compared to drug free media. In the agar proportion method, a strain is classified as susceptible if its constituent cell population contains below a critical proportion of resistant cells, and as resistant if above this proportion (Canetti et al., 1963, Canetti et al., 1969, Vareldzis et al., 1994). The proportion varies with different drugs for example 1% for INH or RIF or 5% for PZA. In practical terms, the proportion of drug-resistant mutants is obtained from the ratio of the number of colonies growing on drug-containing medium and number of colonies on drug-free medium (Canetti et al., 1963, Canetti et al., 1969, Vareldzis et al., 1994). The ratio of growth indices obtained by inoculation of the test isolate in drug-containing medium and inoculation of a 100-fold dilution (1%) of the isolate in drug-free medium (Canetti et al., 1963, Canetti et al., 1969, Vareldzis et al., 1994). Standardization of the inoculation, although essential, is not as critical in this method (Canetti et al., 1963, Canetti et al., 1969). The proportion method is currently the most commonly used method for the determination of drug susceptibility worldwide.

2.15.1.4 Disadvantages of conventional methods

Although conventional methods have been used widely, a low reproducibility of a procedure have been reported due to poor standardization (Shiferaw et al., 2007, Richter et al., 2009). Higher sensitivities and specificities are found for INH and RIF than for EMB and SM testing (cumulative sensitivities: 99% for INH, 97% for RIF, 91% for SM and 89% for EMB; cumulative specificities: 98% for INH, 97% for RIF, and 94% for both EMB and SM) have been reported (Shiferaw et al., 2007, Richter et al., 2009). For second-line drugs mostly the convenctional DST method has many drawbacks, the critical concentrations used in convenctional DST are not as clear cut, often owing to the fact that the MICs are close to the assumed critical concentrations (Shiferaw et al., 2007, Richter et al., 2009). The main disadvantage of conventional DST is the long turnaround time, as these methods depend on primary culture to retrieve an isolate for inoculation and DST results may take an additional 2 to 6 weeks (Shiferaw et al., 2007, Richter et al., 2009). This adds up to a 1-3 month delay in the determination of drug resistance (Shiferaw et al., 2007, Richter et al., 2009).

2.16 RAPID DRUG SUSCEPTIBILITY TESTING METHODS FOR *M. TUBERCULOSIS*

To overcome the drawbacks of conventional methods, numerous new techniques have become available with the aim of providing more rapid detection of resistance which includes the liquid culture and molecular methods (Kim, 2005, Krüüner et al., 2006, Lin et al., 2009, Moore and Shah, 2011).

2.16.1 Liquid culture-based DST methods

Rapid liquid culture-based techniques have been established that can detect growth-dependent changes such as CO_2 production (BACTEC 460 and MB/BacT) or oxygen consumption (Mycobacteria Growth Indicator Tube [MGIT] and VersaTREK) (Ängeby et al., 2003, Woods et al., 2007, Moore and Shah, 2011). Other techniques rely on the ability of living cells to convert an oxidation–reduction indicator dye into the reduced state (resazurin and tetrazolium bromide) or on the very specific property of *M. tuberculosis* to reduce nitrate to nitrite) (Ängeby et al., 2003,

Woods et al., 2007, Moore and Shah, 2011). Detection of growth can also be achieved by microscopic observation of liquid cultures in tissue-culture plates (microscopic-observation drug-susceptibility [MODS] assay) (Piersimoni et al., 2006, Martin et al., 2008, Van Deun et al., 2010). Since mycobacteriophages only replicate in living cells, phage-based tests have also been developed for speeding up DST (Tortoli et al., 1997, Scarparo et al., 2004, van Klingeren et al., 2007).

2.16.1.1 BACTEC 460 TB system

BACTEC 460 TB system, based on the determination of radiolabelled ^{14}C, was one of the first liquid based methods and was regarded as the standard for rapid and reliable DST results (Rohner et al., 1997, Woods et al., 2007). Costly machines together with needles (with the hazard of needle-stick injury/infection) are required to set up the system (Rohner et al., 1997, Woods et al., 2007). Precautions for the handling and waste disposal of radioactive substances render this system fraught with risk and impractical (Rohner et al., 1997, Woods et al., 2007). Because of these problems, newer methods have been established with non-radiometric techniques for growth detection.

2.16.1.2 BACTEC MGIT 960 systems

The manual and automated *Mycobacterium* Growth Indicator Tube (MGIT) systems (Becton Dinckinson Microbiology System, Sparks, NV,USA) are commercial tests based on liquid medium introduced to replace radioactive BACTEC 460 systems (Piersimoni et al., 2006, Martin et al., 2008, Van Deun et al., 2010, Alqahtani and Asaad, 2014). The MGIT system uses 7H9 broth together with a fluorescence quenching–based oxygen sensor (Piersimoni et al., 2006, Martin et al., 2008, Van Deun et al., 2010, Alqahtani and Asaad, 2014). When inoculated with *M. tuberculosis*, consumption of oxygen occurs owing to bacterial growth which produces fluorescence when illuminated by an ultraviolet light source (Van Deun et al., 2010). The fully automated MGIT system can incubate up to 960 samples for *M. tuberculosis* and can be used for identification through culture of samples (pulmonary and others), and drug sensitivity testing. It offers the possibility of performing DST using prepared kits, which are available for susceptibility

testing of INH, RIF, EMB and SM, as well as pyrazinamide (PZA) (Piersimoni et al., 2006, Martin et al., 2008, Van Deun et al., 2010, Abuali et al., 2012, Alqahtani and Asaad, 2014). The test is based on detection of growth in the antibiotic-containing media compared with an antibiotic free tube, which is inoculated with 1:100 dilution of the TB strain, or a 1:10 in case of PZA dilution of *M. tuberculosis* growth (Martin et al., 2008, Rodrigues et al., 2008, Van Deun et al., 2010, Alqahtani and Asaad, 2014).

The MGIT system reduces the turnaround time for culture and DST results from 6-12 weeks to 3-4 weeks, respectively (Martin et al., 2008, Van Deun et al., 2010, Alqahtani and Asaad, 2014, Ravibalan et al., 2016). The MGIT has a high throughput capacity, is automated, and has the convenience of standardized reading of samples (Van Deun et al., 2010, Alqahtani and Asaad, 2014). A disadvantage of the MGIT is that it needs continuous, stable electricity to maintain a constant incubator temperature and prevent loss of data, often requiring a back-up generator (Piersimoni et al., 2006, Martin et al., 2008, Van Deun et al., 2010). Technical support and maintenance are essential and some expensive reagents have shelf-lives of half a year or less upon arrival (Piersimoni et al., 2006, Martin et al., 2008, Van Deun et al., 2010, Alqahtani and Asaad, 2014). In addition, liquid media are prone to contamination, leading to failed testing and unreliable results (Piersimoni et al., 2006, Martin et al., 2008, Van Deun et al., 2010, Alqahtani and Asaad, 2014, Ravibalan et al., 2016).

2.16.1.3 BacT/Alert 3D

The BacT/Alert 3D system (formerly known as MB/BacT; bioMerieux, Marcy L'Etoile, France) is also a non-radiometric, fully automated, continuously monitoring liquid culture system. Growing microorganisms produce CO_2, which induces a color change in a sensor (Colditz et al., 1995, Garrigó et al., 2007, Wilson et al., 2016). It was introduced not only for the primary isolation of mycobacteria, but also for susceptibility testing (Garrigó et al., 2007, Wilson et al., 2016). Several studies have evaluated the MB/BacT 3D system for rapid DST of *M. tuberculosis* to first-line and second-line drugs (Woods et al., 2007, Garrigó et al., 2007, Martinez et al., 2014, Wilson et al., 2016). In these studies, performance values for all drugs were high (Garrigó et al., 2007, Martinez

et al., 2014, Wilson et al., 2016). However, there is no commercially available kit and no standardized protocol for performing DST (Garrigó et al., 2007, Wilson et al., 2016).

2.16.1.4 The VersaTREK system

VersaTREK system (TREK Diagnostic Systems, Cleveland, OH, USA) is also an automated, non-radiometric liquid culture system (Ruiz et al., 2000, Palomino et al., 2008, Wilson et al., 2016). The detection of growth is based on gas-related pressure changes resulting from multiplying bacteria (Wilson et al., 2016). A DST kit is available for testing INH, RIF and EMB. Only very few studies are available on the performance of this system (Ruiz et al., 2000, Palomino et al., 2008, Palomino and Martin, 2016, Wilson et al., 2016).

2.16.2 Colorimetric Assays:

Colorimetric methods use either an oxidation-reduction indicator or nitrate reduction detection. “ln- house assays” have been developed which are based on metabolic activity of the mycobacteria, which can be detected much earlier than the visual identification of colonies (Martin et al., 2008). Since the color change can be detected visually, no additional devices are necessary (Martin et al., 2008). Two dyes are currently available; the resazurin, an oxidation–reduction indicator dye, which is marketed as alamarBlue®, and tetrazolium bromide 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-^{2}H-tetrazoliumbromid, an indicator which is reduced by dehydrogenases of living cells (Abate et al., 2004, Montoro et al., 2005, Martin et al., 2005, Raut et al., 2008, Martin et al., 2008). For both dyes, assays have been established in microtiter plate format. The dyes are added to control wells after several days of incubation (Abate et al., 2004, Montoro et al., 2005, Martin et al., 2005, Raut et al., 2008). If a change in color indicates sufficient growth, the dye is also added to the antibiotic-containing wells. A color change in these wells then indicates resistance of the strain. In many studies, the assays were performed in microtiter plates, which need to be opened after several days of incubation for the addition of the indicator dye. This procedure poses a risk to the laboratory personnel since the use of liquid medium in microtiter plates is prone to create aerosols (Abate et al., 2004, Montoro et al., 2005, Martin et al., 2008, Raut et al., 2008, Shikama et al., 2009). To prevent this risk, some studies have been performed using

tubes instead of microtiter plates, which also gave comparable results. This more expensive and time-consuming than working with microtiter plates (Martin et al., 2008, Raut et al., 2008, Shikama et al., 2009). Another disadvantage of these tests is the variety of protocols used in the studies, so more standardization is needed for the performance of these assays (Abate et al., 2004, Montoro et al., 2005, Martin et al., 2008, Raut et al., 2008, Shikama et al., 2009) .

2.16.3 Microscopic-observation drug-susceptibility Assay

Microscopic observation broth-drug susceptibility assay (MODS) is based on the observation of the characteristic cord-formation of *M. tuberculosis* in liquid culture (Middlebrook 7H9) medium using an inverted light microscope (Caviedes et al., 2000, Park et al., 2006, Moore et al., 2006). The MODS assay can be performed from cultures and directly from decontaminated sputum samples (Moore et al., 2006, Mello et al., 2007, Ha et al., 2010). The strains or the sputum samples are inoculated into microtiter plate wells and drug containing and drug-free control wells allow DST for several test drugs. Results of DST are obtained in 15 to 29 days from direct sputum analysis (Bwanga et al., 2009). The method is approximately 90% sensitive and has a high specificity of approximately 99% for INH and RIF (Minion et al., 2010).

The MODS assay guarantee a low material and running costs for the test compared with the automated liquid culture systems growth (Caviedes et al., 2000, Park et al., 2006, Moore et al., 2006). However, potential challenges include the need for meticulous technique since MODS requires delicate inoculation and plate handling to prevent cross-contamination (Caviedes et al., 2000, Park et al., 2006, Moore et al., 2006). The requirement of an inverted microscope and biosafety concerns related to extensive handling of liquid cultures may restrict its use in laboratories (Caviedes et al., 2000, Park et al., 2006, Richter et al., 2009). Also, reading of the plates has to be performed on a daily basis and is therefore, laborious and time-consuming (Park et al., 2006, Bwanga et al., 2000, Minion et al., 20109). Furthermore, the identification of *M. tuberculosis* can be ambiguous since typical cording but can sometimes be seen in non-tuberculous *Mycobacterium* species (e.g *M. kansansii*) (Bwanga et al., 2009, Richter et al., 2009). Use of ziplock plastic bags which is opened after the whole procedure also presents a safety risk (Caviedes et al., 2000, Park et al., 2006, Richter et al., 2009).

2.16.4 Slide DST method

The slide DST method is similar to MODS, but is safer and requires less equipment. The requirement of the decontamination process and Ziehl-Neelsen staining render it potentially more error proof than MODS. Quality assurance constitutes a challenge since control strains cannot be used (Kim et al., 2008, Alqahtani and Asaad, 2014).

2.16.5 Micro-colony method

The micro-colony method, also known as the thin-layer agar (van der Heijden et al. 2013) method is performed on Middlebrook 7H11/7H10 agar for the rapid detection of mycobacterial micro-colonies by conventional microscopy (Martin et al., 2009, Satti et al., 2010). Its sensitivity for detection of *M. tuberculosis* has been reported in one study as comparable to MGIT system (Satti et al., 2010). This method is rapid and simple, allowing simultaneous detection of TB and resistance to RIF and INH, but more evaluation is still required. The excessive workload as a result of repeated microscopic reading of all plates and the need for a CO_2 incubator is disadvantages (Leung et al., 2012). The pooled sensitivity of TLA was found to be 87%, while sensitivity was found to be 98% (Leung et al., 2012).

2.16.6 Nitrate-reduction assay

The Nitrate Reductase Assay (NRA) also known as the Griess method is based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite for convenctional biochemical identification of *M. tuberculosis* (Martin et al., 2008). By incorporating potassium nitrate (KNO_3) into media, the reduction of nitrate can be detected using the Griess reagent, which produces a coloured reaction when reduction occurs (Martin et al., 2008, Van Deun et al., 2010). In the presence of antibiotic at the critical concentration, development of a red-pink colour in the medium represents resistance (Martin et al., 2008, Van Deun et al., 2010). Susceptible strains lose the capacity to reduce nitrate in the presence of the antibiotic, thus produce no colour (Martin et al., 2008, Van Deun et al., 2010). The assay is simple and rapid and can be an inexpensive alternative method for the rapid

and accurate detection of resistance to all first-line drugs in culture isolates (Van Deun et al., 2010). A limitation of the NRA is that it cannot be used for the rare nitrate reductase-negative *M. tuberculosis* or for *M. bovis* strains (Van Deun et al., 2010, Halwai et al., 2018).

2.16.7 Mycobacteriophage-based Assays

The mycobacteriophage assays utilize bacteriophage viruses to infect and detect the presence of viable *M. tuberculosis* in clinical specimens and culture isolates (Muzaffar et al., 2002, Albert et al., 2004). In the presence of a drug at critical concentration, the phages are only able to replicate if a drug-resistant strain is present (Wilson et al., 1997, Albert et al., 2001, Pai et al., 2009, Wilson, 2013). Two main approaches have been developed: i) amplification of phages after their infection of *M. tuberculosis* followed by detection of progeny phages using sensor bacteria and measuring plaque formation and ii) detection of light produced by lucifease reporter phages after their infection of live *M. tuberculosis* (visualization of the amplified phages can be performed either by counting the plaques or by determining of the light emission) (Wilson et al., 1997, Albert et al., 2001, Pai et al., 2009, Wilson, 2013). Most evaluation studies using phage assays are restricted to the detection of RIF resistance in culture isolates and also in direct patient specimens (Albert et al., 2001, Pai et al., 2009). The mycobacteriophage method allows for rapid, accurate and simple, DST detection showing high sensitivity and slightly lower specificity.

However, the mycobacteriophage assay requirement of engineered phages (e.g Luciferase reporter phage) and the detection format (photographic film or luminometer) hampered its wide application in diagnostic clinical laboratories, and it seems more appropriate for use in research laboratories (Wilson et al., 1997, Albert et al., 2001, Albert et al., 2002, Pai et al., 2009, Wilson, 2013).

2.17 GENETIC BASED TECHNIQUES

The detection of genetic mutations that are linked with resistance to certain antibiotics using molecular tests has become acknowledged as tools for research and diagnostic purposes (Wilson et al., 1997, Albert et al., 2002, Pai et al., 2009, Wilson, 2013, Sharma et al., 2016a). These methods offer several advantages which include faster turnaround times and the opportunity of omitting the

cultures (Boehme et al., 2011, Alqahtani and Asaad, 2014, Sharma et al., 2016a, Sharma et al., 2016b). Resistance to anti-TB agents develops by sequential acquisition of mutations in target genes owing to the selection of mutants arising from natural mutations occurring at known mutation rates in genomic DNA (Maus et al., 2005). As a result, the knowledge of specific mutations that are associated with resistance are a pre-requisite to using molecular techniques (Richter et al., 2009). The most commonly used commercially available methods include the line probe assay (Hain Lifescience , Nehren, Germany) and Xpert® MTB/RIF (Cepheid, Sunnyvale, CA) (Richter et al., 2009, Blakemore et al., 2010, Ryu, 2015).

2.17.1 Line-probe Assays

Line-probe assays use a conventional multiplex PCR and reverse-hybridization to probes immobilized on a plastic strip (Mäkinen et al., 2006, Miotto et al., 2006). The absence of a wild-type band or the appearances of bands representing specific mutations indicating the existence of a resistant strain (Ling et al., 2008, Hillemann et al., 2005, Mäkinen et al., 2006, Miotto et al., 2006, Rahman et al., 2016, Paramasivan and Raizada, 2016). Currently there are two commercially available line-probe assays, the INNO-LiPA® Rif. TB assay (Innogenetics, Ghent, Belgium) and the GenoType® MTBDR*plus* assay (Hain Lifescience, Nehren, Germany). The newer version of the GenoType® MTBDR*plus* assay, the GenoType® MTBDR*plus VER2*, is recommended for smear positive pulmonary samples as well as smear negative samples while the former is recommended for smear positive specimens. The INNO-LiPA® Rif TB kit targets the *rpoB* for RIF resistance and GenoType® MTBDR*plus* targets *rpoB*, *katG* and *inhA* for RIF and INH resistance (Hillemann et al., 2005, Mäkinen et al., 2006, Miotto et al., 2006, Hillemann et al., 2007, Hillemann et al., 2009).

The INNO-LiPA assay, when performed on cultures, was found to have high sensitivity and specificity values (>95 and 100%, respectively) but a lower sensitivity (80–100%) when performed directly on clinical specimens (Hillemann et al., 2005, Mäkinen et al., 2006, Miotto et al., 2006, Hillemann et al., 2007, Hillemann et al., 2009). The pooled sensitivity and specificity values of the MTBDR*plus* assays for RIF resistance were found to be 98.1% and 98.7%, respectively, and consistent across all subgroups, assay versions and specimen types (clinical specimens or culture

isolates) (Lin et al., 2009). The accuracy for INH was variable with lower and more inconsistent sensitivity (84.3%), despite high specificity (99.5%) (Hillemann et al., 2005, Mäkinen et al., 2006, Miotto et al., 2006). Based on these findings, the WHO released a policy in 2008, recommending the use of the GenoType MTBDR *plus* assay for the rapid screening of MDR-TB in low and middle income settings (WHO, 2009).

The GenoType® MTBDR*sl* assay (Hain Lifescience, Nehren, Germany) was developed for detection of resistance to second-line drugs fluoroquinolones, capreomycin, amikacin, kanamycin and viomycin resistance. The GenoType® MTBDR*sl* assay is commercially available in two version 1.0 and later version 2.0. Version 2.0 detects mutations in gyrB and *eis* in addition to *gyrA*, *rrs* mutation of ver 1.0. However, *emb*B mutation for EMB resistance is excluded in version 2.0 kit. (Hillemann et al., 2007, Hillemann et al., 2009, Richter et al., 2009, Tomasicchio et al., 2016). The GenoType® MTBDR*sl* assay aims for the rapid identification of patients with XDR-TB (Richter et al., 2009, Tomasicchio et al., 2016). The assay is performed with the same equipment and protocols as the GenoType MTBDR*plus* assay and can thus be easily combined with this assay in the laboratory workflow (Tomasicchio et al., 2016). High specificity values were obtained (100% for OFX, AMK and EMB; and 99.1% for capreomycin) (Hillemann et al., 2007, Hillemann et al., 2009, Tomasicchio et al., 2016). However, the sensitivity varied markedly from 90.2% for OFLX, 86.8% capreomycin, and 83.3% AMIK to 59.0% EMB (Hillemann et al., 2007, Hillemann et al., 2009).

A major limitation of line probe is the limited number of mutations that can be detected with a single test (Hillemann et al., 2007, Hillemann et al., 2009, Tomasicchio et al., 2016). Line-probe assays only allow the detection of the most frequent mutations leading to resistance. In the case of mutations outside the targeted regions, assay may be incorrectly read as susceptible results (Richter et al., 2009, Tomasicchio et al., 2016, Bai et al., 2016, Rahman et al., 2016, Paramasivan and Raizada, 2016). If mutations are only indicated by the omission of wild-type bands, valuable knowledge of the specific genetic alteration(s) involved is sometimes misleading to non-interpretable results or unexplained heterogeneity (Bai et al., 2016, Rahman et al., 2016, Paramasivan and Raizada, 2016).

2.17.2 Real-time PCR techniques for susceptibility testing of *M. tuberculosis:*

At present, a number of real-time PCR instruments are available, together with several fluorescence formats for correlating the amount of PCR product with fluorescence signals emitted (Boehme et al., 2011). All real time systems have advantages of running the reaction as a closed system and therefore diminishing the chances of contamination (Richter et al., 2009, Boehme et al., 2011).

In 2010 WHO endorsed real-time based PCR for the direct identification of *M. tuberculosis* complex bacteria with simultaneous detection of RIF resistance from specimens (Xpert® MTB/RIF, Cepheid) (WHO, 2011). The Xpert MTB/RIF is a cartridge based nucleic acid amplification test that simultaneously identifies *M.tuberculosis* DNA and resistance to RIF in less than two hours (Vadwai et al., 2011, Kaur et al., 2016). The system has the additional feature of being fully automated from DNA extraction to the PCR and post-PCR analysis (Blakemore et al., 2010, Boehme et al., 2011, Alqahtani and Asaad, 2014). For this reason, this assay is one of the most common molecular which has seen its use in more than 120 countries whether or not strict safety standards for culturing mycobacteria have been realized in the laboratory (Maus et al., 2005). Xpert MTB/RIF assay can be used directly on the unprocessed clinical specimens, either raw sputum samples or sputum sediments obtained after decontamination of the sample and concentrating it (Blakemore et al., 2010, Kaur et al., 2016). Xpert MTB/RIF is less prone to contamination and is more biocompatible as it has tuberculocidal properties and does not pose a biosafety concern lab workers (Steingart et al., 2006, Kaur et al., 2016). The Xpert MTB/RIF has better sensitivity than sputum smear microscopy and in South Africa in 2011 a policy was made to replace sputum microscopy with Xpert MTB/RIF as the first-line test for tuberculosis across the entire national health laboratories (Blakemore et al., 2010, Vadwai et al., 2011, Meyer-Rath et al., 2012).

The, development of the Xpert MTB/RIF assay was a major step forward for improving the diagnosis of tuberculosis (TB) and rifampicin resistance detection globally. However, Xpert MTB/RIF sensitivity has been found to be low, particularly in smear-negative and HIV-associated TB and some limitations remain in its determination of RIF resistance (Nicol et al., 2011, Theron

et al., 2011, Dorman et al., 2018). In addition, for detection of RIF resistance, Xpert MTB/RIF can give false-positive results for stains that carry phenotypically silent mutations or if the bacillary burden is very low, although this is rare (WHO.XpertMRT, 2017).

The Xpert MTB/RIF Ultra was developed as the next generation assay to improve assay sensitivity in the detection of *M. tuberculosis* complex. It incorporates two different multi-copy amplification targets (IS6110 and IS1801) and in addition, the Ultra has larger DNA reaction chamber than Xpert MTB/RIF (50µl PCR reaction in Ultra versus 25µl in Xpert MTB/RIF which incorporates fully nested nucleic acid, more rapid thermal cycling, and improved fluidics and enzymes. This has resulted in Ultra having a limit of detection of 16 bacterial colony forming units per ml compared to 114 per ml for Xpert MTB/RIF (WHO.XpertMRT, 2017). A total of four probes identify RIF resistance mutations in the RIF resistance determining region of the *rpo*B gene by shifting the melting temperature away from the wild type reference value. All the improvements as resulted in an approximately 1-log in improvements in the lower limit of detection compared with Xpert (Dorman et al., 2018). Analytical laboratory data also demonstrated improved differentiation of certain silent mutations, improved detection of rifampicin resistance in mixed infections, and avoidance of false-positive results for detection of rifampicin resistance in pauci-bacillary specimens (Chakravorty et al., 2017, Dorman et al., 2018, WHO.XpertMRT, 2017).

2.17.3 Chip-based assays

Chip-based assays for the detection of resistance are commercially available, such as the Combichip Mycobacteria chip or the DNA microarray (LCD array), detecting specific mutations in the *rpoB*, *katG* and *inhA* genes (Aragon et al., 2006, Park et al., 2006, Sharma et al., 2016a). The TB-Biochip oligonucleotide microarray system is designed to identify 29 codon substitutions and one codon deletion in *rpoB* while, the high-density DNA probe array is for the detection of 11 distinct *rpoB* mutations (Sougakoff et al., 2004, Caoili et al., 2006), The biological microchips detects eight mutations in the *gyrA* gene (Antonova et al., 2008, Sharma et al., 2016b). However, these assays have not been adequately validated in different settings. (Sougakoff et al., 2004, Caoili et al., 2006) .

2.17.4 DNA Sequencing

DNA sequencing based approaches are considered to be reference molecular assays for the detection of mutations, providing the highest level of information. It can be performed by both manual and automated procedures (Abdelaal et al., 2009). First-generation generally refers to "sanger sequencing" while, next generation is generally used to refer to any of the high-throughput methods which were developed after Sanger sequencing (Abdelaal et al., 2009, Tyler et al., 2016).

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The main problems of the Sanger sequencing was dealing with larger sequence output when the using of gels or polymers as separation media, the limited number of samples which could be handled in parallel and the difficulties with complete automation of the sample preparation (Abdelaal et al., 2009, Tyler et al., 2016). These limitations and problems triggered the initial efforts to develop new techniques which would have the capacity to sequence a large number of samples in parallel (Abdelaal et al., 2009, Tyler et al., 2016). Next generation sequencing encompass second-line and third generation sequencing. Second-generation generally encompass illumina and Roche SOLiD (use fluorescent labels). While third generation means single-molecule methods and include Ion Torrent and single-molecule technologies like Helicos ("post light") (Tyler et al., 2016).

2.17.4.1 Whole genome sequencing

Whole genome sequencing (WGS) is a laboratory process which involves determining the complete DNA sequence of an organism's entire chromosomal DNA at a single time (Walker et al., 2013, Kohl et al., 2014). Whole genome sequencing provides comprehensive information on the genetic basis of XDR, MDR and TDR isolates and promises to transform clinical microbiology practice and to become a viable technology in reference laboratories (Walker et al., 2013, Kohl et al., 2014, Walker et al., 2015). Automation has simplified the process, bringing sequencing within the capability of large academic and reference centers, although automated analyzers are relatively expensive (Roetzer et al., 2013). Studies using Illumina sequencing-by-synthesis technology dominate the field of bacterial WGS (Hatherell et al., 2016, Ssengooba et al., 2016). In the case of microbial WGS, the majority of studies use Illumina MiSeq technology employing either Nextera

XT or TruSeq (TS) Sample Preparation Kits (Illumina, San Diego, USA) for library construction prior to sequencing (Hatherell et al., 2016, Ssengooba et al., 2016). Each of these relies on construction of NGS libraries, but by different mechanisms (Tyler et al., 2016). The NX kit fragments genomic DNA (gDNA) employing a proprietary transposon/transposase-mediated cleavage mechanism, with genomic fragments subsequently amplified using primers targeted to adaptor sequences linked to the transposon (Walker et al., 2013, Kohl et al., 2014, Walker et al., 2015). In contrast, in the TS protocol gDNA is first fragmented by mechanical shearing, followed by end-repair of the fragments and adaptor ligation (Walker et al., 2013, Kohl et al., 2014, Walker et al., 2015). Advantages to using the NX kit include the requirement for only 1ng of input DNA and significantly faster preparation time (Tyler et al., 2016).

2.17.5 Limitations of Molecular Tests

The sensitivity of molecular tests for all drugs is generally lower than phenotypic DST methods (Maus et al., 2005, Robledo et al., 2008, Richter et al., 2009). This is due to the fact that none of the established molecular tests target all possible genes or mechanisms involved in resistance (some are not identified yet) and consequently, a variable proportion of resistant strains will not be detected (Maus et al., 2005, Robledo et al., 2008, Richter et al., 2009). Moreover, it has not been clarified for all identified genetic changes whether specific mutations are resistance-associated or manifest as single nucleotide polymorphisms (Richter et al., 2009). Another limitation of molecular assays is the identification of hetero-resistance, which is defined as a mixture of resistant and susceptible strains (Kumar et al., 2014, Kansal et al., 2014). The detection limit is approximately 10% or more mutant DNA in a mixture of wild-type and mutant DNA (Richter et al., 2009, James et al., 2014). If the proportion of resistant cells in an isolate is less than that amount, it may not be detected by molecular methods, while the classical susceptibility testing might give a more sensitive test result in these cases (Richter et al., 2009, Kansal et al., 2014).

Whole genome sequencing has been shown to provide a rapid and comprehensive view of the genotype of *Mycobacterium tuberculosis*, with the potential to reliably predict drug susceptibility within a clinically relevant timeframe (Walker et al., 2013, Kohl et al., 2014, Walker et al., 2015). In addition, it provides the highest resolution when investigating outbreaks. However, the lack of

bioinformatics expertise among clinical microbiologists is a potential barrier for clinical adoption. It is evident that the WGS, despite its great potential, may be hampered by the complexity of data and its analysis (Walker et al., 2013, Kohl et al., 2014, Walker et al., 2015). Hence, there is need for a simpler software tools, able to analyze data with basic functionalities all in-one.

2.18 QUANTITATIVE DRUG SUSCEPTIBILITY TESTING OF *M. TUBERCULOSIS*

In diagnostic laboratories DST of *M. tuberculosis* strains is notably different from the standard procedures in clinical microbiology for non-mycobacterial species (Schon et al., 2009). Classically, in the case of many other microorganisms, a series of drug dilutions is used to determine the MIC required to inhibit bacterial growth in-vitro (Kim et al., 2008, Schon et al., 2009). Currently established DST procedures of *M. tuberculosis* classify clinical isolates as either drug "resistant" or "susceptible" on the basis of their ability to grow in the presence of a mostly single concentration named the "critical concentration" (Kim et al., 2008, Ängeby et al., 2012). Critical concentration is defined as the lowest concentration of the drug that inhibits ≥95% for all other TB drugs and 90% for pyrazinamide of wild-type strains of bacilli that have not been exposed to the drug previously while, at the same time not inhibiting clinical strains of *M. tuberculosis* (Kim et al., 2008, Ängeby et al., 2010b). Thus defined, the critical concentration is an epidemiological parameter used to distinguish "wild type" isolates from "non-wild type" strains that are able to grow in the "wild type" presence of higher drug concentrations; it correspond to what is defined as the epidemiological "cut off" concentration (Kim et al., 2008, Schon et al., 2009).

The use of critical concentrations has two major problems : i) an isolate will be categorized uniformly as resistant in the diagnostic laboratory regardless of whether high level, moderate level or low-level resistance is present (Kim et al., 2008). However, scientific evidence has established unequivocally, that the chromosomal loci responsible for resistance in *M. tuberculosis* does not consist of a single genetic locus, such as up-regulation of an efflux pump or induction of transcriptional regulator, but rather by an accumulation of multiple different mutations (Kim et al., 2008). These studies have also established that resistance in *M. tuberculosis* is by no means a

standardized biological entity, but on the contrary, is quite heterogeneous (Kim et al., 2008, Ängeby et al., 2010b). Such heterogeneous resistance in the case of *M. tuberculosis* involves low level, moderate level and high level drug resistance phenotypes (Woods et al., 2007, Böttger, 2011).

ii) There is limited scientific background for the use of critical concentrations particularly for second-line drugs which may be too close to the assumed critical concentrations (Ängeby et al., 2010b, Angeby et al., 2012). The critical concentrations are based on specialists' consensus rather than scientific evidence (Kim et al., 2008, Ängeby et al., 2010b, Ängeby et al., 2012). Specialists committees standardize critical concentrations using evidence obtained through clinical trial sciences, pharmacokinetics and pharmacodynamics, population simulation tools, resistance mechanisms, antimicrobial susceptibility testing methods and bacterial populations dynamics (Kim et al., 2008, Ängeby et al., 2010b, Ängeby et al., 2012). Two such committees are the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute of the United States of America (CLSI) (Kim et al., 2008, Ängeby et al., 2010b, Angeby et al., 2012).

Thus scientists have questioned critical concentrations credibility in defining susceptibility or resistance of *M. tuberculosis* (Kim et al., 2008, Ängeby et al., 2010b, Angeby et al., 2012, Burke et al., 2017). The EUCAST does not publish recommendations for mycobacteria breakpoints for most drugs (EUCAST, 2016) of clinical *M. tuberculosis* isolates as resistant or susceptible on basis of susceptibility testing at "single concentration" may need to be revised and supplemented with quantitative measures of resistance testing to reflect and accommodate the biological complexity of drug resistance (Kim et al., 2008). Limitations in mycobacterial drug susceptibility testing were noted as early as 1960s, when the test procedures currently in place where established (Canetti et al., 1969, Lee et al., 2013). Canetti in 1969 wrote "We consider that the best type of sensitivity test if a fully quantitative determination in which the organisms' capability of growth on medium containing a wide range of drug concentration is known" this type of test would provide full information on the degree of resistance. "However, since such a test requires large amounts of medium and is time consuming, it cannot be recommended as a routine procedure" (Canetti et al., 1969, Lee et al., 2014b).

Thus utilization of MICs has long been overdue and needs to be implemented in a way that reduces cost and time required to obtain MIC results. Most important are standardized protocols for quantitative drug susceptibility testing of both first-line and second-line drugs as a prerequisite for prospective studies addressing the impact of resistance heterogeneity on treatment results i.e correlating the data from quantitative resistance testing with clinical outcome.

A number of techniques have been used for MIC testing (quantitative measuring of resistance) of *M. tuberculosis* which includes i) agar based MIC which is antimicrobial gradient method which encompass the Epsilometer test (Etest) (Hausdorfer et al., 1998, Joloba et al., 2000, Freixo et al., 2004) ii) agar-based MIC based which includes the 7H10 or Loweistein-Jensen medium (L-J medium) (van Klingeren et al., 2007) iii) macro-dilution MIC based tests which has the MGIT 460 and 960 systems iv) micro-dilution MIC based methods which has the Sensititre MYCOTB plate (TREK Diagnostic Systems) (Abuali et al., 2012a, Hall et al., 2012b, Lee et al., 2013, Mpagama et al., 2013) and Alamar blue plates (Franzblau et al., 1998) which are newer microtitre plates that are cost effective, methods that allow a range of drugs to be tested in parallel.

