Differences in the Susceptibility of *Mycobacterium tuberculosis* to the 1st and 2nd Line Antituberculosis Drugs under Aerobic and Anaerobic conditions

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Submitted in fulfilment of the requirements for the degree of Master of Medical Science (Medical Microbiology) in the Department of Medical Microbiology and Infection Prevention and Control, College of Health Sciences, University of KwaZulu-Natal

As the candidate's supervisor I agree to the submission of this dissertation.

Signed: _____Date:_____

Declaration

Plagiarism:

I, Zethembiso Brightness Ngcobo declare that:

(i) The work described in this dissertation has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification.

(ii) The laboratory work described in this dissertation was conducted by the candidate, where the work by others was used, it is stated and they are acknowledged and referenced,

(iii) All the written work is the candidates own work and words. Where other written sources have been quoted, then the author's words have been rewritten but the general information attributed to them has been referenced.

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Presentations from this Dissertation

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List of abbreviations

AIDS	Acquired Immunodefiency Syndrome
TB	Tuberculosis
ANTI-TB	Anti-tuberculosis
ARV	Antiretrovirals
CAP	Capreomycin
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DMF	Dimethylformamide
DST	Drug Susceptibility Testing
EDTA	Ethylenediaminetetraacetic Acid
EMB	Ethambutol
HIV	Human Immunodeficiency Virus
INH	Isoniazid
KAN	Kanamycin
LIN	Linezolid
MDR	Multi Drug Resistant
MIC	Minimum Inhibitory Concentration
MODS	Microscopic Observation Drug Susceptibility
MTT	(3-(4, 5-Dimethylthiazoli-2-yl) -2, 5- Diphenyltetrazolium Bromide)
NADH	Nicotinamide Adenine Dinucleotide
N/T	Not Tested
OADC	Oleic Acid Dextrose Complex
OFL	Ofloxacin
PAS	p-Aminosalicylic Acid
RIF	Rifampicin
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription – Polymerase Chain Reaction

SDS	Sodium Dodecyl Sulphate
SUS	Susceptible
STR	Streptomycin
TAE	Tris Base, Acetic Acid and EDTA
XDR	Extensively Drug Resistant
WHO	World Health Organisation

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Ethics

This study was approved by the Biomedical Research Council of the University of KwaZulu – Natal under ethics number **BCA274/09.**

Abstract

Although *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), is now considered a facultative anaerobe, and bacilli isolated from sputum specimen possess morphologies identified from bacilli growing aerobically and under oxygen deprived conditions, most of the targets for the antituberculosis drugs are readily found on bacilli that are thriving aerobically. This raises questions on the efficiency of antituberculosis drugs on eradicating the pathogen from the host during treatment. In this study to determine whether the antituberculosis drugs that are used currently for the treatment of TB have similar effect of these different populations of this mycobacterium, we grew this organism under aerobic and oxygen deprived environments and then subjected them to the antimicrobial agents. The minimum inhibitory concentration (MICs) of these isolates against nine antituberculosis drugs were determined under aerobic conditions for the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and under both aerobic and anaerobic conditions using the Microscopic Observation Drug Susceptibility (MODS) assays. In addition the bactericidal activities of isoniazid, rifampicin, kanamycin and ofloxacin were tested and compared amongst MDR isolates that were growing aerobically and anaerobically.

There were some differences in the MICs determined by the MTT assay and the MODS assay for some isolates. For the susceptible isolates the MICs from the MTT assay were higher than the MICs from the MODS assay. The reverse was true for the drug resistant isolates. The reference strain H37Rv was resistant to some of the antimicrobial agents that were tested in this study. This was under both methods. However, MICs measured under anaerobic conditions with anaerobic bacilli did not yield viable results due to absence of growth as the bacilli are known to

replicate at a negligible rate under anaerobic conditions. The bacilli in the inoculum were viable as following 40 days of anaerobic incubation but upon aerobic incubation of these cultures, growth was observed. And again with the bactericidal assays that were conducted on the multidrug resistant (MDR) isolates proved this. Rifampicin was the most potent antimicrobial agent against the anaerobic *M. tuberculosis* as susceptibility to this antimicrobial agent increased under anaerobic conditions.

Chapter 1: Introduction

Mycobacterium tuberculosis is a facultative intracellular bacterial pathogen (van Crevel *et al.*, 2002), that is associated with the world's number one infectious killer disease tuberculosis (TB) (Sherman *et al.*, 2001; Sasseti *et al.*, 2003 Rachman *et al.*, 2006). One third of the world's population is latently infected with TB and in 2014, 1.5 million deaths caused by this disease were reported (WHO, 2015).

Tuberculosis is transmitted through aerosols produced during coughing, sneezing and talking by symptomatic individuals infected with *M. tuberculosis*. The aerosolised organisms can infect any exposed individual independent of whether they are already infected or not. Following infection, one of three possibilities may occur. Firstly the infected individual develops active TB immediately. Such people are usually immunocompromised (Gumbo *et al*., 2004). The second option is that the immune system fights off the pathogen, totally eradicating it. This accounts for only 10 % of all infected individuals (Ahmad, 2011). The third possibility is that of latent infection, which occurs when an individual is infected but the bacilli are neither killed nor cause symptomatic disease. The bacilli are contained in the infected individual and can persist for decades with no symptoms (Kana *et al*., 2008). This last possibility is the one that is the most problematic as it is hard to eradicate latent *M. tuberculosis.* The large group of latently infected persons serves as a reservoir for symptomatic infections in the future. When conditions are favourable for the microbe, it can resuscitate and gain back its ability of causing symptomatic infection (Gumbo *et al*., 2004; Koul *et al*., 2008) that is contagious.

Although antimicrobials for the treatment of TB have been available since the middle of the 20th century, this disease still remains a major public health concern (Sasseti *et al.*, 2003). This

situation has worsened over the years as drug resistant strains have emerged. Although there are treatment protocols available for susceptible and resistant TB, which are applied globally (WHO, 2004), the number of drugs to which *M. tuberculosis* is resistant has increased over time and this is still continuing.

Treatment regimens for TB vary from 6 months for patients infected with a susceptible strain to 2 years for those infected with an extensively drug resistant (XDR) strain. Therefore there is still a need for more effective drugs that will also provide shorter duration of treatment (Wayne and Hayes, 1996; Ahmad *et al.*, 2006).The currently used anti-tuberculosis (anti-TB) drugs are not effective in eradicating latent *M. tuberculosis.* This is at least partly due to the lack of information on latent *M. tuberculosis* (Ashiru *et al.*, 2012). And also a number of studies have reported that in sputum specimens of individuals suffering from TB, a mixed population of bacilli thriving aerobically, microaerophilically and anaerobically are present in a specimen collected from each patient. This may mean that aerobic and anaerobic bacilli exist inside the same host (Fattorini *et al*., 2013). Clearly, for the eradication of TB, the treatment regimen must include antibiotics that are able to kill all three subpopulations that are inhabiting the host.

Our study aimed to grow *M. tuberculosis* clinical isolates of different drug susceptibilities to survive under anaerobiosis and thereafter determine their survival outcome after being exposed to antibiotics that are currently used for the treatment of TB. These results would then be compared to the outcomes with aerobically thriving bacilli. We also conducted a gene expression assay to validate that the bacilli that we were working with were indeed under anaerobiosis. Work that has been done on gene expression assays on *M. tuberculosis* have shown that some of this organisms' genes are up regulated under anaerobic conditions while others are down

regulated (Saxena *et al.*, 2008). More information on clinical isolates will be useful in the development of drugs that are active against both active and latent *M. tuberculosis*.

Aim:

To determine the minimum inhibitory concentration (MICs) of the 1st and 2nd line drugs for treatment of *Mycobacterium tuberculosis* under aerobic and anaerobic conditions.

Objectives:

1) To culture and grow *M. tuberculosis* anaerobically by means of standing broth cultures.
2) To confirm that 18 *M. tuberculosis* isolates that were cultured using the standing culture method, did indeed survive anaerobically using the gene expression method.
3) To determine MICs of the 1st and 2nd line drugs aerobically and anaerobically.
4) To compare differences between MICs of aerobically and anaerobically growing *M. tuberculosis* isolates (n=18).

Chapter 2: Literature Review

2.1 Pathogenesis

Tuberculosis is an airborne infectious disease that is caused by *Mycobacterium tuberculosis.* During transmission of this pathogen, it is inhaled and engulfed and ingested by dendritic cells, type II alveolar pneumocytes, adipocytes and by alveolar macrophages. This may then result in the infection of the host (Ashiru *et al.*, 2012). If infection occurs, macrophages are activated. The

activated macrophages then destroy most *M. tuberculosis* cells while others are able to survive. A series of biological events occurs which include the production of chemokines by the infected macrophages, which leads to the recruitment and migration of immune cells which later develop into matured macrophages, lymphocytes and neutrophils. The newly recruited and developed macrophages engulf the small number of surviving *M. tuberculosis*. These new macrophages internalize *M. tuberculosis* cells without destroying them (van Crevel *et al.*, 2002; Koul *et al.*, 2008).

As *M. tuberculosis* is inside the macrophage, the fusion of the phagosome with the lysosome and other regulatory activities are inhibited. This helps the microbe to survive inside the macrophage and also protects it from the immune system (Kaufmann and Britton, 2008). This is followed by exponential growth of *M. tuberculosis*, until the macrophages are activated by T-cell mediated immunity (van Crevel *et al.*, 2002). Upon this activation, which is accompanied by the emergence of unfavourable growth conditions, that include low pH; limited nutrition, toxic fatty acids and low oxygen levels which exist in the granulomas and necrotic tissues, *M. tuberculosis* stops replicating i.e. the bacilli enters a stationary phase (van Crevel *et al.*, 2002; Aly *et al.*, 2006; Koul *et al.*, 2008). The low oxygen level causes the bacilli to survive anaerobically (Wayne and Hayes, 1996). All of this is a gradual process.

Growth of the bacilli is arrested when the host is immunocompetent; this means that the host will be latently infected with TB until they become immunocompromised. If the latently infected individual doesn't become immunocompromised they will harbour the bacilli for decades, without any symptoms or spreading the disease (Wayne and Hayes, 1996). In human beings immunosuppression leads to conditions inside the host that permit persistence of replicating *M. tuberculosis*. With the HIV pandemic increasing the number of immunocompromised individuals

(Wang *et al*., 2011) as well as with one third of the world's population being latently infected with *M. tuberculosis* (Kaufmann and Britton, 2008), the number of patients in which resuscitation of oxygen deprived *M. tuberculosis* occurs is increasing and has led to TB being the number one killer infectious disease in the world (Gumbo *et al.*, 2004; Koul *et al.*, 2008).

Under anaerobiosis, *M. tuberculosis* undergoes several physiological changes during which they transform from vegetative state that metabolises and multiplies under aerobic conditions to non-replicating bacilli which persist under low or oxygen depleted environment (Papadopoulou *et al.*, 2007; Parrish *et al.*, 2009). The latter together with low redox potential is known to be a characteristic of the granuloma. Hence, *M. tuberculosis* has been classified as a facultative anaerobe (Ramcharandra and Sturm, 2010; Ashiru *et al.*, 2012). The bacilli that is surviving under anaerobic conditions (in the granuloma or necrotic tissue), then shits to a different pathophysiological pathway. The bacillis' overall behaviour changes, including its virulence and macrophage invasion capacity, which are higher than of the bacilli thriving aerobically (Ashiru *et al.*, 2012). These behavioural and physiological changes aid the bacilli in becoming resistant or tolerant to the macrophages' bactericidal mechanisms (Wayne and Hayes, 1996; Aly *et al.*, 2006; Deb *et al*., 2009).

2.2 Anti-tuberculosis Therapy

The TB pandemic continues to be a conundrum despite the existence of the pathogen for centuries. The causative agent of TB is an obligate pathogen (Warner and Mizrahi, 2006). Even after more than 40 years of usage of vaccines and antibiotics against this disease (Böttger, 2011; Keshavjee and Farmer, 2012), TB continues to be one of the biggest killer infectious diseases globally (WHO, 2015).

2.2.1 Association of tuberculosis with Human Immunodeficiency Virus

Studies have shown that the TB pandemic is enhanced by the HIV epidemic and limited availability of effective TB treatment (Ducati *et al*., 2006; Keshavjee and Farmer, 2012). The HIV-TB co-infection is a lethal association as the two diseases have a mutualistic relationship as they enhance each other's progress in the infected individual (Razafimahefa *et al*., 2005; Schlipköter and Flahault, 2010). It has been witnessed that HIV infected individuals have higher chances of acquiring TB (Asif, 2013). This maybe because HIV compromises the activity of the immune system of infected individuals (Ducati *et al*., 2006). The compromised immune system is a favourable, conducive environment for acquiring new infections as well as for resuscitation of the latent bacilli into a vegetative form which then causes active disease (Kana *et al.*, 2008). It has also been noted that infection from *M. tuberculosis* can accelerate progression of HIV infection to AIDS (Ahmad, 2011), hence TB is the number one killer infectious disease in individuals infected with HIV (Schlipköter and Flahault, 2010).

In the mid 1980s, an increase in the number of deaths caused by TB occurred mainly because of AIDS, poverty and inadequate medical care. This has continued over the years, despite the discovery and use of vaccines and various antibiotics over the past decades (Ducati *et al*., 2006).

2.2.2 Tuberculosis therapy and drug resistance

Another factor in fueling the TB pandemic has been the emergence of drug resistant strains. Drug resistant strains are believed to have developed as the result of failure to implement proper TB control programmes, poor management of TB cases and the long treatment duration (Hu *et al.*, 1998; Parrish *et al*., 2009; Calver *et al.*, 2010; Schlipköter and Flahault, 2010). The emergence of *M. tuberculosis* strains that are drug resistant and the pathogens success in being the dominating pathogen globally has necessitated an increased intensity in the search for new anti-TB drugs (McDonough *et al.*, 1993; Ducati *et al*., 2006), as the currently used drugs are fast becoming irrelevant as this organism has become tolerant and resistant to them. Such strains are the multidrug resistant (MDR) and the XDR) strains. MDR strains are resistant to at least rifampicin (RIF) and isoniazid (INH) while XDR strains are resistant to RIF, INH and fluoroquinolones and at least one second-line injectable drug (WHO, 2015).

2.2.3 Current tuberculosis chemotherapy

The current standard TB chemotherapy is a minimum of six months for infections with susceptible strains, while it is up to 20 months for infections with the MDR strains (WHO, 2015). During this period multidrug therapy is applied to suppress emergence of drug resistance (Zhao and Drlica, 2001), to treat extra-and intra-cellular bacteria in the actively metabolising and in the semi-dormant state and to shorten the treatment duration (Wayne and Hayes, 1996; Böttger, 2011; Drusano *et al.*, 2011).This method is favoured over monotherapy which gives rise to resistant strains (Keshavjee and Farmer, 2012). The primary advantage of treatment with multiple drugs is that if an individual is infected with two or more strains that have different drug

susceptibilities, chances are that these strains will be susceptible to two or more of the applied combination therapy drugs, in that way no strain will survive. This leaves little chance for survival of the mutant strains, hence no emergence of drug resistance.