2.18.1 The agar based MIC on antimicrobial gradient

The antimicrobial diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility. The Etest (bioMerieux) is a commercial test based on version available for TB DST (Hausdorfer et al., 1998, Joloba et al., 2000, Freixo et al., 2004). The Etest was popularised for susceptibility testing of *M. tuberculosis* by Wanger and Mills (Wanger and Mills, 1994, Wanger and Mills, 1996). It employs thin plastic strips that are impregnated with a dried antibiotic concentration gradient and are marked on the upper surface with a concentration scale (Wanger and Mills, 1994, Wanger and Mills, 1996). Pre-defined, stable gradient of 15 antibiotic concentrations on a plastic strip and is used for MIC determination for a variety of antibiotics (Hausdorfer et al., 1998, Joloba et al., 2000, Freixo et al., 2004). The strip is read by determining the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip (Hausdorfer et al., 1998, Joloba et al., 2000, Freixo et al., 2004). The E-test has been evaluated against the agar proportion method and

the BACTEC system for DST of first-line drugs anti TB drugs and for OFX (Hausdorfer et al., 1998, Joloba et al., 2000, Freixo et al., 2004). Good overall agreement with conventional DST has also been reported for INH and RIF (Hausdorfer et al., 1998, Joloba et al., 2000, Freixo et al., 2004), but lower agreement for EMB and SM. The E-test is easy to perform and fast, results could be obtained within 5-15 days (Hausdorfer et al., 1998, Joloba et al., 2000, Freixo et al., 2004). The Etest has the flexibility of being able to test the drugs the laboratory chooses. This feature is most suited in situations in which an MIC determination for only 1 or 2 drugs instead of a whole panel is performed (Hausdorfer et al., 1998, Joloba et al., 2000, Freixo et al., 2004). These features make the gradient diffusion test attractive for use in routine mycobacteriology laboratories (Hausdorfer et al., 1998, Joloba et al., 2000, Freixo et al., 2004). However, a major concern of this methodology is the associated bio-safety risks. A high amount of TB bacteria grow on standard culture plates, since a McFarland turbidity of about 3 is required for testing in a single plate which cannot be adequately closed (Richter et al., 2009). Closing those plates using adhesive tapes is laborious and not performed routinely and puts the laboratory staff at risk of infection (Fegou et al., 2006, Palomino et al., 2007, Richter et al., 2009). In addition, there is some difficulties in achieving uniform inocula in terms of the true numbers of bacilli plated prior to the placement of Etest strips and the subsequent misinterpretation of the susceptibility profile (Palomino et al., 2008, Richter et al., 2009). Determining the zone of the ellipse for some isolates may be difficult which may lead to erroneous results (Fegou et al., 2006, Palomino et al., 2007, Richter et al., 2009). Due to high rate of contamination, insufficient growth of particular isolates, the Etest often requires retesting which makes it expensive and laborious (Fegou et al., 2006, Palomino et al., 2007, Richter et al., 2009). In some isolates growth is seen within the zone of inhibition which may be due to presence of isolated *M. tuberculosis* isolates which increases the difficulty in interpreting results (Fegou et al., 2006, Palomino et al., 2007, Richter et al., 2009). In addition, there are some systemic biases toward higher or lower MICs (Fegou et al., 2006, Palomino et al., 2007, Richter et al., 2009). These difficulties, experienced along with the expense of the media, made the Etest strips less useful for TB DST (Fegou et al., 2006, Palomino et al., 2007).

2.18.2 Agar dilution MIC determination method:

The agar dilution based MIC determination involves culture medium plates with wells containing either 7H10 medium or Lowenstien-Jensen (L-J) medium. The technology involves preparation of serial dilutions of anti-TB drugs in the respective media (Das et al., 2003, Schaaf et al., 2007, Springer et al., 2008, Schönfeld et al., 2012). The plates are then inoculated with a standardized *M. tuberculosis* inoculum and after incubation for about 21 days the MICs are determined (van Klingeren et al., 2007). The MIC can be determined by a comparison with the 1/100 control well (Schaaf et al., 2007, Springer et al., 2008, Schönfeld et al., 2012). The reading of the plates is carried out when the bacterial growth in the control well without anti-TB drugs is sufficient i.e when colonies are clearly visible (Schaaf et al., 2007, Springer et al., 2008, Schönfeld et al., 2012). The interpretation of the MIC readings i.e the decision as to whether a strain is susceptible or resistance is based on the lowest concentration of antibiotic that prevents growth of *M. tuberculosis* in comparison to the 1/100 control well (Schaaf et al., 2007, Springer et al., 2008, Schönfeld et al., 2012).

2.18.3 Broth Macro dilution MIC determination method

The macro-dilution MIC based tests were one of the earliest antimicrobial susceptibility testing methods. The assays involves preparing serial fold dilutions in the liquid medium used for MGIT testing (Bastian et al., 2001, Pfyffer et al., 2002, Huang et al., 2004). This procedure involved preparing two-fold dilutions of antibiotics (e.g. to obtain final concentrations of 1, 2, 4, 8 and 16µg/ml) in a liquid growth medium dispensed in test tubes (Bastian et al., 2001, Pfyffer et al., 2002, Huang et al., 2004). The antibiotic-containing tubes will then be inoculated with a standardized bacterial suspension and examined for growth (Bastian et al., 2001, Pfyffer et al., 2002, Huang et al., 2004). The lowest concentration of antibiotic that prevents growth of *M. tuberculosis* represents the minimal inhibitory concentration (MIC) (Bastian et al., 2001, Pfyffer et al., 2002, Huang et al., 2004). The precision of this method is considered to be plus or minus one fold concentration due in large part to practice of manually preparing serial dilutions of antibiotics (van Klingeren et al., 2007). The disadvantage of macro-dilution method is the tedious, manual task of preparing the antibiotic solutions for each test and the possibility of errors in preparation of

the antibiotic solutions (van Klingeren et al., 2007). Furthermore, both invisible contamination and overgrowth with atypical mycobacteria affect the reliability of the tests (van Klingeren et al., 2007). The macro-dilution also have the drawback of involving multiple tubes or bottles which require relatively large amount of reagents and space for each batch of tests rendering the procedure expensive (van Klingeren et al., 2007). All these factors made the macro-dilution using in liquid media system undesirable and practically impossible to do routinely.

2.18.4 Broth micro-dilution MIC determination method

To overcome the limitations of macro-tube dilution method, the technology was miniaturized and mechanized by use of small disposable, plastic "micro-dilution" trays (Wallace et al., 1985, Swenson et al., 1982, Wallace et al., 1986). These techniques have been described by a number of authors in the 1980s (Wallace et al., 1985, Swenson et al., 1982, Wallace et al., 1986). Swenson and colleagues in 1982 described the micro-dilution MIC method in which Mueller-Hinton broth was used (Wallace et al., 1985, Swenson et al., 1982, Wallace et al., 1986). In 1986 Wallace and colleagues changed the medium from Mueller-Hinton broth to 7H9 Middlebrook liquid medium (Wallace et al., 1986). A colorimetric micro-dilution method for determination of MICs was also described which utilizes oxidation-reduction of the Alamar Blue dye (Franzblau et al., 1998, Vanitha and Paramasivan, 2004, Martin et al., 2003, Li et al., 2013). MIC results which showed good agreement with results obtained by the APM were obtained with high agreement of > 97% between the two methods. Unfortunately, the colometric method using the Alamar microtiter plate requires opening of the plate after several days of incubation for addition of the indicator dye. This procedure poses risk to laboratory personnel and thus should not be used for routine purposes (Abate et al., 2004, Montoro et al., 2005, Mengatto et al., 2006).

Currently the micro-dilution trays plate is available commercially as the Sensititre® MYCOTB supplied by (Trek Diagnostic Systems, Cleveland, OH, USA (Abuali et al., 2012a, Lee et al., 2013). This MIC based antimicrobial susceptibility testing method comprises a micro-dilution plate with 96 wells and accommodates 12 lyophilized first and second-line anti-mycobacterial drugs (Abuali et al., 2012a). The Sensititre plate offers the advantage of miniaturization which

allows optimum use of available space and economy of reagents (Abuali et al., 2012a, Hall et al., 2012b, Lee et al., 2013).

The Sensititre plate uses small, disposable, plastic micro-dilution trays which makes the micro-dilution test a practical method of susceptibility testing (Abuali et al., 2012a, Hall et al., 2012b, Lee et al., 2013, Mpagama et al., 2013). The Sensititre micro-dilution panels are typically prepared using dispensing instruments that aliquot precise volumes of pre-weighed and diluted antibiotics, offering reproducibility and convenience (Abuali et al., 2012a, Hall et al., 2012b, Lee et al., 2013, Mpagama et al., 2013). MICs are determined using an automated viewing device for inspection of each of the panel wells for growth (Abuali et al., 2012a, Hall et al., 2012a, Lee et al., 2013). The use of dispensing instruments and automated viewing device makes the test less tedious and easy to perform and these are features that were absent in 1963 when *M. tuberculosis* susceptibility testing was standardized and implemented (Canetti et al., 1969, Lee et al., 2013). The Sensititre plates utilize non-radiometric liquid material that makes its disposable easy (Abuali et al., 2012a, Hall et al., 2012a, Lee et al., 2013). The main disadvantage of the micro-dilution commercialized plate is limited flexibility of drug selections available in standard commercial panels. The whole drug panel has to be tested as one or two drugs cannot be tested individually (Lee et al., 2013).

2.19 ASSOCIATION OF MIC WITH GENETIC POLYMORPHISM

It has been established that not all genetic mutations in *M. tuberculosis* affect phenotypic resistance to drugs equally and a number of published studies indicate that MIC levels strongly correlate with the position and nature of the encoded amino-acid substitution (Jamieson et al., 2014a, Ocheretina et al., 2014). Thus whilst molecular tests have greatly reduced the turnaround time to obtain results, one may need to know the MICs related to a particular type of mutation as not all mutations are associated with resistance or the level of resistance (Jamieson et al., 2014a, Ocheretina et al., 2014). Correlating specific mutations conferring drug resistance with specific MICs for a given drug class is essential before successful implementation of these technologies (Kambli et al., 2015). This information is particularly useful for rifamycins, INH, fluoroquinolones (FLQ), and the injectable anti-TB drugs and is exemplified by the definitions of

MDR-and XDR-TB which are based on these compounds (van Ingen et al., 2011b, Sirgel et al., 2012b). The heterogeneous MIC levels observed in drug resistant *M.tuberculosis* may have important therapeutic implications (Sirgel et al., 2012b, Jamieson et al., 2014a).

For rifampicin more than 95% of RIF-resistant isolates have been shown to possess mutations within the RRDR region of the *rpo*B gene. However not all *rpo*B mutations are associated with RIF or RFB resistance (Sirgel et al., 2012b, Jamieson et al., 2014a). Mutations in the RRDR at codons 450, 531, 526 and 513 are generally associated with high-levels of RIF resistance. in contrast amino acid substitutions resulting from specific changes at codons 511, 514, 515, 516, 518, 522 and 533 are correlated with lower levels of RIF resistance (Sirgel et al., 2012b, Jamieson et al., 2014a). In addition, it has been shown that mutations in the *rpo*B that confer high-level of resistance to RIF (MICs >32ug/ml) in *M. tuberculosis* have been associated with cross-resistance to RFB (MICs >4) (Sirgel et al., 2012b, Jamieson et al., 2014a) . Conversely isolates that exhibit lower levels of RIF-resistance (MICs > 8 ug/ml) were found to remain phenotypically susceptible to RFB based on a critical concentration of 0.5 ug/ml (Sirgel et al., 2012b, Jamieson et al., 2014a). For isoniazid and ETH high-level resistance in *M. tuberculosis* are found to be associated with mutations in the *kat*G gene which include mutations S315T, S315G, while lower levels of resistance are associated with mutations in the *Inh*A region which among others include G-17T, T-8A and C-15T (Sirgel et al., 2012b, Jamieson et al., 2014a).

For FLQs it has been shown that there is a clear correlation between the various *gyr*A mutations observed and the levels of phenotypic resistance seen to OFX and MXF by MGIT testing (Böttger, 2011, Sirgel et al., 2012b, Kambli et al., 2015, Witney et al., 2016). Mutation with codons 89, 90 and 91 were linked with lower levels of resistance while, codons 88 and 94 were linked to higher levels of FLQ resistance (Böttger, 2011, Sirgel et al., 2012b, Kambli et al., 2015, Witney et al., 2016). Thus these results suggests that clinical *M. tuberculosis* isolates with *gyr*A mutations Ala90Val and Ser91Pro have potential to be treated with higher levels of FLQs, while mutations ASP94 Aspn/Tyr/Gly may require an alternative drug to FLQs (Böttger, 2011, Sirgel et al., 2012b, Kambli et al., 2015, Witney et al., 2016). As a result a report summarized the clinical implications of mutations detected by molecular methods and provided clarity on how genotypic data can be interpreted in terms of TB regimens (Domínguez et al., 2016).

2.20 RELATIONSHIP BETWEEN MIC AND PK/PD VALUES

Pharmacokinetics describes the way the human body handles a medicine, the time course of concentrations of a drug resulting from administration of a dosage regimen and accounts for its absorption, distribution, metabolism and excretion in the body (Boshoff and Barry, 2005, Gloede et al., 2010, Vaddady et al., 2010). Protein binding in in-vivo tends to decrease the concentration of free drug concentration or the unbound fraction that is relevant to the patient and can distribute into target tissue and undergo metabolism and excretion (Boshoff and Barry, 2005, Gloede et al., 2010, Vaddady et al., 2010, Chigutsa et al., 2015). Most drugs bind to plasma proteins such as albumin, lipoprotein and cellular blood components such as erythrocytes (Boshoff and Barry, 2005, Gloede et al., 2010, Vaddady et al., 2010, Chigutsa et al., 2015). On the other hand, pharmacodynamics (PD) describes the intensity of drug effect in relation to its concentration which defines the effect of the drug on the pathogen residing in the host organism (Boshoff and Barry, 2005, Gloede et al., 2010, Vaddady et al., 2010, Chigutsa et al., 2015). The PK/PD analysis, provides the complete time courses of the drug effect in response to concentration-time course encountered in-vivo and thus seems to be more adequate and informative for selecting optimal dosing than the use of a point concentration (Peloquin, 2002, Heysell et al., 2010, Chigutsa et al., 2015). Furthermore, PK/PD tends to accurately reflect the complex interaction among the three major players, the drug, the micro-organisms and the host over a concentration-time course (Chigutsa et al., 2015). In-vitro drug testing experiments comprising MIC and minimum bactericidal concentration (MBC) determinations tend to assume that there is no distribution involved and the drug has direct access to the bacteria (Boshoff and Barry, 2005, Gloede et al., 2010, Vaddady et al., 2010).

Although MIC and MBC appear to be good measures of the potency of a drug and it has interaction with a pathogen, they do not provide the course of the antimicrobial activity (Boshoff and Barry, 2005, Gloede et al., 2010, Vaddady et al., 2010). Limited bioavailability can be caused among other things by PK variability, mal-absorption, altered metabolism and drug degradation in the stomach (Sotgiu et al., 2012, Srivastava et al., 2013, Zhang et al., 2014, D'Ambrosio et al., 2015). All these changes can only be monitored in vivo with PK/PD and not in vitro by means of

MIC or MBC testing (Vaddady et al., 2010, Zhang et al., 2014, D'Ambrosio et al., 2015). Laboratory MIC data and PK modeling can be used to calculate PK/PD characteristics and to provide measures of drug availability, such as area under the curve over MIC, the maximum concentration over MIC, and the time above MIC (Vaddady et al., 2010, Zhang et al., 2014, D'Ambrosio et al., 2015).

Although the use of PK/PD seems crucial, MIC determination tends to be very useful because i) although MICs are measured in-vitro the antimicrobial dosing are chosen to achieve concentrations above the MIC of the pathogen being treated (Peloquin, 2002, Heysell et al., 2010, D'Ambrosio et al., 2015). Doses are chosen to obtain antibiotic plasma concentrations that are above the MIC for a given pathogen throughout the dosing interval thus providing for an effective course of antimicrobial activity in the body (Peloquin, 2002, Heysell et al., 2010, D'Ambrosio et al., 2015) ii) therapy for TB comprises a combination of three or four drugs administered simultaneously (Peloquin, 2002, Heysell et al., 2010, D'Ambrosio et al., 2015). Thus, whereas in-vitro DST results apply to each of the drugs in the combination, and not to the entire regimen, the outcome of anti-TB therapy is a composite outcome (additive/synergistic fashion), owing to the use of multiple drugs (Peloquin, 2002, Heysell et al., 2010, D'Ambrosio et al., 2015). The efficacies of non-antagonistic drugs in combination therapy will be at least addictive, effectively reducing the MIC of the drugs used in the combination (Peloquin, 2002, Heysell et al., 2010, D'Ambrosio et al., 2015). Thus, depending on PK/PD indices sufficiently high drug concentration may be achieved in in-vivo, despite low-level or moderate-level drug resistance in in-vitro (Vaddady et al., 2010, Böttger, 2011) iii) obtaining PK/PD information is not easy owing to the expensive models that are needed to simulate the human body (Boshoff and Barry, 2005, Gloede et al., 2010, Vaddady et al., 2010). There is often limited information on PK/PD available owing to a lack of inexpensive suitable animal models to predict efficacy and a poor understanding of the relationship between PK and PD for novel compounds (Katsube et al., 2008). In-vitro systems (hollow fibers) are then used to determine PK/PD. Although models can provide similar growing conditions for bacteria as encountered in the human host and thus imitate the characteristics of human infection, the potential differences in PK such as rate and extent of drug metabolism and drug delivery to the species-specific tubercular lesions, such as organized granulomas in humans might limit the extrapolation of information from animals to

humans (Boshoff and Barry, 2005, Gloede et al., 2010, Vaddady et al., 2010). iv) the cost of setting up the human models is often prohibiting which make the PK/PD determination impractical in developing countries and thus use of MICs is the preferred predictor of effective treatment in humans (Boshoff and Barry, 2005, Gloede et al., 2010, Vaddady et al., 2010). v) The WHO held a technical consultation to discuss priority clinical issues related to clinical management of drug-resistant TB. The experts acknowledged that numerous gaps remain in our understanding of the relationship between PK/PD and clinical-relevant end-points, such as culture conversion and relapse, which limits the quest for the optimal dose, frequency and duration of each medicine to maximize efficacy and minimize harm (WHO.PK/PD, 2018). Considering all the drawbacks of using the PK/PD the use of MICs remains crucial and most practical method to determine appropriate dosages.

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CHAPTER 3

EVALUATION OF SENSITITRE® MYCOTB MIC PLATE METHOD AGAINST AGAR DILUTION MIC METHOD FOR SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS*

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Running Title: Evaluation of Sensititre MYCOTB MIC plate against agar dilution MIC method for *M. tuberculosis*

Abstract

Determination of the minimum inhibitory concentration (MIC) of anti-TB drugs provides valuable information for optimizing individual patient management and facilitate therapeutic drug monitoring. The Sensititre MYCOTB plate (MYCOTB) method is a quantitative testing for MIC testing of first- and second-line anti-TB drugs. Using 138 *M. tuberculosis* isolates, the performance of the MYCOTB MIC method was evaluated against the Agar Dilution Method (ADM). The categorical, essential and as well as sensitivity and specificity of MYCOTB were determined in comparison with ADM. Strains showing discordant results between MYCOTB and ADM after repeat testing, had next generation sequencing and drug resistance related genes analysed. Categorical agreement between the two methods ranged from 88% to 98%. The sensitivity of MYCOTB varied between the drugs, ranging between 60-100% (except for para-aminosalicylic acid). Specificity however, was high for all drugs tested, ranging from 94% to 100%. Sequencing resolved 70% of discrepant results in favor of the MYCOTB plate method. The overall agreement between the MYCOTB and ADM methods was ≥80% for 8/11 drugs at ±1 doubling dilution, and demonstrated ≥90% agreement for 8/11 drugs within ±2 doubling dilution. The MYCOTB plate method is quantitative method and could be excellent alternative to conventional drug susceptibility testing and it is relativley simple and rapid method and could be suitable for use in resource-limited settings.

3.1 INTRODUCTION

Drug resistant tuberculosis (TB), particularly multidrug-resistant-TB (MDR-TB), defined as resistance to isoniazid (INH) and rifampicin and extensively drug-resistant TB (XDR-TB), defined as MDR-TB plus resistance to at least one fluoroquinolone and a second-line injectable agent (WHO, 2016, WHO, 2018). is a matter of concern worldwide and poses many therapeutic challenges. Individualized treatment is the standard of care in areas with adequate expertise and laboratory capacity, however in developing countries, empiric treatment is common due to unavailability of rapid and reliable drug susceptibility testing assays (Jaramillo, 2008, Falzon et al., 2011). Conventional phenotypic methods are mostly qualitative DST methods and reporting of susceptibility of *M. tuberculosis* is based on testing of strains at single drug concentrations termed a critical concentrations (Böttger, 2011). It has been shown that *M. tuberculosis* resistance is heterogeneous and varies from low, borderline and high level resistance (Ängeby et al., 2010, Ängeby et al., 2012). In addition, there is limited published minimum inhibitory concentration (MIC) data to inform the choice of critical concentrations, especially for those of second-line drugs with decisions often based on consensus of limited data rather that robust scientific evidence (Kim, 2005, Ängeby et al., 2010). As a result, simple binary categorization of clinical *M. tuberculosis* isolates as resistant or sensitive on basis of susceptibility testing at single concentration may need to be revised and supplemented with quantitative methods of resistance testing to reflect and accommodate the biological complexity of drug resistance in TB (Kim, 2005).

Minimum inhibitory concentration testing using the agar dilution method is very time-consuming, it is rarely performed in routine laboratories. The Trek Sensititre MYCOTB (Trek Diagnostics Systems, USA) is a standardized commercial assay developed as a simpler alternative for MIC determination of first- and second-line anti-TB drugs. It is based on dry micro-dilution plate with wells containing serial amounts of lyophilized antibiotics. The assay has been previously evaluated only against CC based DST methods such BACTEC MGIT 960 and the indirect agar proportion method (Abuali et al., 2012, Hall et al., 2012, Lee et al., 2013); however, none of the studies compared the assay against MIC based methods. Furthermore, studies are scarce in an

African setting. This study is the first to assesse the performance of the MYCOTB MIC plate method against the agar dilution MIC (ADM) method in a high TB burden setting.

3.2 MATERIALS AND METHODS

3.2.1 *Mycobacterium tuberculosis* strains used in study

A total of 138 available *M. tuberculosis* isolates from stock cultures stored at repository of the Centre for Tuberculosis, National Institute for Communicable Diseases (NICD) Johannesburg, South Africa were included in this study. These isolates were collected as part of routine surveillance of *M. tuberculosis* drug resistance conducted from 2012 to 2014 in South Africa. Drug susceptibility patterns were previously determined by MGIT 960 method (Table 1). The isolates included had different susceptibility profiles. Ethics approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of the Free State, South Africa (Ref: 230408-011).

3.2.2 Sample preparation

Purity of *M. tuberculosis* cultures used for this study was checked prior to MIC testing by inoculation on 5% sheep blood agar which was then incubated at 37^0C for 48h and checked for bacterial or fungi contamination.

3.2.3 Determination of *M. tuberculosis* MICs using the Sensititre MYCOTB plate

The MICs were determined using the MYCOTB plate method. This assay comprises a 96 well microtitre plate containing 12 lyophilized antibiotics, configured for determination of MICs to first- and second-line TB drugs. The MIC ranges for the 12 drugs included were as follows: 0.5 µg/ml to 32µg/ml for ethambutol streptomycin (SM), and ofloxacin (OFX), 0.12 to 16 µg/ml; for rifabutin (RFB), amikacin (AMI) and rifampicin 0.3 to 40 µg/ml and 0.6 to 40 µg/ml; for ethionamide (ETH) and kanamycin (KAM), 0.5 to 64 µg/ml; for p-amino salicylic acid (PAS),

0.06 to 8 μg/ml; for moxifloxacin, (MXF), 2 to 256 μg/ml; for cycloserine (CYC) and 0.03 to 4 μg/ml for isoniazid (INH)].

The MIC testing was performed according to the manufacturer's instructions: Briefly, *M. tuberculosis* colonies were scraped off the surface of 7H11 plates and vortexed for 30 to 60 seconds in a sterile saline-tween-tube containing glass beads (Trek Diagnostics Systems). The suspension was allowed to settle for 15 to 30 min and then adjusted to a turbidity of 0.5 McFarland standard, using a nephelometer (Becton Dickinson Diagnostics, USA). A 100 μg of suspension was transferred to 11ml of Middlebrook 7H9 broth supplemented with oleic acid-albumin dextrose-catalase (OADC). A Sensititre AIM™ Automated Inoculation Delivery System (Trek Diagnostics systems, USA) was used to dispense 100 μl of the OADC-containing inoculum to each well. The MYCOTB plates were sealed and incubated at 37^0C. Plates were checked after 48 hours for contamination. The MYCOTB plates were read at days 10, 14 and 21 of incubation. Initially the plates were read by visual inspection for growth and subsequently via a computerized Vizion system (Trek Diagnostic Systems, Cleveland, USA). For each antibiotic, the lowest concentration with no visible growth was considered to be the MIC.

3.2.4 Determination of *M. tuberculosis* MICs using ADM

The ADM was performed according to CLSI guidelines (CSLI, 2003) using 7H11. Briefly, 11 plates were used for each isolate representing all drugs on MYCOTB except for CYC which was not tested. Each plate incorporated two control wells and eight drug-containing wells that represented each drug at sequentially increasing concentrations corresponding to the same concentration as the MYCOTB plate. Each well contained two ml of 7H11 broth.

Mycobacterium tuberculosis suspensions were prepared as described for the MYCOTB plate method, by scraping *M. tuberculosis* colonies from 7H11 plates into a sterile saline-tween-tube containing glass beads, followed by vortexing for 30 to 60 sec. The suspension was allowed to settle for 15 to 30 min and the supernatants adjusted to reach a 1.0 McFarland turbidity standard, using a nephelometer (Becton Dickinson Diagnostics, USA). Two different drug-free control wells were inoculated with 100 μl of undiluted sample and 100 μl of 10^{-2} dilution of the sample.

The plates were allowed to stand until the inocula were adsorbed onto the medium surfaces and put in plastic bags to reduce drying of media. The inoculated plates were incubated in 5-10% CO_2, 35-37^{0}C incubator. The agar plates were checked weekly for contamination by incubating a subset of freshly made agar plates at 35-37^{0}C in 5-10% CO_2 incubator. Plates were read after 21 days of incubation with the aid of an inverted microscope to help visualize colonies. For valid results, the control well with 10^{-2} dilution should have between 10 and 50 colonies which verified that the bacterial suspension was within the targeted amount (~10^5 CFU/ml). The MIC was read at the well where growth of *M. tuberculosis* was less than number of colonies in the 10^{-2} diluted drug-free control well.

3.2.5 Resolution of discordant isolates by next generation sequencing

All isolates with categorical discordant results between the MYCOTB and ADM methods were re-tested by both methods. Whole genome sequencing (WGS) using next generation sequencing was also performed on isolates that remained discordant after repeat testing.

DNA extraction was performed using the Nuclisens EasyMag (Biomeriux, France) system, following the manufacturer's instructions, and quantified using a Qubit fluorometric assay (Invitrogen). Next generation sequencing was performed using a MiSeq Next-Generation Sequencing System (Illumina) at the sequencing facility of the NICD, South Africa. Genomic libraries were prepared according to recommendations, using Nextera XT DNA sample preparation kit (Illumina). During the library preparation input, DNA was tagmented (tagged and fragmented) by Nextera XT transposome. The Nextera XT transposome simultaneously fragments the input DNA and adds adaptor sequences to the hydroxyl end, allowing amplification by PCR in subsequent steps. The resulting DNA was amplified using Nextera XT Indexing tags, to generate multiplexed paired-end libraries (2 x 300 bp) which allowed pooling of samples on a single MiSeq run.

Sequencing data was imported to CLC Genomics Workbench v7.5.2 (CLC bio, Aarhus, Denmark) software. Sequence reads of the *M. tuberculosis* isolates were mapped against the reference genome of *M. tuberculosis* strain H37Rv. Variant for genetic targets associated with resistance to

different TB drugs were linked to their respective mutations as follows INH and ETH was linked to *inh*A and *kat*G mutations, *rpo*B mutation to RIF and RFB mutations, *rrs* and *rps*l mutations where linked to SM, KAN and AMI, *gyr*A and *gyr*B mutations were linked OFX and MXF and EMB was linked to the *emb*B mutation. Single nucleotide polymorphisms (SNPs) were identified for genes of interest, previously known to be related to drug resistance, according to the online drug resistance mutation database (TBDReaM database). If the mutation was not described in this database, the literature was reviewed.

3.2.6 Definitions and Statistical Analysis

As the MYCOTB has no established CC, analysis was conducted using agar proportion break points as reported in other studies (Abuali et al., 2012, Hall et al., 2012, Lee et al., 2013). An isolate was considered susceptible if the MIC was lower than or equivalent to the APM critical concentration and resistant if the MIC was higher than APM critical concentration. The susceptibility status of an isolate was considered to be in categorical agreement if both MYCOTB and ADM methods characterized the isolate as susceptible or both MYCOTB and ADM methods characterized the isolate as resistant. Essential agreement was reported as the percentage of isolates for which the MYCOTB MIC was the same or one two-fold dilution (±1 doubling dilution) apart from the reference ADM method.

Analyses were performed using STATA version 14 statistical software. Descriptive statistics were used to describe the data in terms of frequencies and percentages. The MIC results of the MYCOTB were compared with the MICs obtained with ADM. The ADM was used as the reference standard for evaluating the test performance of MYCOTB method. Categorical, essential and ±2 doubling dilution agreement, as well as sensitivity and specificity indices with 95% confidence intervals of the MYCOTB assay were calculated for each drug.

3.3 RESULTS

Of the total 138 *M. tuberculosis* isolates tested, 14 isolates were excluded due to insufficient growth on either ADM (10/14) or MYCOTB plate (4/14) methods. Therefore, 124 isolates with

valid results for both MYCOTB and ADM methods were analyzed. The MGIT DST profile of the 124 isolates is shown in Table 3. 1.

Table 3.1: MGIT 960 DST profile of clinical *M. tuberculosis* isolates used in the study (n=124)

Drugs	Susceptible	Resistant	Unknown
RIF	41	83	
AMI	101	8	15
INH	54	58	12
KAN	87	24	13
RFB	70	54	
OFX	99	13	12
MXF	97	5	22
ETH	93	13	18
SM	68	41	15
PAS	94	2	28
EMB	86	22	16

ADM method was considered as gold standard. The majority of MYCOTB results were obtained between 10 to 14 days after incubation, as compared to 21-28 days using ADM. The categorical agreement of MYCOTB method compared to ADM method is shown in Figure 3. 1 and Table 3.2.

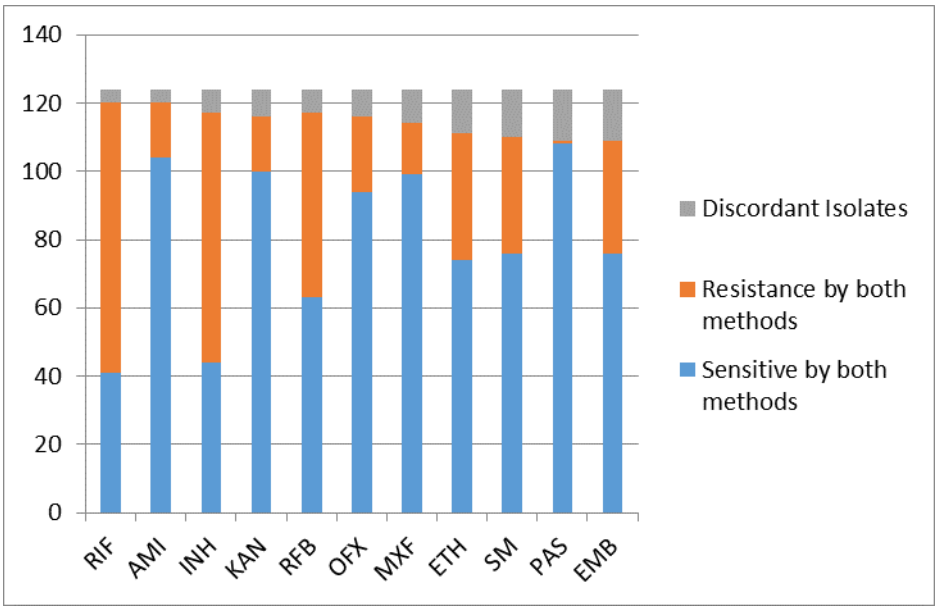


Figure 3. 1: categorial agreement between MYCOTB and ADM methods+

Categorical agreement between ADM and MYCOTB method ranged between 88% and 98%. The highest categorical agreement between the methods was for AMI and RIF with 98% and 97% respectively while, the lowest categorical agreement was for EMB and PAS both with 88% (Table 2). Of the 1364 MIC results (11 drugs x 124 isolates), 103 (7.6%) were categorically discordant, with the majority 89 (86.4%) were found resistant with ADM and susceptible with MYCOTB and the remaining 15 (14.5%) strains were resistant by MYCOTB and susceptible with ADM.

The sensitivity of MYCOTB compared to the ADM varied by drug (Table 3.2). For RIF, there were four discordant results and seven for INH (Figure 1), resulting in a sensitivity of 95% and 92%, respectively. For AMI, RFB, OFX, ETH, the sensitivity ranged between 81% to 89% while, MXF, KAN, EMB showed lower sensitivity ranging between 60 to 73% (Table 3.2). The lowest sensitivity was found for PAS, which was 9%, with 15 discordant results. The specificity, however, was high between 94% to 100% for all drugs.

Table 3.2: Results of testing of 124 *M. tuberculosis* isolates, using the MYCOTB and ADM methods

Drug	CC	No (%)		No (%) Agreement			% (95% CI)	
		S	R	Categorical	±1	±2	Sensitivity	Specificity
RIF	1	41 (33)	83 (67)	120 (97)	108 (87)	117 (94)	95 (88.1-98.7)	100 (91.4-100)
AMI	4	105 (85)	19 (15)	121 (98)	77 (62)	92 (74)	84 (59.5-95.8)	99 (94.0-99.9)
INH	0.25	45 (36)	79 (65)	117 (94)	100 (81)	116 (94)	92 (83.6-96.8)	98 (86.7-99.9)
KAN	5	102 (82)	22 (18)	116 (94)	87 (69)	111 (89)	73 (49.6-88.4)	98 (92.4-99.7)
RFB	0.5	63 (51)	61 (49)	117 (94)	103 (83)	113 (91)	89 (77.1-94.9)	100 (92.8-100)
OFX	2	97 (80)	27 (22)	116 (94)	102 (82)	120 (96)	81 (61.2-92.9)	97 (90.6-99.2)
MXF	0.5	99 (80)	25 (20)	114 (92)	101 (81)	119 (96)	60 (38.9-78.2)	99 (93.7-99.9)
ETH	5	82 (66)	42 (36)	112 (90)	104 (82)	117 (94)	88 (73.5-95.5)	94 (85.7-97.7)
SM	2	77 (62)	47 (38)	110 (89)	104 (84)	114 (92)	72 (57.1-83.9)	99 (91.9-99.9)
PAS	2	113 (91)	11 (9)	109 (88)	104 (84)	109 (88)	9 (0.47-42.9)	95 (89.4-98.3)
EMB	5	79 (64)	45 (36)	109 (88)	87 (70)	112 (90)	73 (57.8-84.9)	96 (88.5-99.0)

The individual MIC results of MYCOTB compared to those obtained by ADM is shown in Figure 3.2. For comparison of the MICs, the doubling dilution difference in the MIC was calculated. The MYCOTB method tended to give MICs consistently lower (+1 to 2 doubling dilutions) than ADM method. The essential agreement (±1 doubling dilution) between the two methods differed depending on the individual drug. Eight out of the 11 drugs had ≥80% essential agreement. Amikacin, KAN and EMB demonstrated lower essential agreement of 62%, 69% and 70%, respectively. Within the ±2 doubling dilution, the agreement was ≥90% for all the drugs (except for AMI, KAN, and PAS). A small percentage had differences more than 3 dilutions.