First line treatment of TB is divided into the intensive and the continuation phase. In the intensive phase four antibiotics are administered and are thought to kill the majority of the vegetative bacilli responsible for the clinical manifestation of the disease. In the continuation phase, two antibiotics are employed to sterilize the tissue by eliminating the minimally metabolically active bacilli (Ducati *et al*., 2006). A minimum of six months treatment duration is necessary to totally eradicate the disease in the infected individual (Böttger, 2011), infected with a susceptible strain. If the patient is not compliant or doesn't complete the treatment this may lead to selection of drug resistant mutants and treatment failure or relapse of active disease (Rustad *et al.*, 2008). Drugs such as fluoroquinolones, macrolides, aminoglycosides and various other second line drugs are in use for treatment of MDR and latent *M. tuberculosis* infections, but these are less effective, more toxic and more expensive than most first line drugs (Ducati *et al.*, 2006; Özkütük *et al.*, 2008). There has been success with the above mentioned drugs, but drug resistance has emerged to these drugs as well (García-García *et al*., 2004). Despite the success of combination chemotherapy and new drugs on the market, development of drug resistance continues to be a problem as more drug resistant strains continue to emerge (Keshavjee and Farmer, 2012). This may be due to the fact that the currently used drugs have little effect on anaerobic bacilli; even the combination of the two most potent anti-TB drugs, i.e. INH and RIF have minimal effect (Drusano *et al*., 2011).

2.2.4 Failure of the current anti-tuberculosis chemotherapy

Emergence of drug resistance is not only caused by ineffectiveness of some of the drugs used, but also by failure of patients to comply with treatment which in turn is caused by the long treatment duration (Joshi, 2011). The lack of information on metabolism of *M. tuberculosis* especially its metabolism under anaerobic conditions has made this pathogen hard to eradicate. Targets of most of the currently used drugs are readily found on vegetative *M. tuberculosis* and therefore they may have minimal effect on bacilli that's surviving under anaerobiosis as *M. tuberculosis* surviving anaerobically lacks the usual drug targets (Marttila *et al.*, 1999; Miki *et al.*, 2001; Ramcharandra and Sturm, 2010).

During latency it is known that bacteria enter a state of no or minimal metabolic activity (Ducati *et al*., 2006) as a result of being inside the hosts' bactericidal macrophages (Zhang and Yew, 2009). This is a problem as *M. tuberculosis* does not only do this but it also enters a state of anaerobiosis once it is inside the hosts' body if the hosts' immune system is competent. In a study done by Garton *et al*., (2002), they discovered that bacilli isolated from sputum specimens were phenotypically different from bacilli that are cultured in the laboratory. They hypothesised that this may be due to the different environmental conditions that these two bacilli populations are thriving under. The structures described by Garton *et al*., in 2002 as lipid bodies on the bacilli seen in sputum specimens have been observed in bacilli that are surviving under anaerobiosis in macrophages (Peyron *et al*., 2008). The phenotypic similarity of the different bacilli populations present in the sputum samples and the bacilli grown in the laboratory at different oxygen gradient concentrations (Rustad *et al.*, 2008), emphasising the need for more drugs that show dual activity against both aerobic and anaerobic bacilli.

The lack of knowledge on *M. tuberculosis*' anaerobic pathophysiology has hampered the development of more drugs that are active against anaerobic bacilli. Most of the pathogenesis studies and the development of new anti-TB drugs are conducted using the aerobically grown cultures. These do not give a complete indication about *M. tuberculosis*' adaptation ability and characteristics, hence scientific advancement in eradicating TB is slow (Rachman *et al.*, 2006; Ashiru *et al.*, 2012).

2.2.5 The Current anti-tuberculosis drugs

2.2.5.1 Isoniazid

Isoniazid (INH) is one of the first line drugs used for the treatment of TB (Özkütük *et al.*, 2008) and is one of the two drugs considered to be the most powerful anti-TB drugs (WHO, 2015) and the backbone of anti-TB chemotherapy (Ducati *et al*., 2006). It is also one of the most affordable anti-TB drugs (Kelley *et al*., 1997). Isoniazid was first used for the treatment of TB in 1951 (Böttger, 2011; Keshavjee and Farmer, 2012).

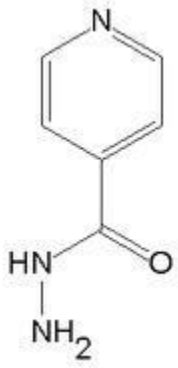

Figure 2.2.5.1: Structure of Isoniazid

This nicotinamide analogue (Figure 2.2.5.1) is bactericidal and is mostly effective against vegetative bacilli (Ducati *et al*., 2006; Kolyva and Karakousis, 2012). Isoniazid targets mycolic acids biosynthesis (Hazbo´n *et al.*, 2006) and nucleic acids biosynthesis (Timmins and Deretic, 2006) by being activated by the KatG enzyme (catalase-peroxidase) produced by *M. tuberculosis* (Ducati *et al.*, 2006).It is a pro-drug that is activated through oxidation in the intra-cellular environment of the bacilli. The resulting metabolites then inhibit the bacilli's mycolipid synthesis which is essential for mycobacterial survival (Kolyva and Karakousis, 2012).

Although INH has proven to be the most effective drug for the treatment of TB, it has also become the most affected drug in terms of drug resistance development (Kolyva and Karakousis, 2012). The development of MDR and XDR strains as well as dormant bacilli are proof of this (Zhang and Yew, 2009; Filippini *et al*., 2010) as the latter are harder to eradicate due to their reduced susceptibility to the routinely used anti-TB drugs such as RIF and INH (Wayne and Hayes, 1996).

Resistance to INH is a result of mutations in either *katG*, *inhA*, *ahpC, kasA, ndh* and/or *oxyR* genes (Silva *et al*., 2003; Ducati *et al.*, 2006; Hazbo´n *et al*., 2006; Kolyva and Karakousis, 2012), with the *katG* mutations being most frequent (Silva *et al*., 2003). The *katG*, *inhA, ndh* and *ahpC* genes encode enzymes, namely catalase peroxidise, putative mycolic acid synthesis enzyme, NADH dehydrogenase and alkyl hydroxiperoxidase respectively (Silva *et al.*, 2003; Kolyva and Karakousis, 2012). Mutations in the *inhA* gene interrupt the synthesis of essential mycolic acids (Hazbo´n *et al*., 2006), while mutations in the *ahpC* gene cause an over expression of this gene (Kelley *et al.*, 1997). A quarter of the clinical strains that have been confirmed as INH resistant, do not have mutations in any of the above mentioned known genes. This suggests

other mechanisms that may be responsible for this trait in these strains (Kolyva and Karakousis, 2012).

2.2.5.2 Rifampicin

Rifampicin (RIF) (Figure 2.2.5.2) is a broad spectrum bactericidal antibiotic (Campbell et al., 2001) and its spectrum includes *M. tuberculosis* (Campbell *et al*., 2001; Louw *et al*., 2011). This anti-TB drug belongs to the rifamycin group (Kolyva and Karakousis, 2012). Antibiotics from the rifamycin group have a lipophilic profile. This trait makes the diffusion of RIF across the membrane of the *M. tuberculosis* cell to be easy (Kolyva and Karakousis, 2012).

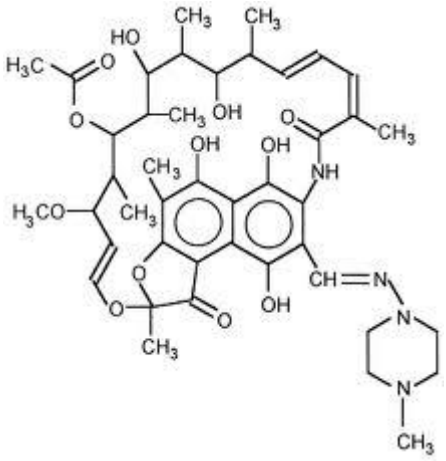

Figure 2.2.5.2: Structure of Rifampicin

After penetrating the mycobacterial cell envelope, the drug binds to DNA-dependant RNA polymerase, forming a drug-enzyme complex thereby inhibiting transcription of DNA to RNA hence protein synthesis is inhibited (Miki *et al.*, 2001; Gumbo, 2010; Kolyva and Karakousis, 2012; Asif, 2013). Because of these characteristics RIF has become one of the best drugs for the

treatment of TB and forms with INH the backbone for successful TB control programmes (Jayaram *et al*., 2003; Keshvjee and Farmer, 2012). RIF was first used as anti-TB drug in 1957 (Keshvjee and Farmer, 2012). Its addition to the treatment regimen of TB shortened the treatment duration from 18 months to 9 months (Joshi, 2011; Kolyva and Karakousis, 2012). This concentration dependant drug is the most effective drug when it comes to killing anaerobic bacilli (Jayaram *et al*., 2003; Joshi, 2011). Although RIF has become one of the major drugs for the treatment of TB, its interaction with ARV drugs when used in HIV-TB co-infected individuals has limited the usage of this drug, as its concentration drops to sub-therapeutic levels when used together with ARVs (Diaz *et al*., 2003; Asif, 2013).

Mono-resistance to RIF is not common, as most (90%) of the RIF resistant strains are also resistant to INH (Kolyva and Karakousis, 2012). Resistance to this drug occurs when there are mutations in the *rpoB* gene which encodes for the β subunit of the RNA polymerase (Campbell *et al*., 2001; Hwang *et al*., 2003; Louw *et al*., 2011; Kolyva and Karakousis, 2012). Mutations in the *rpoB* gene are the only known mechanism of resistance to this drug, as less than 5% of the known RIF's resistant strains do not have mutations in the *rpoB* gene (Campbell *et al*., 2001; Kolyva and Karakousis, 2012).

2.2.5.3 Ethambutol

Ethambutol (EMB) (Figure 2.2.5.3) is a narrow spectrum (Chopra and Brennan, 1998; Jadaun *et al*., 2007) bacteriostatic anti-TB drug (Faugeraux *et al*., 2007; Salgado-Moran *et al*., 2013) and is one of the four drugs currently used for first line treatment of TB (WHO, 2015). This drug that has poor sterilizing activity, was first reported to have anti-TB activity in 1961 (Yendally and

Lee, 2008; Kolyva and Karakousis, 2012). Due to its moderate activity against the bacilli, it is used in combination with other anti-TB agents to supplement its activity (Yendapally and Lee, 2008). It inhibits the synthesis of the mycobacterial cell wall by binding to the arabinosyltransferases, the enzymes that are responsible for the synthesis of arabinogalactan and lipoarabinomannan. These two compounds are essential components of the cell wall (Faugeroux *et al*., 2007; Salgado-Moran *et al*., 2013).

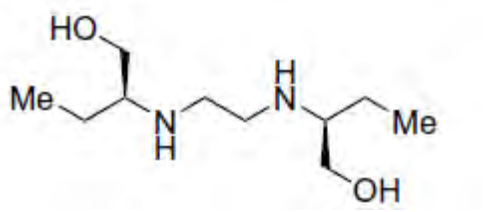

Figure 2.2.5.3: Structure of Ethambutol

Resistance to EMB is caused by point mutations in the *emb*CAB operon, specifically in the EmbB subunit of the arabinosyltransferase (Kolyva and Karakousis, 2012; Salgado-Moran *et al*., 2013). Once this mutation has occurred the activity of arabinosyltransferases is reduced, eventually leading to the mycobacterial cell wall being compromised (Salgado-Moran *et al*., 2013).

2.2.5.4 Aminoglycosides

Aminoglycosides normally act in synergy with other antibiotics (Kotra *et al*., 2000), and bind to the 30S ribosomal subunit, which affects polypeptide synthesis resulting in the inhibition of translation during protein synthesis (Honroé and Cole, 1994; Chopra and Brennan, 1998; Zhang and Yew, 2009; Kolyva and Karakousis, 2012). Over the years there have been a number of reports on resistance to aminoglycosides (Kotra *et al*., 2000).

Kanamycin (KAN) is an aminoglycoside that is used for the treatment of MDR-TB. This antibiotic inhibits protein synthesis. Resistance to KAN (Figure 2.2.5.4.1) is associated with mutations in the 1400 region of the *rrs* gene (Alangaden *et al*., 1998; Zhang and Yew, 2009) and mutations in the *eis* promoter region. Mutations in the *eis* promoter region cause an overexpression of aminoglycoside acetyltransferase which inactivates kanamycin (Zaunbrecher *et al*., 2009). There is usually cross-resistance between this drug and amikacin which is also an aminoglycoside. This cross-resistance is more likely to be caused by mutations in the *rrs* gene rather than mutations in the *eis* promoter region as the aminoglycoside acetyltransferase enzyme inactivates KAN but not amikacin (Zaunbrecher *et al*., 2009). There have been strains that are resistant to these two antibiotics but did not have any mutations in the above mentioned gene (*rrs* gene); this hints that there may be other mutation/s or mechanisms that account for resistance to either of the two drugs (Alangaden *et al*., 1998).

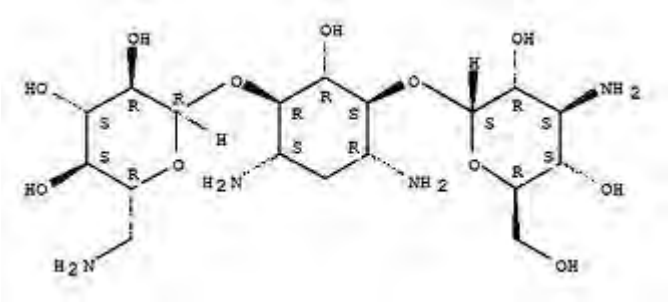

Figure 2.2.5.4.1: Structure of Kanamycin

Streptomycin (STR) (Figure 2.2.5.4.2), a broad spectrum antibiotic (Chopra and Brennan, 1998) was the first antibiotic to be used for TB chemotherapy (Honroé and Cole, 1994; Kolyva and Karakousis, 2012). It was discovered in the 1940s, with the first patient to use it in 1944 (Keshavjee and Farmer, 2012).

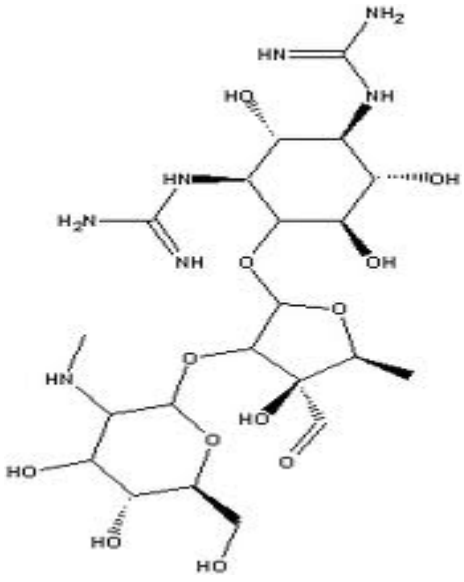

Figure 2.2.5.4.2: Structure of Streptomycin

M. tuberculosis strains with mutations in the *rps*L, *rrs*, and *gid*B genes encoding the S12 protein, 16S rRNA and 7-methyl-guanosine methyltransferase respectively have been found to be resistant to STR (Honroé and Cole, 1994; Meier *et al*., 1994; Chopra and Brennan, 1998; Zhang and Yew, 2009). Efflux pumps have also been cited as the other mechanism contributing resistance to this antibiotic (Zhang and Yew, 2009).

2.2.5.5 Linezolid

Linezolid (LIN) (Figure 2.2.5.5) is a synthetic drug that belongs to the class of antibiotics called oxazolidinones (Vardakas *et al*., 2009; Long and Vester, 2012). It was the first drug from this group of antibiotics to be approved for clinical use (Kolyva and Karakousis, 2012). It is one of the latest additions in the market of antibiotics as it was first introduced in year 2000 (Long and Vester, 2012). It is used for treatment of serious infections that are caused by gram positive bacteria. It also has good activity against *M. tuberculosis* (Diaz *et al*., 2003; Long and Vester, 2012).

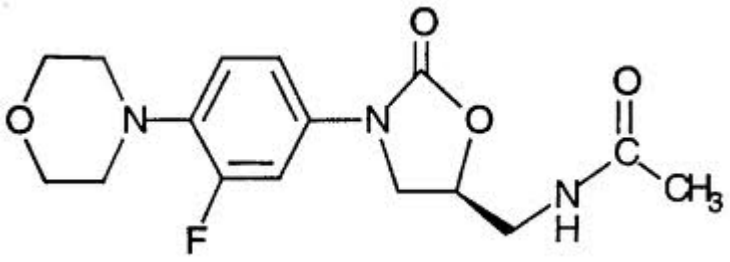

Figure 2.2.5.5: Structure of Linezolid

It inhibits protein synthesis by binding to the bacterial 23S rRNA of the 50S subunit during the formation of the 70S initiation complex (Vardakas *et al*., 2009; Long and Vester, 2012; Kolyva and Karakousis, 2012). Its use is limited due to its toxic effects; hence it is only used for a limited period of time in treatment regimens or resistant TB (Kolyva and Karakousis, 2012). Its use was first reported in 2003 by von der Lippe *et al*., (2006). So far, resistance to this drug in *M. tuberculosis* is rare although it has been observed with other pathogens (Vardakas *et al*., 2009).

2.2.5.6 p-aminosalicylic acid

The discovery of *p*-aminosalicylic acid (PAS) (Figure 2.2.5.6) was as early as the 1800s, and its antimycobacterial activity was recognized in 1946 (Chopra and Brennan, 1998). It was first prescribed for treatment of TB in 1948 (Kolyva and Karakousis, 2012). Despite the early discovery, its mechanism of action is still not well understood, but it is thought to inhibit the biosynthesis of folic acids and the uptake of iron (Chopra and Brennan, 1998; Kolyva and Karakousis, 2012).

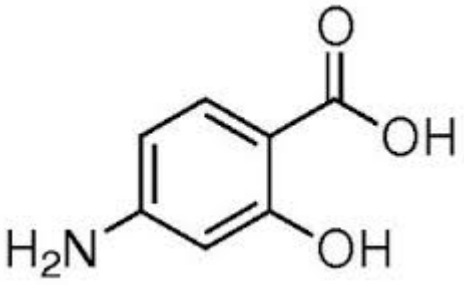

Figure 2.2.5.6: Structure of *p*-aminosalylic acid

Mutations in the *thy*A gene which encodes for an enzyme involved in the folate biosynthesis pathway have been identified in a third of *M. tuberculosis* strains that are resistant to this antibiotic (Kolyva and Karakousis, 2012).

2.2.5.7 Ofloxacin

Ofloxacin (OFL) (Figure 2.2.5.7) is a broad spectrum bactericidal antibiotic that belongs to a group of antibacterial agents called the fluoroquinolones (Chopra and Brennan, 1998; Kolyva and Karakousis, 2012). Ofloxacin is a synthetic derivative of nalidixic acid (Chopra and Brennan; 1998) and is used as part of the second line treatment regimen for TB andrecommended by WHO for the treatment of MDR (Shandil *et al*., 2007). It kills bacteria by inhibiting the activities of DNA gyrase and DNA topoisomerase IV, thereby preventing DNA replication and its resolution and transcription (Chopra and Brennan, 1998; Piddock, 1998; Zhang and Yew, 2009). Since *M. tuberculosis* doesn't have DNA topoisomerase IV, DNA gyrase is ofloxacin's only target in this organism (Kolyva and Karakousis, 2012).

Figure 2.2.5.7: Structure of Ofloxacin

M. tuberculosis strains that have mutations in *gyrA* and *gyrB* which encode the A and B subunits of the DNA gyrase are resistant to this drug (Zhang and Yew, 2009; Kolyva and Karakousis, 2012). There have been strains that are reported to be resistant to OFL, but do not have mutations in these two genes. This observation suggests that there are other genes or mechanisms that may be responsible for resistance to this drug that are yet to be elucidated (Kolyva and Karakousis, 2012).

2.2.5.8 Capreomycin

Capreomycin (CAP) (Figure 2.2.5.8) is a macrocyclic polypeptide that is isolated from *Streptomyces capredus* and is used in the second line regimen of treatment of TB (Zhang and Yew, 2009; Kolyva and Karakousis, 2012; Asif, 2013). Its spectrum is restricted to species of the genus *Mycobacterium* (Chopra and Brennan, 1998; Kolyva and Karakousis, 2012).

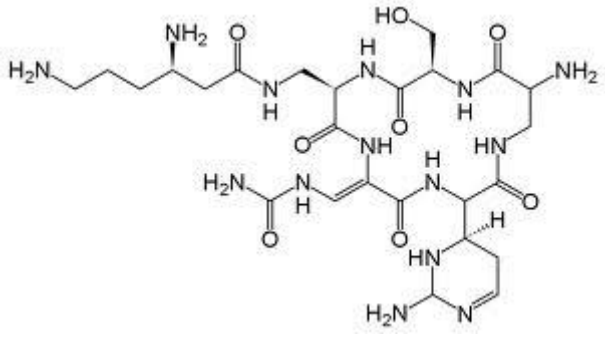

Figure 2.2.5.8: Structure of Capreomycin

Its mode of action lies with its ability to inhibit protein synthesis (Kolyva and Karakousis, 2012). A similar mode of action is exhibited by viomycin which is structurally related to CAP and also shares co-resistance with this anti-tuberculosis agent (Chopra and Brennan, 1998). Strains

resistant to CAP have been found to have mutations in the *tly*A gene which encodes rRNA methyltransferase (Zhang and Yew, 2009) and the *rrs* gene which encodes 16S rRNA (Kolyva and Karakousis, 2012). It shares this with the aminoglycosides and therefore co-resistance with KAN and AMK is often found.

2.2.6 The 'Ideal' anti-tuberculosis drug

From the description of the above mentioned drugs it is clear that each of the anti-TB drugs has its specific mechanism for fighting the invading *M. tuberculosis* (Marttila *et al.*, 1999; Miki *et al.*, 2001) and that most of the currently used drugs have bactericidal activity mostly against replicating bacteria, which in turn makes them more effective in killing actively growing bacilli, while latent bacilli are tolerant to these drugs (Zhang *et al*., 2012). This, together with the growing emergence of strains resistant to a multitude of drugs enforces the growing need for new drugs that are active against both actively growing and latent bacilli (Koul *et al*., 2008).

According to Koul *et al.*, (2011), an ideal drug for the treatment of TB needs to be able to shorten the treatment duration, be active against drug resistant strains, and be taken less frequently and together with medications for other diseases without complications. None of the drugs that are currently used as part of treatment of TB fit the above mentioned criterion completely. Since most antimicrobial drugs interfere with metabolic pathways of the microbes, these drugs act on metabolically active bacilli and their activity is likely to be related to the level of metabolic activity. This is problematic as *M. tuberculosis* is known to enter into a phase which is non-replicating and shows minimal metabolic activity e.g. following macrophage invasion as this is its survival strategy against the hosts' immune response. Although drugs such as RIF,

CAP and some other anti-TB drugs have shown some activity against dormant *M. tuberculosis* (Filippini *et al*., 2010), their activity is still not satisfactory.

Studies have shown that the emergence of resistant strains is a result of the long treatment duration. The long treatment duration in turn is caused by the presence of dormant *M. tuberculosis* that take long to be eradicated as these bacilli are not killed rapidly enough during short treatment (Hu *et al.*, 1998; Rustad *et al.*, 2008; Parrish *et al*., 2009). During the long treatment duration it has been observed that cases of inadequate supply and taking of anti-TB drugs give the resistant mutants the opportunity to develop, thereby giving rise to drug resistant strains (Zhang and Yew, 2009; Singh *et al*., 2011). Dormant *M. tuberculosis* is able to return to active growth and cause active TB (Deb *et al*., 2009). This necessitates development of drugs that are active against the actively growing and the slow metabolising, dormant *M. tuberculosis*. The general assumption is that mutation will hardly or not at all occur during dormancy since such events occur only when replication takes place. However, recent evidence suggests otherwise. Ford *et al*., (2011) showed that in the lungs of experimentally infected macacques, the mutation frequency was higher in animals with latent infection compared to active infection. In a not yet published study from our department it was shown that the mutation frequency in F15/LAM4/KZN and Beijing isolates was higher under anaerobic incubation as compared to aerobic incubation (Joseph and Sturm, in preparation).

2.3 Anaerobiosis

Anaerobiosis is a biological metabolic pathway that a microbe shifts to in order to survive under anaerobic conditions (Drusano *et al*., 2010). This ability is reserved or seen in obligate and

facultative anaerobes. For facultative anaerobic microorganisms this process is usually accompanied by several morphological and physiological changes on the affected organism. These changes help the organism to adapt and survive in environmental conditions that are detrimental to aerobic organisms that do not have an ability of metabolizing anaerobically. *Mycobacterium tuberculosis* as a facultative anaerobe is no different in this behaviour. When *M. tuberculosis* is exposed to an anaerobic environment, it alters its metabolism to anaerobiosis, rather than sporulating as observed in some other bacteria (Drusano *et al*., 2010, Ramcharandra and Sturm, 2010).

2.3.1 Environmental conditions inside the granuloma

During pathogenesis as *M. tuberculosis* is engulfed by macrophages or is inside the granuloma it survives anaerobically. Under anaerobiosis, metabolism of *M. tuberculosis* is minimal, in such a way that it is assumed that it does not replicate or if it does, it replicates at a negligible rate (Parrish *et al.*, 2009). The characteristic conditions of the granuloma include low redox potential, acidic environment, minimal oxygen concentration and minimal nutrients amongst others (Ahmad, 2010). These conditions have been mimicked *in vitro* by a number of methods. The Wayne model is one of the most widely used ones. The conditions in the granuloma that are thought to be beneficial for the successful survival of the *M. tuberculosis* cells inside the host are not conducive for the normal aerobic metabolism and growth of the bacterium (Nickel *et al*., 2012). *Mycobacterium tuberculosis* therefore has to adapt to ensure survival in such an environment; it therefore enters a state of anaerobiosis, during which it stops replicating while

remaining viable, changing its gene expression pattern resulting in a different phenotype (Daniel *et al.*, 2011).

Although all the above mentioned conditions are responsible for the bacilli to enter the state of anaerobiosis inside the granuloma, oxygen limitation is the one characteristic that is thought to contribute the most to this metabolic change as the availability of nutrients is not limited during infection (Wayne and Lin, 1982). Upon availability of oxygen the dormant bacilli reverts back to thriving aerobically and multiplying (Voskuil *et al*., 2004; Nickel *et al*., 2012). The changes that are brought about by the harsh environmental conditions aid the bacterium to be tolerant to antibiotics that target aerobic biological pathways as these are either absent or minimal during anaerobiosis (Watanabe *et al*., 2011). This has been proven as much higher concentrations of anti-TB drugs are needed to eradicate latent bacilli than the concentrations used to eradicate vegetative bacilli, while some anti-TB drugs have shown better activity against latent bacilli than they have against multiplying bacilli (Filippini *et al*., 2010).

2.3.2 The *in vitro* survival of *Mycobacterium tuberculosis* under anaerobiosis

Achieving anaerobiosis *in vitro* by growing *M. tuberculosis* in liquid media in sealed tubes for certain duration is one of the established methods. There are several methods that are used to achieve this with *M. tuberculosis*, which include the Wayne model. In the Wayne model *M. tuberculosis* is inoculated in liquid media in tubes. The tubes are then sealed tightly to inhibit the introduction of oxygen into the tube. The tube is then incubated without agitation. During incubation access to oxygen is only limited to the oxygen which is already inside the tube, and over time as the oxygen and nutrients are utilized by the bacilli, these get depleted and the

environment in the tube becomes anaerobic and with limited nutrients (Rustad *et al*., 2008; Filippini *et al*., 2010; Watanabe *et al.*, 2011). An accumulation of waste and acidification of media have also been observed (Rustad *et al*., 2008; Watanabe *et al.*, 2011). As soon as the available oxygen becomes limited, the bacilli adopt low metabolic activity, with slow or no replication at all (Voskuil *et al*., 2004; Khan and Sarkar, 2006; Nickel *et al*., 2012). The bacilli then become dormant, triggering bacilli at the bottom of the tube to change their metabolism in order to survive under anaerobiosis (Wayne and Hayes, 1996).

Once the bacilli are anaerobic, energy generation, RNA and protein synthesis decreases (Rustad *et al*., 2008; Drusano *et al*., 2010; Watanabe *et al*., 2011) and then a switch to anaerobic metabolism occurs (Voskuil *et al*., 2004). The bacilli also undergo structural changes such as the thickening of the cell wall and development of inclusion bodies (Anuchin *et al*., 2009).

2.3.3 Survival and changes in gene expression in *Mycobacterium tuberculosis* inside the granuloma

Bacilli surviving under anaerobiosis have thickened cell walls and there is an induction and repression of various genes (Zahrt, 2003). This is ensured by the ability of pathogens of being able to regulate a cascade of genes in response to the different antibacterial stresses encountered inside the host (Srivastava *et al*., 2008). Successful inhabitation of the granuloma by *M. tuberculosis* and persistence of the organism requires the bacilli to undergo changes morphologically (Christophe *et al*., 2009) and genetically (Li *et al*., 2002; Ahmad, 2010; Wang *et al*., 2011). All these changes require the bacilli to decrease and increase the expression of certain genes. Up regulation and down regulation of these genes depends on the function of the

gene. For *M. tuberculosis* the genes that are up-regulated are thought to aid the bacilli in remaining viable but not replicating or replicating at a slower rate (Saxena *et al*., 2008; Ahmad, 2010; Shleeva *et al*., 2011).