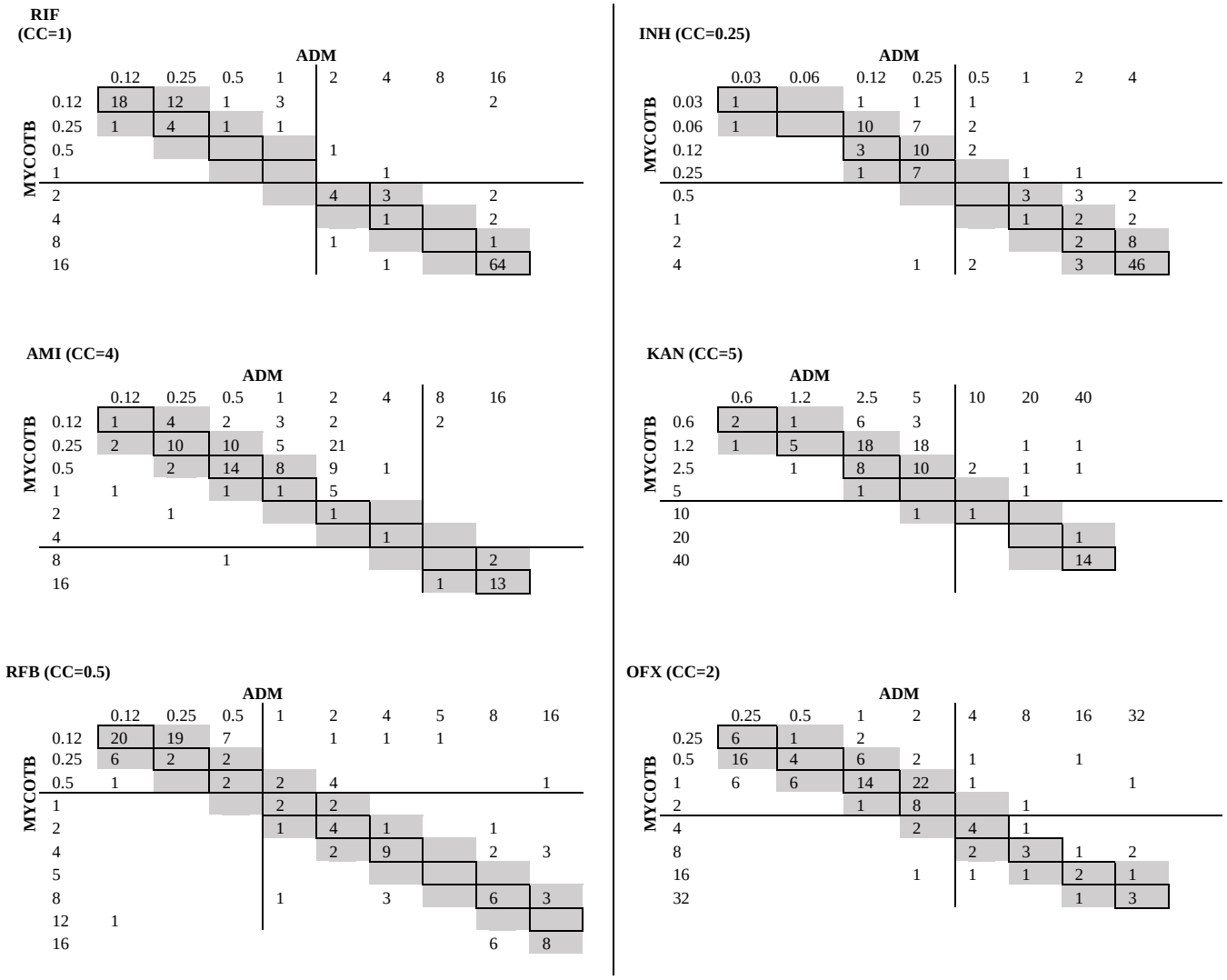
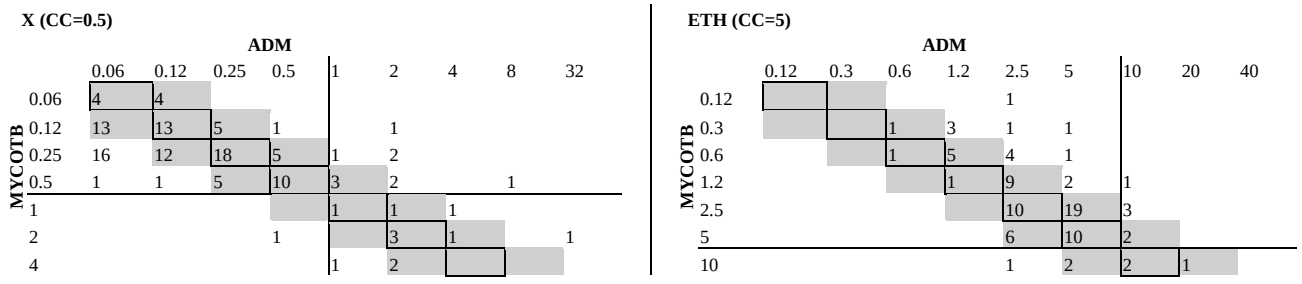


Figure 3.2: Comparison of MIC results of MYCOTB and reference ADM method. MICs within essential agreement (within ± 1 dilution of reference MICs) are highlighted in grey and MICs identical with reference MICs are within boxes.



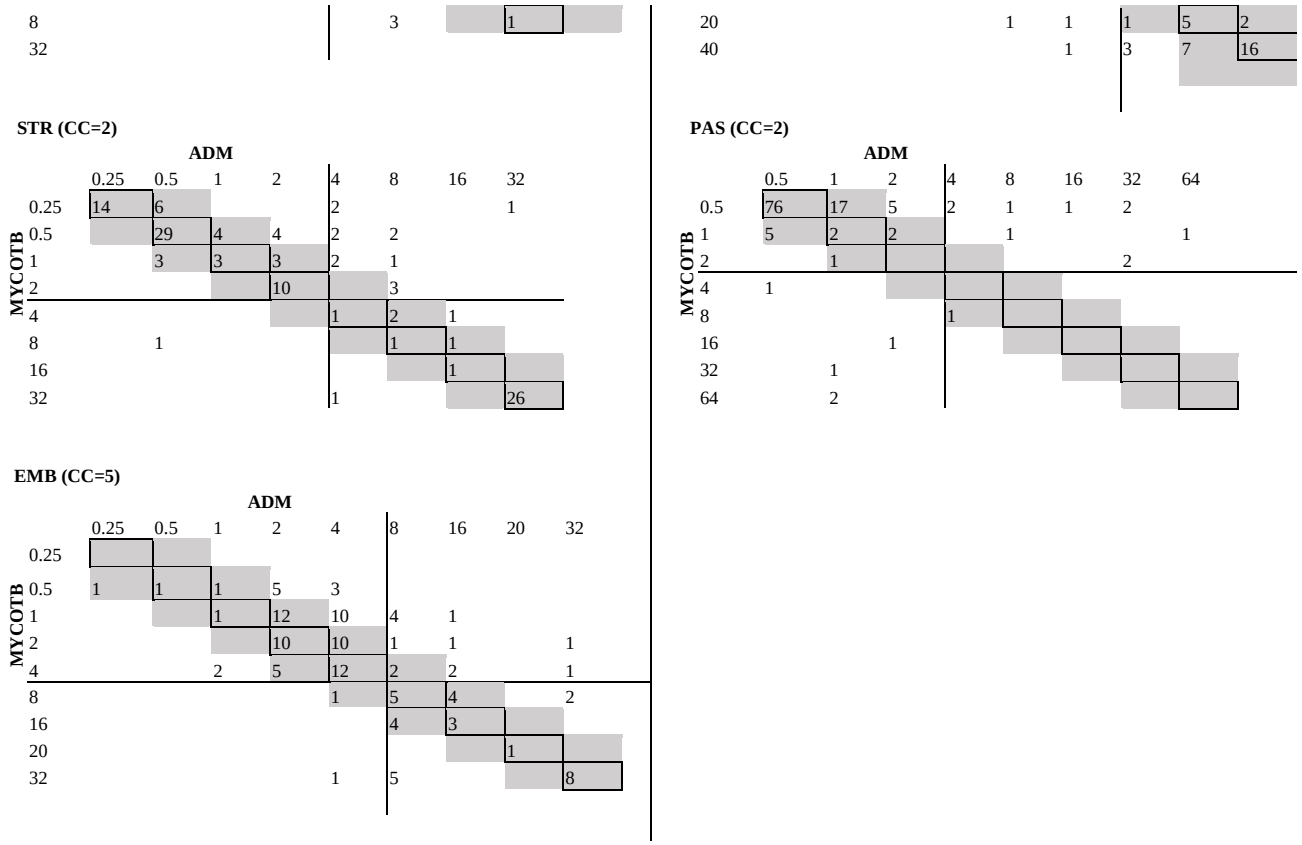


Figure 3.2 (continued)

3.3.1 Sequencing results for discordant isolates between ADM and MYCOTB methods

Overall the next generation sequencing resolved 70% of discrepant results in favor of the MYCOTB method (Table 3.3). Only 12/62 (19.3%) sequenced ADM resistant and MYCOTB susceptible strains had mutations associated with resistance. However, none of the 14 sequenced ADM susceptible and MYCOTB resistant had resistance conferring mutations in the sequenced genes. Discordant results for eight drugs including KAN, MXF, OFX, EMB, SM, AMI, ETH, INH, and PAS were resolved in more than 50% of the cases in favor of MYCOTB plate method, with exception of RIF and RFB which were mostly resolved in favor of ADM.

Table 3.3: Resolution of discordant isolates using next generation sequencing

Drug	Number of discordant	Number of isolates	Isolates in agreement with

Sequenced	Isolates	sequenced	MYCOTB results
RIF	4	3	1(33)
INH	7	6	4(67)
RFB	7	6	2(33)
OFX	8	6	4(67)
MXF	10	8	8(100)
AMI	3	3	2(67)
KAN	8	6	5(83
ETH	13	7	5(71)
EMB	15	14	10(71)
SM	14	10	9(90)
PAS	15	14	8(57)
TOTAL	**104**	**83**	**58(70)**

3.4 DISCUSSION

Quantitative methods for DST are widely used in general microbiology and are lacking in mycobacteriology. The need for such an approach is clearly evidenced by the high proportion of cases that had an MIC around the CC for many drugs in this study. Thus the simplistic approach of categorizing isolates as susceptible or resistant loses the granularity to inform clinical decision making and even more relevant with the limited therapeutic options for patients with drug resistant TB. Our study has shown that the MYCOTB method holds promise and provides an enhancement over current phenotypic DST methods. It is standardized commercially available microtiter plate based method for MIC determination and thus can be suitable for routine diagnostic laboratories.

Compared to the ADM method, the categorical agreement of MYCOTB method ranged between 88% to 98%, which was within the range of previously reported studies (72% to 100%) (Lee et al., 2014, Abdel-Rahman et al., 2016, Xia et al., 2017). The majority of discordant results in this study were categorized as resistant by ADM (86.4%) and susceptible by MYCOTB (13.6%) method. The next generation sequencing resolved the discrepancies in favor of MYCOTB for 70% of the discordant results. This finding is important as questions have been raised about the appropriateness of the critical concentrations in use and although the WHO has recently reviewed the CCs for selected drugs, data was often lacking for 7H11.

When analyzed by individual drug, the categorical agreement between the two methods was high for core first-line drugs, RIF (97%) and INH (94%), though slightly lower than previously reported (98% to 100%) (Hall et al., 2012, Lee et al., 2014, Xia et al., 2017). For second-line drugs, the performances were found to be good with categorical agreements ranging from 92% to 98%. The aminoglycosides, AMI (98%) and KAN (94%) performed slightly better, than the fluoroquinolones OFX (94%) and MXF (92%).

The MYCOTB performed moderately for SM (89%) and EMB (88%), which is consistent to that reported previously (SM, 83% to 96% and EMB, 72% to 98%) (Hall et al., 2012, Lee et al., 2014, Xia et al., 2017). Interestingly, the performance of MYCOTB was good for ETH (90%) and higher than reported previously. Also for PAS, the categorical agreement was 88% was higher than the previously reported study by Salee and McArthur (Salee et al., 2014) which was 76%. Drugs such

as PAS, EMB and ETH are known to be problematic for DST, often yielding less reliable results. It is documented that EMB and ETH susceptibility testing with the APM gave the most variable and inconsistent results (Madison et al., 2002, Abuali et al., 2012, Angra et al., 2012), and the majority of MYCOTB errors occurred with EMB, ETH and SM.

The proportion of strains yielding results within an accuracy limit of ±1 doubling dilution by the MYCOTB and ADM was low due to the tendency of the MYCOTB to produce lower MICs as compared to the ADM method. The essential agreement was >80% for eight of the 11 drugs, but was relatively low for EMB (70%), KAN (69%) and AMI (62%). The difference in the MICs between ADM and MYCOTB could be due variations in methodology including medium, inoculum preparation, interpretation and colony heterogeneity as testingwas done with more than one colony. Also the discrepancies could be attributed to the considerable number of isolates having MIC near the APM critical concentration. In this study a high proportion of isolates had MICs at or ±1 doubling dilution than the APM CC (Figure 3.2). This likely explains the variable performance of both tests and highlights the important value of quantitative DST for determining MICs. Furthermore, the current WHO approach of having only S and R categorically leads to inconsistent results and consideration for an intermediate category needs to be considered to reduce major errors in testing. Within the ± 2 doubling dilution the agreement between the two methods improved (>90%) for majority of the drugs (except for AMI, RFB, and PAS) (Table 3.2).

The MYCOTB had above 90% sensitivity for RIF (95%) and INH (92%). For RFB, OFX, AMI, ETH, the sensitivity was moderate ranging between 81 to 89%, but was relatively lower for MXF, KAN, EMB ranging between 60-73%. The sensitivity was lowest for PAS (9%). However, high specificity was obtained for all the drugs, the lowest being 94% for ETH. Thus over all a MYCOTB susceptible result for core first and second-line drugs provides robust information for patient management.

The MYCOTB method has a shorter turnaround time (10-14 days) as compared to ADM method requiring three to four weeks to obtain results, with the obvious benefit that results can be communicated to the clinicians in the earliest possible time. In addition, the storage conditions of the reagents for MYCOTB plate is mostly at room temperature for up to two years with the

exception of OADC which requires refrigeration (2 to 8 ^{0}C). These easy storage conditions and long shelf life make the use of MYCOTB plate a very attractive option for DST, particularly in resource limited areas where electricity and availability of fridges is scarce. In addition, the 96-well microplate format is a huge advantage since it optimizes the use of incubator space and minimizes costs in terms of equipment requirement. However, similar to other liquid based methods, MYCOTB cannot differentiate growth of contaminants or overgrowth with atypical Mycobacteria from that of *M. tuberculosis*, since it lacks the ability to check for colony morphology of the bacterial cultures. Thus to overcome the challenge, one would need to perform a purity test on agar before embarking on the susceptibility testing procedure. Unlike the MYCOTB, the ADM constituents for test performance are not commercially available which may in part be as a result of a lack of standardization of methods, equipment and reagents due to in house preparation of drug stocks and agar plates and could have been a source of variability in our results (Schön et al., 2009).

Customizations of MYCOTB plate to reflect drugs most used in a particular region could be beneficial. In South Africa the incorporation of drugs such as bedaquiline, linezolid, clofazimine would be advantageous while rarely used drugs like CYC and drugs such as PAS with methodological problems in DST performance, can be removed.

The limitation of the present study the number of resistant strains for all the drugs (except RIF, INH, RFB and SM) were relatively low which might have affected the precision for the sensitivity calculation.

3.5 CONCLUSION

The MYCOTB gave reliable MIC results for both core first- and second-line drugs as compared to ADM in terms of categorical agreement. The essential agreement was low in this study and need further evaluation. Nevertheless, the MYCOTB offers faster turnaround-time, throughput and quantitative MIC information of first- and second-line drugs on a single platform.

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CHAPTER 4

CORRELATION OF *RPOB* MUTATIONS WITH MINIMAL INHIBITORY CONCENTRATION OF RIFAMPIN AND RIFABUTIN IN *MYCOBACTERIUM TUBERCULOSIS* IN AN HIV/AIDS ENDEMIC SETTING, SOUTH AFRICA

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Correlation of *rpoB* Mutations with Minimal Inhibitory Concentration of Rifampin and Rifabutin in *Mycobacterium tuberculosis* in an HIV/AIDS Endemic Setting, South Africa

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Key Words: *Mycobacterium tuberculosis*, *rpo*B, rifampicin, rifabutin, MICs, HIV/AIDS, South Africa, point mutation

Running Title: Rifampin and Rifabutin MIC for *M. tuberculosis*

Abstract

Treatment of tuberculosis (TB) and HIV co-infections is often complicated by drug-drug interactions between anti-mycobacterial and anti-retroviral (ARV) agents. Rifabutin (RFB) is an alternative to rifampin (RIF) for TB regimens and is recommended for HIV patients concurrently receiving protease inhibitors because of reduced induction of CYP3A4. This study sought to determine the proportion of RFB susceptible isolates among RIF resistant strains in a high HIV prevalence setting in South Africa. In addition, the study explored the association between *rpoB* mutations and minimum inhibitory concentration (MIC) levels of RIF and RFB. A total of 189 multidrug resistant (MDR) *M. tuberculosis* isolates from the Centre for Tuberculosis (CTB) repository were analyzed. The MICs were determined using the Sensititre MYCOTB system and the *rpo*B gene was sequenced. Of the 189 MDR isolates, 138 (73%) showed resistance to both RIF and RFB, while 51 (27%) isolates were resistant to RIF but RFB susceptibility. S531L was the most frequent *rpo*B mutation in 105/189 (56%) isolates, followed by H526Y in 27/189 (14%) isolates. Resistance to both RIF and RFB was found predominantly in association with mutations S531L (91/105, 87%), H526Y (20/27, 74%), and H526D (15/19, 79%), while D516V (15/17, 88%) and L533P (3/4, 75%) were found in RFB susceptible isolates. This study has shown that up to 27% of MDR-TB patients in South Africa may benefit from a treatment regimen that includes RFB.

4.1 INTRODUCTION

Tuberculosis (TB) is responsible for 25% of HIV/AIDS related mortality worldwide, with sub-Saharan Africa accounting for 79% of HIV-associated TB cases (WHO, 2015). In South Africa, 65% of TB patients are HIV-positive, and TB remains the leading cause of death among HIV-infected individuals. Treatment of TB in the context of HIV co-infection is challenging, due to the high potential for drug-drug interactions in combined antimicrobial and anti-retroviral (ARV) chemotherapy. There is an urgent need to harmonize TB and HIV treatment through development of compatible ARV regimens. Moreover, multidrug-resistant (MDR) TB, defined by resistance to rifampin and isoniazid (INH), is emerging, particularly within high burden countries, such as South Africa (WHO, 2015). MDR-TB is associated with poor treatment outcomes and greatly elevated health costs.

Due to its sterilizing capacity, the inclusion of RIF in TB treatment regimens is crucial for achievement of high cure rates coupled with low relapse rates (CDC, 2013). However, RIF is a potent inducer of CYP3A4 and other cytochrome P450 enzymes, leading to reduced serum levels of protease inhibitors, used in treatment of HIV/AIDS (CDC, 2013). Rifabutin (RFB) is an alternative rifamycin, which has less impact on CYP3A4 activity and improved pharmacokinetics compared to RIF (Regazzi et al., 2014). Although the activity of RFB is comparable to that of RIF for treatment of drug-susceptible TB, current guidelines recommend limited use of RFB only in drug-susceptible adult TB patients with HIV/AIDS or adults experiencing intolerance to RIF. Widespread use of RFB has also been limited by its cost and absence from most commercial susceptibility testing systems (Horne et al., 2011). However, RFB costs have been lowered by its addition to the WHO Essential Medicines List, while the recently validated the Sensititre MYCOTB system includes RFB in its drug panel (Lee et al., 2014).

Resistance to both RIF and RFB is largely associated with mutations in an 81-bp RIF resistance determining region (RRDR) within the *rpoB* gene of *Mycobacterium tuberculosis* (Jamieson et al., 2014). Although high-level cross-resistance between the two rifamycins is reported, some studies have shown RFB susceptibility in RIF-resistant strains of *M. tuberculosis* in association with specific *rpoB* mutations (Yoshida et al., 2010, Cavusoglu et al., 2004, Jamieson et al., 2014,

ElMaraachli et al., 2015). Thus, it has been argued that knowing the type of *rpoB* mutation may have clinical implications for guiding rifamycin-based therapeutic regimens (Sirgel et al., 2013, Berrada et al., 2016).

Studies from low HIV settings have reported that 13 to 26% of MDR-TB isolates show sensitivity to RFB (Chen et al., 2012, Jo et al., 2013, Schon et al., 2013). However, there is limited information on the frequency of RFB susceptibility among MDR-TB isolates in an HIV endemic region. This study aimed to determine the proportion of MDR strains with RFB susceptibility in Gauteng Province, South Africa. Gauteng is the economic hub of South Africa, with a large migrant workforce, where 73% of TB patients are also co-infected with HIV. In addition, we examined correlations between specific *rpoB* mutations and the minimum inhibitory concentration (MIC) of RIF and RFB among clinical MDR-TB isolates.

4.2 MATERIALS AND METHODS

4.2.1 Clinical specimens and Ethics

A total of 211 available MDR-TB isolates from the Centre for Tuberculosis (CTB) repository were included. These isolates were collected as part of a cross-sectional MDR-TB study of patients diagnosed from January to June 2010 in Gauteng Province of South Africa. . Drug susceptibility patterns were previously determined by MGIT 960 method. The isolates included had different susceptibility profiles. Ethics approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of the Free State, South Africa (Ref: 230408-011).

4.2.2 Primary isolation and identification of MTB

All specimens were collected from hospitals settings. Specimens were processed within 24 h of collection Sputa were decontaminated using a standard NALC-sodium hydroxide-sodium citrate method with a 1.5% final NaOH concentration. Following the centrifugation, sediments were

reconstituted with 2.5 ml of phosphate buffer, pH 6.8, Sputum smear slides for AFB examination were prepared from these reconstituted sediments. All highly positive AFB smears grade 3+ or 4+ were enrolled to maximize positivity. The 7H10 agar was inoculated with 0.2 ml of the AFB positive sediments. The agar were incubated at 37^0C and examined for growth weeky up to 36 weeks. Plates were sealed and incubated at 5-10% CO_2. All media were examined for colonies of typical MTB morphology. Positive cultures were confirmed as MTB using the Ziehl-Neelsen method and the Capilla (Tauns, Numazu, Japan) lateral flow MPB64 antigen detection method.

Ethics approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of the Free State (Ref: 230408-011).

4.2.3 Minimal Inhibitory Concentration Determination of *M. tuberculosis* isolates

MICs were determined using a commercially available Sensititre MYCOTB plate (TREK Diagnostics, Cleveland, Ohio), following the manufacturer instructions. The MIC test range for both RIF and RFB was from 0.12 to 16 mg/L. Resistance and sensitivity to RIF were defined as MIC >1 mg/L and MIC ≤1 mg/L, respectively, and to RFB as MIC >0.5 mg/L and MIC ≤0.5 mg/L, respectively, based on laboratory standards (CSLI, 2011).

4.2.4 DNA extraction, PCR and Sanger Sequencing

All isolates were grown on 7H10 agar; genomic DNA was extracted using the phenol-chloroform (CTAB) method (Said et al., 2016). Six primer sets were used for PCR amplification of the entire *rpo*B gene (Table 4.1). Following Sanger sequencing of amplicons, mutations in *rpo*B were identified by alignment to H37Rv reference strain (NCBI Accession number AL123456) using ClustalW2.

Table 4.1: *rpoB* primers used to amplify RRDR region

Primer Set	Forward Primers (5'-3')	Reverse Primers (5'-3')	Amplicon Size (nt)
rpoB-RRDR	GGGAGCGGATGACCACCCA	GCGGTACGGCGTTTCGATGAAC	350
rpoB-2	ATGACGTACGCGGCTCCACTGTTCG	GGTGGTCATCCGCTCCCGGACCAC	840
rpoB-3	CGCGGCGAACGGGCCCGTGGGCA	CGGGATCACCTTGACGCTGTGCAG	675
rpoB-4	CTGTCGGTGTACGCGCGGGTCAA	GGGACCGTCGGCGATCACCTGACC	621
rpoB-5	CCACGGCACTTGCGCCAACCAG	CATCCGTCGCGGCACGCCGTGGGT	742
rpoB-6	CCGGTTGAGGACATGCCGTTC	TCCCTTTCCCCTAACGGGTTTAGT	879

4.2.5 Statistical analysis

Kruskal-Wallis (KW) tests were used to determine whether mutations were associated with differences in RIF and RFB MICs. A Dunn test incorporating the Benjamini-Hochberg false discovery rate correction was performed to identify pairwise differences in RFB MICs.

4.3 RESULTS

Of the 211 MDR isolates in the collection, MIC and sequencing data were available for 189 (90%). The remaining 22 (10%) isolates were excluded from analysis due to RIF resistance not confirmed on MIC testing, contamination or loss of viability.

Among the 189 MDR isolates analyzed, S531L was the most frequently observed *rpoB* RRDR mutation, found in 105/189 (56%) isolates, followed by H526Y in 27 (14%), H526D in 19 (10%), D516V in 17 (9%), L533P in four (2%) and D516G_L533P in three (2%) isolates (Table 1). Of

the 189 isolates, 138 (73%) showed resistance to both RIF and RFB, while 51 (27%) were RIF resistant but exhibited RFB susceptibility. Resistance to both RIF and RFB was predominantly associated with S531L (91/105, 87%), H526Y (20/27, 74%), and H526D (15/19, 79%) mutations. Rifabutin susceptibility was most commonly observed for isolates carrying D516V (15/17, 88%) and L533P (3/4, 75%), although two of three isolates with the double mutation D516G_L533P were moderately resistant to RFB. Nine (5%) RIF-resistant isolates had no mutations in the RRDR. However, three of the nine had a mutation outside the RRDR. Two of these (V276L and V276F) were RFB susceptible, while one (V252E) was RFB resistant; the remaining six had no mutations in *rpo*B outside the RRDR.

The Kruskal-Wallis test showed significant association between mutations and MICs for both rifamycins (KW for RIF: H=67.699, p=0.0001; for RFB: H=42.988, p=0.0003). Dunn's pairwise comparison with Benjamini-Hochberg false discovery rate correction showed that RFB MICs in D516V and L533P isolates were not significantly different from wild type (q=0.1317), nor from each other (q=0.4118), although the numbers were small (WT n=9; L533P n=4; D516V n=17). The RFB MICs for H526D H526Y and S531L were not significantly different from each other. However, there were significant differences between RFB MICs in D516V isolates and those in H526D, H526Y and S531L (all q<0.0001). Among isolates with the most common RRDR mutations, there was a significantly lower median MIC for RFB compared to RIF, with at least a 3-fold lower median MIC for S531L, H526Y and H526D; and 5- and 7-fold lower medians for D516V and L533P, respectively (Figure 4.1). Additionally, higher levels of resistance to RIF correlated with higher resistance to RFB (Spearman's correlation coefficient r=0.4511, p<0.000001).

Table 4.2: Mutations in *rpo*B RRDR and MICs of RIF and RFB for all MDR isolates

Mutation	Total Number	Number of RFB S isolates (% of total)	MIC (ug/ml)		Total number of isolates with the mutation	Median MIC:RIF	Median MIC:RFB
			RIF	RFB			
S531L	105	14 (13)	16	16	4	16	2
			16	8	5		
			16	4	21		
			16	2	31		
			16	1	28		
			16	0.5	10		
			16	0.25	2		
			8	2	1		
			8	0.12	1		
			4	8	1		
			4	0.5	1		
H526Y	27	7 (26)	16	16	5	16	2
			16	8	2		
			16	4	3		
			16	2	6		
			16	1	4		
			16	0.5	5		
			16	0.25	2		
H526D	19	4 (21)	16	16	2	16	2
			16	8	4		
			16	4	2		

Table 4.2: Continued…….

			16	2	5		
			16	1	2		
			16	0.5	4		
D516V	17	15 (88)	16	4	1	4	0.12
			16	1	1		
			16	0.25	1		
			16	0.12	1		
			8	0.25	1		
			8	0.12	3		
			4	0.12	5		
			2	0.25	1		
			2	0.12	3		
L533P	4	3 (75)	16	1	1	16	0.31
			16	0.5	1		
			16	0.12	2		
D516G-L533P	3	1 (33)	16	4	1	16	1
			16	1	1		
			16	0.5	1		
S531Q	5	2 (40)	16	2	1	16	2
H526P			16	4	1		
H526R			16	4	1		
H526L			4	0.12	1		
ins 3bp(CTT) c514			8	0.5	1		
WT	9	5 (56)	16	4	1	16	0.5
			16	1	3		
			16	0.5	1		
			16	0.25	2		
			4	0.25	1		
			4	0.12	1		
Total					189		

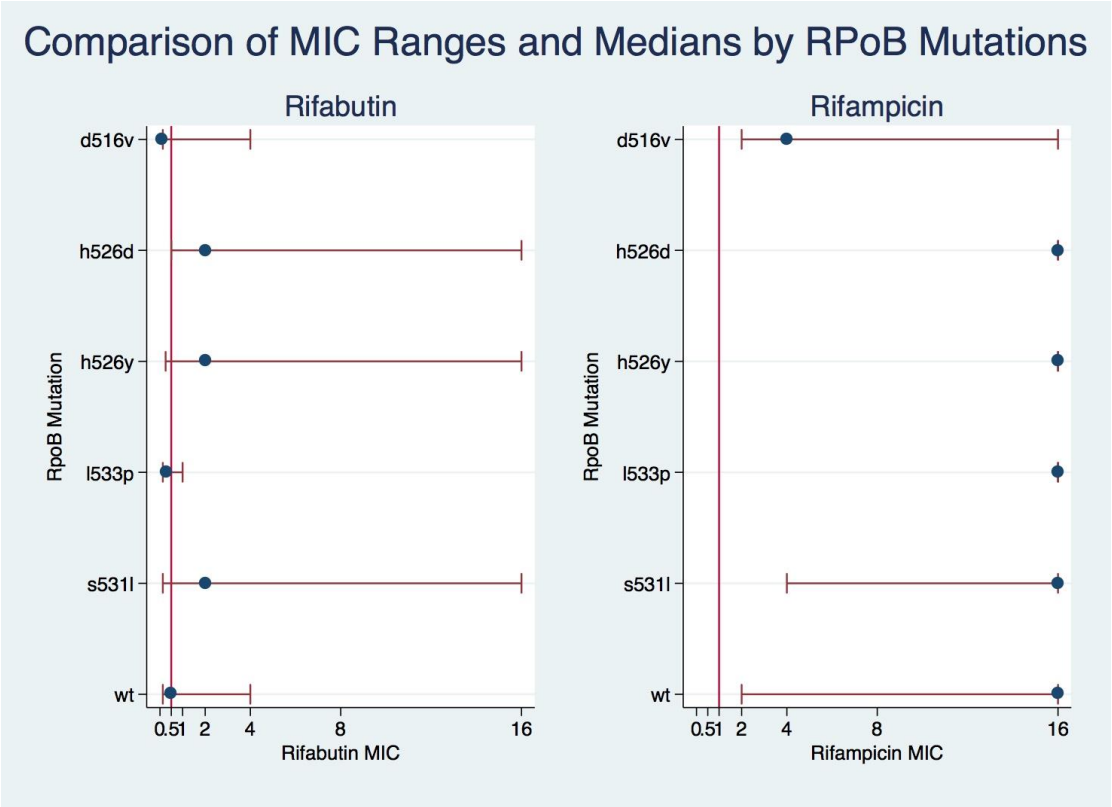

Figure 4.1: MIC range showing *rpoB* mutations with levels of RIF and RFB resistance

4.4 DISCUSSION

This study sought to determine the potential usefulness of RFB for MDR-TB patients in a high HIV prevalence setting. In our study population, 27% of MDR clinical isolates retained sensitivity to RFB. This is in agreement with previous studies conducted in Turkey, Canada, Bangladesh and Taiwan, reporting RFB susceptibility in 13% to 28% of RIF-resistant isolates (Jamieson et al., 2014, Cavusoglu et al., 2004, van Ingen et al., 2011, Heysell et al., 2015). Moreover, the strong association we observed between levels of resistance to RIF and RFB is consistent with another published report (Jamieson et al., 2014). The mutations S531L, H526Y, and H526D were primarily associated with resistant to both drugs, while D516V and L533P were mainly RFB susceptible, as also previously reported (Cavusoglu et al., 2004, Sirgel et al., 2013, van Ingen et al., 2011). Rifabutin MICs for D516V isolates were significantly lower than those associated with the most common RRDR mutations (S531L and D526Y). Moreover, no significant differences were found between RFB MICs of isolates with D516V or L533P mutations and those with wild type *rpo*B sequences. Interestingly, a double mutation at positions D516V-L533P was found in isolates with lower levels of RFB resistance. The treatment option may need to be investigated since increased RFB concentration are not currently recommended due to possible toxicity issues (Sirgel et al., 2013).

Current guidelines recommend RFB for treatment of TB patients with HIV co-infection or poor tolerance to RIF, only after safety and efficacy of the drug has been demonstrated (Horne et al., 2011, Nettles et al., 2004, Li et al., 2005). However, despite the known sterilizing properties of rifamycins, no randomized controlled trials (RCT) have investigated the use of RFB in treatment of MDR-TB. The results of our study show that a proportion of MDR-TB cases may potentially benefit from the inclusion of RFB in chemotherapeutic regimens. Relevant clinical studies are needed to establish appropriate RFB-based regimens for achieving improved clinical outcome, particularly in HIV endemic regions. Our findings additionally support an association between specific *rpo*B mutations and RFB susceptibility, which can have implications for expanding therapeutic options in MDR-TB. For example, while *rpo*B mutations would not provide definitive

classification of RFB susceptibility, molecular assays could be used as a basis for targeted susceptibility testing in MDR-TB patients.

The limitations of our study include the use of CLSI recommended clinical breakpoints for susceptibility testing of RFB (CSLI, 2011). However, a recent review and a study from a low HIV-TB prevalence setting have both suggested that the current breakpoint of 0.5 mg/L may be in need of revision (Schon et al., 2013, Crabol et al., 2016). In addition, as our study was focused on MDR-TB, our population of clinical isolates did not include any RIF-susceptible strains, and our study design did not enable us to determine HIV status of individual cases.

The study identified three novel mutations outside of the RRDR (V276L, V252E and V276F) in RIF resistant isolates, supporting inclusion of the 5'-end of *rpoB* in molecular testing for RIF/RFB resistance, as previously suggested (Tan et al., 2012). Moreover, the wide range of MICs seen in isolates with the same *rpo*B mutations suggests that other, as yet unidentified, genes may be contributing to the observed resistance levels. The use of whole genome sequencing of clinical isolates of *M. tuberculosis* would be helpful in expanding our understanding molecular mechanisms of drug resistance.

4.5 CONFLICT OF INTEREST:

The authors declare no conflict of interest.

4.6 AUTHOR CONTRIBUTION

IR, DF and HS performed the experiments analyzed results, drafted the manuscript. MA, HM statically analyzed the paper and edited the paper. GK, OS, HK, AD edited and drafted the manuscript. NI conceived the study, analysed results and drafted manuscript. All authors read and approved the final manuscript.

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CHAPTER 5

RESISTANCE TO ANTI TB DRUGS IN SOUTH AFRICA: ASSOCIATION BETWEEN MIC AND GENETIC RESISTANCE DETERMINANTS

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Running Title: Resistance to anti TB drugs in South Africa: Association between MIC and genetic resistance determinants

Summary

Genotypic studies have shown that different genetic mutations affect phenotypic resistance in different ways. Determination of MICs have shown to accomodate and accurately assess the biological effects of genomic variation in understanding the mechanism of resistance. Thus the study compared MICs with drug resistance mutations to predict the level of resistance. MICs for first-and second-line drugs were determined using MYCOTB method and relevant genes including: *kat*G and *inh*A (INH and ETH), *gyr*A and *gyr*B (OFX and MXF), *rrs* and *rps*l (AMI, KAN and SM) and *emb*B (EMB and ETH) were sequenced. The Kruskall Wallis (KW) static was used to determine the association between MICs and the different mutations. The *kat*G mutations S315T and S315G was significantly associated with INH resistance (p=0.0001) but not with ETH resistance (0.832). However, *inh*A mutations C-15T, T-8A and G-17T were significantly associated with both ETH (p=0.001) and INH resistance (p=0.013). The *rrs* mutations; A1401G, C492T_A514C and A514C_A1401G were associated with high level of resistance to KAN (p=0.0001), AMI (p=0.0001) and SM (p=0.001). The *rps*l mutations K43R, K88R and K88M was significantly associated with SM resistance (0.001), but were not statically associated with AMI (p=0.822) or KAN (p=0.1078) resistance. The MXF and OFX *gyr*A mutation at codon 90 and 91 were associated with lower MIC compared to isolates with at codon 94 (p=0.0001). While *gyr*B mutations were not significantly associated with both OFX (p=0.0554) and MXF (p=0.1422). The *emb*B mutations codon 306, 405 or 406 were not significantly associated with EMB resistance (p=0.448). In conclusion our results indicated that different drug resistance mutations were associated with different MIC ranges. Such information can be useful in acurately predicting resistant phenotypes, which can be used to guide clinical decision-making and the development of new and improved diagnostic assays.

5.1 INTRODUCTION

Global surveillance data suggest that in 2017, there was an estimated 460.000 new cases of multi-drug resistant tuberculosis (MDR-TB) [i.e resistant to at least isoniazid and rifampicin] and that 8.5% of these cases were extensively drug-resistant (XDR) [i.e also resistant to at least one fluoroquinolone (FLQ) and one injectable drug] (WHO, 2018). It is estimated that more than half (55%) of these cases are detected, and an even smaller proportion receive appropriate treatment (WHO, 2018). One of the main problems in the control of drug-resistant TB (DR-TB) is the lack of laboratory capacity to diagnose resistance. This has necessitated initiatives to improve diagnostic testing capabilities and more efficient detection of drug resistance. Convenctional drug susceptibility testing (DST) methods are time consuming, labour intensive and methods for DST of many of the second-line drugs have not yet been fully standardized (Kim, 2005, Böttger, 2011, Ängeby et al., 2012

The introduction of molecular techniques improved diagnostics for detection drug-resistance and reduced the lengthy delays to receive DST results using conventional culture techniques. However, prediction of drug resistnce using molecular methods depends on correlation of mutation with resistant phenotype. Previous studies on genetic association for drug resistance in *M. tuberculosis* mainly relied on phenotypes defined by DST performed at a single CC. Although, studies on molecular drug resistance mechanisms in *M. tuberculosis* have shown that different genetic mutations affect phenotypic resistance in different ways with minimum inhibitory concentration (MIC) levels strongly correlating with the position and nature of the encoded amino-acid substitution (Sirgel et al., 2013, Jamieson et al., 2014, Lee et al., 2014, Rukasha et al., 2016).. Minimum Inhibitory concentration more appropriate to assess the biological effects of genomic variation in understanding the mechanism of resistance. This way the susceptibility status of such strains can be predicted accurately by molecular assays which could play a crucial role in modern patient management. Hence the study aimed to determine association of genetic polymorphism and MICs of first and second-line drugs in MDR-TB isolates to predict the level of resistance.

5.2 MATERIALS AND METHODS

5.2.1 *Mycobacterium tuberculosis* strains used in the study and Ethics

Multidrug resistant TB isolates collected between January and June 2010, as part of a cross-sectional MDR-TB studies in Gauteng Province of South Africa were included. Drug susceptibility patterns were previously determined by MGIT 960 method. Ethical approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of the Free State (Ref: 230408-011).

5.2.2 Determination of *M. tuberculosis* MICs using the Sensititre MYCOTB plate

The MICs were determined using the Sensititre MYCOTB plate (MYCOTB) method. Purity of *M. tuberculosis* cultures used for this study was checked prior to MIC testing by inoculation on 5% sheep blood agar which was then incubated at 37^0C for 48 h and checked for contamination. The MYCOTB assay comprises a 96 well microtitre plate containing 12 lyophilized antibiotics, configured for determination of MICs to first- and second-line TB drugs. The MIC ranges for the 12 drugs included were as follows: for ethambutol, streptomycin (SM), and ofloxacin (OFX),0.12 to 16 μg/ml; for rifabutin (RFB), amikacin and rifampicin 0.3 to 40 μg/ml and 0.6 to 40 μg/ml; for ethionamide (ETH) and kanamycin (KAM) respectively 0.5 to 64 μg/ml; for p-amino salicylic acid 0.06 to 8 μg/ml; for moxifloxacin, (MXF) and 0.03 to 4 μg/ml for isoniazid].

The MIC testing was performed according to the manufacturer's instructions: Briefly, *M. tuberculosis* colonies were scraped off the surface of 7H11 plates and vortexed for 30 to 60 sec in a sterile saline-tween-tube containing glass beads (Trek Diagnostics Systems). The suspension was allowed to settle for 15 to 30 min and then adjusted to a turbidity of the supernatant equivalent to a 0.5 McFarland standard, using a nephelometer (BD, Diagnostics). A 100ul of suspension was transferred to 11ml of Middlebrook 7H9 broth supplemented with oleic acid-albumin dextrose-catalase (OADC). A Sensititre AIM™ Automated Inoculation Delivery System (Trek Diagnostics systems) was used to dispense 100 μl of the OADC-containing inoculum to each well. The

MYCOTB plates were sealed and incubated at 37^0C. Plates were checked after 48 hours for contamination. The MYCOTB plates were read at 7, 14 and 21 days of incubation. Initially the plates were read by visual inspection for growth and subsequently via a computerized Vizion system (Trek Diagnostic Systems, Cleveland, Ohio, USA). For each antibiotic, the lowest concentration with no visible growth was considered to be the MIC.

5.2.3 DNA extraction, PCR and Sanger Sequencing

Genomic DNA was extracted using the phenol-chloroform (CTAB) method (Van Embden et al., 1993). PCR amplification was performed using primer sets shown in Table 5.1. A singleplex PCR was performed for each of the genes using primer sets shown in Table 5.1. The amplification conditions were the same for all genes and consisted of a 5 min denaturation step at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 62°C and 50 sec at 72°C and a final extension step at 72°C for 2 min. PCR products were sequenced by the Sanger method using 3500xl. Genetic Analyzer (Applied Biosystems). Following sequencing, mutations were identified by alignment of nucleotide sequences to H37Rv reference strain (NCBI Accession number AL123456) (Cole, 2002) using ClustalW2 (Li et al., 2015).

Table 5.1: Primers sets used to amplify mutation regions

Drugs	primer set	Forward Primers (5'-3')	Reverse Primers (5'-3')
KAN,AMI,SM	rrs	CCATCGACGAAGGTCCGGCTTC	CGCGTCCTGTGCATGTCAAACC
KAN,AMI,SM	rrs -2	GTAGCTAACGCATTAAGTACC	CATACAGACAAGAACCCCTCACGG
KAN,AMI,SM	*Rps*l	GGCCGACAAACAGAACGT	GTTCACCAACTGGGTGAC
OFX, MXF	gyrA	CCGATCGAACCGGTTGACATC	GGGTTCGGTGTACCTCAT
OFX,MXF	*gyr*B	AACACCGAGGTCAAATCGTT	CTGAATGCGTCTTCCTTTCCTTGT
EMB	embB	TCCACCCGGCCGACCACGCT	AGCGCCGCCGGTGTGAGGCCG
ETH, INH	*inh*A	CCTCGCTGCCCAGAAAGGA	ATCCCCGGTTTCCGG
ETH,INH	katG	CCCATGGCCGCGGCGGTCGACATT	CGCCGCCTTGGCGGTATTGCC

5.2.4 Statistical Analysis

To determine the association between MICs and mutation, the MICs of different TB drugs were linked to their respective mutations as follows INH and ETH was linked to *inh*A and *kat*G mutations, *rrs* and *rps*l mutations where linked to SM, KAN and AMI, *gyr*A and *gyr*B mutations were linked OFX and MXF and EMB was linked to the *emb*B mutation. Graphs were drawn to show the relationship between MICs and mutation (Figure 5.1 to Fig 5.14). To determine the association between MICs and the different mutations, the Kruskall-Wallis (KW) statistic was applied.

5.3 RESULTS

A total of 211 isolates with MIC and sequencing results were used for analysis. Interpretation of MIC results was done using the MIC breakpoints established by indirect agar proportion method (Lee et al., 2013). The association of MICs and different muations is summarized in Table 5.2 and figures 1 to 14.

Table 5.2: Association of genetic mutations and MICs for anti-TB drugs

Drug	Total resistant isolates No (%)	Mutation	Point mutation	Resistant isolates No (%)	MIC Geometric Mean	MIC Range of mutation	Kruskal-Wallis with 2 degrees of freedom	P Value
INH	194 (92)	*inh*A	C-15T T-8A G-17T wt	49 (25) 28 (14) 2 (1) 114 (59)	3.29 4 3 3.58	0.5- 4 4- 4 2- 4 0.5- 4	14.46	0.013
		*kat*G	S315T S315G Double peak - S315T Wt	149 (77) 1(0.5) 1(0.5) 43(22)	3.9 4 4 2	2- 4 4- 4 4- 4 0.5- 4	80.50	0.0001
ETH	109 (52)	*inh*A	C-15T T-8A G-17T Wt	44 (40) 27 (25) 1 (1) 37 (34)	35 39 40 32	10- 40 10- 40 40- 40 10- 40	57.61	0.001
		*kat*G	S315T S315G Wt	80 (73) 1 (1) 29 (27)	36 40 32	10- 40 40- 40 10- 40	1.519	0.832
OFX	35(17)	*gyr*A	A90V D94G D94N S91P Wt	13 (37) 13 (37) 1 (3) 2 (6) 6 (17)	7 14 1 6 9	4- 8 8- 16 32- 32 4- 8 4- 16	68.65	0.0001
		*gyr*B	L566F D472H Wt	2 (6) 1 (3) 32 (91)	8 8 11	8- 8 8- 8 4- 32	10.805	0.0554
MXF	30(14)	*gyr*A	D94G A94V D94N S91P Wt	17 (57) 7 (23) 1 (3) 1 (3) 4(13)	2 1 1 1 2	1- 8 1- 8 1- 8 1- 1 1- 4	72.62	0.0001
		*gyr*B	L566F Wt	2(7) 28(93)	1 2	1- 1 1- 8	8.27	0.1422
KAN	13(6)	*Rrs*	A1401G C492T C492T_A1401G A514C_A1401G Wt	6 (46) 2 (15) 1 (8) 1 (8) 2 (15)	40 10 40 40 33	40- 40 10- 10 40- 40 40- 40 20- 40	44.69	0.0001
		*rps*l	T39T Wt	1 (7) 12 (92)	10 36	10- 10 20- 40	9.032	0.1078

Table 5.2: continued…….

AMI	13(6)	*Rrs*	A1401 A514C_A1401 G C492_A514C Wt	6 (46) 1 (8) 1 (8) 5 (39)	16 16 16 16	16- 16 16- 16 16- 16 16- 16	42.36	0.0001
		*rps*l	K43R wt	1 (8) 12 (92)	16 16	16- 16 16- 16	2.190	0.822
SM	82(39)	*rrs*	A514C C517T G878A C492T A906G A514C_A1401 G A1401G Wt	10 (12) 9 (11) 6 (7) 4 (5) 2 (2) 1 (1) 1 (1) 49 (60)	29 16 20 14 6 32 4 23	16- 32 4- 32 8- 32 4- 32 4- 8 32- 32 4- 4 4- 322	66.36	0.001
		*rps*l	K43R K88R K88M Wt	32 (39) 5 (6) 1 (1) 44 (54)	29 19 32 17	4- 32 8- 32 32- 32 4- 32	68.42	0.001
EMB	92 (44)	*emb*B	M306V M306I A405_A409P E378A D328G G406A G406S M306L M306V_E378 A wt Not sequenced	22 (24) 20 (22) 11 (12) 4 (4) 2 (2) 1 (1) 1 (1) 1 (1) 1 (1) 27 (29) 2 (2)	13 15 17 22 8 16 8 32 8 19 20	8- 32 8- 32 8- 32 8- 32 8- 8 16- 16 8- 8 32- 32 8- 8 8-32 8- 32	210	0.448

Figures 5.1 to 5.16: showing the relationship between mutations and MICs

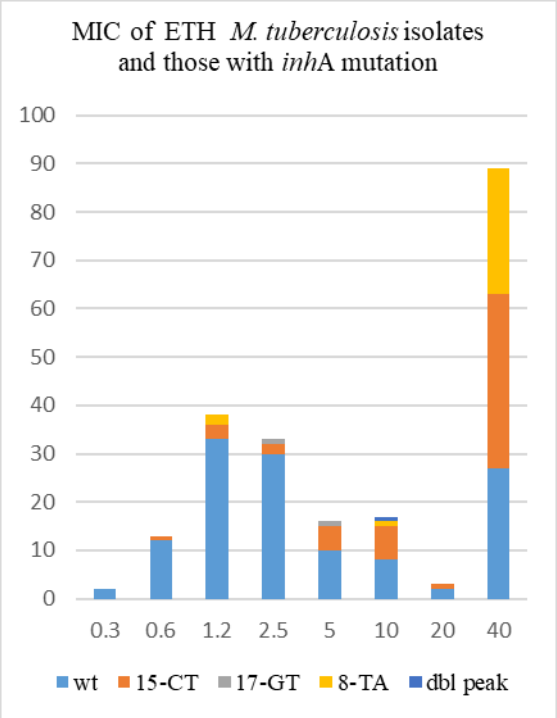

Figure 5.1

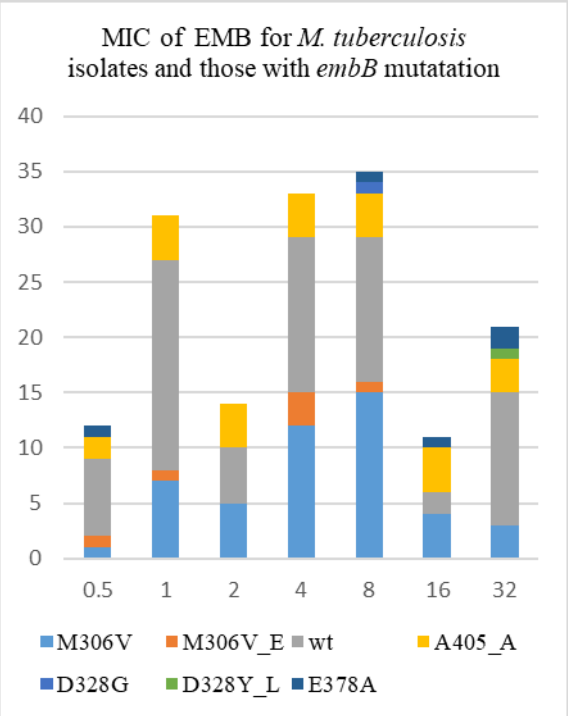

Figure 5.2

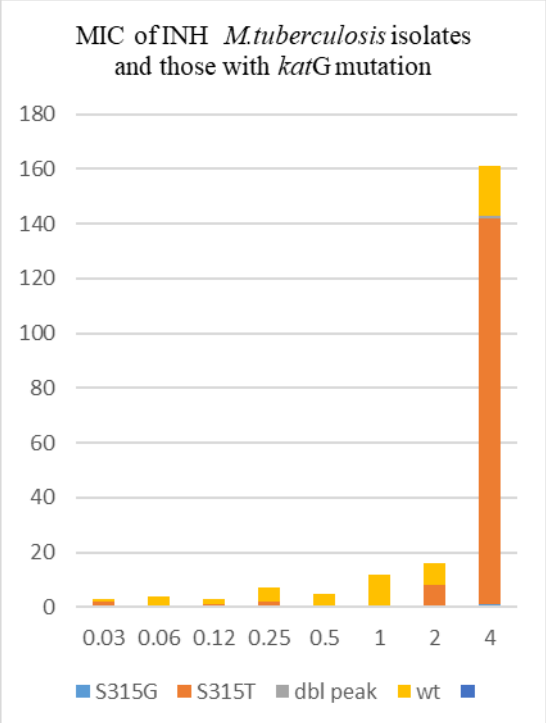

Figure 5.3

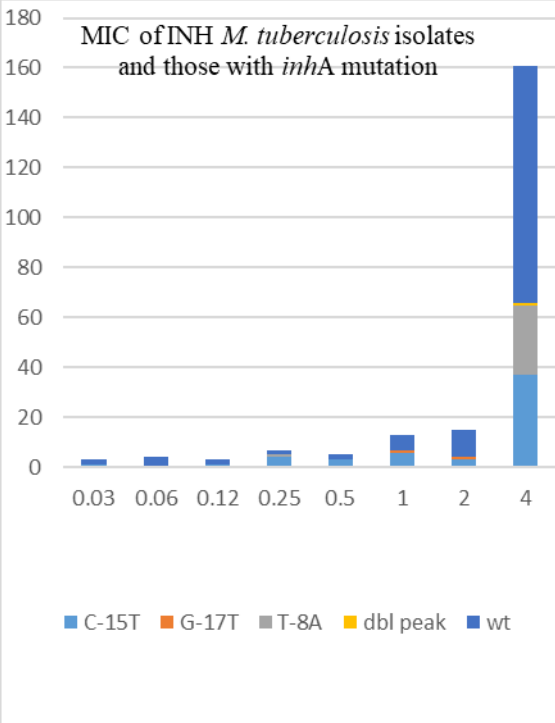

Figure 5.4

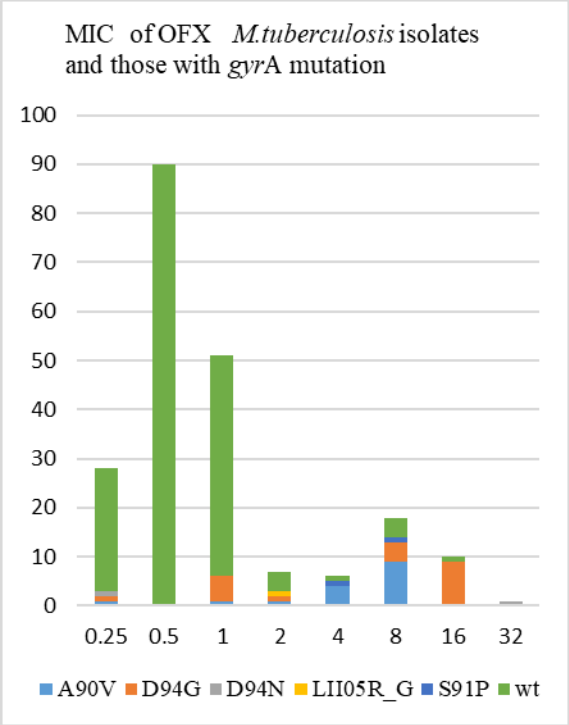

Figure 5. 5

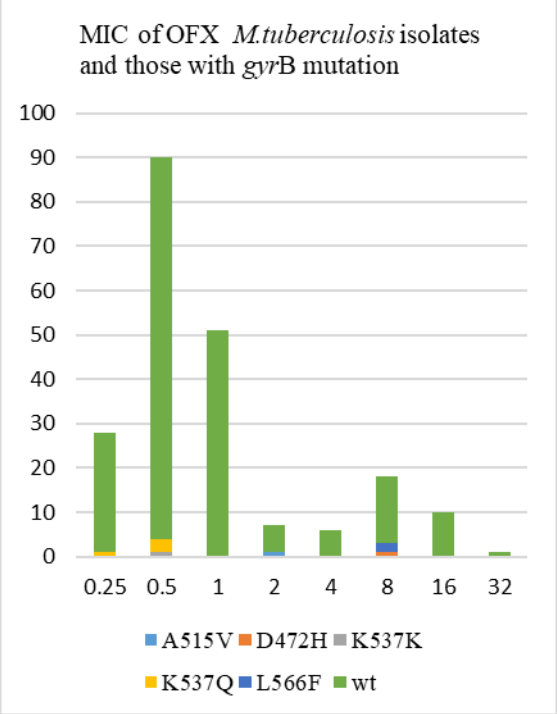

Figure 5. 6

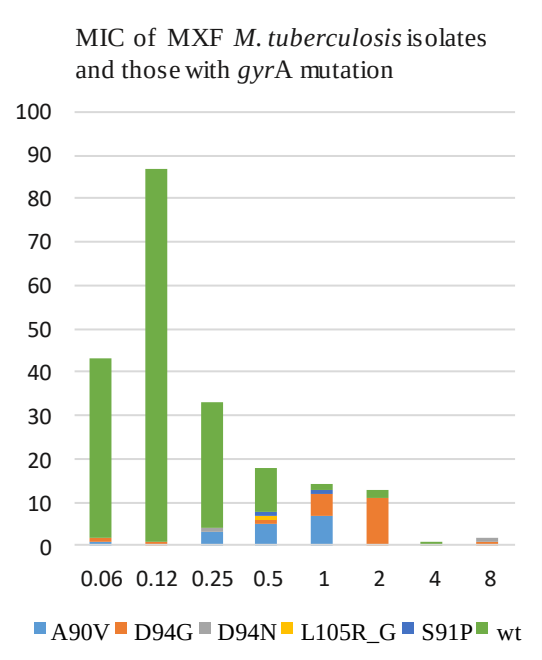

Figure 5. 7

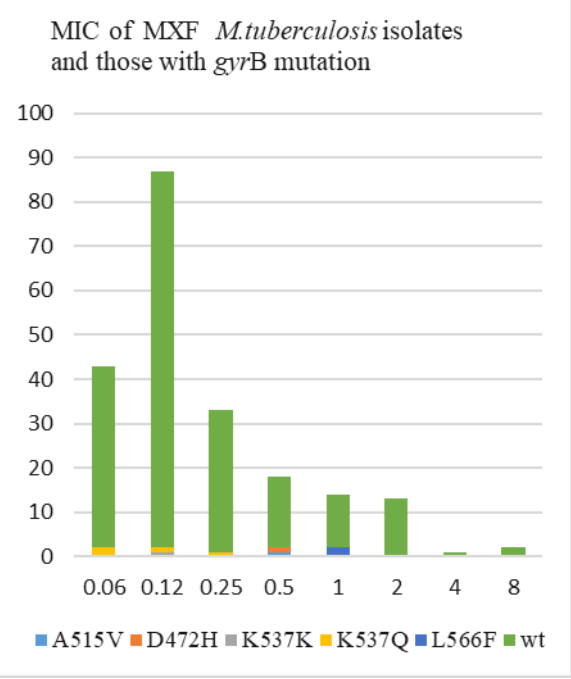

Figure 5.8

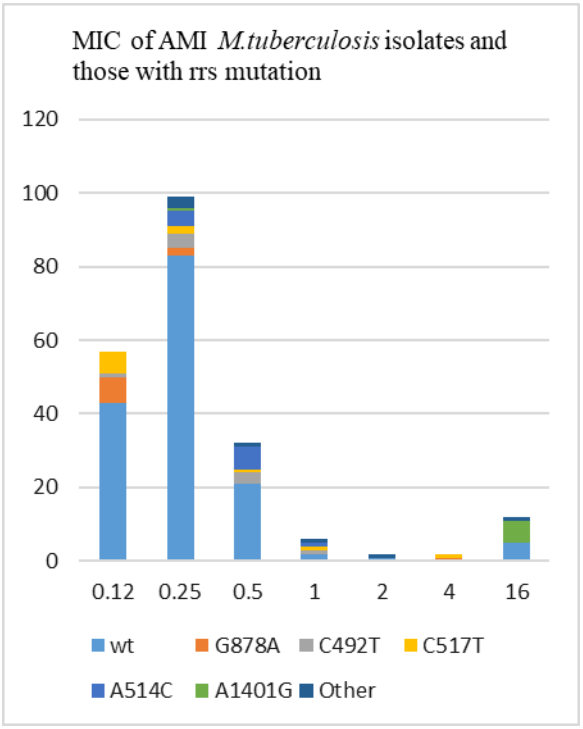

Figure 5. 9

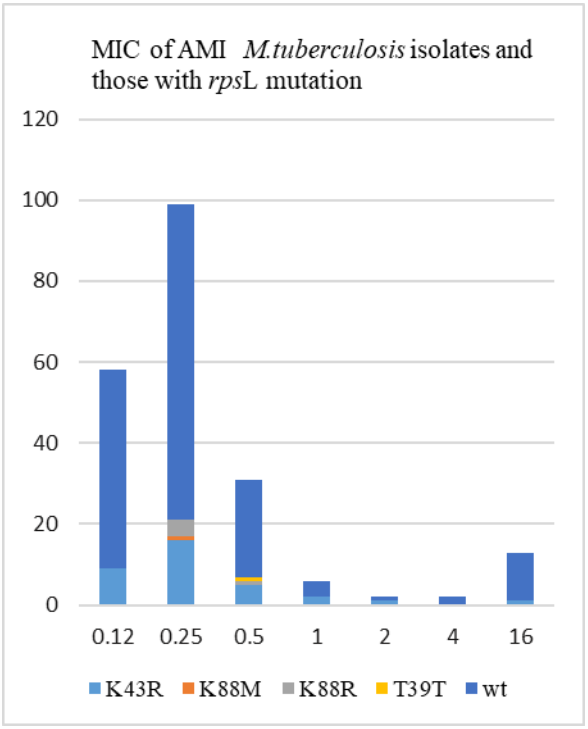

Figure 5.10

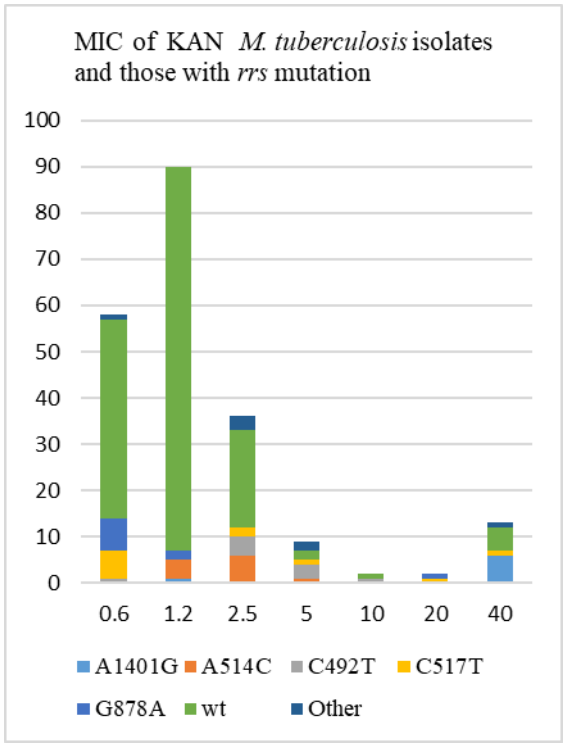

Figure 5.11

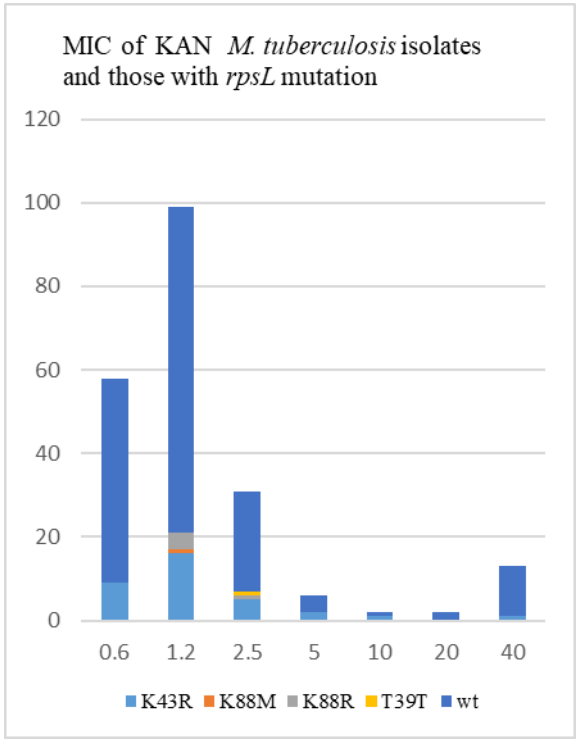

Figure 5.12

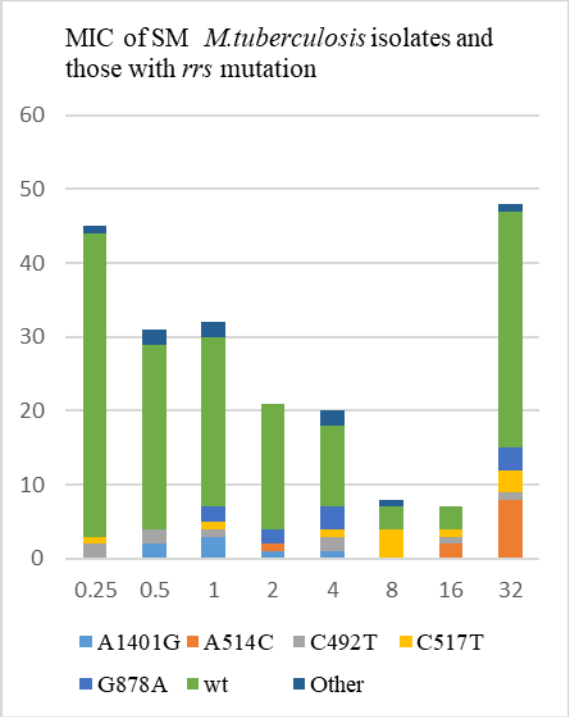

Figure 5. 13

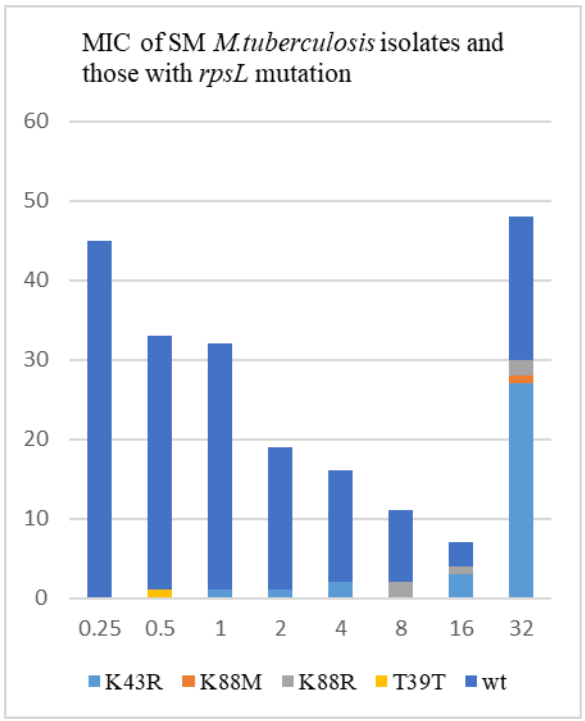

Figure 5. 14

INH and ETH: A total of 194/211 (92%) isolates were INH resistant at breakpoint of >0.25 µg/ml, while 109/211 (52%) isolates showed ETH resistance at breakpoint >5 µg/ml. The most common *kat*G mutation was S315T which was found in 149/211 (77%) (MIC 2 to 4g/ml) for INH and 44/ 109 (40%) (MIC 10 to 40ug/ml) for ETH isolates, followed by S315G found in 1/194/ (0.5%) (MIC 4ug/ml) of INH and 1/109 (1%) (MIC 40ug/ml) of ETH. While for the *inh*A promoter region the most prevalent mutation C-15T was exhibited by 49/194 (25%) (MIC: 0.5 to 4 µg/ml) isolates, followed by T-8A with 28/194 (14%) (MIC >4 µg/ml) and G-17T in 2/211 (1%) (MIC; 2 to 4 µg/ml) isolates (Table 2). A total of 69 isolates showed cross resistance between ETH and INH. A statistically significant association was found between *kat*G and INH (KW =80.50; p value = 0.0001) and *inh*A and INH (KW=14.46: p= 0.013). Similarly, a significant association was found between *inh*A mutations and ETH (KW=57.61: p=0.001). However, the association was not significant between *kat*G and ETH (KW=1.519; p =0.832).

MXF and OFX: In total, 35/211 (17%) isolates were resistant to OFX at breakpoint >2 µg/ml, while 30/211 (14%) isolates were resistant to MXF at >0.5 µg/ml. Mutations in *gyr*A at the codon

90 (n=13) and 94 (n=13), were detected in the majority of OFX-resistant isolates. Isolates with mutation at codon 90 and 91 had lower MIC ranging between 4 to 8 µg/ml as compared to MIC range of 8 to 32µg/ml for isolate with a mutation at the codon 94. The *gyr*B mutations, L566F and D472H were found in 2 and 1 isolates, respectively and both had MIC of 8 µg/ml.