Mycobacterium tuberculosis isolated from sputum differs phenotypically to the bacilli that are propagated aerobically *in vitro* as a subpopulation of the bacilli isolated from it has triacylglycerol lipid bodies that are produced by a gene that is part of a regulon (Dos R regulon) which is associated with the anaerobic respiration (Garton *et al*., 2008). The Dos R regulon consists of a minimum of 48 genes (Voskuil *et al.*, 2004; Leistikow *et al*., 2010; Kumar *et al*., 2012). The activity of this regulon is induced *in vitro* when the bacilli are exposed to environmental conditions that are similar to those found in the granuloma such as low oxygen concentration (Nickel *et al*., 2012). This regulons' mutants are not able to adapt in a hypoxic environment, showing the importance of this regulon for adaptation to anaerobic conditions (Fang *et al*., 2012).

Studies have shown that when *M. tuberculosis* is exposed to different environmental conditions that are all thought to be encountered by the bacilli in the granuloma, these bacilli gave different gene expression profiles (Figure 2.3.3) (Deb *et al*., 2009). This indicates that environmental conditions that are encountered by the bacilli have an influence on expression of the genes by the bacilli. This in turn indicates that bacilli inhabiting different habitats may have different drug susceptibility profiles (Deb *et al*., 2009) and pathophysiologies (Ashiru *et al*., 2012).

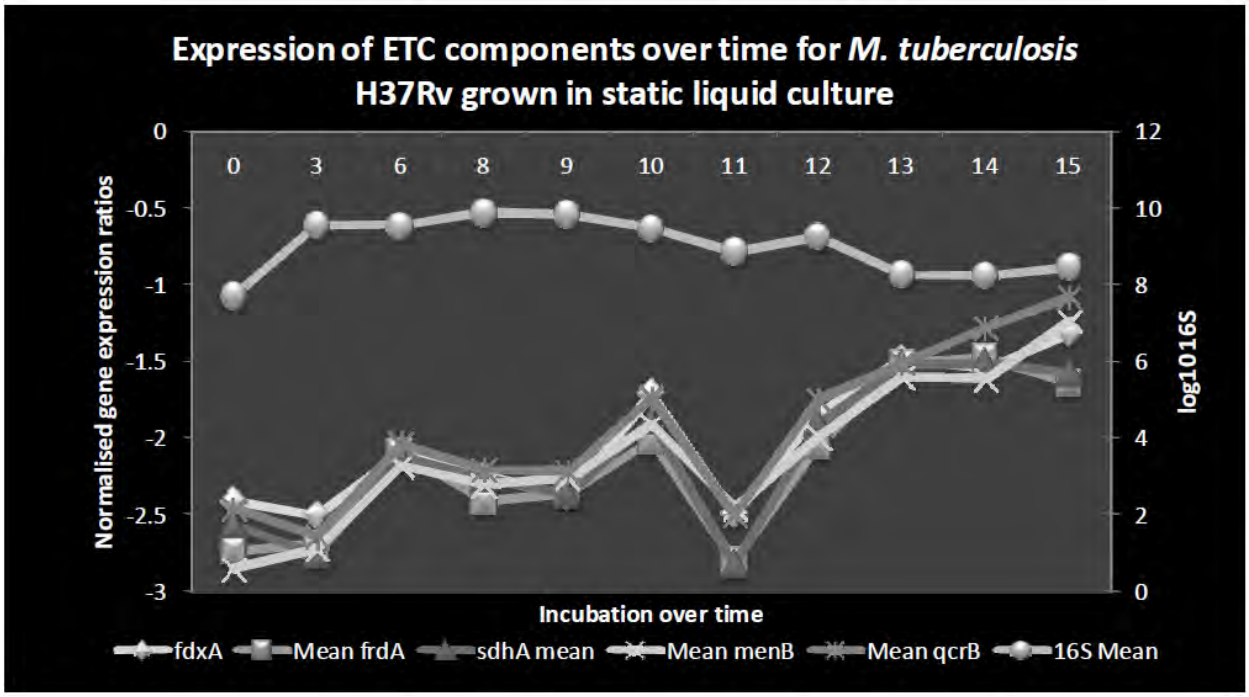

Figure 2.3.3: Expression of *M. tuberculosis* genes as the bacilli is surviving and adapting under anaerobiosis (adapted from a thesis by Ramcharandra and Sturm)

Chapter 3: Methodology

3.1 Preparation of antimicrobial dilution series

Nine different anti-tuberculosis agents were used. For each of these, stock solutions were prepared in triplicate. For each drug an appropriate solvent was chosen as shown in table 3.1. The amount of each drug depended on the range of dilutions to be tested and the antimicrobial effect of the solvent. The drug solutions were sterilized through a 0.22 µm polyethersulfone membrane filter (Millipore, United Kingdom), except for rifampicin as for this compound methanol was used as a solvent. This alcohol is known to damage the filter. At the concentration used, methanol acts as a sterilising disinfectant. The stock solutions were stored in cryovials at -20ºC in aliquots of 500 µl.

To prepare the dilution series to be tested, the antibiotic stock solutions were thawed at room temperature (± 25ºC). Of each of the drug solutions, 0.5 ml was added to 4.5 ml of OADC supplemented Middlebrook 7H9 broth (BD, Difco Laboratories, USA) (Based on the media that was used for the assay conducted). From this two-fold serial dilutions were prepared in a tissue culture plate with the appropriate number of the wells for each test. Each well contained twice the final concentration for each drug as shown in table 3.1. The test dilution series for each drug were determined based on published minimal inhibitory concentrations (MIC) of susceptible *Mycobacterium tuberculosis* (Table 3.1). This included two dilutions below and eight dilutions above this MIC.

Table 3.1: Stock solutions and test range of the anti-tuberculosis agents

Drug	Solvent	Stock solution concentration (mg/L)	Concentration range (mg/L)	MIC (mg/L) of susceptible *M. tuberculosis*
Isoniazid	Distilled water	1280	64 – 0.0625	0.25
Rifampicin	Methanol: distilled water (30%)	1280	64 – 0.0625	0.01
Streptomycin	Distilled water	2560	128 – 0.125	0.5
Kanamycin	Distilled water	20480	1024 – 1	4
Ofloxacin	6.7% acetic acid: distilled water (1:1)	5120	256 – 0.25	1
Capreomycin	Distilled water	20480	1024 – 1	4
Para-aminosylic acid	Distilled water	2560	128 – 0.125	0.5
Linezolid	Distilled water	2560	128 – 0.125	0.5
Ethambutol	Distilled water	20480	1024 – 1	4

3.2 Preparation of Inoculum

Eighteen clinical *M. tub*erculosis isolates from patients admitted to the Church of Scotland Hospital in Tugela Ferry, province of KwaZulu-Natal were used for this study. All isolates had been formerly fingerprinted by means of IS*6110* RFLP and their resistance profile was determined with 1% proportional method on Middlebrook 7H10 agar. The genotypes and resistance profiles of these isolates are shown in table 3.2. Reference strain *M. tuberculosis* H37Rv ATCC 27294 was included as a control. All organisms were inoculated into supplemented Middlebrook 7H9 (BD, Difco Laboratories, USA) broth (see appendix A.1.1).

These broth cultures were incubated aerobically in an incubator with a horizontally rotating plate (shaker-incubator) at 37^0C until the log phase was reached, defined as an OD_{600nm} reading of 0.8 - 1. To prepare cultures with single colonies two to three drops from these cultures were inoculated with a Pasteur pipette onto OADC supplemented Middlebrook 7H11 (BD, Difco Laboratories, USA) (see appendix A.1.4). The plates contained 200.000 units/L of Polymixin B (Sigma-Aldrich, USA); 20 mg/L of Amphotericin B (Sigma-Aldrich, USA); 100 mg/L of Carbenicilin (Sigma-Aldrich, USA) and 20 mg/L of Trimethoprim (Sigma-Aldrich, USA) (PACT) to avoid contamination. The culture was spread using a four way streak technique. The plates were sealed in gas permeable plastic bags and incubated aerobically at 37ºC in 5% CO_2 for 3 weeks. One single colony of each isolate was picked and inoculated in fresh OADC supplemented Middlebrook 7H9 (BD, Difco Laboratories, USA) (see appendix A.1.1) broth which was incubated aerobically in the shaker-incubator at 37ºC until visual turbidity was observed. Two to three drops of each broth culture were spread onto two OADC and PACT supplemented Middlebrook 7H11 (BD, Difco Laboratories, USA) plates to get a lawn of *M. tuberculosis* colonies. These plates were sealed in gas permeable plastic bags and incubated aerobically at 37ºC in 5% CO_2 for 3 weeks, after which the culture was harvested and stored in 1 ml aliquots at -80ºC in storage media consisting of protease peptone (BD, Difco Laboratories, USA) and 12.5% glycerol (see appendix A.1.5).

Table 3.2: Genotypes and drug susceptibilities of the isolates used in the study

Strain	Genotype	Classification*
H37Rv	Reference Strain	SUS
TF1582	Beijing	SUS
TF1413	Beijing	SUS
TF832	F11 variant	SUS
TF1538	Unique	SUS
TF1001	Unique	SUS
TF2034	Unique	MDR
MODS682	Unique	MDR
TF36480	F28	MDR
TF2889	F28	MDR
TF3203	F28	MDR
MODS11	F15/LAM4/KZN	MDR
TF3228	F15/LAM4/KZN	XDR
MODS370	F15/LAM4/KZN	XDR
TF1762	F15/LAM4/KZN	XDR
TF1497	F15/LAM4/KZN	XDR
TF80164	F15/LAM4/KZN	XDR
TF3334	F15/LAM4/KZN	XDR
TF3229	F15/LAM4/KZN	XDR

*Classified according to resistance profile with 1% proportional method on Middlebrook 7H10 agar

To prepare the inoculum for MIC determination, stored cultures were thawed and a 100 µl volume was inoculated into 10 ml of OADC supplemented Middlebrook 7H9 broth (see appendix A.1.2). This culture was incubated for approximately two weeks at 37ºC in a shaker-incubator until an optical density of 0.8 – 1.0 at OD_{600nm} was obtained compatible with the log phase of growth.

Oxygen deprived *M. tuberculosis* cultures were prepared according to Ramchandra and Sturm, (2010). A 2 ml volume of log phase culture was inoculated into 8ml of OADC supplemented Middlebrook 7H9 (BD, Difco Laboratories, USA) broth (see appendix A.1.2) in a 15 ml culture

tube (Sterilin Limited, Cambridge, UK). The tubes were tightly closed with a screw cap and sealed with parafilm. The cultures were incubated undisturbed for a minimum of two months at 37ºC.

To confirm the presence of *Mycobacteria* and the absence of contaminants the Ziehl-Neelsen Staining Method was applied. Smears were made on microscopy slides after each incubation step. The smears were heat fixed on a hot plate for 2 hours and stained. The slide was then air dried at room temperature (± 25ºC) and viewed at 1000X magnification using a Nikon bright light microscope.

3.3 Determination of the Minimum Inhibitory Concentrations of aerobic of *M. tuberculosis*

Bacterial cultures that had been grown aerobically in OADC supplemented Middlebrook 7H9 broth (see appendix A.1.2) were sonicated once at 15 Hz for 10 seconds, and the OD was measured at 600nm. Cultures with an OD of 0.7 to 1.4, containing approximately 10^8 colony forming units (cfu) per ml were used for the preparation of the inoculum to be used for the experiment. This suspension was diluted in supplemented Middlebrook 7H9 broth (see appendix A.1.2) to a concentration of 10^3cfu/ml and a volume equal to that of the antibiotic solution was added to each well of the tissue culture plates that contained the antibiotic dilutions (see 3.1).

3.3.1 (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) Assay

A 96-well flat bottom microtitre plate was used for the (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) assay. The assay was done three times for each drug per isolate. Two hundred microlitres of sterile triple distilled water was added to all the wells on the edge of the plate. Fifty microlitres of OADC supplemented Middlebrook 7H9 broth (see appendix A.1.2) was added to columns 3-11 from rows B-G and two fold serial dilutions of the drug to be tested were made in these rows, discarding 50 µl from the well with the lowest concentration. An equal volume of the inoculum was added to all the wells except the ones with water and the control wells. Experimental controls were as follows: broth only, drug only, culture only and MTT solution only (added on the day the MTT solution was added onto the rest of the place). The plates were sealed in gas permeable plastic bags and incubated at 37ºC aerobically for 7 days for susceptible strains and H37Rv; while drug resistant strains were incubated for 14 days. After the incubation period, 7.5 µl of MTT (Sigma-Aldrich, USA) solution (5 mg/ml) was added to each experimental well. The plates were sealed again in gas permeable bags and incubated overnight at 37ºC aerobically. After overnight incubation, 25 µl of Sodium Dodecyl Sulphate (Sigma-Aldrich, USA)-Dimethylformamide (Sigma-Aldrich, USA) (SDS-DMF) solution [which was 20% SDS and 50% aqueous DMF (1:1 v/v)] was added to all wells. This was followed by another overnight incubation period at 37ºC, aerobically in a sealed gas permeable plastic bag and colour change from yellow to purple, indicating growth, was noted. The minimum inhibitory concentration (MIC) was read as the lowest concentration that showed no colour change.

3.3.2 Microscopic Observation Drug Susceptibility Assay: Aerobic

A 24–well flat bottom tissue culture plate was used for the Microscopic Observation Drug Susceptibility (MODS) assay. The assay was done three times for each drug per isolate. Five hundred microlitres of supplemented Middlebrook 7H9 broth (see appendix A.1.3) was added to all wells except wells A1 and C1. Onto these two wells 500 µl of the drug working solution was added and two-fold serial dilutions were made in each row starting from wells A2 and C2, discarding 500 µl from the well with the lowest concentration. Following this, 500 µl of the inoculum was added to all the wells except the controls. This resulted in final drug concentrations as summarised in table 3.1. Experimental controls were as follows: broth only, drug only and culture only. The plates were sealed in gas permeable plastic bags and incubated at 37ºC aerobically for 14 days. After 14 days of incubation the contents of the wells were viewed under the Olympus CKX 41 inverted microscope (100X magnification). The MIC was interpreted as the lowest concentration that had no growth of *M. tuberculosis* as determined by the visualisation of whorls of bacteria due to *M. tuberculosis* typical cording.

3.3.3 Microscopic Observation Drug Susceptibility Assay: Anaerobic

For the anaerobic MODS assay, a similar procedure was followed except that all the work as well as the incubation (37ºC) was done in a Forma Scientific anaerobic chamber. Four isolates (TF1538; TF3203; TF3334 and TF3229) and H37Rv were chosen for the determination of the MICs under anaerobiosis.

Two different experiments were performed. In the first experiment, the MODS assay was done as described above using the cultures grown under oxygen deprivation (section 3.2).