The MXF D94G *gyr*A mutation was found in the majority (n=17) of MXF resistant isolates followed by A94V (n=7) (Table 2). All MXF-resistant isolates with mutations at codon 91 had MIC of 1 µg/ml while, isolates with mutation at codon 94 had higher MIC ranging from 1 to 8 µg/ml. The KW was significant between *gyr*A mutations (D90G, D94N and A94V) and MICs to both OFX (68.65; p=0.0001) and MXF (KW=72.62; p=0.0001). The L566F *gyr*B mutation were found in only two MXF resistant isolates both with MIC ≥8 µg/ml. The association was not significant between *gyr*B mutations and both MXF (KW=8.27; p=0.1422) and OFX (KW=10.805; p=0.0554) for MXF (

KAN, AMI and *SM*: A total 13/211 (6%) isolates were found to be resistant to KAN and AMI at breakpoints of 5 µg/ml and 4 µg/ml, respectively. Eleven out of 13 (85%) isolates showed cross resistance between the two drugs. For AMI the *rrs* mutations A1401G, A514C_A1401G and C492T_A514C were found in 6, 1 and 1 isolates respectively. All AMI isolates with *rrs* mutation had MIC of 16µg/ml. Only one isolate had K43R *rps*l mutation with MIC 16 µg/ml while the remaining (12/13) samples had wt pattern (Table 2). For KAN the *rrs* mutations A1401G, C492T, C492T_A1401G and A514C_A1401G were found in 6, 2, 1 and 1 resistant isolates respectively. All isolates with *rrs* mutations had high MICs of 20 to 40µg/ml except for 2 isolates harbouring the C492T which had MIC of 10 µg/ml. The association was significant between *rrs* mutations and both KAN (KW=44.69; p=0.0001) and AMI (42.36; p=0.0001). Whereas, the association was not significant between the *rps*l mutations and AMI (KW=2.190; p=0.822) and KAN (KW =9.032; p=0.1708).

In total, 82/211 (39%) isolates with resistant to SM at >2µg/ml. The most prevalent *rrs* mutation was A514C (n=10), followed by C517T *rrs* mutations (n=9) and C878A *rrs* mutations (n=6) (Table 2). Whereas, the K43R was the most prevalent *rps*l mutation (n=32), followed by the K88R *rps*l mutation (n=5). The MIC range for mutations A514C and A514C_A1401G was higher at 16

to 32 µg/ml while, MIC range for mutations C517T, C492T, A906G, and A1401G were lower ranging from 4 to 32ug/ml. The KW statistic for SM and *rrs* and SM and *rps*l mutation was significant (KW= 66.36; p=0.00; KW=68.42; p=0.001, repectivley) with MIC of 4 to 32.

EMB: In total there were 92/211 (44%) isolates with resistant to EMB at breakpoint >5 µg/ml and 44/211 (21%) at breakpoint >10 µg/ml. The *emb*B mutation at codon M306V was the most prevalent mutation (n=22), followed by M3061 (n=20) and A405_A409P (n=11). The *emb*B mutations were associated with MIC ranging from 8 µg/ml to 32 µg/ml. The association, however was not significant (KW= 210.000: p = 0.448).

5.4 DISCUSSION

Drug resistance in *M. tuberculosis* is quite heterogeneous and genotype-phenotype studies have revealed that different sites of the mutation (SNPs) are associated with distinct phenotypic resistance levels. Determination of MIC levels associated with different genetic resistance could help in predicting accurate level of resistance as well as development of new and optimizing the existing molecular methods. In the present study, we investigated the association between MICs and mutations in the *kat*G and *inhA* (INH and ETH), *gyr*A and *gyr*B (OFX and MXF), *rrs* and *rps*l (AMI, KAN and SM) and *emb*B (EMB and ETH).

A high (92%) proportion of isolates were resistant to INH as compared to ETH (52%). Mutations in the *kat*G, were the predominant cause of INH resistance, found in 80% of the isolates and was significantly associated (p=0.0001) with INH resistance (MIC range of 2 to 4 µg/ml). These findings are consistent with previous reports which showed that between 40% and 95% of clinical *M. tuberculosis* isolates resistant to INH have mutations in *kat*G (Ramaswamy et al., 2003, Ramaswamy et al., 2004, Zhang et al., 2005, Hazbón et al., 2006). The majority (77%) of the mutations were located on codon 315 which is comparable to 64% to 90%, reported in previous studies (Ramaswamy et al., 2003, Ramaswamy et al., 2004, Zhang et al., 2005, Hazbón et al., 2006, Bolotin et al., 2009). These results support the hypothesis that *kat*G mutations provide a more favourable environment for the development of INH resistance than do *inh*A promoter mutations. For ETH, however, the *kat*G was not significantly associated to ETH resistance (p=0.8232). On the other hand, the *inh*A mutation was significantly associated with both INH and ETH resistance (MIC range 0.5 to 4 µg/ml; P =0.012 and MIC range of 10 to 40 µg/ml; P=0.001, respectively).

In this study, more than 40% of OFX-resistant isolates had *gyr*A mutations at codons 90, 91 and 94. For MXF the prevalence of *gyr*A mutations at codons 90 and 91 was lower (27%), but higher for codon 94 (51%). Previous studies showed that codon 94 for OFX is the most common mutation, with mutation D94G being associated with high resistance. In this study mutation D94G was found in 37% and 57% of the OFX and MXF resistant isolates, respectively and was associated with high MICs ranging between 1 to 8 µg/ml. Mutation D94G is thought to provide the

greatest advantage for the cell with regard to increased resistance and the minimum loss of fitness (Hu et al., 2013, Chaoui et al., 2018). Resistant OFX isolates with a mutation at codon 90 and 91 had lower MIC of 4 to 8 µg/ml compared to *gyr*A mutations at codon 94 (MIC range 8 to 32 µg/ml).

Moxifloxacin resistant isolates with a mutation at codon 94 had higher MICs (1 to 8 µg/m) than isolates with mutation at the 90 and 91 (1 µg/ml). The MICs were found to be consistently lower for MXF than for OFX among isolates with the same *gyr*A mutation. For OFX and MXF resistant isolates with wt *gyr*A isolates, it may be important to screen for mutations outside the QRDR region of *gyr*A genotype and in other genes, such as mfpA or active efflux pump (Rv2626c_Rv2687c-Rv2688c operon associated with fluoroquinolone resistance) (Pasca et al., 2004, Mokrousov et al., 2008, Hillemann et al., 2009, Chaoui et al., 2018).

The *gyr*B mutations were less common in both OFX and MXF resistant isolates. In addition, the association between MIC and *gyr*B mutation was not significant for both OFX and MXF (P=0.00554 and P=0.1422, respectively). Thus, based on the present study finding, in developing countries with limited resources and high drug resistance, sequencing of the *gyr*B gene might not be a great importance for fluoroquinolone resistance determination. Although its acclaimed to consider the *gyr*B gene mutation when screening for resistance (Takiff et al., 1994, Feuerriegel et al., 2009, Malik et al., 2012, Chaoui et al., 2018)

In this study, A1401G was the most common *rrs* mutation found in 54% of KAN and AMI resistant isolates and the mutation was associated with high MIC of >16µg/ml. This is consistent with previous reports which found that the A1401G *rrs* mutation was associated with resistance and was the most common mutation reported in 30% to 90% of isolates (Maus et al., 2005, Georghiou et al., 2012, Sirgel et al., 2012). In addition, mutations C492T_A514C and A514C_A1401G were found in 15% isolates and were associated with high MIC (>16 µg/ml). Three isolates with wt pattern showed high-level resistance to KAN (MIC > 20 µg/ml), indicating that other resistance determinants may be involved in their resistance phenotype. In this study, high cross resistance was found with 85% of KAN and AMI resistant isolates showing cross resistance. This is in line with previous reports that found between 70% to 90% cross-resistance

between KAN and AMI (Maus et al., 2005, Georghiou et al., 2012). All isolates with A1401G *rrs* mutation showed cross-resistance, indicating the association of this mutation with cross-resistance. The *rps*l mutation, however, was not associated with AMI and KAN resistance (p =0.822 and P=0.1078, respectively).

The *rrs* mutations was higher (60%) than *rps*l mutation (46%) among SM resistant isolates. Previous studies had reported a prevalence of 12% to 90% and 13.2% to 80.4% for *rrs* and *rps*l mutation, respectively. The most common *rpsl* mutation was the K43R which was found in 39% of the isolates with MIC ranging between 4 to 32 µg/ml. This finding is comparable to previous studies which showed a prevalence between 24% to 64%. In this study mutation K88M was found in only 1% of isolates and has been reported to play a minor role in resistance and occurs much less frequently than K43R. Previous results have shown that *rrs* mutation being associated with intermediate to low SM drug resistance while *rps*l had been associated with high resistance (Sirgel et al., 2012). However, in this study no difference was found in the association between MIC and *rrs* and *rps*l mutation.

The *emb*B mutation was found in 69% of the EMB resistant isolates, with majority (91%) of the *emb*B mutation being either in codon 306, 405 or 406. These isolates had MIC ranging between 8 to 32 µg/ml suggesting that the sequencing of this region can be used for rapid screening and detection of EMB resistance. The most common *emb*B mutation was M306V which was found in 36% of the resistant isolates. Previous studies had found prevalence of mutation at the codon 306 ranging between 27% to 87 % (Ramaswamy et al., 2000, Plinke et al., 2006, Zhao et al., 2014, Yin et al., 2016, Sun et al., 2018). The M306I mutation had been reported to have lower MIC than do M306V or M306L. However, no such association was found in this study, all mutation were linked with MICs ranging between 8 µg/ml to 32 µg/ml. In addition, the presence of *emb*B mutation was not significantly associated with MICs (p=0.448). Previous studies in South Africa had found *emb*306 to be associated with discordant EMB susceptibility test results, the region could not be linked significantly with resistance or sensitivity (Sirgel et al., 2012).

The limitation of the study include limited regions of genes associated with drug resistance were sequenced. Additional regions (e.g *gid*B, *eis* promoter, *tly*A) or whole genome sequencing could provide more information on association of genotypic and different levels of phenotypic drug

resistance particularly in isolates with wt pattern. The number of isolates used in the study was also relatively small. Whole genome sequencing on large number of strains collected worldwide, coupled with MIC results, drug treatment and clinical outcomes data could provide more information on the role of different mutations in determining MIC levels.

5.5 CONCLUSIONS

The results of this study showed that the different drug resistance mutations were associated with different MIC ranges; *kat*G, *inh*A *gyr*A and *rrs* mutation were associated with high MICs of their respective drugs while *rps*L mutation was only associated with high MIC to SM but not KAN and AMI. The *emb*B mutation was not significantly associated with MIC ranges in this study.The MICs of MXF were found to be consistently lower compared to OFX MICs, suggesting MXF may be used in the treatment of OFX-resistant TB. Mutation at A1401G of the *rrs* gene was found in isolates resistant to KAN and AMI, suggesting association with cross resistance between KAN and AMI. Different mutations lead to distinct MICs, this knowledge that can help in guiding clinical decision-making and facilitate therapeutic drug monitoring.

5.6 REFERENCES

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CHAPTER 6

ASSESSMENT OF MIC TRENDS TO FIRST AND SECOND-LINE ANTI-TUBERCULOSIS DRUGS IN MULTI-DRUG RESISTANCE ISOLATES IN SOUTH AFRICA

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Running Title: Assessment of MIC trends to first and second-line anti-tuberculosis drugs in Multi-drug resistance isolates in South Africa

Summary

Despite the problem of drug resistance and poor cure rates of DR-TB in South Africa, the changes/shifts in MIC levels of anti-TB drugus has not been monitored. Methods categoriseing drug susceptibility into resistance or sensitive may mask small changes in resistance that might be occurring with anti-TB drugs until a major shift into the next category. The geometric mean of minimum inhibitory concentration (MIC) is more sensitive marker and can accurately reflect changes in MIC distributions when compared with conventional methods. Thus the study evaluated MIC changes or trends for first and second-line anti-tuberculosis drugs over a 3-year period and investigated possible MIC creep and the role of mutations in MIC changes over time. The MICs were determined using the Sensititre MYCOTB plate method and relevant genes were sequenced. The distributions of individual MICs were plotted against time to evaluate changes over the study period and the MIC_{50}, MIC_{90} and MIC range, modal MIC, geometric mean (GM) and median MIC were determined over the three-year period. MIC creep was assessed by evaluating the changes in number of resistance mutations and statistically using the Paired-T-test. The increase in geometric mean, was significant for RFB (GM=2.160 fold; P = 0.001), MXF (GM=3.84 fold; P=0.0001) and OFX (GM=0.990 fold: P=0.0001) over the study period. In contrast, there was a decrease in the geometric mean for INH (GM=-0.330; P=0.0218) and EMB (GM=-2.310; P = 0.0067) over the study period. However, the changes in mean MIC was not statistically significant for PAS (GM=0.132 fold; P =0.903), RIF (GM=0.400 fold; P=0.4290), SM (GM=-0.164 fold; P=0.8940), KAN (GM=-0.370 fold; P=0.8226), CYC (GM=-1.860 fold; P=0.1244), AMI (0.250 fold; P = 0.5023) and ETH (0.710 fold; P=0.6749). The study have shown evidence of MIC creep in three of the anti-TB drugs (OFX, MXF and RFB), highlighting the need for monitoring MIC changes over time. Shift in MIC population distributions may have important implications for treatment.

6.1 INTRODUCTION

The increasing number of people with multidrug-resistant tuberculosis (MDR TB) (i.e., resistant to at least, rifampin (RIF) and isoniazid (INH) and extensively drug resistant (XDR) (i.e., MDR plus resistance to at least one fluoroquinolone and second line injectable agent) represents a threat to national TB control efforts (WHO, 2017). Treatment choices for drug-resistant TB (DR-TB) are limited and treatment outcomes are often sub-optimal. Currently, only half of all global MDR patients that are started on treatment achieve a successful outcome and it is even lower for XDR-TB. The treatment success rate for adult MDR and XDR-TB remains low in South Africa (less than 54% and 27% for MDR and XDR-TB, respectively).

The association of treatment failure and a phenomenon called minimum inhibitory concentration (MIC) "creep or "drift" has been reported in many bacteria (Diaz et al., 2017, Sader et al., 2009). Shifts and changes in antimicrobial resistance to several antibiotics occur over time. This phenomenon has not been investigated for *M. tuberculosis* due to lack of appropriate MIC testing methods. Most of the established procedures for susceptibility testing of *M. tuberculosis* classify clinical isolates as either drug resistant or drug susceptible on the basis of the organism ability to grow in the presence of a (mostly single) critical concentration of a drug (Böttger, 2011). The simple categorization of clinical *M. tuberculosis* isolates as resistant or sensitive on basis of susceptibility testing at single concentration may mask important changes in MIC distributions until the mode shifts to the next category. Therefore, MIC based methods are needed to reflect any changes in trend of MIC of drug resistance.

Hence, the present study was conducted to evaluate the trend of anti-TB MIC for clinical MDR-TB isolates over a three years period and to observe MIC creep, if any and investigate the role of mutations in MIC changes over time.

6.2 MATERIALS AND METHODS

6.2.1 Clinical specimens and Ethics

Multidrug resistant TB isolates collected between January 2010 to June 2013 as part of two cross-sectional MDR-TB studies in Gauteng Province of South Africa, were included in this study. Drug susceptibility patterns were previously determined by MGIT 960 method. Ethics approval for this study was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of the Free State (Ref: 230408-011).

6.2.2 Determination of *M. tuberculosis* MICs using the Sensititre MYCOTB plate

The MIC testing was performed using the Sensititre MYCOTB plate (MYCOTB) method. Purity of *M. tuberculosis* cultures used for this study was checked prior to MIC testing by inoculation on 5% sheep blood agar which was then incubated at 37^0C for 48 h and checked for contamination. The MYCOTB assay comprises a 96 well microtitre plate containing 12 lyophilized antibiotics, configured for determination of MICs to first- and second-line TB drugs. The MIC ranges for the 12 drugs included were as follows: for ethambutol, streptomycin (SM), and Ofloxacin (OFX),0.12 to 16 µg/ml; for rifabutin (RFB), amikacin (AMI) and rifampicin 0.3 to 40 µg/ml and 0.6 to 40 µg/ml; for ethionamide (ETH) and kanamycin (KAN) respectively 0.5 to 64 µg/ml; for p-amino salicylic acid 0.06 to 8 µg/ml; for Moxifloxacin, (MXF) 2 to 256 µg/ml; for cycloserine (CYC) and 0.03 to 4 µg/ml for isoniazid (INH)].

The MIC testing was performed according to the manufacturer's instructions: Briefly, *M. tuberculosis* colonies were scraped off the surface of 7H11 plates and vortexed for 30 to 60 sec in a sterile saline-tween-tube containing glass beads (Trek Diagnostics Systems). The suspension was allowed to settle for 15 to 30 min and then adjusted to a turbidity of the supernatant equivalent to a 0.5 McFarland standard, using a nephelometer (BD, Diagnostics). A 100µl of suspension was transferred to 11ml of Middlebrook 7H9 broth supplemented with oleic acid-albumin dextrose-catalase (OADC). A Sensititre AIM™ Automated Inoculation Delivery System (Trek Diagnostics systems) was used to dispense 100 µl of the OADC-containing inoculum to each well. The

MYCOTB plates were sealed and incubated at 37^0C. Plates were checked after 48 hours for contamination. The MYCOTB plates were read at days 10, 14 and 21 of incubation. Initially the plates were read by visual inspection for growth and subsequently via a computerized Vizion system (Trek Diagnostic Systems, Cleveland, Ohio, USA). For each antibiotic, the lowest concentration with no visible growth was considered to be the MIC.

6.2.3 DNA extraction, PCR and Sanger Sequencing

All isolates were grown on Löwenstein–Jensen agar; genomic DNA was extracted using the phenol-chloroform (CTAB) method (Van Embden et al., 1993). Primer sets which were used for PCR amplification of the genes are shown in Table 6.1. The PCR amplification protocol consisted of a 5 min denaturation step at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 62°C and 50 sec at 72°C and a final extension step at 72°C for 2 min. The PCR products were sequenced by Sanger method using3500xl Genetic Analyzer (Applied Biosystems). Following Sanger sequencing of amplicons, mutations were identified by alignment to H37Rv reference strain (NCBI Accession number AL123456) (Cole, 2002) using ClustalW2 (Li et al., 2015).

Table 6.1: Primers used to amplify different mutation region

Drugs	**primer set**	**Forward Primers (5'-3')**	**Reverse Primers (5'-3')**
KAN,AMI,SM	rrs	CCATCGACGAAGGTCCGGCTTC	CGCGTCCTGTGCATGTCAAACC
KAN,AMI,SM	rrs -2	GTAGCTAACGCATTAAGTACC	CATACAGACAAGAACCCCTCACGG
KAN,AMI,SM	*Rps*l	GGCCGACAAACAGAACGT	GTTCACCAACTGGGTGAC
OFX, MXF	gyrA	CCGATCGAACCGGTTGACATC	GGGTTCGGTGTACCTCAT
OFX,MXF	*gyr*B	AACACCGAGGTCAAATCGTT	CTGAATGCGTCTTCCTTTCCTTGT
EMB	embB	TCCACCCGGCCGACCACGCT	AGCGCCGCCGGTGTGAGGCCG
ETH, INH	*inh*A	CCTCGCTGCCCAGAAAGGA	ATCCCCGGTTTCCGG
ETH,INH	katG	CCCATGGCCGCGGCGGTCGACATT	CGCCGCCTTGGCGGTATTGCC

6.2.4 Statistical Analysis

The distributions of individual MICs were plotted against time to evaluate changes over the period. Additionally, the MIC_{50} (MICs required to inhibit the growth of 50% of organisms), MIC_{90} (MICs required to inhibit the growth of 90% of organisms) and MIC range, modal MIC, geometric mean (GM) and median MIC were determined over the three-year period using Stata 14 software. The MIC trends over the three years and the significance of changes was assessed using paired T-test using STATA 14 software. A P-Value <0.05 was considered as significant. The T-tests were used to determine whether mutations were associated with any differences in MICs in 2010 and 2013.

6.3 RESULTS

Over the three years' period, 439 MDR-TB isolates were collected; including 211 isolates from 2010 and 228 isolates from 2013. The number of isolates resistant by MGIT 960 system is shown in Table 6.2

Table 6.2: MGIT 960 drug susceptibility profiles of isolates from 2010 (n=211) and 2013 (n=228)

Drug	No (%) resistant isolates in 2010	No (%) resistant isolates in 2013
RIF	190 (90)	207 (91)
INH	194 (92)	192 (84)
SM	82 (39)	84 (37)
EMB	92 (44)	76 (33)
ETH	109 (52)	121 (53)
OFX	35 (17)	65 (29)
MXF	30 (14)	73 (32)
KAN	13 (6)	11 (5)
AMI	13 (6)	16 (7)
RFB	140 (66)	154 (68)
PAS	16 (8)	23 (10)
CYC	62 (29)	53 (23)

Comparing the isolates between 2010 and 2013, the number of resistant isolates increased for OFX, MXF, AMI and RFB (Table 6.3), while the number of resistant isolates decreased for INH and EMB.

The difference in geometric mean of MIC between 2010 and 2013 resistant isolates was not statistically significant for PAS (GM=0.132 fold; P =0.903), RIF (GM=0.400 fold; P=0.4290), SM (GM=-0.164 fold; P=0.8940), KAN (GM=-0.370 fold; P=0.8226), CYC (GM=-1.860 fold; P=0.1244), AMI (GM=0.250 fold; P = 0.5023) and ETH (GM=0.710 fold; P=0.6749). The increase in geometric mean, however was significant for only three drugs over the study period including RFB (GM=2.160 io1`fold; P = 0.001) MXF (GM=3.84 fold; P=0.0001) and OFX (GM=0.990 fold: P=0.0001). Contrary, there was a decrease in the geometric mean for INH (GM=-0.330; P=0.0218) and EMB (GM=-2.310; P = 0.0067) over the study period (Table 6.2). The MIC population distributions for each drug are shown in Figures 1 to 11. The MIC_{50} and MIC remained stable over the three-year period for RIF, INH, SM, EMB, PAS, ETH, MXF and CYC. However, there was an increase in MIC_{50} and MIC_{90} for AMI, KAN, OFX and RFB (Table 6. 3).

Figure 6.1 to 6.12 showing the distribution of MICs and mutations in 2013 and 2010

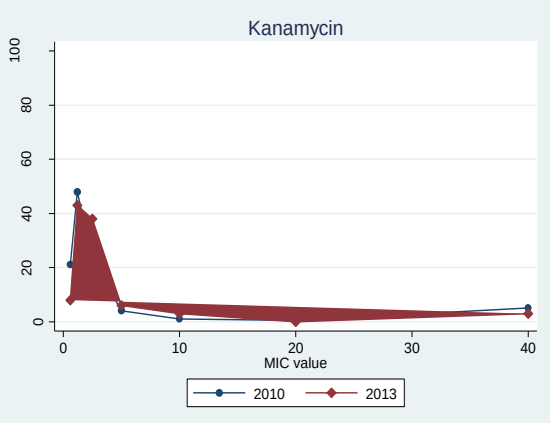

Figure 6.1

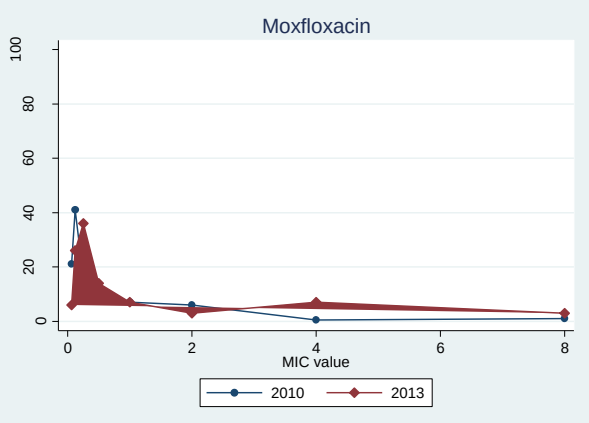

Figure 6.2

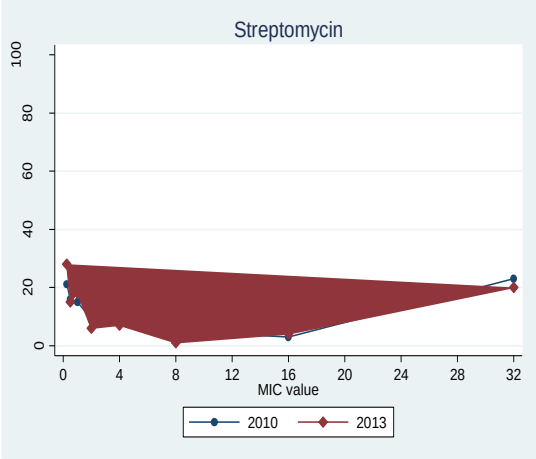

Figure 6. 3

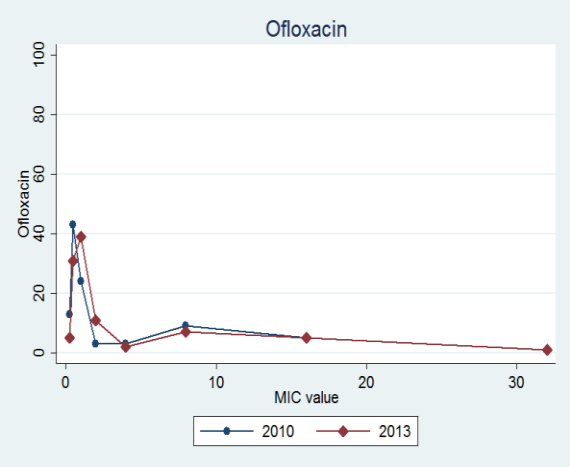

Figure 6.4

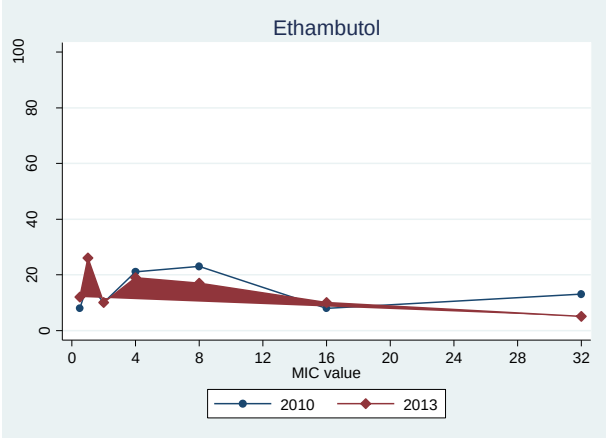

Figure 6.5

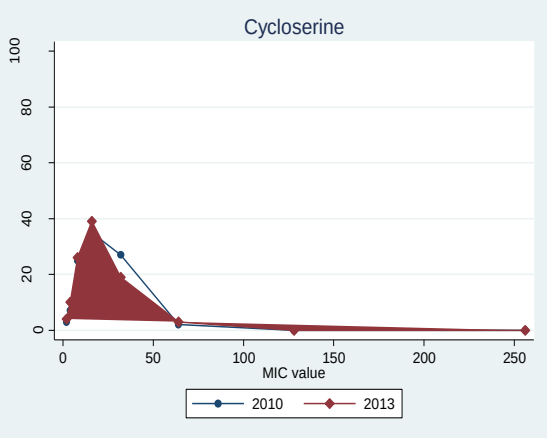

Figure 6. 6

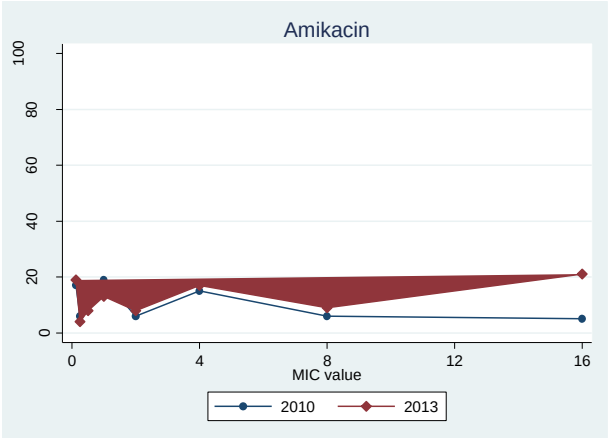

Figure 6. 7

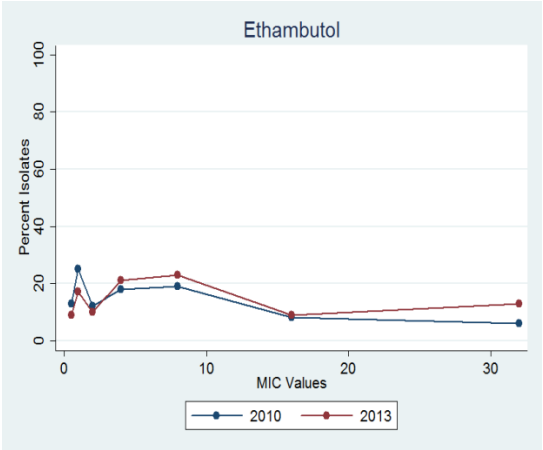

Figure 6. 8

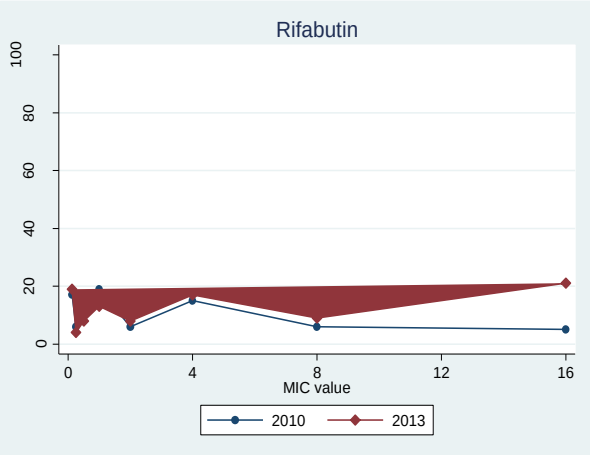

Figure 6. 9

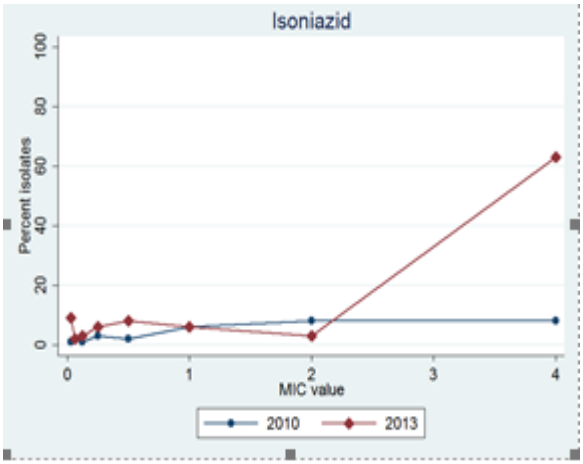

Figure 6. 10

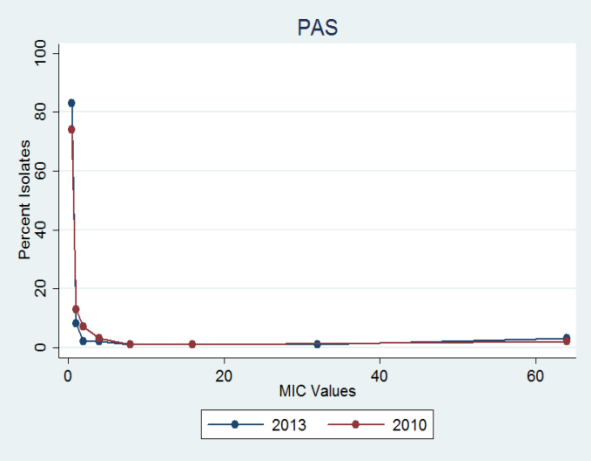

Figure 6. 11

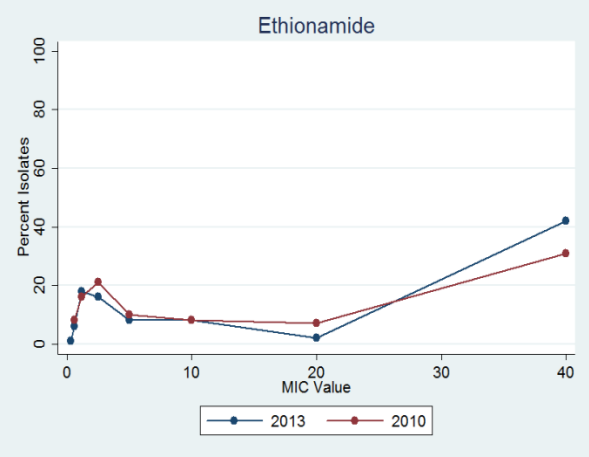

Figure 6. 12

Table 6.3: Table showing summary statistics for TB drugs in 2010 and 2013

Drug	Year	Geometric Mean (CI)	Geometric mean difference and confidence interval between 2010 and 2013	Modal MIC	MIC Range	MIC50	MIC90
RIF	2010	13.35	0.400 (CI -0.59 - 1.39, P=0.43)	16	0.12-16	16	16
	2013	13.75		16	0.12-16	16	16
INH	2010	3.23	-0.330 (-0.6118--0.0482 P=0.0218)	4	0.03-4	4	4
	2013	3		4	0.03-4	4	4
SM	2010	8.99	-0.164 (-2.599-2.27 P=0.895)	0.25	0.25-32	1	32
	2013	8.83		32	0.25-32	1	32
EMB	2010	8.46	-2.310 (CI -3.985--0.645 P=0.0067)	1	4.0-32	4	16
	2013	6.16		8	0.5-32	4	16
ETH	2010	18.98	-7.10 (CI -4.35-2.62, P=0.675)	40	0.3-40	10	40
	2013	18.28		40	0.6-40	10	40
OFX	2010	2.26	0.990 (CI 0.68 - 1.30, P =0.0001)	0.5	0.25-32	0.5	8
	2013	4.27		1	0.25-32	1	16
MXF	2010	0.43	3.84 (CI 3.53 - 4.15, P= 0.0001)	0.12	0.06-8	0.25	4
	2013	1.42		0.25	0.06-8	0.25	4
KAN	2010	3.49	-0.370 (-3.611-2.871 P=0.823)	1.2	0.6-40	1.2	2.5
	2013	3.12		1.2	0.6-40	2.5	5
AMI	2010	1.3	0.250 (CI -0.48 - 0.98, P = 0.5023)	0.25	0.12-16	0.25	1
	2013	1.55		0.25	0.12-16	0.50	1
RFB	2010	2.58	2.160 (CI 1.22-3.10, P=0.0001)	4	0.12-16	1	8
	2013	4.74		16	0.12-16	2	16
CYC	2010	18.19	-1.860 (-4.234-0.514 P=0.12)	16	2.0-64	16	32
	2013	16.33		16	2.0-64	16	32
PAS	2010	3.09	0.132 (CI -1.99 to 2.26 P=0.903)	0.5	0.5-64	0.5	1
	2013	3.22		0.5	0.5-64	0.5	4

6.3.1 Impact of mutation on MIC changes over time

Mutations associated with resistance to the anti-TB drugs tested were compared over the three years period. The role of mutations in MIC changes was assessed (Table 6.4).

Table 6.4: Mutations and MIC changes over the three years

		2010				2013				
			Geometric mean				Geometric mean			
Gene	**Mutation**	**T. isolates**	**AMI**	**KAN**	**SM**	**T. isolates**	**AMI**	**KAN**	**SM**	**P Values**
rrs	A1401G	7	13.75	35	1.43	2	16	40	9	ND, 0.50
	A514C	11	0.45	1.33	26.27	12	1.78	5.18	22.75	0.34; 0.26; 0.50
	C492T	9	0.4	3.73	6.5	5	0.55	5.48	1.55	0.37; 0.48; 0.34
	C517T	11	0.61	1.22	13.57	4	0.12	1.2	14	0.42; 0.65; 0.96
	G878A	10	0.534	1.16	12.3	1	4	1.2	1	ND
	Other	8	4.48	10.84	6.03	8	0.5	6.7	6.5	ND
	Wt	155	0.78	2.14	7.87	132	1.04	2.24	6.89	0.82 0.45; 0.50
	NS					64	2.37	2.93	10.72	ND
rpsl	K43R	34	0.81	1.34	27.14	19	1.78	3.48	23.8	0.67; 0.17; 0.32
	K88R	5	0.3	1.46	19.2	11	2.21	2.15	25.55	0.39; 0.27; 0.33
	Other	2	0.38	7.5	16.25	2	0.38	3.1	0.38	ND
	Wt	170	1.43	3.94	4.98	191	1.59	3.13	6.39	0.71; 0.33; 0.20
	NS					6	0.5	3.53	10.88	ND
Gene	**Mutation**	**T. Isolates**	**RFB**	**RIF**		**T. Isolates**	**RFB**	**RIF**		**P Values**
*rpo*B	S531L	109	2.71	15.07		114	5.01	14.84		0.0003; 0.53
	H526Y	29	4.42	15.45		28	6.62	15.3		0.0001; 0.83
	H526D	19	4.43	15.16		18	10.17	16		0.0067; 0.34
	D516V	19	0.39	6.59		14	1.14	16		0.0001; 0.009
	D516G_L533P	4	1.44	12.13		4	1.14	16		ND
	L533P	6	0.33	10.77		4	0.435	5.06		0.68; 0.30
	Q513P					4	8.28	16		ND
	H526L	5	0.12	1.17		2	2	16		ND
	H526C	1	0.12	1		3	0.16	6		ND
	I572F	1	0.12	0.12		3	5.41	11.3		ND
	S531L_F584S					2	1.5	16		ND
	Other	5	2.5	14.4		11	1.07	13.1		ND
	Wt	13	0.68	9.27		22	2.26	4.49		0.25; 0.067
Gene	**Mutation**	**T. Isolates**	**INH**	**ETH**		**T. Isolates**	**INH**	**ETH**		**P Values**
*kat*G	S315T	154	3.78	19.33		116	3.83	18.7		0.96; 0.60
	S315N					1	4	1.2		ND
	G299R					2	2.25	21.3		ND
	G285R					1	1	2.5		ND
	V320L					1	1	5		ND
	SF272F									ND
	S315G	1	40	4						ND
	dbl pea.	1	2.5	4						ND
	Wt	55	1.9	17.94		102	1.97	18.1		0.98; 0.94
	Other					6	3.34	21		ND
*inh*A	15 C-T	55	2.96	28.45		66	3.04	29.5		0.78; 0.72
	17 G-T	2	3	2.25		4	3.25	14.4		ND
	8-TA	29	3.87	36.29		19	3.24	28.2		0.05; 0.037
	P12R					1	4	40		ND
	dbl peak	1	1	10						ND
	Wt	124	3.31	11.07		134	2.81	11.4		0.0097; 0.85
	9 G-A					1	4	40		ND
	NS					4	4	10.9		ND

Table 6.4:continued……..

Gene	Mutation	T. Isolates	MXF	OFX		T. Isolates	MXF	OFX		P Values
*gyr*A	A90V	16	0.64	5.7		34	3.87	11.7		0.0001; 0.023
	D94G	20	1.78	9.16		20	4.41	11.6		0.0001; 0.058
	Other	5	2.05	9.25		6	2.42	8.71		ND
	Wt	170	0.2	0.92		169	0.53	1.74		0.85
*gyr*B	K537Q	4	0.12	0.44		5	3.35	13.7		0.0922; 0.073
	S458F					5	3.3	13.2		ND
	Other	3	0.37	3.5		1	4	16		ND
	L566F	2	1	8						ND
	NS					30	1.37	4.97		ND
	Wt	202	0.43	2.22		188	1.31	3.6		0.0001; 0.073
Gene	**Mutation**	**T. Isolates**	**GM**			**T. Isolates**	**GM**			**P Values**
*emb*B	M306V	47	7.35			19	11.21			0.0823
	M306I	44	8			11	9			0.74
	E378A	5	17.7							ND
	M306V_E	6	3.58			4	10			0.002
	M306L_	2	16.25							ND
	A405D_A	25	8.84							ND
	G406S	2	4.5							ND
	M306L_	2	16.25							ND
	G406A	2	10							ND
	Wt	72	8.45			50	4.48			0.0035
	Other	3	14.67			3	12.67			ND
	NS					142	5.6			ND

*NS - Not Sequenced, wt - wild type, T. Isolates -Total Isolates, GM – Geometric Mean ND - Not determined

rpoB mutation: For RIF, no significant change was found in the geometric mean difference between mutations in *rpo*B gene between 2010 and 2013 (GM=0.400, CI [-0.59 to 1.39]; P=0.43) while, for RFB the mean difference was significant (GM=2.160, CI [1.22 to 3.10]; P=0.0001) (Table 6. 4).

The geometric mean for RIF resistant isolates with S531L *rpo*B mutation was relatively similar between 2010 and 2013 (GM=15.07 vs 14.84; P= 0.53, respectivley), while, the geometric mean increased almost twice for RFB (GM=2.71 to 5.01; P=0.0003) (Table 6.4). Similarly, the geometric mean doubled for mutations, H526D (GM=4.43 to 10.17; P=0.0067) and D516V (GM=0.39 to 1.14; P=0.0001) for RFB between 2010 to 2013.

Increase in RFB geometric mean was also noticed for mutations H526Y (GM=4.42 to 6.62; P=0.0001) and L533P (GM=0.33 to 0.435; P=0.68) (Table 6.4). In contrast, there was a significant

decrease in mean MIC for isolates with mutations H526Y (GM=15.45 to 15.30; P=0.0001), and H526Y (GM=15.45 to 15.29 ; P=0.0001). However, the mean MIC decreased (GM=10.77 to 5.06) for isolates with L533P, although it was not significant (p=0.068). Two new mutation *rpo*B mutations S53IL-F582S and Q513P were found in 2013 which were not identified in 2010 isolates. There was no significant increase in geometric mean for isolates with no known mutation for *rpo*B (P=0.25) (Table 6.4).

*emb*B *mutation*: The MIC geometric mean difference between *emb*B mutations in 2010 and 2013 was not significant (GM=-2.310 CI [-3.985 to -0.65]; P=0.0067) (Table 6. 4). For *emb*B mutation at codon M306V and M306I, the geometric MIC mean increased between 2010 and 2013 (GM=7.35 to 11.21; P= 0.0823 and GM=8.00 to 9.00, P=0.74) respectively (Table 6.3). However, the MIC geometric mean increase was significant for M306V_E mutation (GM=3.58 to 10.00; P=0.002). The geometric mean MIC for isolates with wt pattern for *emb*B showed a significant decrease between 2010 and 2013 (GM=8.45 in to 4.48 in 2013; P=0.0035).

*gyr*A *and gyr*B *mutations*: Between 2010 and 2013, the geometric mean for isolates with A90V *gyr*A mutation increased significantly for both MXF (GM=0.64 to 3.87; P=0.0001) and OFX (GM=5.7 to 11.68; P=0.023); . Also the geometric mean significantly increased for isolates with D94G *gyr*A mutation) for both MXF (GM=1.78 to 9.16, P=0.0001 and OFX (GM=4.41 to 11.63; P=0.050). However, the increase in geometric mean for isolates with K537Q *gyr*B mutation was not significant for either MXF or OFX (GM=0.12 and 0.44; P=0.092 in 2010 to 3.35 and 13.7 P=0.073 in 2013, respectively). Interestingly, there was significant increase in geometric mean for isolates with wt *gyr*B for MXF and OFX, from 0.43 and 2.22 in 2010 to 1.1 and 3.6 in 2013 (P=0.0001), respectively.

*kat*G *mutation*: The geometric mean difference for INH and ETH mutations between 2010 and 2013 showed a significant decrease for INH (GM=-0.330 CI -0.6118 to -0.0482; P=0.022), while the difference was not statistically significant for ETH (GM=-7.10 CI [-4.35 to -2.62]; P=0.675). No significant change in mean MIC was observed for isolates with S315T *kat*G mutations from 2010 to 2013 for both INH (GM=3.78 to 3.83 P=0.96) and ETH (GM= 19.33 to 18.7 CI, P=0.60) (Table 6.3). However, the mean MIC for isolates with 8-TA *inh*A mutaions decreased significantly

for INH (GM=3.87 to 3 .24; P=0.05) and ETH (GM=36.29 to 28.2; P=0.037). Also mean MIC for isolates with wt *inh*A decreased signifcanly for INH (GM=3.31 to 2.81; P=0.0097), while the mean MIC deacrease for ETH was not significant (GM=11.07 to 11.4, P=0.85).

rrs and rpsl mutations: Geometric mean difference between 2010 and 2013 for AMI increased by 0.250 (CI [-0.48-0.98]; P=0.50) whereas for KAN the geometric mean decreased by (GM=-3.70 (CI [-3.611 to 2.871]; P=0.82). There was no significant increase in geometric mean for isolates with A1401G and A514C *rrs* mutations, for AMI, KAN and SM between 2010 and 2013 (P>0.05)) Similarly, there was no significant difference (P>0.05) in MIC mean for K43R and K88R *rpsl* mutations as well as for isolates with wt *rrs* region for all three drugs (Table 6.4).

6.4 DISCUSSION

In a given microbial population, the central MIC tendency can increase over time, and the phenomenon is termed, MIC creep. Even a subtle shift in an MIC population within the susceptible range has been reported to have potential to affect the pharmacodynamics efficacy of a drug in methicillin resistance staphylococcus aureus (MRSA) and outcomes of patients (Miyazaki et al., 2014). Thus the occurrence of this phenomenon need to be monitored. Unlike other microbial populations the phenomenon of *M. tuberculosis* MIC creep or shift has not been investigated due to lack of MIC testing methods for *M. tuberculosis*. However, the introduction of MYCOTB plate method facilitates monitoring of MIC trends of first and second line anti-TB drugs.

The present study was done to evaluate the possible MIC creep of *M. tuberculosis* isolates over a three-year period. In this study a significant increase in geometric mean was found for RFB (2.160 fold; P=0.001), MXF (3.84 fold; P=0.0001), and OFX (0.990 fold; P=0.0001). Also, increase in prevalence of resistance between 2010 and 2013 was observed, 14% to 32% for MXF, 17% to 29% OFX and 66% to 68 % for RFB. Factors involved in the development of creep or reduced susceptibility are not entirely known, although misuse, clonal replacement, suboptimal dosing and poor adherence may be key factors (Niveditha and Sujatha, 2015). Recognition of the creep phenomenon, is important since it maybe a precursor to XDR or total drug resistance. Clinically poorer treatment outcomes has been shown to be linked to the number of drugs to which a patient is resistant. Thus improving treatment success rate requires thus each patient receive an individual antimicrobial regimen formulated to maximize efficacy.

The A90V and D94G *gyr*A mutation for MXF increased by 2.5 fold (P=0.0001) and 6 fold (P=0.0001) respectively. For OFX the A90V *gyr*A mutation also increased by 2 fold (P=0.023). This increase in geometric mean for the mutations may explain the increase in MIC in 2013 compared to 2010. Increase in geometric mean for MIC was noted over the study period for all the MXF resistant isolates with *gyr*A mutations. A 3.84-fold increase in mean MICs was seen with MXF compared to 0.990-fold geometric mean for OFX. However, increase in geometric MIC mean was not significant for K537Q *gyr*B mutation for both OFX (P=0.092) and MXF (P=0.073).

Fluoroquinolones are considered an important part of MDR-TB regimens. Although OFX is still being used for (M/X)DR-TB treatment the later generation fluoroquinolones (MXF and levofloxacin) are the preferred drugs for treatment of M(X)DR TB. In 2010 OFX was replaced by MXF as part of MDR-TB treatment regimen in South Africa due to high level of resistance, (NDOH, 2013). This observation may explain the increased geometric mean which was observed with MXF compared to OFX. In addition, increase of MICs may also be explained by presence of other mutations in other regions which were not tested in the study for example DNA topoisomerase IV which is composed of ParC and ParE subunits mutations which also form part of the quinolone resistance determining regions which also may contribute to increase in MICs (Jian et al., 2018)

For RFB, although the actual cause of MIC increase are unknown there was a two-fold increase in geometric mean for mutations S531L (2.71 to 5.01; P=0.003); H526Y (4.42 to 6.62; P=0.0001); D516V (0.39 to 1.14; P=0.0001) and H526D (4.43 to 10.17; P=0.0067) isolates for RFB. Significant decrease in geometric mean was seen for INH (GM= -0.330 fold: P=0.0218) and EMB (GM=-2.310 fold: P=0.0067). The number of INH and EMB resistant isolates was 92% and 44% in 2010 compared to 84% and 33% in 2013. The decrease in mean MIC for INH could be partially explained due to the changes in prevalence of *inh*A mutations where the total number of isolates harbouring the mutation 8-TA decreased from 29 in 2010 to 19 in 2013. Also, the *kat*G wt isolates doubled in number which may have contributed to decrease in MIC. Previouse studies reported mutations in codon 306 of *emb*B gene as major resistance mechanism in clinical isolates. The *emb*B mutations M306V, M306V_E and M306I exhibited a 2-fold increase in EMB MIC in 2013 compared to the wt while in 2010 the mutations geometric mean was the same to wt isolates. However, mutations E378A, M306L_, A405D_A, G406S, M306L, and G406A which were found in 2010 were not found in 2013 which may have contributed to a decrease in MIC experienced in 2013. In addition, decrease with INH and EMB may also be because these drugs has been part of first-line treatment in South Africa thus not used at an MDR-TB facility. Four new *kat*G mutations (S315N, G299R, G285R and V320L) and two new *inh*A mutation (P12R and 9GA), were found in 2013 which were not previously identified in 2010.

In contrast no significant change in geometric mean of MICs was found for RIF (0.40 fold; P=0.43), SM (0.164; P=0.8940), ETH (-0.710 fold; P=0.6749), PAS (0.132 fold; P=0.9030), CYC (-1.860; P: 0.12), KAN (-0.370; P=0.823) in 2010 compared to 2013. Similarly, no significant change in resistance pattern or associated mutations to these drugs was observed (figures 1 to 11). There was no statistically significant difference in RIF mutations S531L (15.97 to 14.84; P=0.53), H526Y (15.45 to 15.3; P=0.83) and H526D (15.16 to 16; P=0.34). The prevalence of RIF-resistant isolates was relatively similar in 2010 (91%) and 2013 (90%). The same trend was seen in prevalence of resistance for SM (39% versus 37%), KAN (6% versus 5%), CYC (29% versus 23%), PAS (8% versus 10%), AMI (6% vs 7%) and ETH (52% vs 53 %) in 2010 compared to 2013. New *rpo*B mutations Q513P, S531L and_F584S were found in 2013 but were not found in 2010.

Though it is difficult to explain the of lack of increase in geometric mean for drugs RIF, PZA, SM, and ETH in 2013 there was a policy change in South Africa whereby there was a shift from MDR-TB centralization to decentralization and deinstitutionalisation of MDR-TB treatment. The decentralisation policy was prompted by insufficient numbers of hospital beds to provide hospitalisation for all MDR-TB patients. Community based treatment through satellite MDR-TB units at provincial level was implemented. Since 2011, South Africa had begun a progress towards treatment decentralisation and by 2015, all provinces were reported to have decentralised sites. This was associated with increased proportions of number of diagnosed patients being treated successfully.

The study has a number of limitations. The data was based on one MDR-TB hospital namely, Sizwe Tropical hospital located in Gauteng Province of South Africa. A study involving different settings from diverse geographical areas may give an overall national trend in MIC of drug resistant isolates. In addition, the MICs were analysed over a fairly short period of time (3-years), longer periods may have given a better picture of the MIC trends over time.

In conclusion, there was a general increase in the proportion of resistant strains over the study time for 9 of the 11 drugs in this study with statistically significant MIC creep evident in three of the drugs (RFB, OFX and MOX). The study thus recommends the need for monitoring anti-TB drugs

MIC trends and creep to prevent gradual loss of drug activity and, consequently, the compromise of clinical utility of available anti-drugs.

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CHAPTER 7

7.1 CONCLUDING REMARKS

Chapter 3: The heterogeneous nature of *M. tuberculosis* involving low level, moderate level and high level drug resistance phenotype necessitates minimum inhibitory concentration (MIC) testing (Böttger, 2011, Ängeby et al., 2012). The current standard methods for DST binary classify *M. tuberculosis* isolates as either drug resistant or drug susceptible on the basis of determination of critical concentrations (CCs). Many of the CC defining resistance is often very close to the MIC, particularly secondline anti-TB drugs, increasing the probability of misclassification of susceptibility or resistance (Ängeby et al., 2010, Ängeby et al., 2012). The Sensititre MYCOTB plate (MYCOTB) broth microdilution method has been introduced in the recent years. However, the methods has been validated only at categorical level against methods such as BACTEC MGIT 960, the indirect agar proportion method, and genotypic tests (Abuali et al., 2012, Abdel-Rahman et al., 2016, Xia et al., 2017) and have not yet been validated at MICs level. The present study validated the MYCOTB plate method against agar the dilution method (ADM). A total of 138 *M. tuberculosis* isolates, were used. The categorical agreement, essential agreement (± one-two fold dilution) and modified agreement (± two two-fold dilution), of MYCOTB were assessed in comparison with ADM. For strains with discordant results between MYCOTB and ADM, whole genome sequence (WGS) was performed. The categorical agreement between two methods was fairly good for all the drugs (88% to 98%) tested. The sensitivity and specificity of MYCOTB ranged from 11% to 95% and 94% to 100%, respectively. Sensitivity was highest for RIF (95%) and INH (92%) and was lowest for PAS (11%), while specificity was highest for RIF and MXF both with 100% and lowest for PAS and ETH with 94%. Whole genome sequencing resolved 70% in favour of MYCOTB plate. The MYCOTB is a standardized and high-throughput method for determination of MIC of first and second line anti-TB drugs. It could be a good alternative method in high-TB burden settings such as South Africa for therapy guidance, therapeutic drug monitoring to optimize regimen efficacy and surveillance of drug resistance. Additionally, the MYCOTB plate allows modification of the design of the microtitre plate to reflect locally recommended regimens.

Chapter 4: It has been reported that certain point mutations in *rpo*B RRDR are more likely to confer, higher levels of RIF resistance while exhibiting susceptibility to RFB. In clinical practice it is argued that RFB may be used to treat RIF-resistant isolates and is an alternative to RIF (Sirgel et al., 2013, Jamieson et al., 2014, ElMaraachli et al., 2015). The association of RIF and RFB MICs with *rpo*B mutations has been investigated, but all the studies were conducted in low-TB burden settings and most of those studies included a relatively low number of isolates. The present study aimed to determine the prevalence of RFB susceptible isolates among RIF-resistant strains in a high HIV burden setting in South Africa using 189 MDR-TB isolates. In addition, the study correlated the association between *rpo*B mutations and MIC of RIF and RFB. The MICs were determined using the MYCOTB plate method and the RRDR region of *rpo*B gene was sequenced. Cross-resistance to both RIF and RFB was seen in 73% of the isolates while 27% isolates were resistant to RIF but retained susceptibility to RFB. Mutation, S531L was the most common mutation and was found in 56% isolates, followed by H526Y in 14% isolates. Mutations S531L, H526Y and H526D was found in 70% of cases which were resistant to both RIF and RFB. Whereas mutations D516V and L533P was found in 88% and 75% of the RIF-resistant but RFB-susceptible isolates, respectively. Three novel mutations (V276L, V252E and V276F) were found outside of the RRDR, suggesting the need for inclusion of the 5'-end of *rpo*B in molecular testing for RIF/RFB resistance. The study has shown that in 27% of MDR-TB patients in South Africa may be treated with RFB. Our findings also showed that there was a statistically significant association between r*po*B mutations and the MICs of RIF and RFB and specific *rpo*B mutations (D516V and l533P) can predict RFB susceptibility among MDR-TB patients. Therefore, identification of these mutation could be used for rapid diagnosis of RFB susceptible cases.

Chapter 5: Genetic polymorphisms among *M. tuberculosis* are known to confer differential levels of resistance (Böttger, 2011, Sirgel et al., 2013, Rukasha et al., 2016). Linking different genotypic mutations with specific phenotypic resistance is key for the development of rapid molecular methods and accurately assessing their performance for detecting drug resistance. Also the level of resistance, reflected by the MIC and genetic polymorphism is most important for clinicians in optimizing regimen efficacy. We investigated the association between MIC levels and mutations in *kat*G and *inh*A (INH and ETH), *gyrA* and *gyrB* (OFX and MOX) *rrs* and *rps*l (AMI KAN and SM) and *emb*B (EMB) genes.

KAN, AMI and *SM*: Kanamycin mutations A1401G, C492T, C492T_A1401G and AMI mutations, A514C_A1401G were associated with high MICs (20 to 40 µg/ml and 16ug/ml, respectively ($p<0.005$). While, the *rrs* mutations (A514C, C517T, G878A, C492T, A906G) were resistant ($p=0.001$). Also, *rps*l mutations K43R, K88R and K88M were significantly associated with SM resistance ($P=0.001$). This finding suggested that both *rrs* and *rps*l mutations were important in predicting SM resistance.

OFX and MXF: Resistant isolates with *gyr*A mutation at codon 90 and 91 signifcantly lower ($p=0.0001$) MICs for OFX (OFX: 4 to 8 µg/mland MXF: 1µg/ml), w as compared to isolates with *gyr*A mutations at codon 94 of for OFX (OFX: 8 to 32 µg/ml and for MXF: 1 to 8 µg/ml). The *gyr*B mutations, however, were not significantly associated with resistantce to either OFX or MXF ($p>0.05$). Thus, in the current study *gyr*A mutations are more predictive level OFX and MXF resistance as opposed to *gyr*B mutation.

EMB: Mutations at codons M306V, M306I, M306LA405_A409P, E378A and D328G and 406 had MIC range of 8 to 32µg/ml but were not significantly associated with resistance ($P>0.05$). In this study *emb*B mutation was not a good predictor of EMB resistance.

INH and ETH: *kat*G mutations S315T and S315G were associated significantly associated with INH resistance ($P= 0.0001$) but not with ETH resistance ($P=0.832$). While, C-15T, T-8A and G-17T inhA mutations were significantly associated with both INH and ETH resistance (MIC range: 0.5 to 4µg/ml; $P<0.05$). Thus the study showed, different drug resistance mutations were associated with different MIC ranges; *katG*, *inh*A *gyr*A and *rrs* mutation were associated with high MICs of their respective drugs while mutations such as *rps*L (for KAN and AMI) and *emb*B were not significantly associated with MIC ranges.

Chapter 7: The evidence of gradual increase in MIC over time a phenomenon called MIC creep or drift has been reported in many bacteria (Diaz et al., 2017, Sader et al., 2009). This phenomenon has not been previously investigated for *M. tuberculosis* due to lack of an appropriate MIC testing methods. Thus in this study we evaluated the MIC trend over a three years' period to observe MIC creep, if any and investigate the role of mutations in MIC changes over time. The MIC testing

was performed using the MYCOTB plate method and relevant genes were sequenced. Significant increase in geometric mean of MIC was seen for three drugs; RFB, MXF and OFX ($p>0.05$) suggesting MIC creep. Contrary, significant decrease in geometric mean was found for EMB and INH ($p<0.05$). The finding of this study shows the importance of continuous monitoring of the MIC of individual drugs in order to prevent gradual loss of drug activity which can consequently compromise of the clinical utility of available anti-drugs.

7.2 FUTURE RESEARCH

More research is needed to customize the current MYCOTB plate method in order include the relevant drugs used in treatment regimen in South Africa. The UKMYC5 were designed after the MYCOTB by Comprehensive Resistance Prediction for Tuberculosis (CRyPTIC Consortium) determining MIC 14 different anti-TB drugs, including several new drugs such as Delamind and Bedaquiline as well as repurposed drugs such as clofazamine and linezolid. The developments represent an advancement in the paradigm shift from categorical determination resistance to MICs based methods. Whilst, significant progress has been made in elucidating the role of mutations in resistance mechanisms, further WGS based studies with large number of strains collected worldwide are required, to generate more data on association of specific mutations with MICs and their impact on treatment outcomes. Whole genome sequencing could provide full spectrum of genes involved in resistance. Future research on increasing the current sequencing coverage and the bioinformatics algorithms are necessary to identify hetero-resistant insertions and deletions. It is also important to note that a number of factors in addition to the already known genotypic and phenotypic results may be due to yet unknown mechanisms. Factors such as role of channels (efflux pumps), inadequate limits-of-detection of sequencing, random errors need to be investigated.

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APPENDICES

Appendix A
DETAILED SUSCEPTIBILITY TESTING METHODOLOGY

1.1 Determination of MICs using the Sensititre MYCOTB plate method

The Sensititre MYCOTB MIC plate is a dry micro-dilution plate containing lyophilized antibiotics, with concentrations and configured for determination of MIC to first and second-lie TB drugs. The layout of the Sensititre plate is shown in Table 1.1. The MICs were determined using a commercially available Sensititre MYCOTB plate (TREK Diagnostics, Cleveland, Ohio). Tests were performed according to the manufacturer's instructions. The Sensititre MYCOTB assay is a 96 well microtitre plate containing 12 lyophilized antibiotics and configured for determination of MICs to first and second-line TB drugs. The MIC ranges for the 12 drugs were: for ethambutol streptomycin (STR), and Ofloxacin (OFX), (0.12 to 16 μg/ml) for rifabutin (RFB), AMK and RIF (0.3 to 40 μg/ml and 0.6 to 40 μg/ml) for Ethionamide (ETH) and Kanamycin (KAN) respectively, 0.5 to 64 μg/ml for P-amino sacylic acid (PAS), (0.06 to 8 μg/ml) for moxifloxacin, (MXF) (2 to 256 μg/ml) for cycloserine (CYC) and (0.03 to 4 μg/ml) for INH (Table 1.2).

Briefly, the inoculum for the Sensititre MYCOTB/MYCOTBI plate (Trek Diagnostic Systems, Cleveland, OH, USA) was prepared from a *M. tuberculosis* positive MGIT tube within three to five days of signaling positive. An aliquot of three to five ml from the positive MGIT bottle was inoculated into a sterile saline-tween tube containing glass bead and vortexed for 30-60 seconds. The inoculum was allowed to settle for 15 to 30 min. Using a 1 000 μl pipette and a long aerosol resistant pipette tip, 100 μl of inoculum from the top 1/3rd of the saline-tween tube, was inoculated into a Middlebrook 7H9-OADC broth-containing tube, and then vortexed for 20 seconds. A Sensititre AIM™ Automated Inoculation Delivery System (Trek Diagnostics systems, Ohio, USA) was used to add 100 μl of the inoculum to each well. Inoculum purity checks was performed by streaking out 50 μl from the Middlebrook 7H9 tube onto a 5% sheep blood agar which was then incubated at 35-37^{0}C for 48 h and checked for contamination. The Sensititre MYCOTB plate

(Trek Diagnostic Systems, Cleveland, OH, USA) was sealed and incubated at 35-37^{0}C for 21 days. The positive growth control wells were used to determine the testing read time point.

Table 1.1:**MYCOTB microtiter plate format and the range of MIC dilution**

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

*OFL-Ofloxacin, MXF – Moxifloxacin, RIF – Rifampicin, AMI – Amikacin, STR – Streptomycin, RFB – Rifabutin, PAS – Para-aminosalicylic acid, ETH – Ethionamide, CYC – Cycloserine, INH-Isoniazid, KAN – Kanamycin, EMB - Ethambutol

The MYCOTB plates were read at days 7, 10, 14 and 21 of incubation. The MYCOTB plates were read both manually and via a computerized Vizion system (Trek Diagnostic Systems, Cleveland, OH, USA). Manually by visually inspecting the plate and via a computerized Vizion system which projects an image of the plate onto a computer screen, allowing the user to select the MIC value for each drug. Images of the plates along with recorded MIC readings were stored on the Vizion computer system.

1.2 DETERMINATION OF *M. TUBERCULOSIS* MICS USING THE AGAR DILUTION METHOD (ADM):

The APM method was determined using a MICs based Agar Dilution Method

Table 1.2: Layout of the Agar dilution plate showing MICs at increasing dilutions

32	16	8	4
1	0.5	0.25	0.125
Control	**Control**		

*Plate showing a typical layout for Ofloxacin or Streptomycin

A total of 11 plates were used for each isolate representing all drugs on Sensititre except] for Cycloserine which was not tested. Each plate incorporated two control wells and eight drug-containing wells that represented each drug at sequentially increasing concentrations similar dilutions to the Sensititre MYCOTB plate dilution (Trek Diagnostics Systems) [(0.5 to 32 μg/ml) for EMB, STR, and OFLX, (0.12 to 16 μg/ml) for AMI, RFB and RIF (0.3 to 40 μg/ml and 0.6 to 40 μg/ml) for ETH and KAN respectively, 0.5 to 64 μg/ml for PAS, (0.06 to 8 μg/ml) for MXF, (2 to 256 μg/ml) for CYC and (0.03 to 4 μg/ml) for INH]. Each well contained two millimeters of 7H11 broth.

Mycobacterium tuberculosis colonies were scrapped off from 7H11 plates and vortexed for 30 to 60 sec in a sterile saline-Tween-tube containing glass beads (Trek Diagnostics Systems). The suspension was allowed to settle for 15 to 30 min and then adjusted to reach a 1.0 McFarland standard using a nephelometer (BD, Diagnostics). Two drug-free control wells were inoculated

with 100 µl of undiluted sample and the other 100 µl of 10^{-2} dilution of the undiluted sample. Wells five to twelve contained different drug concentrations and were similarly inoculated with 100 µl of neat sample (undiluted sample). The plates were allowed to stand until the inocula was absorbed and put in plastic bags to reduce media from drying. The inoculated medium was be incubated side down at 35-37^{0}C in 5-10% CO^2 (Olympus, USA). The plates were checked weekly for contamination and read at days 10, 14 and 21 days of incubation with the aid of a inverted microscope (Olympus, USA) to help visualize the colonies. For valid result, the control well with 10^{-2} dilution should have between 10 and 50 colonies. The MIC was read at the well where the growth of *M. tuberculosis* was ≥1% of colonies to the 10^{-2} drug-free control well. Media was tested for sterility by incubating a subset of freshly made agar plates at 35-37^{0}C in 5-10% CO^2 (Olympus, USA). Inoculum purity checks was performed by streaking out 50 µl from the Middlebrook 7H9 tube onto a 5% sheep blood agar which was then incubated at 35-37^{0}C for 48 h and checked for contamination (Olympus, USA).

Appendix B

DETAILED MOLECULAR METHODOLOGY

2.1 Deoxyribonucleic acid (DNA) extraction of *M. tuberculosis* isolates using the phenol-chloroform method

1) One millilitre of the cell suspension in a BHI (Merck, Darmstadt, Germany) was taken after 48h of incubation (Labcon, Sepsci, South Africa) and inoculated into a sterile 2 ml Eppendorf tube (Eppendorf AG, Hamburg, Germany) and centrifuged (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 4 930 x g for 15 min at 40C to obtain a pellet.

2) The supernatant was discarded and the pellets were placed on ice.

3) Pellets were re-suspended in 1 m sodium STE buffer [0.1 M NaCl (Merck, Darmstadt, Germany), 1 mM EDTA (pH 8.0) (Merck, Darmstadt, Germany) and 10 mM Tris-HCl (pH 7.5) (Sigma-Aldrich, St. Louis, USA)] by careful up and down aspiration with a pipette

4) Ten microlitres of lysozyme (50 mg.m-1) (Roche Applied Science, Germany) was added to each of the cell suspensions and incubated (AccuBlockTM, White Scientific, South Africa) at 37°C for 1 h.

5) A volume of 50 µl of a 20% SDS solution (Promega, Madison, USA) was added to each tube, followed by 10 µl proteinase K (20 mg.m-1) (Roche Applied Science, Germany).

6) The Eppendorf tubes (Eppendorf AG, Hamburg, Germany) were incubated (Grant instrument, Cambridge, England) at 56°C for 1 h or until the suspension was clear.

vii) Several gentle extractions were performed thrice with equal volumes of mixed phenol: chloroform:isoamylalcohol (Merck, Darmstadt, Germany) in the ratio of 25:24:1, respectively.

7) The suspensions was centrifuged (Spectrafuge 24D, Labnet International, Inc.,New Jersey, USA) at 4 930 x g for 5 min at 25°C.

8) The supernatant was transferred to a sterile 2 m Eppendorf tube (Eppendorf AG, Hamburg, Germany) and an equal volume of chloroform:isoamylalcohol (24:1) (Merck, Darmstadt, Germany) was added to the suspension.

9) The suspension was centrifuged (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 4 930 x g for 20 min at 15°C to remove any traces of phenol from the DNA suspension.

10) The supernatant (DNA-containing aqueous phase) was removed and added to a sterile 2 ml of Eppendorf tube (Eppendorf AG, Hamburg, Germany) and the concentration of the aqueous phase was adjusted using a stock solution of 3 M sodium acetate (Merck, Darmstadt, Germany).

11) Equal volumes of ice-cold, 100% absolute ethanol (-20°C) (Merck, Darmstadt, Germany) was added and mixed, the tubes were stored overnight at -20°C (Kelvinator, Gauteng) to precipitate the DNA.

12) The DNA was pelleted by centrifugation (Spectrafuge 24D, Labnet International, Inc., New Jersey, USA) at 2 682 x g for 10 min at 4°C and the supernatant was discarded.

n) The pellets were desalted with 400 µl of ice-cold, 70% absolute ethanol (-20°C) (Merck, Darmstadt, Germany) and the supernatant was discarded, the tubes were inverted to dry.

13) The DNA pellet was resuspended in 100 µ of TE buffer [10 mM Tris HCl, 1 mM EDTA (pH 8)] (Sigma-Aldrich, St. Louis, USA) and stored at -20°C (Kelvinator, Gauteng) until further analysis.

2.2 Determination of the extracted DNA concentration using the Qubit® fluorometer spectrophotometer.

The concentration of the extracted DNA was determined by the Qubit® fluorometer (Invitrogen, Thermo Fisher Scientific, USA). The Qubit fluorometer uses fluorescent dyes to determine the concentration of the nucleic acid (DNA) in a sample. Each dye is specific for one type of molecule (DNA, RNA or protein). There have extremely low fluorescence until bound to their target molecule. The difference in fluorescence between bound and unbound dye is several orders of magnitude. Upon binding to DNA, by intercalation between bases, it assumes a more rigid shape and become intensely fluoroscent. The Qubit dye binds to the DNA within seconds and reaches equilibrium in less than two minutes. The Qubit fluorometer picks this fluorescence signal up and coverts it into DNA concentration measurement. In DNA concentration determination, the absorbance peak is at 260 nm and the ratio of 260/280 should be 1.8-2.0 to indicate pure and high quality DNA. The concentration of the DNA was found to range between 22.4 ng/ul and 499.7 ng/ul.