In the second experiment, six isolates (TF1538; TF1001; TF3203; TF3228; TF3229; TF3334) and H37Rv were cultured aerobically in OADC supplemented Middlebrook 7H9 broth (see appendix A.1.3) followed by MIC determination under anaerobic conditions. When the log phase of growth was reached, the MODS assay was performed as describe above with an inoculum containing 10^3cfu/ml. Isolates (TF1538 & TF1001) were tested for susceptibility to only INH and RIF; isolates (TF3203 & TF3228) to KAN and OFL and isolates (TF3229 & TF3334) to PAS and LIN and susceptibility of H37Rv was tested against the all six anti-TB drugs. The gas-mix used in the anaerobic chamber was as follows: 5% of carbon dioxide, 10% hydrogen and 85% nitrogen. The actual incubation gas mix had an undetermined lower hydrogen contents since this is converted to water during the removal of oxygen. To check culture viability, 5 µl of culture from the inoculum dilutions containing 1 $\times 10^3$, 1 x 10^4 and 1 x 10^5 bacteria / ml was spread onto a 65 mm Middlebrook 7H11 agar plate. The plates were incubated aerobically in sealed gas permeable plastic bags at 37ºC in 5% CO_2 for 3 weeks, after which the number of colonies on the plate were counted.

3.3.4 Killing Experiments

Following the analysis of the results from the MODS Assay, five MDR isolates were chosen for the time kill experiments with INH, RIF, KAN and OFL. If anaerobic conditions would increase the susceptibility to drugs, the MDR isolates should show kill at lower dilutions with INH and RIF. If the anaerobic conditions would make the organisms more drug tolerant, the opposite

would be observed with KAN and OFL. The test was done at different concentrations for each drug, for each isolate, as determined by the MIC obtained in the aerobic MODS assay. The assay was done three times for each drug per isolate under aerobic conditions while under anaerobic conditions the assay was done in duplicates for each drug per isolate. These concentrations were 0.25X MIC, 1X MIC and 4X MIC. The concentrations for each drug per isolate are shown in table 3.3.

Table 3.3: Drug concentrations used for the killing experiments for each isolate

Isolates	INH			RIF			OFL			KAN		
TF2034	2	8	32	16	>64	256	0.125	0.5	2	0.5	2	8
MODS682	8	32	128	16	>64	256	0.25	1	4	8	32	128
TF36480	4	16	64	16	>64	256	1	4	16	256	>1024	4096
TF2889	2	8	32	16	>64	256	0.125	0.5	2	2	8	32
MODS11	16	64	256	2	8	32	0.25	1	4	0.5	2	8

A suspension of 100µl *M. tuberculosis* stock culture was inoculated into 10 ml of OADC supplemented Middlebrook 7H9 broth (see appendix A.1.2). The suspension was incubated at 37ºC in a shaker-incubator, until the culture reached the log phase of growth (see section 3.2). Upon reaching this phase, 1ml of the culture was inoculated into 9 ml OADC supplemented Middlebrook 7H9 broth (see appendix A.1.2) and was placed back in the same incubator under the same conditions. Another 1ml of the inoculum was inoculated into 9 ml OADC supplemented Middlebrook 7H9 broth (see appendix A.1.2) in a 15 ml culture tube (Sterilin

Limited, London, UK). The conditions for incubation were similar to section 3.2, with an incubation period of4 weeks.

Once the aerobic cultures were in the log phase and following 4 weeks of incubation for the anaerobic cultures the following procedure was done for both experiments:

All the anaerobic work was done inside the anaerobic chamber, while the aerobic work was done in the Biosafety Level 2 Cabinet. Briefly, 3 ml of each culture was inoculated into 42ml of OADC supplemented Middlebrook 7H9 broth (see appendix A.1.2). This suspension was divided into aliquots of 4.5 ml in 30 ml white capped tubes (Sterilin Limited, London, UK) for aerobic cultures and in 15 ml white capped tubes (Sterilin Limited, London, UK) for the anaerobic cultures. The drug working solutions (see section 3.1) with the desired concentrations (Table 3.3) were added to these aliquots. All the drug dilutions were done in triplicates. The aerobic cultures were incubated at 37ºC, in an incubator with a shaker-incubator for a week. The anaerobic cultures were incubated at 37ºC in the anaerobic chamber for a week as well. At day 0, 2 days and 7 days, 100 µl each antibiotic-bacterial isolate combination was transferred into a 1.5 ml microcentrifuge tube (Eppendorf, Germany) with 900 µl of phosphate buffer saline (PBS). The contents of the tubes were vortexed, followed by centrifugation at 12128 xg for 10 minutes. The supernatant was decanted, and the pellet was resuspended in 100 µl PBS, which was then vortexed and spread onto a 65 mm Middlebrook 7H11 agar (see appendix A.1.4) plate. The plates were dried for 30 minutes and then incubated aerobically for 3 weeks in 5% CO_2 at 37ºC. One plate was inoculated for each dilution. Following aerobic incubation of the plates at 37ºC for 3 weeks, the plates were checked and the percentage of growth was estimated in comparison with the growth on day 0 for each antibiotic-bacterial isolate combination.

N.B.: All the anaerobic work was done in the anaerobic chamber (as illustrated in section 3.2) except for the subculturing and the incubation of the Middlebrook 7H11 plates (see appendix A.1.4).

Bacteriodes fragilis ATCC 25282 and *Propionibacterium acnes* ATCC 11827 were inoculated onto blood agar plates and incubated for 5 days aerobically and anaerobically, while a clinical isolate of *Pseudomonas aeroginosa* was inoculated onto nutrient agar aerobically and anaerobically. The plates were incubated overnight. These three organisms were used as biological controls as *B. fragilis* is a strict anaerobe and its growth in the anaerobic chamber indicated the presence of anaerobic conditions, whereas *P. acnes* as facultative anaerobe grew at a slower rate when it was incubated in the anaerobic chamber compared to when it was incubated aerobically. *P. aeroginosa* is a strict aerobe. No growth under anaerobic conditions confirms oxygen depletion inside the chamber while its growth aerobically confirmed viability of the test isolate. Methylene blue indicator strips were also used to confirm the absence of oxygen in the chamber. All controls confirmed anaerobiosis inside the chamber during the full operational period.

3.4 Gene Expression

3.4.1 RNA Extraction

RNA was extracted from aerobically incubated as well as oxygen deprived cultures (refer to section 3.2) using the Trisure Nucleic Acid extraction protocol. The RNA was extracted from aerobically incubated cultures once the cultures were at the log phase growth, while from the

oxygen deprived cultures the RNA was extracted after 12 months of incubation, except for isolate TF3203 whose extraction was done after 10 months as it was inoculated and incubated later than the other isolates. Briefly, the culture was centrifuged at 3000xg at 4ºC for 30 minutes and the supernatant was discarded. The pellet was resuspended in 1ml of Trisure solution (Bioline, United Kingdom) and mixed by pipetting up and down. The mixture was then transferred into a 2 ml conical screw cap microtube with a cap that had an o-ring (Quality Scientific Products, USA) containing 0.1 mm diameter Zirconia/silica beads (BioSpec Products, Bartlesville, USA). The mixture was vortexed three times for 2 minutes with a 2 minutes break in between the vortexing steps during which the tubes were placed on ice. The Trisure and nucleic acid mixture was stored at -80ºC until the subsequent RNA extraction steps were carried out.

The frozen Trisure mixture was thawed and centrifuged at 12000xg at 4ºC for 10 minutes. The supernatant was transferred into a new tube and incubated at room temperature for 10 minutes. Cold chloroform (Sigma-Aldrich, USA) was then added and mixed vigorously by hand for 30 seconds. The chloroform mixture was centrifuged at 12000xg at 4ºC for 10 minutes. The colourless upper aqueous phase was transferred into a new tube. Cold isopropanol (Sigma-Aldrich, USA) was added and mixed with the aqueous phase by inverting the tube, followed by a rest phase of 15 minutes at room temperature. The liquid was centrifuged at 12000xg at 4ºC for 10 minutes. The supernatant was carefully decanted without disturbing the RNA pellet. The pellet was then resuspended in 75% ethanol in Diethylpyrocarbonate (DEPC)-treated water (Sigma- Aldrich, USA) and agitated by hand. The solution was centrifuged at 7500xg for 5 minutes at 4ºC. Following that the supernatant was discarded. The pellet was air-dried for 10 minutes, followed by resuspension of the pellet in RNA Secure and incubation at 60ºC in a water

bath for 10 minutes. The RNA was quantified using the NanoDrop 2000c (Thermo Fischer Scientific, Inc., USA), with RNA Secure being used as a blank. This was then followed by the DNAase treatment according to the manufacturer's instructions, using the RNA concentration of each sample as the reference for the amount of the reagents to be added. Following the DNAse treatment the RNA was once again quantified using the NanoDrop 2000c with DEPC treated water as the blank. After quantification, the extracted RNA was stored at -80ºC until the subsequent cDNA synthesis step.

3.4.2 Synthesis of Complimentary DNA (RT-PCR)

For each sample 3 µl of loading dye was mixed with 10 µl of the RNA sample. This was loaded into the wells of a 2% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer (Sigma-Aldrich, USA). Electrophoresis was performed at 100V for 1 hour. On completion of the run, the bands were viewed under UV light.

Complementary DNA (cDNA) was synthesised using the ABI High capacity cDNA synthesis kit (Life Technologies, USA).The 2x RT master mix was prepared with volumes of ingredients shown in table 3.4.2. These were mixed and gently vortexed. Ten microlitres of 2x RT master mix was mixed with 10 ml of the prepared RNA. The mixture was vortexed, briefly centrifuged in a microcentrifuge and placed on ice until it was processed in the GeneAmp 9700 thermal cycler under the following conditions: 25ºC for 10 minutes followed by 37ºC for 120 minutes; which was followed by annealing for 5 seconds at 85ºC and finally at 4ºC indefinitely. Once the PCR reaction was complete the cDNA was stored at -20°C until further use.

Table 3.4.2: The quantities of the High-Capacity Reverse Transcription Kit reagents as used per reaction for the synthesis of cDNA synthesis

Reagents	Amount per reaction (µl)	Concentration
10x Buffer	2.0	-
25x dNTP mix	0.8	100Mm
10x Reverse transcriptase random primers	2.0	-
MultiScribereverse transcriptase	1.0	50U/µl
RNase Inhibitor	1.0	20U/µl
Nuclease free water	3.2	N/A
RNA sample	10	≤ 2µg
Total	20	N/A

3.4.3 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) using the ABI 7500 system

Applied Biosystems TaqMan® Gene Expression Assays (Life Technologies, USA) were used. Reaction volumes of 25 µl per well were used in a 96-well plate: 12.5 µl of TaqMan® Gene Expression PCR Master Mix (2✕) (Life Technologies, USA) was mixed with 1.25µl Assay Mix (20✕) (Life Technologies, USA) for each target (*16SrRNA; men*B*; sdh*A and *fdx*A); 2.5µl cDNA (diluted to a concentration of 50ng/µl) and 8.75µl of nuclease free water. The mixture was centrifuged briefly to remove air bubbles. The 96-well plate was placed into the ABI 7500

system (Life Technologies, USA) as per systems programme. Gene expression levels were normalized with 16SRNA levels of each sample tested. Table 3.4.3 displays the sequences of the primers and probes applied in the gene expression assays. The assay was done three times for each gene per isolate.

Table 3.4.3: Sequences of the primers and probes of the genes used for qRT-PCR

Gene	Forward primer	Reverse primer	Probe
*men*B (Rv0548c)	CGGCCGGTTGCACATC	CTGATCCGGTTCATGCCCA	CTGGAGGTGCAGCGG
*fdx*A (Rv2007c)	GCGTCGAGGCGATCTACTG	AGCATCTGGGGGACAACG	CCCGACGATCAACACC
*sdh*A (Rv3318)	CGCCCGCTCGATGGT	CTCAAGGACTACGTCTACATCGAC	TGGAAGTGCTGGAGGG
16S rRNA (Rv2907c)	GGGTCTCTGGGCAGTAACTG	AGGATTAGATACCCTGGTAGTCCAC	AAAGCGTGGGGAGCGA

Chapter 4: Results

The critical concentrations proposed by WHO (WHO, 2009) for drug susceptibility testing (DST) in liquid media were used to evaluate the activity of the antimicrobial agents against the 18 *M. tuberculosis* isolates used in this study. As there are no standard critical concentrations for DST against the antimicrobial agents used when performed in home-made Middlebrook 7H9 broth the proposed critical concentrations for BACTEC460 and the MGIT960 were the closest concentrations that could be applied for the evaluation of the results from both the (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) and Microscopic Observation Drug Susceptibility (MODS) assays. These critical concentrations are shown in table 4.

Table 4: Critical concentrations of anti-tuberculosis drugs as proposed by WHO for MGIT960 and BACTEC460

Antibiotics	Critical concentrations (mg/L) as proposed by WHO	
	MGIT960	BACTEC460
Isoniazid	0.1	0.1
Rifampicin	1	2
Ethambutol	5	2.5
Streptomycin	1	2
4-aminosalicylic acid	-	2
Ofloxacin	2	2
Kanamycin	-	4
Capreomycin	2.5	1.25
Linezolid	1	1

Guidelines for Surveillance of Drug Resistance in Tuberculosis, 4th edition, WHO/HTM/TB/2009.422

4.1 Minimum Inhibitory Concentrations obtained with the MTT test

When the WHO proposed (WHO, 2009) breakpoints (Table 4) for both the MGIT960 and BACTEC460 methods were used, H37Rv was found to be resistant to all drugs tested using the MTT assay except for ofloxacin (OFL), if the MGIT960 breakpoint for STR is used (Table 4.1). Isolate TF1582 was the only one within the group of five susceptible isolates tested (Table 4.1) that was found to be susceptible to ethambutol (EMB) in the MTT assay if the breakpoint from the BACTEC460 was used.3 of the 5 of the susceptible isolates had an MIC of 0.25 mg/L for isoniazid (INH). Both H37Rv and TF1582 were susceptible to streptomycin (STR), while all the resistant isolates that were tested (TF36480 and TF3203, both MDR) had an MIC that was greater than 128 mg/L and XDR isolates TF1497 and TF80164 had an MIC of 16 mg/L. In the MTT assay, all the isolates were susceptible to OFL except for the XDR isolates (TF1497; TF80164 and TF3334) that had an MIC of 8 mg/L.

Table 4.1: The MIC (mg/L) results of anti-tuberculosis agents of *M. tuberculosis* isolates under aerobic conditions as determined by the MTT Assay

Strain	Classification*	INH	EMB	STR	OFL
H37Rv	SUS	0.125	4	1	1
TF1582	SUS	N/T	2	0.5	≤0.25
TF1538	SUS	0.25	N/T	N/T	0.5
TF832	SUS	0.25	N/T	N/T	N/T
TF1001	SUS	0.25	2	N/T	0.25
TF36480	MDR	N/T	32	>128	1
TF3203	MDR	N/T	16	>128	0.25
TF1497	XDR	N/T	32	16	8
TF80164	XDR	N/T	N/T	16	8
TF3334	XDR	N/T	16	N/T	8

*Classified according to resistance profile with 1% proportional method on Middlebrook 7H10 agar

N/T – Not Tested, as the experiments were being carried, it was established through literature that the MTT assay would not work under anaerobic conditions as *M. tuberculosis* has low metabolic activity.

4.2 MICs obtained with the MODS assay under aerobic conditions

With the MODS assay a total of nine antimicrobial agents were tested. As illustrated in table 4.2, H37Rv showed unexpected resistance to several of the antimicrobial agents. TF1582 was the only isolate that was susceptible to all the anti-tuberculosis agents. The minimal inhibitory concentration for streptomycin (STR) for four of the six MDR isolates that were tested, was > 128 mg/L since there was still growth at this concentration, being the highest concentration that was tested. All the MDR-F15/LAM4/KZN (KZN) isolates except for isolate MODS11 were resistant to ofloxacin (OFL). All the drug resistant isolates were resistant to kanamycin (KAN) except for KZN isolate MODS11 as well as TF2034, which was an isolate that was not from an identified genotype cluster. All the other KZN isolates had an MIC that was > 1024 mg/L. Isolate TF2034 was an exception in the group of the MDR isolates as it was the only one that had an MIC that was $\leq$ 1 mg/L for capreomycin (CAP). All resistant isolates together with H37Rv had an MIC of 16 mg/L or above for EMB. Resistance to OFL was only seen in the KZN isolates.

Table 4.2: The MIC (mg/L) results of anti-tuberculosis agents of *M. tuberculosis* isolates under aerobic conditions as determined by the MODS Assay

Isolate	Classification*	INH	RIF	EMB	STR	KAN	CAP	LIN	OFL	PAS
H37Rv	SUS	0.125	0.25	16	0.5	8	4	0.5	1	2
TF1582	SUS	≤0.0625	0.25	≤1	0.5	4	≤1	0.25	≤0.25	1
TF1413	SUS	≤0.0625	0.125	8	0.5	2	≤1	0.25	0.5	2
TF832	SUS	≤0.0625	0.125	8	2	8	≤1	0.25	0.25	16
TF1538	SUS	≤0.0625	0.125	8	0.25	2	≤1	0.25	0.25	0.25
TF1001	SUS	<0.0625	<0.0625	8	0.5	4	8	0.5	0.25	1
TF2034	MDR	8	>64	32	>128	2	≤1	0.5	0.5	64
MODS682	MDR	32	64	16	2	32	16	0.25	1	4
TF36480	MDR	16	>64	16	>128	>1024	16	0.5	4	>128
TF2889	MDR	8	64	16	>128	8	8	0.25	0.5	0.5
TF3203	MDR	16	>64	64	>128	8	4	0.5	0.5	0.25
MODS11	MDR	64	8	16	0.5	2	4	0.25	1	8
TF3228	XDR	32	>64	64	2	>1024	8	0.25	4	2
MODS370	XDR	64	>64	64	4	>1024	8	≤0.125	4	4
TF1762	XDR	16	>64	64	8	>1024	8	0.5	8	32
TF1497	XDR	32	>64	32	32	>1024	32	0.5	8	8
TF80164	XDR	>64	>64	64	4	>1024	8	0.5	8	0.5
TF3334	XDR	64	>64	32	4	>1024	8	0.25	8	2
TF3229	XDR	64	>64	64	4	>1024	16	2	4	1

***Classified according to resistance profile with 1% proportional method on Middlebrook 7H10 agar**

4.3 Comparison between MTT and MODS assays

Overall, the MICs that were determined by the MODS assay for the group of isolates classified as susceptible were either equal to or lower than the MICs determined by the MTT assay, with MICs for EMB being the exception (Table 4.3). For INH only the isolates grouped as susceptible and H37Rv were tested in the MTT assay. All these isolates' MICs for INH were two folds higher compared to the MIC in the MODS assay, while the MIC for H37Rv was the same at 0.25 mg/L. With this drug, four isolates showed identical results in both assays while for the other three antibiotics the MICs were higher in the MODS than in the MTT assay. The opposite was witnessed with the resistant isolates, with an exception of isolates TF36480 for EMB and TF80164 for STR (Table 4.3). Minimum inhibitory concentrations of STR from both the MTT and MODS assays were the same for isolates TF1582; TF36480 and TF3203, although with the latter two isolates that could change as 128 mg/L was the highest concentration that was tested for both assays. The minimum inhibitory concentrations in the MTT assay of this drug for isolate TF80164 and H37Rv were higher than the MICs in the MODS assay. TF1497 had an MIC of 32 mg/L for STR in the MODS assay which was double the MIC obtained with the MTT assay. All the isolates whose susceptibility to OFL that were tested through both assays and showed similar susceptibility profiles, with the exception of TF36480 with a two-fold higher MIC of 1 mg/L in the MTT assay as compared to the MODS assay. All the XDR isolates (TF1497; TF80164 and TF3229) were resistant to OFL as they all had an MIC of 8 mg/L for this antimicrobial agent with both assays. The rest of the isolates that were tested for OFL were susceptible to this drug with MICs for isolates TF1582 and TF1001 and H37Rv remaining the same amongst the two assays. Based on the limited number of isolates tested with both assays, no trend was observed with respect to the level of the MIC between these assays.

Table 4.3: Comparison between MIC (mg/L) results of anti-tuberculosis agents of *M. tuberculosis* isolates under aerobic conditions as determined by the MODS and the MTT Assay

Isolate	Classification*	INH		EMB		STR		OFL	
H37Rv	SUS	0.125	0.125	16	4	0.5	1	1	1
TF1582	SUS	≤0.0625	N/T	≤1	2	0.5	0.5	≤0.25	≤0.25
TF1538	SUS	≤0.0625	0.25	8	N/T	0.25	N/T	0.25	0.5
TF832	SUS	≤0.0625	0.25	8	N/T	2	N/T	0.25	N/T
TF1001	SUS	≤0.0625	0.25	8	2	0.5	N/T	0.25	0.25
TF36480	MDR	16	N/T	16	32	>128	>128	4	1
TF3203	MDR	16	N/T	64	16	>128	>128	1	0.25
TF1497	XDR	32	N/T	32	32	32	16	8	8
TF80164	XDR	64	N/T	64	N/T	2	16	8	8
TF3334	XDR	64	N/T	32	16	4	N/T	8	8

*Classified according to resistance profile with 1% proportional method on Middlebrook 7H10 agar

Red – MIC results with the MODS Assay

Black – MIC results with the MTT Assay

N / T – Not Tested, as the experiments were being carried, it was established through literature that the MTT assay would not work under anaerobic conditions as *M. tuberculosis* has low metabolic activity.

4.4 MICs obtained with the MODS assay under anaerobic conditions

We were unable to determine the MICs for any of the isolates under anaerobic conditions. After 40 days of incubation of the inoculum of the 24 well tissue culture plates in the anaerobic chamber, there was no growth in the growth control. There was lack of growth in the MODS assay with both experimental methods used, i.e. using an inoculum that was grown under oxygen deprived conditions prior to conducting the MODS assay and using an inoculum that was grown aerobically prior to the MODS assay.

The biological controls *B. fragilis* and *P. acnes* grew in the anaerobic chamber while *B. fragilis* did not grow when it was incubated aerobically. Growth of *P. Aeroginosa* was the opposite of *B. fragilis* as this microorganism did not grow when it was incubated in the anaerobic chamber while it grew under aerobic incubation. *P. acnes* produced smaller colonies under aerobic conditions.

4.5 The bactericidal effect of isoniazid, rifampicin, ofloxacin and kanamycin on MDR isolates under aerobic and anaerobic conditions / (Killing experiments)

The results of the killing experiments are summarised in tables 4.5.1 and 4.5.2. Isoniazid had the lowest percentage kill, both aerobically and anaerobically. On day 2, there were no differences in percentage kill for INH when the experiments were conducted aerobically or anaerobically for all isolates except for TF2889 under aerobic conditions. At 0.25XMIC, this antibiotic was only able to kill bacteria from isolate TF2889 under anaerobic conditions on day 2 (Table 4.5.1). The rest of the isolates were not killed under both incubation conditions. At the MIC as determined by the MODS Assay, none of the isolates was killed. This was under both the conditions at which the incubation was carried out. Isolate TF2889 together with MODS11, were the only two isolates where kill was observed at day 7 with INH at a concentration of 4XMIC with a percentage kill of close to 100% each (Table 4.5.2). The killing was achieved under aerobic conditions only. There was no specific killing trend that was observed with this antimicrobial agent.

Out of the four antibiotics that were used for the time-kill experiments RIF was the most successful one. This antimicrobial was the only one that was able to kill a substantial amount of

bacilli from all the isolates that were used, although this occurred at different concentrations and conditions. Under anaerobic conditions on day 2, RIF showed better activity towards isolates TF2034 and TF2889. No kill of these isolates was observed under aerobic conditions. RIF killed ±100% of the inoculum of TF2889 at 0.25XMIC under anaerobic conditions (Table 4.5.1). At the concentration of 0.25X MIC, only one isolate (TF2889) was killed by RIF at day 2 under anaerobic conditions, while at the MIC concentration no kill was observed with any of the isolates. On day 2 under aerobic conditions RIF had no lethal effect on any of the isolates except MODS11. On day 7 this antibiotic was only able to kill isolates not belonging to an identified IS*6110* lineage, namely MODS682 and TF2034 (Table 4.5.2) and this was under both aerobic and anaerobic conditions. The average percentage kill for these two isolates under both conditions was almost equivalent, with the percentage kill for TF2034 under anaerobic conditions being slightly greater, and the opposite was true for MODS682. Overall RIF was more lethal under anaerobic conditions, as by day 7 (Table 4.5.2) at a concentration of 4X MIC 4 of the 5 isolates were killed. At day 7, a similar scenario was observed with isolates TF2889 and TF36480, with ±100 % kill of anaerobically incubated bacilli (Table 4.5.2).

Similar activity was observed with OFL at day 2 (Table 4.5.1) with kill of the inoculum incubated under anaerobic conditions but not when incubated under aerobic circumstances. Ofloxacin at 0.25XMIC was not able to kill any on the bacilli under both aerobic and anaerobic conditions, while at the MIC concentration of this antibiotic only TF2889 was killed. This was on day 2 under anaerobic conditions. At 4XMIC, the potency of OFL showed signs of not being associated with either of the two conditions that were under investigation (Table 4.5.1). This was because on day 2, 3 out of the 5 isolates were killed by this drug under anaerobic conditions but under aerobic conditions kill was observed for these three isolates at day 7 while under anaerobic

conditions these isolates were still alive on that day (Table 4.5.2). Isolates TF2034 and TF36480 were not killed at any drug concentration under both incubation conditions.

KAN showed no major difference in its killing activity whether the bacilli were incubated aerobically or anaerobically at day 2 (Table 4.5.1). The only isolate where there was a difference was isolate TF2889, as at a concentration of 4XMIC of this antibiotic $\pm$ 100% kill was observed with the anaerobically surviving bacilli but no kill was seen with aerobically incubated bacilli or at lower concentrations anaerobically. In contrast, isolate TF36480 was killed at the MIC concentration and at 4XMIC and this occurred under both conditions and at similar percentage kill. Isolates from the F28 family lineage (TF2889 and TF36480) were the only isolates that experienced kill at day 2 at the concentration of 4XMIC (Table 4.5.1). Only a small percentage of the initial population of isolate TF2889 escaped kill. TF36480 was susceptible under both conditions at this concentration, with $\pm$100% death under both aerobic and anaerobic conditions. By day 7 KAN (Table 4.5.2) showed better lethal activity towards aerobically incubated bacilli as at the concentration of 0.25XMIC, this antibiotic was able to kill almost 100% of TF2889 and MODS11 under aerobic conditions. This is the only time that there was any kill of the bacteria by this anti-TB drug at this concentration. Again, at a concentration of the MIC there was killing of isolates MODS682, TF2889 and TF36480 while there was no kill of bacilli under anaerobiosis. With TF2889 and TF36480 at a concentration 4XMIC, the killing was experienced under both conditions with minor differences, while the MODS11 population was killed when it was grown aerobically, but survived under anaerobiosis. Isolate TF2034 was the only isolate that did not experience any killing by KAN.

Table 4.5.1: Comparison of percentage kill at day 2 of antibiotics towards aerobic and anaerobic surviving MDR *M. tuberculosis* isolates at different concentrations

		TF2034			MODS682			TF2889			TF36480			MODS11		
Antibiotic	**Condition of incubation**	0.25X MIC	1X MIC	4X MIC	0.25 X MIC	1X MIC	4X MIC	0.25 X MIC	1X MIC	4X MIC	0.25 X MIC	1X MIC	4X MIC	0.25 X MIC	1X MIC	4X MIC
INH	Aerobic	0	0	0	0	0	0	0	0	99.93	0	0	0	0	0	0
	Anaerobic	0	0	0	0	0	0	99.96	0	0	0	0	0	0	0	0
RIF	Aerobic	0	0	0	0	0	0	0	0	0	0		0	0	100	0
	Anaerobic	0	99.96	99.99	0	0	0	99.97	99.96	100	0	0	0	0	0	0
OFL	Aerobic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Anaerobic	0	0	0	0	0	100	0	0	99.86	0	0	0	0	0	99.8
KAN	Aerobic	0	0	0	0	0	0	0	0	0	0	99.86	99.9	0	0	0
	Anaerobic	0	0	0	0	0	0	0	0	99.86	0	99.92	99.94	0	0	0

Table 4.5.2: Comparison of percentage kill at day 7 of antibiotics towards aerobic and anaerobic surviving MDR *M. tuberculosis* isolates at different concentrations

		TF2034			MODS682			TF2889			TF36480			MODS11		
Antibiotic	**Condition of incubation**	0.25X MIC	1X MIC	4X MIC	0.25 X MIC	1X MIC	4X MIC	0.25 X MIC	1X MIC	4X MIC	0.25 X MIC	1X MIC	4X MIC	0.25 X MIC	1X MIC	4X MIC
INH	Aerobic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	99.81
	Anaerobic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RIF	Aerobic	0	0	99.88	0	0	100	0	0	0	0		0	0	100	0
	Anaerobic	0	0	100	0	0	99.88	0	0	99.97	0	0	99.9	0	0	0
OFL	Aerobic	0	0	0	0	0	99.61	0	0	99.85	0	0	0	0	0	99.8
	Anaerobic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KAN	Aerobic	0	0	0	0	99.79	99.77	0	99.9	99.98	0	99.81	100	0	0	99.87
	Anaerobic	0	0	0	0	0	0	0	0	99.75	0	0	99.95	0	0	0

4.6 Gene Expression

Gene expression levels were measured to establish whether there was metabolic activity under both aerobic and anaerobic incubation and whether the gene expression was in keeping with the presence or absence of oxygen. All the three genes (*menB*, *sdhA* and *fdxA*) that were chosen for the study were expressed at different levels under the two conditions that the bacilli were harvested from. Isolate TF1538 (susceptible) had the highest expression levels for all three genes under aerobic conditions, while these genes were less expressed under anaerobic conditions. With the MDR isolate TF3203; the three genes were detected under aerobic conditions, while under anaerobic conditions there was no amplification of these genes even thou there was amplification of the endogenous control (16S rRNA) and the method that was used for the extraction of the RNA produced intact RNA (Figure 4.6.1). Gene expression levels of the XDR isolates (TF3228 and TF3229) were the lowest amongst the isolates tested in this study. The *sdhA* and *menB* genes were expressed at higher levels under anaerobic conditions for both isolates as compared to under aerobic conditions, with *menB* being expressed at a higher level under anaerobic conditions in isolate TF3229 (Figures 4.6.2 and 4.6.3). The ferrodoxin gene (*fdxA*) was expressed at a highest level under anaerobic conditions in TF3229 (Figure 4.6.4). This was the only time in our experiments that this gene was upregulated under anaerobic conditions.