Amplification of the *rpo*B region of *M. tuberculosis* DNA

Six primer sets were used for PCR amplification of the entire *rpoB* gene (Table 2). The PCR amplification protocol consisted of a 5 min denaturation step at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 62°C and 50 sec at 72°C and a final extension step at 72°C for 2 min. Following Sanger sequencing of amplicons, mutations in *rpo*B were identified by alignment to H37Rv reference strain (NCBI Accession number AL123456) (Cole, 2002) using ClustalW2 (Li et al., 2015).

Table1.1: *rpoB* primers used to amplify RRDR region

Primer Set	Forward Primers (5'-3')	Reverse Primers (5'-3')	Amplicon Size (nt)
rpoB-RRDR	GGGAGCGGATGACCACCCA	GCGGTACGGCGTTTCGATGAAC	350
rpoB-2	ATGACGTACGCGGCTCCACTGTTCG	GGTGGTCATCCGCTCCCGGACCAC	840
rpoB-3	CGCGGCGAACGGGCCCGTGGGCA	CGGGATCACCTTGACGCTGTGCAG	675
rpoB-4	CTGTCGGTGTACGCGCGGGTCAA	GGGACCGTCGGCGATCACCTGACC	621
rpoB-5	CCACGGCACTTGCGCCAACCAG	CATCCGTCGCGGCACGCCGTGGGT	742
rpoB-6	CCGGTTGAGGACATGCCGTTC	TCCCTTTCCCCTAACGGGTTTAGT	879

2.3 Primers used to amplify *M. tuberculosis* mutation regions

Primer sets were designed for *kat*G and *inh*A (INH and ETH), *gyrA* and *gyrB* (OFX and MOX), *rrs* and *rps*l (AMI KAN and SM) and *emb*B using primer sets shown in the Table below. The PCR amplification protocol consisted of a 5 min denaturation step at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 62°C and 50 sec at 72°C and a final extension step at 72°C for 2 min. PCR products were sequenced by the Sanger method using 3500xl Genetic Analyzer (Applied Biosystems).

Drugs	**primer set**	**Forward Primers (5'-3')**	**Reverse Primers (5'-3')**
KAN,AMI,SM	rrs	CCATCGACGAAGGTCCGGCTTC	CGCGTCCTGTGCATGTCAAACC
KAN,AMI,SM	rrs -2	GTAGCTAACGCATTAAGTACC	CATACAGACAAGAACCCCTCACGG
KAN,AMI,SM	*Rps*l	GGCCGACAAACAGAACGT	GTTCACCAACTGGGTGAC
OFX, MXF	gyrA	CCGATCGAACCGGTTGACATC	GGGTTCGGTGTACCTCAT
OFX,MXF	*gyr*B	AACACCGAGGTCAAATCGTT	CTGAATGCGTCTTCCTTTCCTTGT
EMB	embB	TCCACCCGGCCGACCACGCT	AGCGCCGCCGGTGTGAGGCCG
ETH, INH	*inh*A	CCTCGCTGCCCAGAAAGGA	ATCCCCGGTTTCCGG
ETH,INH	katG	CCCATGGCCGCGGCGGTCGACATT	CGCCGCCTTGGCGGTATTGCC

Appendix C

Table C1: List of MDR isolates collected in 2010 and results of the MICs and mutations

Lab Num	AMI	CYC	EMB	ETH	INH	KAN	MXF	OFX	PAS	RFB	RIF	SM	Rpob	katg	inha	rpsl	rrs	pnca	gyra	gyrb	emb
2305	0.25	16	8	40	4	0.6	0.25	0.5	0.5	0.5	3	32	S531L	S315T	wt	K43R	wt	T135P	wt	wt	G406S
2508	0.25	32	32	40	4	2.5	0.12	1	0.5	8	16	4	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	M306V
2511	0.25	4	4	0.6	0.12	0.6	0.12	0.5	1	0.3	8	1	S531L	S315T	wt	K43R	wt	S59P	wt	K537Q	A405D_A409P
2517	0.25	32	32	40	4	1.2	0.12	1	0.5	1	16	1	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	M306I
2518	0.25	8	8	10	4	1.2	0.06	0.25	0.5	1	16	1	L533P	S315T	T-8A	wt	wt	mk	A90V	wt	A405D_A409P
2520	0.25	16	1	1.2	4	1.2	0.12	0.5	0.5	0.1	2	8	D516V	S315T	wt	K88R	wt	wt	wt	wt	wt
2521	0.5	32	4	40	4	1.2	0.12	1	0.5	4	16	32	S531L	S315T	wt	K43R	wt	T135P	wt	wt	WT
2524	0.25	16	8	40	4	1.2	0.12	16	0.5	2	16	1	S531L	S315T	T-8A	wt	wt	V139G	wt	wt	M306I
2526	0.12	16	8	40	4	0.6	0.25	1	0.5	0.5	16	0.25	wt	S315T	T-8A	wt	wt	G132A	A90V	wt	M306V
2527	0.25	16	4	2.5	2	1.2	0.12	1	0.5	0.1	8	32	D516V	S315T	wt	K43R	wt	L151S	wt	wt	M306V
2530	0.25	32	32	40	4	2.5	2	16	0.5	0.5	16	1	H526Y	S315T	C-15T	wt	wt	H71Y	D94G	wt	M306L
2532	0.25	32	32	2.5	4	1.2	0.25	1	1	2	16	1	S531L	S315T	wt	wt	wt	D12Y	wt	wt	A405D_A409P
2534	0.25	16	1	10	4	1.2	0.06	1	0.5	2	16	1	H526D	S315T	wt	wt	wt	G97C	wt	wt	wt
2535	0.12	32	32	40	4	1.2	0.06	0.5	0.5	0.3	16	0.5	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	D328Y_L402V
2536	0.25	16	16	40	4	1.2	0.5	0.5	0.5	1	16	1	S531L	S315T	T-8A	wt	wt	F94L	wt	wt	A405D_A409P
2542	0.25	16	4	10	4	1.2	0.12	1	0.5	0.3	8	32	D516V	S315T	wt	K43R	wt	L151S	wt	wt	M306V
2544	0.25	16	1	1.2	4	1.2	0.06	0.25	0.5	1	16	8	S531L	S315T	wt	K88R	wt	wt	wt	wt	M306V
2552	0.25	16	1	1.2	4	1.2	0.12	2	8	2	16	0.25	S531Q	wt	wt	wt	wt	wt	wt	wt	M306V
2553	0.25	16	4	1.2	0.5	0.6	0.25	0.5	4	1	16	32	S531L	wt	wt	K43R	wt	M1T	wt	wt	WT
2555	0.25	32	32	40	4	1.2	0.06	0.5	0.5	0.5	16	0.5	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	WT
2559	0.25	8	1	10	2	1.2	0.12	0.5	0.5	2	16	0.5	S531L	wt	dbl peak	wt	wt	ins G pos26	wt	wt	M306I

Table C1 continued……..

															wt/C-15T			(codon 9)			
2564	0.5	16	8	10	4	2.5	0.12	1	0.5	4	16	2	S531L	S315T	wt	wt	wt		wt	wt	M306I
2572	0.12	16	2	10	0.5	1.2	0.12	0.5	0.5	0.1	4	0.25	Wt	wt	C-15T	wt	wt	wt	wt	wt	A405D_A409P
2575	0.12	8	2	40	2	0.6	0.12	0.5	0.5	2	16	0.25	S531L	wt	C-15T	wt	wt	wt	wt	wt	WT
2577	0.25	16	32	40	4	2.5	8	32	0.5	8	16	1	S531L	S315T	T-8A	wt	wt	del T pos389 (codon 130)	D94N	wt	WT
2579	0.25	16	4	2.5	4	1.2	0.25	1	0.5	0.1	16	32	D516V	S315T	wt	K43R	wt	L151S	wt	wt	G406A
2580	0.12	32	1	2.5	0.25	1.2	0.12	1	0.5	0.1	0.1	0.25	wt	wt	wt	wt	wt	wt	wt	wt	WT
2587	0.25	8	4	40	4	1.2	0.12	1	0.5	0.5	16	0.5	S531L	S315T	T-8A	wt	wt	V139G	wt	wt	WT
2591	0.25	16	8	5	4	1.2	0.12	1	0.5	0.1	8	32	D516V	S315T	wt	K43R	wt	L151S	wt	wt	WT
2592	0.25	16	1	1.2	4	1.2	0.12	0.5	0.5	8	16	8	S531L	wt	wt	wt	wt	wt	wt	wt	M306V
2598	0.25	16	1	1.2	4	2.5	0.06	0.5	0.5	0.3	16	2	wt	S315T	wt	wt	wt	wt	wt	wt	M306V
2602	16	64	32	40	4	20	0.5	4	64	16	16	32	S531L	S315G	wt	wt	wt	wt	wt	wt	
2603	0.25	32	1	5	4	1.2	0.06	0.5	0.5	1	16	32	H526D	wt	C-15T	K43R	wt	wt	wt	wt	WT
2606	0.12	8	0.5	40	4	1.2	0.06	0.5	0.5	2	16	0.5	H526D	S315T	wt	wt	wt	G97C	wt	wt	M306I
2607	0.12	8	8	40	4	1.2	0.25	4	0.5	2	16	0.25	H526D	S315T	wt	wt	wt	G97C	A90V	wt	M306I
2610	0.5	16	4	10	2	1.2	0.12	0.5	0.5	2	16	32	H526D	wt	C-15T	K43R	wt	A-11G	wt	wt	WT
2614	0.5	16	2	1.2	0.12	1.2	0.06	0.5	0.5	0.5	16	0.25	L533P	wt	C-15T	wt	wt	wt	wt	wt	M306V
2615	0.25	16	2	10	4	0.6	0.12	0.5	1	1	16	0.25	S531L	wt	wt	wt	wt	wt	wt	wt	WT
2617	0.12	16	32	40	4	1.2	0.06	0.5	0.5	0.5	16	0.5	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	WT
2618	0.25	16	8	5	4	1.2	0.12	0.5	0.5	1	16	1	S531L	S315T	wt	wt	wt		wt	wt	M306V
2619	0.25	16	1	2.5	4	1.2	0.06	0.5	0.5	0.5	8	0.25	ins 3bp(CTT) c514	wt	wt	wt	wt	wt	wt	wt	WT
2623	0.5	32	4	40	4	2.5	0.25	0.5	0.5	8	16	0.5	H526D	wt	C-15T	wt	wt	wt	wt	wt	M306V_E378A
2626	16	16	4	2.5	4	1.2	0.12	0.5	0.5	8	16	32	H526Y	S315T	wt	K43R	wt	S104R	wt	wt	M306V
2627	0.12	8	4	40	2	0.6	0.12	1	0.5	2	16	0.25	S531L	wt	wt	wt	wt	wt	wt	wt	M306I
2629	0.25	32	8	40	4	2.5	0.12	0.5	0.5	0.5	16	1	S531L	S315T	T-8A	wt	wt	V139G	wt	wt	M306V
2630	0.12	8	2	1.2	4	1.2	0.12	0.5	0.5	1	16	0.25	wt	S315T	wt	wt	wt	A143P	wt	wt	WT
2634	0.5	8	0.5	1.2	4	1.2	0.12	1	0.5	2	16	32	H526Y	S315T	wt	K43R	wt	wt	wt	Wt	WT

2635	0.25	32	2	2.5	0.12	2.5	0.12	1	0.5	0.3	16	0.5	wt	wt	wt	wt	wt	wt	wt	Wt	A405D_A409P
2637	16	8	16	40	4	1.2	0.5	8	0.5	1	16	0.5	wt	S315T	wt	wt	wt	G97C	S91P	wt	G406A
2641	0.12	8	2	2.5	4	0.6	4	8	0.5	1	16	32	wt	dbl peak wt/S315T	wt	wt	wt	wt	wt	wt	M306I
2650	0.12	32	2	2.5	4	1.2	0.25	1	0.5	4	16	0.25	S531L	S315T	wt	wt	wt	wt	wt	wt	M306V
2652	2	32	32	40	4	5	1	1	64	2	16	32	S531L	S315T	wt	K43R	wt	S67W	wt	wt	M306I
2657	0.25	16	8	40	2	1.2	0.06	0.5	1	2	16	0.25	S531L	wt	wt	wt	wt	wt	wt	wt	M306V
2665	0.25	32	32	40	4	2.5	2	16	0.5	2	16	2	H526Y	S315T	C-15T	wt	wt	H71Y	D94G	wt	WT
2667	16	32	16	40	4	40	2	8	0.5	2	16	1	S531L	wt	wt	wt	wt	wt	wt	wt	M306V
2669	0.25	32	4	1.2	0.5	1.2	0.06	0.5	0.5	0.1	4	0.25	D516V	wt	wt	wt	wt	wt	wt	wt	M306I
2670	0.5	32	8	40	4	2.5	0.12	0.25	16	2	16	1	S531L	S315T	T-8A	wt	wt	G132A	wt	wt	WT
2671	0.12	4	4	2.5	4	1.2	0.12	0.5	0.5	0.1	2	32	D516V	S315T	wt	K43R	wt	L151S	wt	wt	WT
2671	0.12	4	4	1.2	4	1.2	0.25	0.5	0.5	0.1	4	4	D516V	S315T	wt	K43R	wt	L151S	wt	wt	M306V
2675	16	32	32	40	4	40	0.5	8	2	2	16	2	H526Y	S315T	C-15T	wt	wt	H71Y	A90V	wt	WT
2678	0.25	16	4	1.2	4	2.5	0.12	0.5	0.5	1	16	32	S531L	S315T	wt	K43R	wt	Y103stop	wt	wt	M306I
2684	0.12	16	8	40	4	1.2	0.5	8	0.5	1	16	0.5	S531L	S315T	T-8A	wt	wt	del T pos389 (codon 130)	A90V	wt	M306V
2690	0.25	32	32	40	4	2.5	2	16	0.5	4	16	2	H526Y	S315T	C-15T	wt	wt	H71Y	D94G	wt	WT
2692	0.12	16	1	1.2	0.06	0.6	0.12	0.25	0.5	0.5	16	2	H526D	wt	wt	K43R	wt	wt	wt	wt	WT
02693	0.25	32	1	5	4	1.2	0.12	0.5	0.5	0.1	8	32	D516V	S315T	wt	K43R	wt	L151S	wt	wt	M306V
2698	0.12	16	4	2.5	4	0.6	0.12	1	0.5	8	16	32	H526D	S315T	wt	K43R	wt	A134D	wt	wt	M306V
2699	0.12	8	1	2.5	4	1.2	0.06	0.25	0.5	0.5	16	0.25	H526D	S315T	wt	wt	wt	G97C	wt	wt	A405D_A409P
2700	0.5	32	2	1.2	4	2.5	0.12	0.5	4	2	16	1	S531L	S315T	wt	wt	wt	L35R	wt	wt	M306I
2701	0.25	16	1	1.2	4	2.5	0.12	0.5	0.5	0.1	1	0.25	H526L	S315T	wt	wt	wt	wt	wt	wt	WT
2702	0.25	16	2	2.5	4	2.5	0.25	1	0.5	0.1	4	32	H526L	S315T	wt	K43R	wt	wt	wt	wt	M306I
2704	0.25	16	32	10	4	1.2	0.5	8	0.5	0.5	16	1	H526Y	S315T	C-15T	wt	wt	H71Y	A90V	wt	M306I
2706	0.25	8	0.5	2.5	4	1.2	0.06	0.5	0.5	2	16	16	S531L	S315T	wt	K88R	wt	wt	wt	wt	M306I
2708	0.12	8	2	0.6	2	1.2	0.12	1	2	0.1	1	0.5	D516V	S315T	wt	wt	wt	wt	wt	wt	
2709	0.25	64	16	0.6	0.25	1.2	0.06	0.25	0.5	0.3	16	0.5	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	A405D_A409P

2715	0.25	32	32	40	4	2.5	0.12	0.5	0.5	2	16	2	S531L	S315T	C-15T	wt	wt	H71Y	wt	wt	WT
2719	0.25	64	8	20	4	1.2	0.12	0.5	0.5	4	16	32	S531L	S315T	wt	K43R	wt	S67W	wt	wt	M306I
2721	0.25	32	8	2.5	4	1.2	0.06	0.5	0.5	2	16	32	S531L	S315T	wt	K43R	wt	S59P	wt	K537Q	WT
2724	0.12	8	4	10	4	2.5	0.12	0.5	1	2	16	0.25	S531L	S315T	wt	wt	wt	wt	wt	wt	WT
2730	0.25	8	1	1.2	4	1.2	0.12	1	0.5	1	16	0.25	S531L	S315T	wt	wt	wt	wt	wt	wt	M306V
2732	0.25	32	32	40	4	2.5	1	8	0.5	2	16	2	H526Y	S315T	C-15T	wt	wt	H71Y	A90V	wt	WT
2735	0.12	2	1	1.2	4	1.2	0.06	0.5	0.5	0.1	0.1	0.25	H526D	S315T	wt	wt	wt	G97C	wt	wt	A405D_A409P
2745	0.25	4	0.5	0.6	2	0.6	0.12	0.5	0.5	0.3	4	0.25	Wt	S315T	wt	wt	wt	del T pos470 (codon 157)	wt	wt	M306V
3189	0.12	16	8	40	4	1.2	0.06	0.5	0.5	1	16	4	S531L	S315T	T-8A	wt	wt	del T pos389 (codon 130)	wt	wt	M306V
3190	0.12	2	0.5	5	0.5	1.2	0.06	0.25	0.5	0.3	16	0.25	S531L	wt	C-15T	wt	wt	wt	wt	wt	WT
3193	0.12	8	4	40	4	1.2	1	4	0.5	1	16	0.5	S531L	S315T	T-8A	wt	wt	V139G	S91P	wt	M306V
3205	0.5	8	4	10	1	1.2	2	8	0.5	0.5	16	0.25	S531L	wt	C-15T	wt	wt	del A pos441 (codon 147)	D94G	wt	WT
3214	0.12	8	8	40	4	1.2	0.06	0.5	64	0.1	16	0.5	L533P	S315T	T-8A	wt	wt	ins C pos457 (codon 153)	wt	wt	M306I
3228	0.5	16	2	40	4	2.5	0.5	2	64	4	16	2	S531L	S315T	wt	wt	wt	L35R	wt	wt	WT
3247	0.25	8	4	40	1	1.2	0.5	2	0.5	4	16	0.25	S531L	wt	wt	wt	wt	wt	wt	A515V	WT
3316	0.25	32	32	40	4	2.5	0.25	1	0.5	2	16	2	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	WT
3319	0.12	16	8	40	4	0.6	0.12	0.5	0.5	1	16	4	S531L	S315T	T-8A	wt	wt	del T pos389 (codon 130)	wt	wt	WT
3337	0.12	16	32	40	4	2.5	0.12	1	0.5	2	16	1	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	WT
3341	0.25	16	4	40	4	1.2	0.12	1	0.5	8	4	2	S531L	S315T	T-8A	wt	wt	V139G	wt	wt	WT
3342	0.25	8	4	1.2	4	1.2	0.06	0.25	0.5	2	16	32	S531L	S315T	wt	K43R	wt	S59P	wt	K537Q	WT
3344	1	32	32	5	4	2.5	0.25	0.25	4	16	16	32	S531L	S315T	wt	wt	A514C	C14R	wt	wt	E378A

3345	0.25	8	2	1.2	4	1.2	0.06	0.25	0.5	2	16	0.25	S531L	S315T	wt	wt	wt	wt	wt	wt	M306I
3352	0.12	4	0.5	0.6	4	0.6	0.06	0.5	0.5	2	16	16	S531L	S315T	wt	K43R	wt	wt	wt	wt	WT
3356	0.25	32	8	40	4	2.5	0.5	8	0.5	2	16	1	S531L	S315T	T-8A	wt	wt	del T pos389 (codon 130)	A90V	wt	M306V
3369	0.5	16	1	5	0.5	2.5	0.25	0.5	0.5	2	16	0.25	S531L	wt	C-15T	wt	wt	wt	wt	wt	WT
3370	0.25	8	1	0.6	4	0.6	0.06	0.5	0.5	4	16	0.5	S531L	S315T	wt	wt	wt	wt	wt	wt	WT
3371	0.25	16	2	40	4	2.5	0.06	0.5	0.5	4	16	1	D516V	S315T	wt	wt	wt	wt	wt	wt	M306V
3619	0.12	8	2	40	4	0.6	0.5	1	0.5	0.5	16	0.25	H526D	S315T	wt	wt	wt	G97C	D94G	wt	M306I
3662	0.25	16	8	2.5	0.25	1.2	0.12	0.25	0.5	0.5	16	0.25	S531L	wt	C-15T	wt	wt	wt	wt	wt	M306V_E378A
3699	0.25	8	1	0.6	4	1.2	0.25	0.5	0.5	16	16	0.25	H526D	wt	wt	wt	wt	wt	wt	wt	M306V
3700	0.5	32	1	0.6	2	2.5	0.12	0.5	0.5	4	16	0.25	H526R	S315T	wt	wt	wt	wt	wt	wt	M306V_E378A
3701	0.12	16	2	1.2	4	0.6	0.06	0.25	0.5	0.1	0.5	0.25	H526L	S315T	wt	wt	wt	wt	wt	wt	M306I
3711	0.12	4	4	2.5	4	0.6	0.12	0.5	0.5	0.1	1	0.25	H526C	S315T	wt	wt	wt	del A pos64 (codon 22)	wt	wt	A405D_A409P
3715	0.12	16	4	1.2	4	1.2	0.06	0.5	0.5	0.1	0.1	32	I572F	S315T	wt	K43R	wt	H51D	wt	wt	M306V_E378A
3720	0.25	32	32	5	4	1.2	0.12	0.5	0.5	1	16	1	H526D	S315T	wt	wt	wt	G97C	wt	wt	M306I
3721	0.25	16	8	1.2	4	1.2	0.12	0.5	0.5	0.5	16	0.5	S531L	S315T	wt	wt	wt	del T pos515 (codon 172)	wt	K537K	WT
3736	0.12	16	4	1.2	4	0.6	0.12	0.5	0.5	0.1	2	0.5	D516V	wt	T-8A	wt	wt	wt	wt	wt	A405D_A409P
3783	0.12	16	4	40	2	1.2	0.06	0.5	0.5	4	16	0.25	S531L	wt	wt	wt	wt	wt	wt	wt	M306V
3803	0.5	16	8	40	4	0.6	0.12	1	0.5	1	16	4	S531L	S315T	T-8A	wt	wt	del T pos389 (codon 130)	wt	wt	M306V
3808	0.25	32	1	2.5	0.06	1.2	0.12	0.5	0.5	0.1	0.1	0.5	wt	wt	wt	wt	wt	wt	wt	wt	WT
03816	0.12	2	0.5	1.2	0.03	0.6	0.06	0.25	0.5	0.1	0.1	0.25	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	WT
3837	0.25	32	8	40	4	1.2	0.12	1	1	1	16	1	S531L	S315T	T-8A	wt	wt	V139G	wt	wt	WT
3841	0.5	32	32	40	4	2.5	0.5	8	1	1	16	4	H526Y	S315T	C-15T	wt	wt	H71Y	wt	D472H	A405D_A409P

3905	0.25	16	1	2.5	4	1.2	0.25	8	0.5	1	16	0.25	S531L	S315T	wt	wt	wt	wt	wt	wt	A405D_A409P
3908	0.12	32	1	2.5	1	1.2	0.12	0.5	0.5	0.5	16	16	H526D	wt	C-15T	K43R	wt	wt	wt	wt	WT
3909	0.12	32	32	40	4	2.5	1	1	1	0.5	16	2	H526Y	S315T	C-15T	wt	wt	H71Y	D94G	wt	WT
3913	0.12	8	8	5	4	0.6	0.06	0.25	2	4	16	32	S531L	S315T	wt	K43R	wt	Y34D	wt	wt	WT
3914	0.25	8	8	40	4	1.2	0.12	0.25	1	2	16	32	S531L	wt	C-15T	K88R	wt	A102V	wt	wt	M306V
3921	0.12	8	0.5	10	1	2.5	0.06	0.25	0.5	2	16	0.25	H526D_I561I	wt	C-15T	wt	wt	wt	wt	wt	A405D_A409P
3925	0.25	8	4	40	4	2.5	0.06	0.5	0.5	4	16	2	H526D	S315T	wt	wt	wt	G97C	wt	wt	WT
3942	0.25	8	0.5	2.5	0.06	1.2	0.12	0.5	0.5	0.1	0.1	0.25	wt	wt	wt	wt	wt	wt	wt	wt	M306L
3971	0.25	32	8	40	4	1.2	0.12	0.5	0.5	4	16	2	S531L	S315T	T-8A	wt	wt	del T pos389 (codon 130)	wt	wt	A405D_A409P
3975	0.25	8	1	0.6	2	1.2	0.25	0.5	0.5	4	16	4	H526P	S315T	wt	wt	wt	wt	wt	wt	M306I
3988	0.25	32	8	40	4	1.2	0.12	0.5	0.5	4	16	1	S531L	S315T	T-8A	wt	wt	del T pos389 (codon 130)	wt	wt	WT
4010	0.25	8	0.5	10	0.25	5	0.25	0.5	0.5	16	16	0.5	H526Y	wt	C-15T	wt	wt	wt	wt	wt	WT
4012	0.5	8	1	1.2	4	2.5	0.12	0.5	0.5	2	16	0.25	S531L	S315T	wt	wt	wt	wt	wt	wt	WT
4039	0.5	16	16	40	4	2.5	0.25	1	0.5	4	16	32	S531L	wt	C-15T	K88R	wt	A102V	wt	wt	A405D_A409P
4068	0.25	16	16	40	4	1.2	0.12	0.5	0.5	0.1	0.1	0.5	L533P	S315T	T-8A	wt	wt	ins C pos457 (codon 153)	wt	wt	M306V
4085	0.25	4	4	40	4	1.2	0.25	0.5	0.5	0.1	16	0.5	L533P	wt	wt	wt	wt	wt	wt	wt	WT
4159	0.5	16	4	2.5	4	1.2	0.25	1	0.5	8	16	32	H526D	S315T	wt	K43R	wt	A134D	wt	wt	WT
4163	1	32	1	2.5	4	2.5	0.12	0.5	0.5	16	16	32	H526Y	S315T	wt	K43R	wt	wt	wt	wt	WT
4171	0.25	8	1	5	0.25	1.2	0.12	0.5	0.5	1	16	0.25	S531L	wt	C-15T	wt	wt	wt	wt	wt	M306I
4186	0.25	8	0.5	2.5	0.06	2.5	0.06	0.25	0.5	0.1	0.3	0.5	H526L	wt	wt	wt	wt	wt	wt	wt	M306V_E378A
4213	0.25	16	4	2.5	4	5	0.12	0.25	0.5	1	16	32	H526Y	S315T	wt	K88M	wt	A79V	wt	wt	M306I
4234	0.25	16	16	40	4	1.2	0.12	0.5	2	0.1	0.5	1	L533P	S315T	T-8A	wt	wt	ins C pos457 (codon 153)	wt	wt	A405D_A409P

4245	0.5	32	16	40	4	2.5	0.25	0.25	0.5	16	16	32	H526Y	S315T	C-15T	wt	wt	H71Y	D94N	wt	M306I
4248	1	8	4	2.5	4	2.5	0.5	1	32	16	16	4	S531L	S315T	wt	K43R	wt	Y34D	wt	wt	M306V
4250	0.25	8	4	1.2	4	0.6	0.12	1	0.5	4	16	0.25	S531L	wt	wt	wt	wt	S65S	wt	wt	M306I
4254	0.12	8	8	1.2	4	1.2	0.12	0.5	0.5	0.1	4	0.25	D516V	S315T	wt	wt	wt	wt	wt	wt	WT
4255	0.12	8	2	2.5	4	0.6	0.25	0.5	0.5	4	16	32	S531L	S315T	wt	K43R	wt	S59P	wt	K537Q	M306I
4257	0.25	16	32	40	4	2.5	0.12	1	0.5	4	16	2	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	A405D_A409P
4267	0.25	16	4	40	4	1.2	0.12	1	0.5	4	16	1	S531L	S315T	T-8A	wt	wt	V139G	wt	wt	M306V
4279	0.25	16	32	40	4	1.2	0.06	0.5	0.5	16	16	2	H526Y	S315T	C-15T	wt	wt	H71Y	wt	wt	M306V
4298	0.12	16	8	40	4	0.6	0.06	0.5	0.5	1	16	4	S531L	S315T	T-8A	wt	wt	del T pos389 (codon 130)	wt	wt	A405D_A409P
4410	0.5	16	1	40	2	2.5	0.25	1	0.5	2	16	0.5	S531L	wt	C-15T	wt	wt	wt	wt	wt	wt
4457	0.12	8	8	40	4	0.6	1	8	0.5	1	16	0.5	S531L	S315T	T-8A	wt	wt	G132A	A90V	wt	M306V
4467	0.5	32	1	2.5	4	2.5	0.25	1	0.5	0.3	16	4	D516V	S315T	wt	wt	wt	wt	wt	wt	WT
4468	0.5	8	1	1.2	4	1.2	0.25	0.5	0.5	2	16	0.25	S531L	wt	wt	wt	wt	wt	wt	wt	wt
4481	0.25	32	16	40	4	2.5	2	0.25	0.5	1	16	2	H526Y	S315T	C-15T	wt	wt	H71Y	D94G	wt	M306I
4514	0.25	16	4	1.2	4	1.2	0.12	0.25	0.5	8	16	32	H526D	S315T	wt	K43R	wt	A134D	wt	wt	M306V
4533	0.25	16	4	40	4	2.5	0.5	1	0.5	16	16	4	H526D	S315T	wt	wt	wt	G97C	wt	wt	M306I
4575	0.25	8	0.5	0.6	4	2.5	0.06	0.5	0.5	0.1	4	0.5	D516V	S315T	wt	wt	wt	wt	wt	wt	M306I

Appendix C

Table C2: List of MDR isolates collected in 2013 and the results of their MICs and mutations

Lab_Num	rpoB	OFX	MXF	RIF	AMI	SM	RFB	PAS	ETH	CYC	INH	KAN	EMB	katG	inhA	gyrA	gyrB	pncA	rrs	rpsL	embB
14155	S531L	1	0.25	16	0.5	32	8	0.5	40	16	4	1.2	1		wt	Wt		wt	wt		wt
14156	Wt	0.5	0.12	0.12	0.25	16	0.12	1	40	8	0.5	2.5	0.5	wt	-15C>T	Wt	wt	T114M	wt	K88R	wt
14158	I572F	1	0.25	16	1	32	16	0.5	40	16	4	2.5	32	S315T	-8T>A	Wt	wt		wt	wt	M306V
14160	D516V	8	4	16	0.5	4	0.5	4	2.5	32	4	2.5	32	S315T	-17G>T	A90V	wt	ins G pos518 (codon 173)	A514C	wt	M306I
14161	S531L	1	0.25	16	0.25	2	4	0.5	2.5	16	4	1.2	2	S315T	wt	Wt		del G pos50 (codon 17)	wt	wt	L402V
14162	D516Y_N518H	2	0.25	16	0.25	4	0.25	0.5	40	32	1	1.2	8	wt	-15C>T	Wt	wt	wt	wt	wt	M306L_E378A
14164	D516Y	8	4	16	0.25	1	0.12	0.5	40	8	4	1.2	4	S315T	P12R	D94H	wt	F13C	wt	wt	N296Y
14165	S531L	1	0.25	16	0.25	16	16	0.5	2.5	32	4	1.2	4	S315T	wt	Wt	wt	-12T>C	C491T_A514C	wt	M306I
14166	D516V	8	4	16	0.5	4	0.5	4	2.5	32	4	2.5	32	S315T	wt	A90V	wt	ins G pos35	C904A	wt	M306V
14168	D516V	1	0.25	16	0.5	1	4	0.5	10	8	4	1.2	8	S315T	wt	A90V		ins G pos35	C904A	wt	M306V
14169	S531L	16	4	16	0.5	4	1	2	20	32	4	2.5	16	S315T	-8T>A	A90V	wt	V139G	wt	wt	M306V
14170	S531L	16	4	16	0.12	0.5	1	0.5	40	16	4	0.6	4	wt	-15C>T	D94G	wt	T47I	wt	wt	M306V
14171	H526Y	1	0.12	16	0.25	0.25	4	0.5	1.2	8	0.03	2.5	0.5	wt	wt	wt	wt	wt	wt	wt	wt
14172	S531L	1	0.25	16	0.25	0.25	2	0.5	2.5	8	1	1.2	0.5	G285R	wt	wt	wt	wt	wt	wt	wt
14173	D516V	0.5	0.25	16	0.25	0.25	0.25	0.5	1.2	16	4	1.2	2	wt	wt	wt	wt	wt	wt	wt	wt
14175	Wt	1	0.25	1	0.5	0.5	0.12	2	5	16	0.25	2.5	1	wt	wt	wt	wt	wt	wt	wt	wt
14176	S531L	1	0.06	16	0.12	4	0.5	0.5	40	4	1	0.6	8	wt	wt	wt		G97D	C517T	wt	M306L_E378A
14177	H526	16	4	16	0.5	4	1	2	20	32	4	2.5	16	wt		A90V	wt	wt	wt	wt	wt

	D																				
14178	S531L	0.5	0.12	16	0.25	0.5	0.5	0.5	40	16	4	0.6	8	S315T	-8T>A	wt	wt	del T pos389 (codon 130)	wt	wt	M306I
14179	H526Y	0.5	0.12	16	0.5	1	16	0.5	0.6	8	0.5	2.5	0.5	wt	-15C>T	wt	wt	wt		wt	wt
14180	S531L	1	0.25	16	0.12	32	16	0.5	40	8	4	1.2	16	wt	-15C>T	wt		G97D	C517T	wt	M306L_E378A
14181	H526D	0.5	0.12	16	0.5	0.5	16	0.5	0.6	16	4	2.5	1	wt	-15C>T	wt	wt	wt	wt	wt	wt
14297	S531L	1	0.25	16	0.5	0.5	4	0.5	2.5	16	4	5	1	wt	-15C>T	wt	wt	wt	wt	wt	wt
14298	Wt	1	0.25	0.25	0.25	0.25	0.5	1	2.5	8	0.12	1.2	1	wt	wt	wt	wt	wt	wt	wt	wt
14299	S531L	16	4	16	0.5	4	1	2	20	32	4	2.5	16	wt	-15C>T	wt	S458F	wt	wt	wt	M306V
14300	S531L	32	8	16	1	1	4	64	10	64	4	2.5	1	wt	wt	A90V		wt	wt	wt	wt
14303	D516V	1	0.25	16	1	32	0.5	0.5	40	64	4	1.2	8	S315T	wt	wt	wt	ins A pos172	A514C	wt	wt
14304	S531L	0.5	0.25	16	0.5	0.25	8	1	1.2	16	4	1.2	4	S315T	wt	wt	wt	Y103Stop(Hatherell et al.)	wt	wt	M306I
14307	S531L	0.5	0.12	16	0.12	0.25	2	0.5	1.2	4	0.03	0.6	0.5	wt	wt	wt		wt	wt	wt	wt
14308	H526D	1	0.5	16	0.5	0.5	16	0.5	1.2	8	0.12	2.5	0.5	wt	wt	wt	wt	wt	wt	wt	wt
14310	S531L	1	0.25	16	0.25	0.25	0.12	0.5	10	8	0.5	2.5	4	wt	-15C>T	wt	wt	wt	wt	wt	M306V
14311	S531L	0.5	0.12	16	0.12	0.25	0.5	0.5	1.2	4	4	1.2	1	S315T	wt	wt	wt	wt	wt	wt	wt
14312	D516V	32	8	16	1	1	4	64	10	64	4	2.5	1	S315T	-17G>T	A90V	K537Q	ins G pos518 (codon 173)	A514C	wt	M306I
14313	L533P	1	0.25	0.12	0.5	1	0.12	0.5	5	32	4	2.5	2	S315T	wt	wt	wt	wt	wt	wt	wt
14418	L533P	0.25	0.06	0.12	0.12	4	0.12	0.5	0.6	8	4	1.2	2	S315T	wt	wt	wt	wt	A514C	wt	M306I
14420	S531L	1	0.12	16	0.12	16	1	0.5	2.5	2	4	1.2	8	wt	wt	wt	wt	G97D	C517T	wt	M306L_E378A
14422	S531L	0.5	0.12	16	0.5	1	8	0.5	40	32	4	1.2	8	S315T	-8T>A	wt	wt	del T pos389	wt	wt	M306I