Overall all three genes were better expressed under aerobic conditions. *MenB* was expressed more under aerobic conditions in H37Rv; isolates TF1538 and TF3203, while it was down-regulated under anaerobic conditions. The other gene that is involved in energy metabolism in bacteria that was studied was succinate dehydrogenase (*sdhA*). A similar trend is seen with this gene as well.

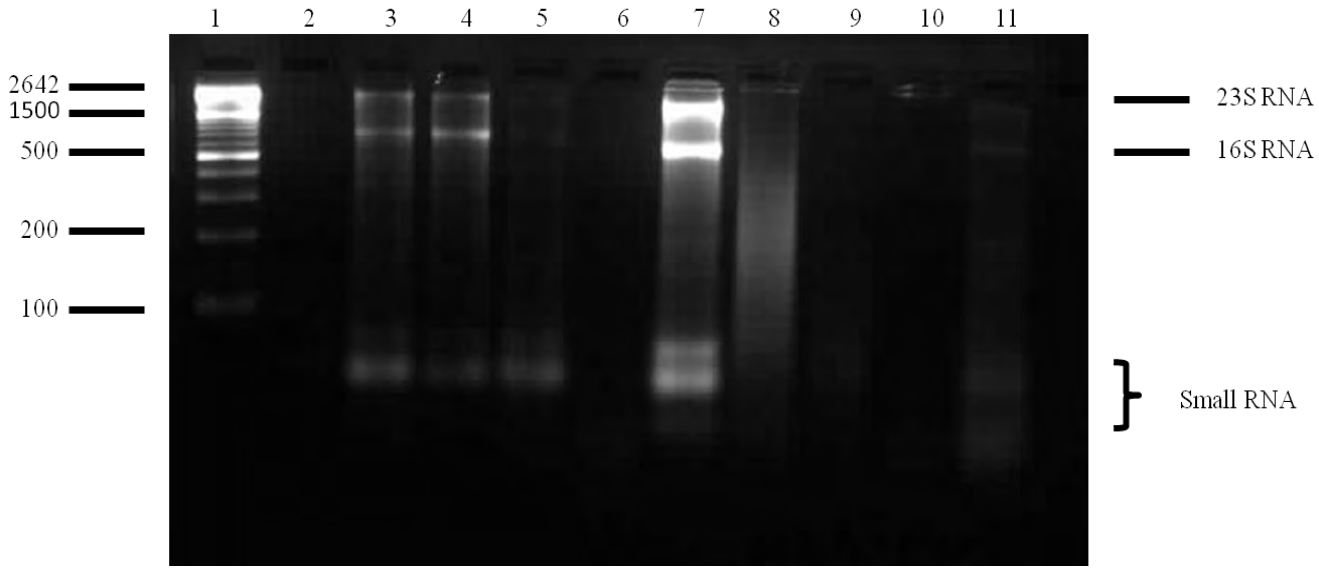

Figure 4.6.1: RNA samples run on a 2% agarose gel at 100V for an hour. Lane 1 – DNA molecular marker XIV (100 bp); Lane 3 – H37Rv; Lane 4 – TF3229; Lane 5 – H37Rv; Lane 7 – TF1001; Lane 11 – TF 1538.

Figure 4.6.1, shows the bands of the RNA that was extracted from the cultures of the isolates in an agarose gel. The 23S rRNA and the 16S rRNA bands are seen to correspond to the known molecular weight sizes of this bacterial RNA.

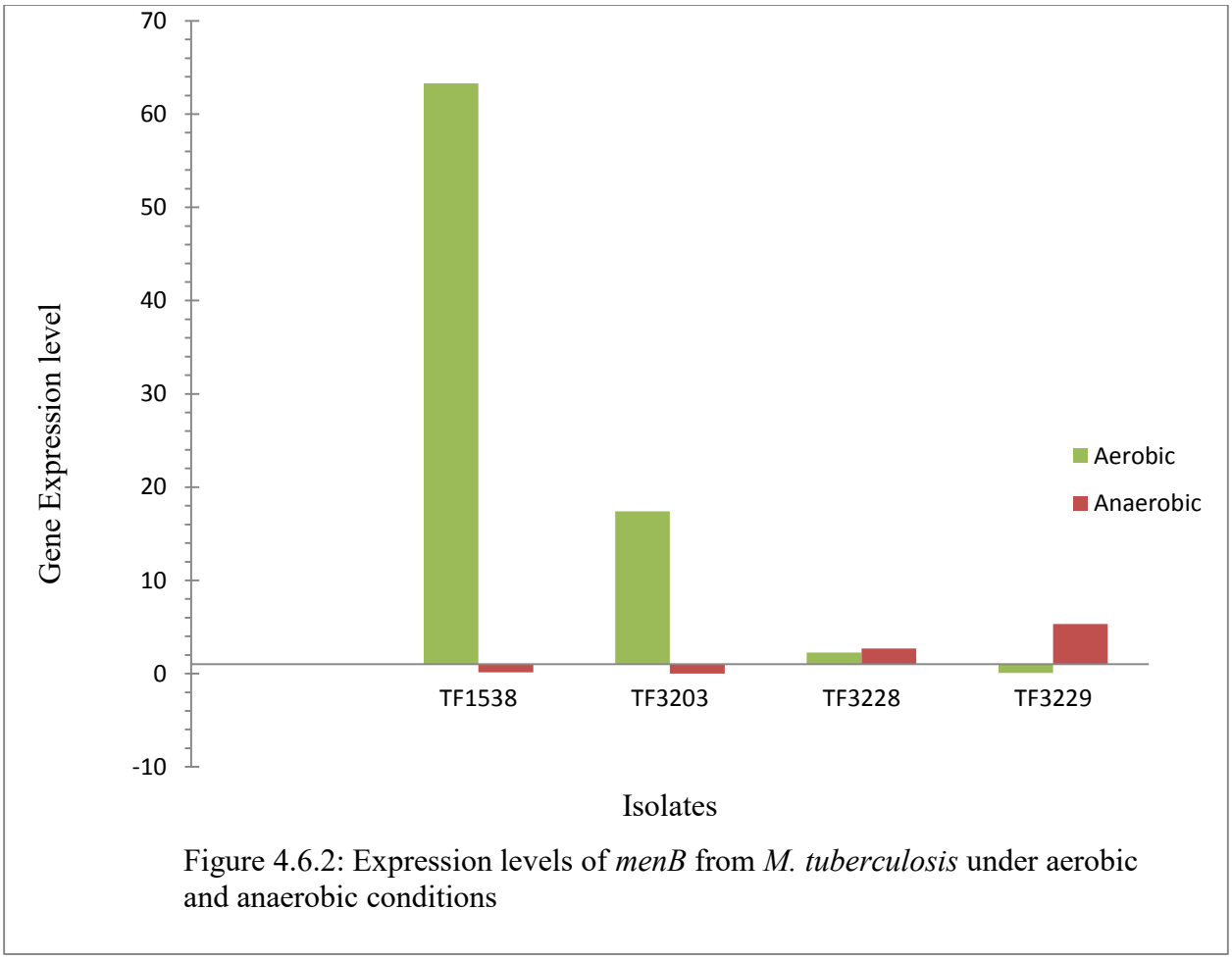

Figure 4.6.2: Expression levels of *menB* from *M. tuberculosis* under aerobic and anaerobic conditions

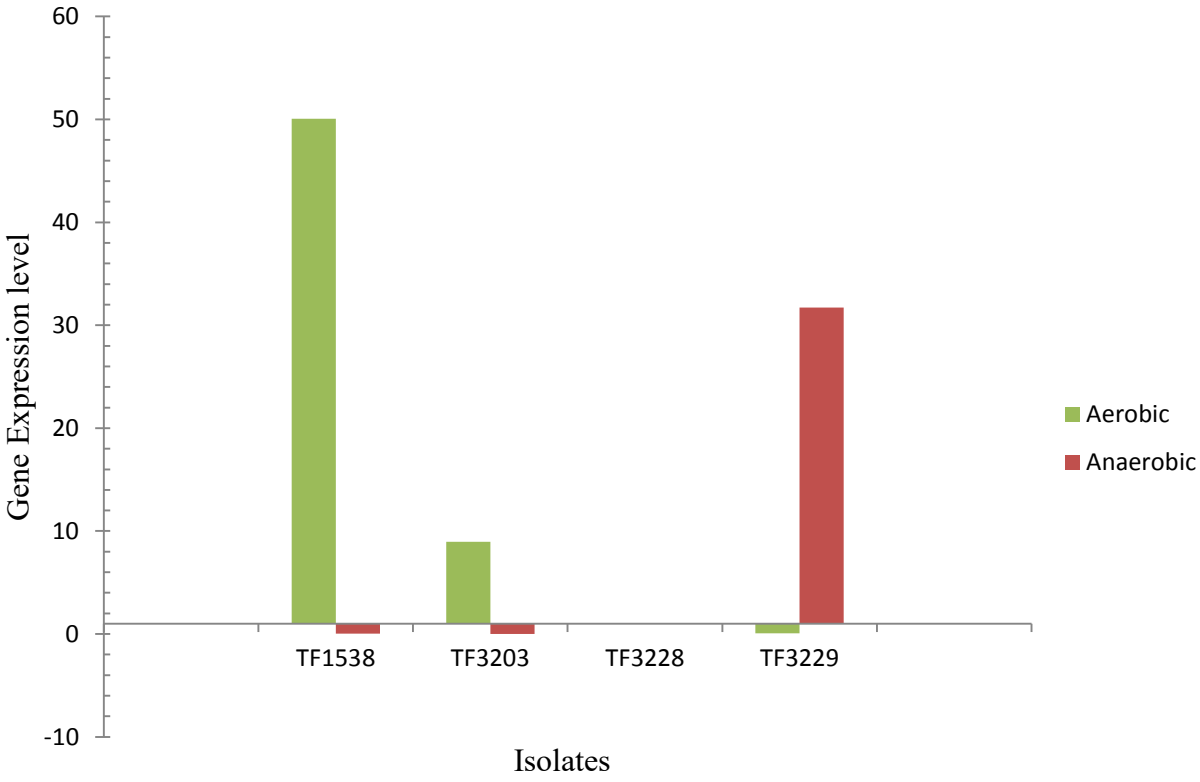

Figure 4.6.3: Expression levels of *sdh*A from *M.tuberculosis* under aerobic and anaerobic conditions.

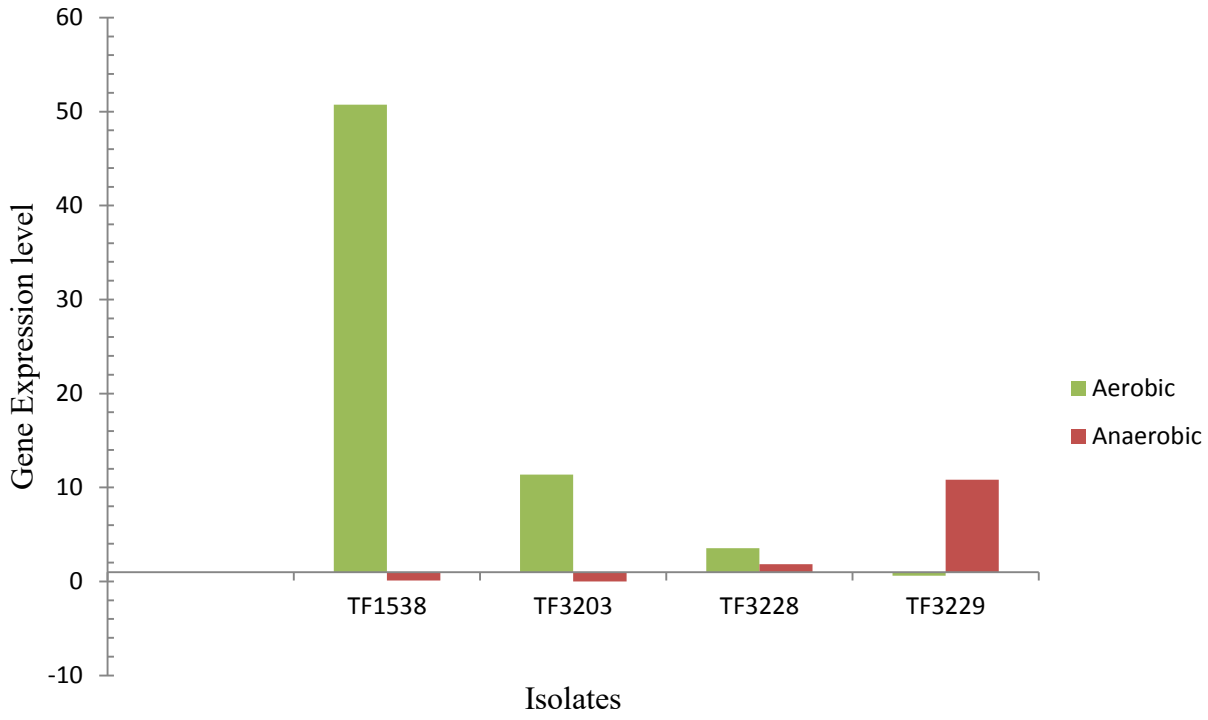

Figure 4.6.4: Expression levels of *fdx*A from *M.tuberculosis* under aerobic and anaerobic conditions.

Chapter 5: Discussion

The low metabolic state, in which some cells of *Mycobacterium tuberculosis* are surviving when inside the host, is thought to be assumed by these bacilli when they encounter stressful environmental conditions that the hosts' immune system utilizes to fight off any potential threats. In this low metabolic state these are referred to as persistent bacilli since they are tolerant to a number of stresses including the anaerobic conditions found in the host's lesions that are not communicating with the bronchial tree (Wayne and Lin, 1982; Wayne and Hayes, 1996). A number of antimicrobial agents that are used for the treatment of tuberculosis (TB) are known to be more active against actively metabolizing, multiplying bacilli as compared to persistent bacteria (Parrish *et al*., 2009). This has been recognized as the possible explanation for the long duration of treatment as research has shown that *M. tuberculosis* found in sputum specimens are exposed to different levels of oxygen saturation and are morphologically different from the bacilli that are grown in the laboratory (Fattorini *et al*., 2013). This is summarized in table 5.1 according to the different locations of the TB lesions in the lungs.