																		(codon 130)			
14423	Wt	0.25	0.06	0.25	0.25	0.25	0.5	0.5	0.6	4	4	1.2	0.5	S315T	wt	wt	wt	wt	wt	wt	wt
14425	D516V	1	0.5	16	16	32	4	2	5	32	4	40	4	S315T	-17G>T	wt	wt	ins G pos518 (codon 173)	A514C	wt	M306I
14426	S531L	0.25	0.25	16	0.5	0.25	4	0.5	2.5	16	4	1.2	1	wt	wt	wt	wt	wt	wt	wt	wt
14672	S531L	0.5	0.12	16	0.25	0.5	1	0.5	40	8	4	1.2	8	wt	-15C>T	wt	wt	wt	wt	wt	M306V
14673	L511P	16	4	16	0.5	4	1	2	20	32	4	2.5	16	S315T	wt	wt	S458F	wt	wt	wt	wt
14674	S531L	16	4	16	0.5	4	1	2	20	32	4	40	4	S315T	wt	wt	wt	V155M	A905G	wt	wt
14675	H526Y	16	4	16	0.5	4	1	2	20	32	4	1.2	8	S315T	wt	wt	T511I	wt		wt	wt
14676	H526Y	16	4	16	0.5	4	1	2	20	32	4	1.2	4	S315T	wt	wt	S458F	wt	wt	wt	wt
14678	S531L	0.5	0.12	16	0.25	0.5	1	0.5	40	8	4	1.2	8	S315T	-8T>A	wt	wt	V139G	wt	wt	M306V
14679	H526Y	16	4	16	0.12	0.5	1	0.5	40	16	4	0.6	4	S315T	wt	wt	K537Q	wt	wt	wt	wt
14907	S531L	0.5	0.25	16	0.25	0.25	16	1	2.5	8	4	1.2	0.5	wt	wt	wt	wt	wt	C491T	wt	wt
14908	S531L	1	0.25	16	0.5	0.25	8	1	1.2	8	4	1.2	2	wt	-15C>T	wt	wt	wt	wt	wt	wt
14909	S531L	4	0.5	16	0.25	32	1	0.5	20	8	4	0.6	8	S315T	wt	D94A	K537Q	D8N	wt	K43R	M306V
14910	D516Y	1	0.25	8	0.5	1	0.12	0.5	2.5	32	4	2.5	2	S315T	wt	wt	wt	wt		wt	wt
14912	H526Y	8	4	16	0.5	4	16	0.5	40	32	4	2.5	32	S315T	-15C>T	A90V	wt	H71Y	wt	wt	
15301	H526D	1	0.5	16	0.25	16	16	0.5	2.5	16	4	2.5	0.5		-15C>T	wt	wt	wt	wt	wt	
15302	Wt	2	0.25	0.12	0.25	0.25	0.12	2	40	4	1	2.5	1	wt	-8T>A	wt	wt	wt	wt	wt	
15303	S531L	0.5	0.12	16	0.25	0.25	4	0.5	40	8	0.5	1.2	1	wt	wt	wt	wt	wt	wt	wt	
15304	Wt	1	0.25	0.12	0.25	0.25	0.12	2	1.2	32	4	2.5	1	wt	wt	wt	wt	wt	wt	wt	
15304	Wt	2	0.5	16	0.5	1	16	1	0.6	16	4	5	1	wt	wt	wt	wt	wt	wt	wt	
15305	H526Y	8	4	16	1	4	16	1	40	32	4	5	32	S315T	-15C>T	A90V	wt	H71Y	wt	wt	
15306	H526Y	0.5	0.12	16	0.25	0.25	4	0.5	40	32	0.5	1.2	1	S315T	wt	wt	wt	D8Y_L35R	wt	S17N	
15307	Wt	0.5	0.12	0.1	0.25	0.25	0.12	0.5	20	16	0.5	2.5	1	wt	-15C>T	wt	wt	wt	wt	wt	

				2																	
15308	S531L	2	0.5	16	0.25	32	4	0.5	40	16	4	2.5	16	wt	-15C>T	wt	wt	A102V	wt		
15309	Wt	2	0.5	0.5	1	2	0.12	0.5	5	16	4	2.5	2	S315T	wt	wt	wt	wt	wt	wt	
15310	H526Y	1	0.5	16	0.5	32	16	0.5	40	4	4	2.5	32	S315T	-15C>T	wt	wt	H71Y	wt	wt	
15312	S531L	1	0.12	16	0.12	4	1	0.5	1.2	2	4	1.2	8	wt	wt	wt	wt	G97D	C517T	wt	
15313	H526Y	16	8	16	16	16	16	1	40	32	4	40	32	S315T	-15C>T	D94G	wt	H71Y	A1401G	wt	
15314	S531L	0.5	0.25	16	0.25	0.25	8	0.5	10	8	2	2.5	8	wt	-15C>T	wt	wt	ins 5nt (CGCGC) pos79	wt	wt	
15315	S531L	1	0.5	16	0.5	32	4	0.5	40	16	4	2.5	16	wt	-15C>T	wt	wt	A102V	wt	K88R	
15316	S531L	2	1	0.25	0.5	1	0.12	1	40	16	0.5	5	2	S315T	wt	wt		wt	wt	wt	
15317	H526D	0.5	0.25	16	0.5	0.25	16	1	1.2	8	0.12	2.5	1	wt	wt	wt	wt	wt	wt		
15317	H526D	0.5	0.25	16	0.5	0.25	16	0.5	1.2	16	0.12	2.5	1	wt	wt	wt	wt	wt	wt		
15318	S531L	0.5	0.25	16	0.5	32	2	0.5	1.2	8	4	1.2	2	S315T	wt	wt	wt	C14R	A514C	wt	
15319	Wt	1	0.5	0.5	0.5	1	0.12	2	40	32	0.5	5	4	wt	-15C>T	wt	wt	wt	C491T	wt	
15320	S531L	0.5	0.25	16	0.25	0.25	1	0.5	40	16	0.25	1.2	1	wt	-15C>T	wt	wt	wt	wt	wt	
15321	S531L	1	1	16	0.25	1	16	0.5	2.5	32	0.5	2.5	0.5	G299R	wt	wt	wt	L172P	wt	wt	
15321	S531L	1	0.5	16	0.5	4	16	0.5	40	32	4	2.5	32	G299R	wt	wt	wt	L172P	wt	wt	
15322	S531L	0.5	0.25	16	0.5	32	4	64	20	16	4	2.5	8	S315T	wt	wt	K537Q	S59P	wt	K43R	
15323	S531L	0.5	0.12	16	0.25	8	0.5	0.5	20	2	4	0.6	0.5	wt	-15C>T	wt	wt	T114M	wt	K88R	
15324	H526C	2	0.25	16	0.25	0.25	0.25	2	1.2	4	4	10	16	S315T	wt	wt	wt	del A pos64 (codon 22)	wt	wt	
15325	H526D	2	1	16	1	1	16	1	0.6	16	1	10	1	wt	wt	wt	wt	L35R	wt	wt	
15325	H526D	2	1	16	1	1	16	2	2.5	16	0.5	10	2	wt	wt	wt	wt	L35R	wt	wt	
15326	S531L	1	0.5	16	1	2	8	4	5	16	4	2.5	4	S315T	wt	wt	wt	wt	wt	wt	
15327	H526D	2	0.5	16	0.5	2	16	64	5	16	4	5	0.5	wt	wt	wt	S458F	wt	wt	wt	

15328	S531L	1	0.25	4	0.5	0.5	0.25	4	40	16	0.12	2.5	4	wt	-8T>A	wt	wt	L172P	wt	wt	
15329	S531L	0.5	0.25	8	1	1	0.25	1	40	16	4	2.5	4	wt	-15C>T	wt	wt	wt	wt	wt	
15329	H526Y	0.5	0.25	4	0.5	1	0.12	0.5	40	16	4	2.5	4	wt	-15C>T	wt	wt	wt	wt	wt	
15330	S531L	1	0.25	4	0.25	0.5	0.12	1	10	16	0.25	2.5	1	wt	-8T>A	wt	wt	L172P	wt	wt	
15331	V276F	1	0.25	16	0.12	0.25	1	0.5	10	4	0.5	1.2	4	wt	wt	wt	wt	T153I	wt	wt	
15332	S531L	0.5	0.25	16	0.25	0.25	8	0.5	20	8	4	2.5	2	wt	-15C>T	wt	wt	wt	wt	wt	
15333	Wt	0.25	0.06	16	0.25	0.25	4	0.5	1.2	8	0.25	1.2	0.5	S315T	wt	wt	wt	wt	wt	wt	
15334	S531L	1	1	16	0.5	0.5	4	0.5	2.5	32	0.25	2.5	1	wt	wt	wt	wt	wt	wt	wt	
15335	H526D	2	1	16	0.5	1	16	0.5	1.2	8	2	2.5	1	S315T	wt	wt	wt	wt	wt	wt	
15336	S531L	4	1	16	4	1	0.12	0.5	10	64	4	1.2	2	S315T	-15C>T	A90V	wt	R154G	G878A	wt	
15337	S531L	1	0.25	16	0.5	0.5	16	0.5	20	16	4	5	8	S315T	-15C>T	wt	wt	L35R_del G pos417 (codon 139)	wt	S17N	
15338	S531L	0.25	0.12	16	0.25	0.25	4	0.5	0.6	2	0.25	1.2	1	wt	wt	wt	wt	wt	wt	wt	
15339	H526Y	0.5	0.12	16	0.25	0.25	16	0.5	1.2	16	0.03	2.5	1	wt	wt	wt	wt	wt	wt	wt	
15340	S531L	0.5	0.25	16	0.5	2	4	0.5	2.5	16	4	1.2	4	S315T	wt	wt	wt	wt	C491T	wt	
15343	S531L	32	8	16	0.5	32	16	1	2.5	16	4	2.5	4	S315T	wt	D94G	wt	C14R	A514C	wt	
15344	H526Y	0.5	0.25	16	0.25	0.25	0.12	1	40	16	0.5	2.5	1	wt	-15C>T	wt	wt	wt	wt	wt	
15345	S531L	1	0.25	16	0.5	32	4	1	1.2	8	4	1.2	8	S315T	wt	wt	wt	S104R	wt	K43R	
15345	S531L	1	0.25	16	0.25	32	4	0.5	2.5	8	4	1.2	8	S315T	wt	wt	wt	S104R	wt	K43R	
15346	S531L	1	0.25	8	0.25	1	2	0.5	2.5	8	4	1.2	8	S315T	-8T>A	wt	wt	V139G	wt	wt	
15347	Wt	0.5	0.25	0.12	0.25	0.25	0.12	0.5	2.5	16	0.25	1.2	1	wt	-15C>T	wt	wt	wt	wt	wt	
15348	S531L	1	0.5	16	0.5	32	16	0.5	2.5	16	4	5	4	S315T	wt	wt	wt	C14R	A514C	wt	
15349	S531L_Y414C	1	0.5	16	0.25	1	4	0.5	40	8	1	1.2	1	wt	-15C>T	wt	wt	wt	wt	wt	
15350	Wt	2	1	2	1	1	2	1	10	16	0.25	2.5	16	wt	-8T>A	wt	wt	L172P	wt	wt	
15351	S531L	0.5	0.25	16	0.25	0.25	0.5	0.5	5	16	1	1.2	8	wt	-15C>T	wt	wt	ins 5nt (CGCG	wt	wt	

																		C) pos79			
15356	H526Y	16	8	16	1	32	16	8	40	32	4	2.5	16	S315T	-15C>T	A90V	wt	H71Y	wt	wt	
15357	S531L	16	8	16	1	32	16	8	40	32	4	2.5	16	wt	-15C>T	D94G	wt	A102V	wt	K88R	
15360	S531L	16	8	16	1	32	16	8	40	32	4	2.5	16	wt	-15C>T	A90V	wt	wt	wt	wt	
15383	S531L	16	8	16	1	32	16	8	40	32	4	2.5	16	S315T	-15C>T	A90V	wt	T100I_T160A	A514C_A1401G	wt	
15403	D516V	1	0.5	16	0.25	4	0.12	0.5	5	32	4	1.2	16	S315T	wt	wt	wt	wt	A514T	wt	M306V
15405	D516V	1	0.12	16	0.5	2	0.12	0.5	5	16	4	1.2	16	S315T	wt	wt	wt	wt	A514T	wt	M306V
15406	S531L	0.5	0.25	16	0.25	32	8	0.5	2.5	16	4	2.5	8	S315T	wt	wt	wt	C14R	A514C	wt	
15407	H526Y	0.5	0.12	16	0.25	0.25	4	0.5	2.5	16	0.03	2.5	1	wt	wt	wt	wt	wt	wt	wt	wt
15408	H526Y	0.5	0.12	16	0.25	0.25	4	0.5	2.5	16	0.03	2.5	1	wt	wt	wt	wt	wt	wt	wt	wt
15409	Q513P	1	0.06	16	0.12	0.25	1	0.5	10	64	2	1.2	1	wt	wt	wt	wt	wt	wt	wt	
15410	H526Y	0.5	0.06	16	0.5	1	16	0.5	0.6	16	0.5	2.5	2	wt	wt	wt	wt	wt	wt	wt	wt
15411	S531L	0.5	0.25	16	0.5	0.5	2	0.5	0.6	16	0.03	2.5	1	wt	wt	wt	wt	wt	wt	wt	
15412	S531L	1	0.12	16	0.5	4	4	0.5	0.6	8	4	2.5	1	wt	wt	wt	wt	wt	A1013G	wt	
15413	S531L	8	4	16	0.5	32	8	1	40	32	4	1.2	4	S315T	wt	D94G	wt	C14R	A514C	wt	M306I
15415	D516V	0.5	0.12	16	0.12	0.25	0.5	0.5	2.5	16	0.06	1.2	1		wt	wt	wt	L151S	wt	K43R	M306V
15415	D516V	1	0.5	16	0.5	32	0.25	0.5	1.2	8	4	1.2	4		wt	wt	wt	L151S	wt	K43R	M306V
15423	H526Y	0.5	0.06	16	0.12	0.25	2	0.5	1.2	16	0.03	2.5	0.5	wt	wt	wt	wt	wt	wt	wt	wt
15424	S531L	0.5	0.12	16	0.25	32	1	0.5	5	16	1	1.2	1	V320L	-15C>T	wt	wt	Y103Stop(Hatherell et al.)	wt	K43R	wt
15427	S531L_F584S	16	4	16	0.25	0.25	2	0.5	10	32	1	2.5	4	wt	-15C>T	D94A	wt	del 9nt pos338-346	wt		M306V
15428	S531L	1	0.12	16	0.25	32	8	0.5	1.2	8	4	1.2	4	S315T	wt	wt	wt	S67W	wt	K43R	M306V
15485	S531L	16	8	16	1	32	16	8	40	32	4	2.5	16	wt	-15C>T	a90V	wt	A102V	wt	K88R	wt

15486	S531L	2	0.5	16	0.25	32	1	0.5	40	4	4	1.2	16	wt	-15C>T	wt	wt	A102V	wt	K88R	wt
15487	Wt	0.5	0.12	0.25	0.12	0.25	0.12	0.5	1.2	4	0.06	2.5	1	wt	wt	wt	wt	wt	wt	wt	wt
15488	S531L	1	0.25	8	0.5	0.5	0.12	0.5	2.5	16	4	2.5	2	S315T	wt	wt	wt	wt	wt	wt	wt
15489	L533P	0.25	0.12	4	0.5	0.25	0.5	0.5	1.2	8	4	2.5	4	S315N	wt	wt	wt	wt	wt	wt	wt
15490	Wt	0.5	0.12	16	0.25	8	4	0.5	40	8	1	1.2	8	wt	wt	wt	wt	wt	A514C	wt	wt
15492	S531L	16	4	16	0.5	32	16	32	40	64	4	2.5	8	wt	-15C>T	a90V	wt	A102V	wt	K88R	wt
15493	S531L	0.5	0.12	16	0.25	0.25	8	2	1.2	8	0.06	1.2	1	wt	wt	wt	wt	wt	wt	wt	wt
15494	S531L	1	0.25	4	1	0.5	0.12	0.5	40	16	2	2.5	1	wt	-15C>T	wt	wt	wt	wt	wt	wt
15496	S531L	0.5	0.12	16	0.25	32	1	0.5	40	8	4	1.2	16	S315T	wt	D94G	wt	C14R	A514C	wt	M306I
15497	S531L	0.5	0.12	16	0.12	0.5	1	0.5	20	16	4	1.2	8	S315T	-8T>A	wt	wt	V139G	wt	wt	M306V
15498	H526Y	1	0.25	8	0.25	0.25	0.12	0.5	40	32	1	2.5	1	wt	-17G>T	wt	wt	wt	wt	wt	wt
15499	S531L	2	0.25	16	0.25	32	16	0.5	40	4	4	2.5	16	wt	-15C>T	a90V	wt	A102V	wt	K88R	wt
15500	H526Y	8	4	16	0.25	1	2	16	40	32	4	2.5	32	S315T	-15C>T	D94G	wt	H71Y	wt	wt	wt
15502	D516G_L533P	8	1	16	16	2	4	1	40	16	4	40	16	S315T	-8T>A	A90V	wt	ins C pos457 (codon 153)	A1401G	wt	M306V
15504	S531L	1	0.25	16	0.25	0.25	1	0.5	40	8	4	1.2	4	wt	-15C>T	wt	wt	wt	wt	wt	wt
15505	S531L	0.5	0.12	16	0.25	0.25	0.5	0.5	5	16	0.25	1.2	8	wt	-15C>T	wt	wt	wt	wt	wt	M306V
15506	S531L_F584S	0.5	0.12	16	0.25	0.5	1	0.5	1.2	8	4	1.2	16	S315T	wt	wt	wt	del T pos515 (codon 172)	wt	wt	M306I
15507	S531L	1	0.12	16	0.25	1	1	0.5	1.2	8	4	1.2	4	S315T	wt	wt	wt	D12Y	wt	wt	wt
15788	H526Y	16	4	16	16	32	2	0.5	40	2	4	1.2	4	S315T	-15C>T	A90V	wt	H71Y	wt	wt	wt
15789	Wt	16	4	16	16	32	2	0.5	40	2	4	1.2	8	wt	-15C>T	D94G		wt	wt	wt	wt
16000	H526D	0.5	0.12	16	0.5	0.5	2	0.5	2.5	4	4	1.2	1	S315T	wt	wt		G97C	wt	wt	
16001	D516V	1	0.25	16	0.25	32	1	0.5	10	16	4	1.2	8	S315T	wt	wt			wt	K43R	
16002	H526Y	0.5	0.25	16	0.25	4	4	0.5	2.5	8	0.03	2.5	1	wt	wt	wt		wt	wt	wt	

16003	H526Y	8	1	16	0.25	0.25	0.12	0.5	20	8	4	0.6	1	S315T	wt	D94G		wt	wt	wt	
16004	S531L	0.5	0.12	8	0.5	1	0.12	0.5	1.2	8	4	1.2	0.5	S315T	wt	wt			wt	wt	
16029	H526Y	16	4	16	16	32	2	0.5	40	2	4	2.5	32	S315T	-15C>T	A90V	S458F	H71Y	wt	wt	E405D_A409P
16054	S531L	8	8	16	1	32	8	4	20	32	4	2.5	8	S315T		D94G	wt	C14R		wt	
16055	S531L	16	4	16	16	32	2	0.5	40	2	4	2.5	0.5	wt	-15C>T	D94G	wt	wt		wt	
16056	H526G	0.5	4	16	2	1	0.5	0.5	2.5	8	4	1.2	8	S315T	wt	wt	wt	wt		wt	
16057	insTTC (c507)_L511P	0.5	0.12	4	0.25	2	0.5	2	40	32	0.5	2.5	8	wt	wt	wt	wt	wt		wt	
16058	Wt	0.5	0.25	0.25	1	0.5	0.12	1	20	16	4	2.5	2	S315T	wt	wt	wt	wt		wt	
16059	S531L	16	4	16	16	32	2	0.5	40	2	4	2.5	4	S315T	-15C>T	A90V	wt	R154G		wt	
16063	S531L	1	1	16	2	32	16	1	2.5	32	4	2.5	16	S315T		wt	wt	C14R		wt	
16116	H526D	2	0.25	16	0.5	0.5	1	1	5	8	4	1.2	4	S315T	wt	wt	wt	G97C		wt	
16117	S531L	8	1	16	0.5	4	16	0.5	40	16	4	2.5	32	S315T	wt	D94G	wt	C14R		wt	
16118	S531L	1	0.12	16	0.25	0.25	8	0.5	2.5	8	4	1.2	1	wt	wt	wt	wt	wt		wt	
16119	D516V	8	2	16	0.5	32	0.12	64	5	16	4	1.2	4	S315T	wt	A90V	wt	L151S		K43R	
16122	H526D	1	0.12	16	0.25	2	4	0.5	2.5	8	4	2.5	0.5	S315T	wt	wt	wt	G97C		wt	
16125	H526D	0.5	0.06	16	0.25	0.25	2	0.5	1.2	16	0.03	1.2	0.5	wt	wt	wt	wt	wt		wt	
16126	S531L	16	4	16	16	32	2	0.5	40	2	4	40	4	S315T	wt	D94G	wt	T135P		K43R	
16127	H526Y	8	2	16	0.5	1	4	0.5	40	32	4	1.2	16	S315T	-15C>T	A90V	wt	H71Y		wt	
16128	S531L	1	0.12	16	0.5	0.5	1	0.5	20	8	4	0.6	8	S315T	-8T>A	wt	wt	V139G		wt	
16130	D516F	8	4	16	0.25	1	0.12	0.5	40	8	4	1.2	8	S315T	wt	A90V	wt	wt		wt	
16131	H526C	1	0.12	1	0.12	0.25	0.12	0.5	2.5	2	4	0.6	4	S315T	wt	wt	wt	del A pos64 (codon 22)		wt	

16133	H526Y	8	4	16	0.25	4	16	0.5	40	32	4	5	32	S315T	-15C>T	A90V	wt	H71Y		wt	
16134	S531L	0.5	0.25	16	0.12	0.5	1	0.5	0.6	4	0.06	0.6	2	wt	wt	wt	wt	wt		wt	
16135	S531L	8	4	16	0.25	1	0.12	0.5	40	8	4	5	2	S315T	wt	A90V	wt	S59P		K43R	
16136	H526D	8	2	16	0.25	1	4	0.5	40	16	4	1.2	4	S315T	wt	D94G	wt	G97C		wt	
16509	H526D	1	0.12	16	0.25	0.25	8	0.5	2.5	16	0.03	2.5	1	wt	wt	wt		wt	wt	wt	
16714	Wt	0.5	0.06	8	0.25	0.5	0.12	0.5	2.5	16	4	1.2	0.5	S315T	wt	wt				wt	
17473	S531L	8	4	16	0.25	1	0.12	0.5	40	8	4	2.5	0.5	S315T	-8T>A	A90V		V139G	wt	wt	
17475	Q513P	2	1	16	0.5	2	16	0.5	10	32	4	2.5	8	S315T	wt	wt		L182S	wt	wt	
17478	Wt	1	0.25	0.25	0.5	0.25	0.12	0.5	40	4	0.25	2.5	1	wt	-15C>T	wt		wt	wt	wt	
17481	S531L	8	4	16	0.25	1	0.12	0.5	40	8	4	2.5	1	S315T	wt	A90V		V155M	A911G	wt	
17485	L533P	0.5	0.12	16	0.5	0.5	1	0.5	2.5	8	0.03	1.2	0.5	wt	wt	wt		wt	wt	wt	
17486	S531L	1	0.12	8	0.5	0.5	0.12	0.5	10	16	0.5	2.5	0.5	wt	-15C>T	wt		wt	wt	wt	
17487	Q513L	1	0.25	4	0.5	0.25	0.12	0.5	40	8	0.25	2.5	1	S315T	wt	wt		wt	wt	wt	
17489	Wt	16	0.5	0.5	0.5	1	1	16	40	64	4	5	1	wt	-15C>T	A90V			wt	wt	
17491	Q513P	8	4	16	0.25	1	0.12	0.5	40	8	4	5	1	S315T	wt	D94G		L182S		wt	
17740	Q513P	2	2	16	0.5	1	16	1	5	32	4	1.2	4	S315T	wt	wt		L182S	wt	wt	
17744	S531L	2	0.5	8	0.5	4	0.12	8	5	32	4	10	2	S315T	wt	wt		wt	C491T	wt	
17745	Wt	0.5	0.25	16	1	0.5	16	64	20	32	4	10	2	wt	-15C>T	wt		wt	C491T		
18143	S531L	1	0.25	16	0.25	0.5	16	1	40	2	4	0.6	1	wt	wt	wt	wt	wt		wt	
18144	D516G_L533P	4	0.25	16	16	0.5	0.25	0.5	10	8	4	40	16	S315T	-8T>A	A90V	wt	ins C pos457 (codon 153)		wt	
18145	S531L	1	0.5	0.12	0.5	32	0.12	0.5	2.5	16	4	1.2	1	S315T	wt	wt	wt	wt		K43R	
18146	S531L	16	8	16	0.5	32	16	4	10	16	4	2.5	4	S315T	wt	D94G	wt	C14R		wt	
18147	S531L	1	0.12	16	0.25	1	1	2	2.5	4	4	1.2	2	S315T	wt	wt	wt	wt		wt	
18148	S531L	0.5	0.12	16	0.25	16	4	0.5	1.2	32	4	0.6	4	S315T		wt	wt			wt	
18149	S531L	2	0.25	0.2	1	1	0.12	8	20	32	0.5	5	1	S315T	wt	wt	wt			wt	

				5																
18154	S531L	0.5	0.25	16	0.5	32	8	0.5	1.2	16	4	1.2	16	S315T	wt	wt	wt	C14R		wt	
18156	S531L	1	0.12	16	0.5	1	16	0.5	40	16	4	1.2	4	S315T	-8T>A	wt	wt	V139G		wt	
18158	S531L	1	0.25	16	0.12	0.25	16	0.5	2.5	8	4	0.6	1	S315T	-8T>A	wt	wt	V139G		wt	
18160	S531L	1	0.25	16	0.25	0.5	2	0.5	2.5	16	4	1.2	8	S315T	wt	wt	wt	ins G pos315 (codon 105)		wt	
18161	S531L	0.5	0.12	16	0.5	0.25	1	0.5	2.5	8	0.25	1.2	4	wt	-15C>T	wt	wt	wt		wt	
18162	S531L	1	0.5	16	0.5	32	16	2	20	4	4	1.2	2	S315T	wt	wt	wt	C14R		wt	
18165	I572F	1	0.25	2	1	32	0.12	1	20	32	4	1.2	4	S315T	wt	wt	wt	H51D		K43R	
18166	S531L	8	4	16	0.25	1	0.12	0.5	40	8	4	2.5	1	S315T	wt	D94G	wt	wt		wt	
18168	S531L	1	0.5	16	0.25	1	2	0.5	2.5	16	4	1.2	8	S315T	wt	wt	wt	S59P		K43R	
18168	S531L	1	0.5	16	0.25	32	8	0.5	2.5	16	4	1.2	4	S315T	wt	wt	wt	S59P		K43R	
18169	S531L	2	0.25	16	4	1	2	64	2.5	16	4	5	8	wt	-15C>T	a90V	wt	A102V	wt	K88R	wt
18171	H526D	0.5	0.12	16	0.25	0.25	16	0.5	2.5	16	0.03	1.2	0.5	wt	wt	wt	wt	wt		wt	
18172	H526D	8	2	16	0.25	2	1	0.5	5	4	0.12	2.5	0.5	wt	wt	D94H	wt	wt		wt	
18173	G523E_H526Y	1	0.25	16	0.25	32	4	0.5	2.5	8	4	1.2	8	S315T	wt	wt	wt	Y99Stop(TAA)		K88R	
18174	H526Y	0.5	0.12	16	0.25	32	4	0.5	5	16	4	1.2	4	S315T	wt	wt	wt	wt		K43R	
18175	S531L	0.25	0.06	16	0.12	0.25	0.5	0.5	2.5	4	0.03	0.6	0.5	wt	wt	S91P	wt	wt		wt	
18177	H526Y	1	0.5	16	0.5	2	2	0.5	2.5	16	0.03	2.5	1	wt	wt	wt	wt	wt		wt	
18178	S531L	0.25	0.06	16	0.12	0.25	0.5	0.5	0.6	4	4	0.6	0.5	wt	wt	wt	wt	wt		wt	
18179	S531L	1	0.12	16	0.25	16	1	0.5	40	4	2	1.2	16	wt	wt	wt	wt	G97D		wt	
18180	H526C	0.5	0.25	1	0.25	1	0.12	0.5	1.2	16	4	2.5	2	S315T	Wt	wt	wt	R123P		wt	
18182	S531L	8	4	16	0.25	1	0.12	0.5	40	8	4	1.2	4	S315T	Wt	D94G	wt	Y34D		K43R	
18184	D516G_L533P	8	4	16	0.25	1	0.12	0.5	40	8	4	1.2	4	S315T	-8T>A	A90V	wt	ins C pos457 (codon 153)		wt	

18189	S531L	8	4	16	0.25	1	0.12	0.5	40	8	4	1.2	4	wt	-15C>T	D94G	wt	wt		wt	
18217	S531L	8	4	16	0.25	1	0.12	0.5	40	8	4	2.5	2	wt	-15C>T	D94G	wt	wt		wt	
18220	D516G_L533P	16	4	16	16	32	2	0.5	40	2	4	1.2	0.5	S315T	-8T>A	A90V	wt	ins C pos457 (codon 153)		wt	
18244	I572F	8	4	16	0.25	1	0.12	0.5	40	8	4	1.2	4	S315T	-15C>T	wt	wt	-11A>G		wt	
18249	H526L	16	4	16	16	32	2	0.5	40	2	4	0.6	0.5	wt	-15C>T	wt	wt	V7L		wt	
18251	S531L	16	4	16	16	32	2	0.5	40	2	4	0.6	0.5		-15C>T	wt	K537Q	A102V	wt	K88R	
18256	S531L	16	4	16	16	32	2	0.5	40	2	4	2.5	0.5	S315T	Wt	A90V+S91P	wt	E111Stop)		wt	
18257	D516V	8	4	16	0.25	1	0.12	0.5	40	8	4	1.2	8	S315T	Wt	A90V	wt	L151S		K43R	
18259	H526L	16	4	16	16	32	2	0.5	40	2	4	2.5	1		-9G>A	wt		wt		wt	
17474	S531L	16	4	16	16	32	2	0.5	40	2	4	1.2	16	wt	-15C>T	A90V		wt	wt	wt	

Table C3: Whole genome sequencing results

Labno	Gene	Amino Acid	Comments		
TRL0024410	gyrA	Glu21Gln , Ser95Thr , Gly668Asp	Naturally occurring mutations		
TRL0024410	gyrB	Pro94Leu	Pro94Leu: phylo		
TRL0024410	rpoB	wt			
TRL0036209	folC	wt			
TRL0036209	thyA	wt			
TRL0036209	ribD	wt			
TRL0036775	katG	wt			
TRL0036775	inhA promoter	wt			
TRL0037003	gyrA	Glu21Gln , Ser95Thr , Gly668Asp	Naturally occurring mutations		
TRL0037003	gyrB	wt			
TRL0037003	rpoB	Ser450Leu			
TRL0038177	katG	wt			
TRL0038177	inhA promoter	wt			
TRL0045713	katG	wt			
TRL0045713	inhA promoter	wt			
TRL0045902	rrs	wt			
TRL0045902	rpsL	wt			
TRL0045902	gidB	wt			
TRL0051498	katG	Tyr337Cys			
TRL0051498	inhA promoter	wt			
TRL0059723	katG	wt			
TRL0059723	inhA promoter	wt			
TRL0060095	rrs	wt			
TRL0060095	eis	wt			

TRL0060765	katG	wt			
TRL0060765	inhA promoter	wt			
TRL0063481	rrs	wt			
TRL0063481	rpsL	wt			
TRL0063481	gidB	wt			
TRL0095023	rrs	wt			
TRL0095023	rpsL	wt			
TRL0095023	gidB	Leu16Arg	Leu16Arg: phylo		
TRL0100824	katG	Arg463Leu , Trp191Gly	Arg463Leu: phylo; Trp191Arg on TBDream		
TRL0100824	inhA promoter	wt			
TRL0101577	folC	wt			
TRL0101577	thyA	wt			
TRL0101577	ribD	wt			
BF01029860	rpoB	wt			
BF01227167	gyrA	Glu21Gln , Ser95Thr , Gly668Asp	Naturally occurring mutations		
BF01227167	gyrB	wt			
BF01227167	rpoB	wt			
JG00256561	rrs	T492C			
KF00065920	gid	Leu16Arg	On TBDream		
KF00065920	rpsL	Lys88Arg			
KF00065920	rrs	T492C			
ND00124877	gyrA	Glu21Gln , Ser95Thr , Gln613Glu , Gly668Asp	Naturally occurring mutations		
ND00124877	gyrB	wt			
ND00124877	rpoB	wt			
OB00122369	inhA promoter	wt			
OB00122369	katG	wt			
OI00115811	gid	wt			

OI00115811	rpsL	Lys88Arg			
OI00115811	rrs	wt			
RD00095112	embA	wt			
RD00095112	embB	wt			
TRL0027406	gyrA	Glu21Gln , Ser95Thr , Gly668Asp	Naturally occurring mutations		
TRL0027406	gyrB	wt			
TRL0027406	rpoB	wt			
TRL0035923	gid	wt			
TRL0035923	gyrA	Glu21Gln	Naturally occurring mutations		
TRL0035923	gyrB	wt			
TRL0035923	rpsL	Lys43Arg			
TRL0035923	rrs	wt			
TRL0065056	eis	wt			
TRL0065056	gid	Leu16Arg	On TBDream		
TRL0065056	rpsL	wt			
TRL0065056	rrs	T492C			
TRL0066006	eis	wt			
TRL0066006	rrs	wt			
TRL0066693	eis	wt			
TRL0066693	rrs	C514A , G1401A			
TRL0073196	embA	wt			
TRL0073196	embB	Met306Ile			
TRL0091775	embA	wt			
TRL0091775	embB	wt			
TRL0101581	rpoB	Ser450Leu			
High-confidence mutation					

On TBDream					