Table 5.1: Replication rate of *M. tuberculosis* under various oxygen concentrations during infection inside the host

Site in the host	Oxygen concentration	Replication Rate
Alveoli	Highly aerobic	Rapid Replication
Inflammation and Necrosis	Microaerophilic	Reduced Replication
Blocked Lesions	Minimal Oxygen	No / Minimal Replication

*Wayne and Lin, 1982; Zhang *et al*., 2012; Fattorini *et al*., 2013

The differences in level of oxygen availability in these three different mycobacterial populations may mean that, they are also physiologically different, which includes the replication rate as well as their metabolic activity. Since antibacterial drugs act by interfering in essential metabolic pathways, such differences may also have an influence on the drug susceptibility of the bacilli if

the drug target is associated with characteristics of bacilli multiplying under specific conditions but not or less active under the other conditions.

In our study, we aimed to expose clinical of isolates of *M. tuberculosis* with different drug susceptibility profiles (e.g. Susceptible, MDR and XDR), to various antimicrobial agents that are currently part of the TB treatment regimens. The isolates were cultured under different environmental conditions with an aim of comparing the level of drug susceptibility of bacilli that are in a similar metabolic state as the different populations of bacilli present in the different lung lesions. We attempted to obtain drug susceptibility profiles of these isolates by determining the minimum inhibitory concentrations (MICs) and compare the values obtained aerobically and anaerobically.

M. tuberculosis is believed to replicate slower or replicate at a negligible rate under anaerobic conditions, low metabolic activities and reduced membrane transport processes (functions associated with ATP generation) are characteristic of *M. tuberculosis* growing under such conditions (Leistikow *et al*., 2010; Turapov *et al*., 2014). This was the reason the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was conducted on a few isolates aerobically and not conducted under anaerobiosis. We believed that since the bacilli would have already be metabolising anaerobically by the time we planned to perform the susceptibility testing (which was also to be done under anaerobiosis), the bacilli would not have produced sufficient ATP to help in converting the MTT substrate into formazan. On a study done by Vistica *et al.*, 1991, they found that cells that are on different phases of growth reduced the MTT differently. As the cells aged, their ability to reduce MTT decreased. They postulated that this was because as the cells age, they produce less NADH / NADPH which is needed in order to reduce the MTT into formazan, therefore there is less of these nucleotides to help in reducing

MTT. Doing an MTT assay under anaerobic conditions would have led to false results in the form of increased MICs under such conditions. Therefore, MICs were performed using the methodology described for the Microscopic Observation Drug Susceptibility (MODS) assay (Caviedes *et al*., 2000) as with this test no indicator needs to be modified for the identification of the MIC and agar based methods would not have worked as previous studies have revealed that *M. tuberculosis* growing under anaerobiosis may lack the ability of forming colonies on agar (Anuchin *et al*., 2009). Once again using this method would have led to false increase of MICs under anaerobic conditions due to underestimating the number of viable bacilli. These concerns were proven to be valid since no growth was obtained in the drug free growth controls under anaerobic circumstances.

MICs under aerobic incubation

Under aerobic conditions, H37Rv had MICs that were greater than the MICs of some of the susceptible isolates. This was the case with both the antimicrobial susceptibility tests (MTT and MODS assays) that were used in this study. Some of these MICs indicated that this strain that was supposed to be fully susceptible was resistant to some of the antimicrobial agents, namely ethambutol (EMB), kanamycin (KAN) and capreomycin (CAP). Resistance of H37Rv to EMB was indicated by both methods that were employed for MIC determination, i.e. MTT and MODS assays, while resistance to KAN and CAP was indicated by the MODS assay only. This reference strain was resistant with an MIC of 16 mg/L to EMB according to the MODS assay, while it had an MIC of 4 mg/L when the MTT assay was employed. 4 mg/L for EMB is susceptible according to the MGIT960 critical concentration while it is resistant according to the BACTEC460 critical concentration. Resistance of this reference strain was unexpected as H37Rv is supposed to be fully susceptible. This observation may be explained by the fact that some

bacilli may have undergone genetic modification in subsequent subcultures (Lakshmi *et al.*, 2012). In a study done by Ioerger and colleagues (2010), it was found that H37Rv from 6 different laboratories had distinct polymorphisms, which they attributed to the different ways these isolates were stored. These modifications may give rise to drug susceptibility changes. Again during the passaging of the bacilli, if a single colony that has a certain mutation (e.g. a mutation that confer drug susceptibility) is selected and propagated, it means that the culture that is later on stored and used from that colony will differ in drug susceptibility from the original culture. It is therefore imperative that the drug susceptibility of this reference strain be evaluated from time to time. The H37Rv strain is used in the Department of Microbiology and Infection Prevention at UKZN for over 10 years. During that time an undetermined number of subcultures have taken place. The discrepancies with the original obtained strain which was fully susceptible, may be based in just one mutation in the *rrs* gene that renders the organism resistant to both KAN and CAP (Alangaden *et al*., 1998; Bastian *et al*., 2001; Zhang and Yew, 2009; Kolyva and Karakousis, 2012). The findings with EMB may be based on the difference in methodologies. This drug is known to be problematic in susceptibility testing.

Overall, the MICs for the susceptible isolates obtained with the MTT assay were either greater than or equal to those determined by the MODS assay. With the drug resistant isolates the reverse of this was true, with exceptions for two isolates, each for a different drug. Variations in the MICs obtained from different drug susceptibility methods are expected as these methods depend on different indicators for the identification of the MIC (Ängeby *et al*., 2012). This observation could be attributed to the fact that the MTT assay is dependent on generation of ATP which is associated with high replication rate. Bacilli that are non-replicating (dormant) produce lower levels of ATP as compared to the actively replicating bacilli (Leistikow *et al*., 2010). The

drug resistant isolates are believed to replicate slower compared to the susceptible isolates due to fitness cost which is caused by the drug resistance trait (Meacci *et al.*, 2005). The MODS assay is based on the growth of the bacilli and the formation of the characteristic whorls of cording bacilli and not on any indicator that changes due to metabolic activity (Caviedes *et al*., 2000). Since drug resistant bacilli replicate slower (Meacci *et al.*, 2005) which may mean that they produce less energy as energy generation is associated with the rate of replication (Leistikow *et al*., 2010), that may mean that the energy may be insufficient to cleave the indicator substrate resulting in colour change, the MICs in the MTT assay should be lower compared to the results from the MODS assay, as it would take more bacteria (drug resistant) than the susceptible bacteria to give sufficient energy to cleave the indicator molecule. With the MODS assay as soon as the typical bacterial growth is visible one can be able to identify the MIC (Caviedes *et al*., 2000). Again the incubation period for the resistant isolates for the MTT assay was double that of the susceptible isolate as there was hardly any colour change when the incubation period was seven days as it was with the susceptible isolates. This further validates our observation about the dependency of the MTT assay on the rate of replication. Although the formation of the microscopically observed bacterial growth in the MODS is also dependent on replication rates, it is unlikely that this would lead to the same delay in observations between the two assays.

The consequences of the differences in the replication rates were also seen in the level of expression of the genes as one susceptible isolate and the reference strain tested showed higher levels of expression of all three genes tested i.e. *menB*, *sdhA* and *fdxA*. Both these organisms replicated at a higher rate in the optimal and conducive aerobic environment which may be accompanied by high level of energy generation and usage of menaquinone and succinate dehydrogenase (products of *menB* and *sdhA* respectively). With both drug resistant isolates, the

expression of *menB* and *sdhA* was lower under aerobic conditions, which may indicate the slower growth rate under this condition compared to the growth rate of the susceptible organisms. Although these genes were down-regulated under anaerobic conditions in the susceptible organism and the MDR isolate and upregulated in the XDR isolates (with both *menB* and *sdhA* being upregulated in one isolate, the other isolate with only *menB* being upregulated), they were still expressed, indicating their essentiality for the survival of the bacilli overall. The products of these genes need to be synthesized as they are important for the maintaining membrane potential in the bacilli (Black *et al*., 2014).

All the MDR and XDR isolates were resistant to both INH and RIF. This was in accordance with the definitions, as MDR isolates are characterized as being resistant to INH and RIF while XDR isolates are MDR isolates with resistance to a fluoroquinolones and an injectable agent (Ahmad, 2011). The XDR isolates together with one classified as MDR, were resistant to ofloxacin (OFL). This potent second line drug is used in the treatment of infections with MDR isolates and is used as a criterion for defining and classifying a strain as an XDR strain (Malik *et al*., 2012). All the XDR isolates that were used in this study were from theF15/LAM4/KZN family while one OFL resistant MDR was from the F28 family. Such isolates are now known as pre-XDR.

The XDR isolates were only susceptible to linezolid (LIN) out of all the 9 antimicrobial agents that were tested. This antimicrobial agent was the only drug tested in this study to which no resistance was found. This was expected as LIN is one of the newest drugs (Long and Vester, 2012) and little drug resistance has been reported to this drug in general (Vardakas *et al*., 2009).

Most of the isolates that were resistant to either KAN or CAP were also resistant to the other. Resistance to both these antibiotics is associated with mutations in the *rrs* gene which may

explain why resistance to one of the two drugs would mean resistance to the other (Alangaden *et al*., 1998; Bastian *et al*., 2001; Zhang and Yew, 2009; Kolyva and Karakousis, 2012). In a study done by Bastian *et al*., (2001) in Europe on MDR strains, they found that 69% of KAN resistant strains had an MIC that was greater than 20 mg/L. In our study similar results were found as 9 of the 13 (69%) drug resistant isolates had MICs that were equal to or greater than 32 mg/L. Eight of these9 (89%) isolates had an MIC that was greater than 1024 mg/L.

Only one isolate from the group of isolates classified as susceptible was resistant to CAP with an MIC of 8 mg/L. This was the only drug that this isolate was resistant to. Ten of the 13 (77%) resistant (both MDR and XDR) isolates showed resistance to STR, with two of these having an MIC of 2 mg/L, a value that is difficult to interpret since 2mg/L is the proposed critical concentration in tests applying the BACTEC460. This means borderline susceptible. However, the critical concentration according to MGIT960 is 1 mg/L which makes these two isolates resistant. Since there is no proposed critical concentration for tests in home-made Middlebrook 7H9, we have labelled them susceptible. This percentage of 77% STR resistance among MDR isolates is slightly lower compared to the findings from studies done among inmates in prisons in the former USSR, where they reported that 97.1% of the MDR strains to be resistant to STR (Bastian *et al*., 2001). All the isolates that were resistant to STR showed resistance to either or both KAN and CAP. Resistance to all three these antimicrobial agents in these isolates could be because of a mutation in the *rrs* gene as such mutations are cited as the reason for resistance to all these structurally related drugs (Alangaden *et al*., 1998; Bastian *et al*., 2001; Zhang and Yew, 2009; Kolyva and Karakousis, 2012).

MICs under anaerobic conditions

Although our primary aim was to assess and compare the MICs performed under aerobic and anaerobic conditions, of selected *M. tuberculosis* isolates performed, we were unable to do this as obtaining MICs under anaerobic conditions was not successful. No growth was observed in the drug free growth controls when the 24-well tissue culture plates used in the MODS assay were incubated anaerobically. For the determination of the anaerobic MICs; representatives from the different genotype families were chosen; namely the F11, F28 and F15/LAM4/KZN families as well as some unique isolates (not clustering according to IS6110 fingerprinting) and H37Rv. No growth was seen with the cultures that were first grown under anaerobiosis (cultures that had been incubated through the standing culture method) and then subjected to MIC determination under anaerobic conditions and the cultures that were first grown aerobically and then subjected to MIC determination under anaerobic conditions.

Although *M. tuberculosis* has been classified as a strict aerobe for centuries and in some literature this still remains the case, Wayne and Hayes in 1996, demonstrated that *M. tuberculosis* can grow under anaerobic conditions. The bacilli grew slowly as witnessed by the slow increase in turbidity and non – significant change in the colony forming units (cfu) counts and the Wayne and Hayes method has been a method that has been adapted by most scientists in culturing *M. tuberculosis* anaerobically. Recent studies confirm that *M. tuberculosis* does replicate under oxygen deprived conditions with as little as 1% (Voskuil *et al*., 2004) and 1.3% oxygen as long as the environment is supplemented with carbon dioxide (Vilchèze *et al*., 2011). We provided a similar environment (mixed gas consisting of 5% carbon dioxide; 85% nitrogen and 10% hydrogen) for the bacilli during the anaerobic incubation of the plates for the

determination of MICs and also extended the incubation period to 40 days, but there was no growth observed.

Interestingly, following the 40 days of no growth under anaerobic conditions, these plates were incubated aerobically, resulting in visible growth within a week. This, together with growth on the agar plates (incubated aerobically with 5% CO_2) that were inoculated at the same time with the same inoculum that was used to inoculate the MODS plate validated that there was viable bacilli in the inoculum and still had the ability to replicate. This was not surprising as *M. tuberculosis* is known that following its dormant state in the non-growth favouring hypoxic environment it resuscitates once it is introduced to an oxygen rich environment (Voskuil *et al*., 2004; Kolyva and Karakonsis, 2012; Nickel *et al*., 2012). These observations also indicated that the lack of oxygen was the hindering factor in producing visible growth under anaerobic conditions as it is the only factor that was different between the two scenarios. The lack of visible growth under anaerobic conditions in the MODS assay with the isolates that we worked with could be because the incubation period was not sufficient to provide visible growth. Although the incubation period under anaerobic conditions was almost three times longer (40 days) than the incubation period under aerobic conditions (14 days), it was still not sufficient to give visible growth. This can be attributed to the fact that, *M. tuberculosis* replicates slower under anaerobic conditions, to an extent that it is negligible compared to an already slow replication rate of this pathogen even under optimal environmental conditions that include high oxygen concentrations. Under normal optimal conditions *M. tuberculosis* takes 16 hours on average to replicate (Beste *et al*., 2009) which is extremely slow compared to most bacteria that divide in 20 to 30 minutes.

We believe that if the plates were incubated for a longer period in the anaerobic environment we may have been able to observe growth on the plates as the lack of visible growth on the plates is not due to the fact the bacilli is dead but it is due to the fact that the bacilli lowers its metabolic activity and may stop replicating (enter the stationary phase) as some studies have suggested (Anuchin *et al*., 2009). In a study done by Anuchin *et al*., 2009, they showed that the longer the bacilli was incubated under anaerobic conditions the lower the colony forming unit counts became. But when they conducted the Most Probable Number assay, the opposite was observed. Hence we believe that in our study if we had incubated the plates for longer than we did we may have been able to observe growth. As with some studies growth has been witnessed in the anaerobic environment, but this was due to an extended incubation period of 6 – 9 months (Zhang *et al*., 2012). *M. tuberculosis* can actually retain its viability, surviving under anaerobiosis for 12 years (Wayne and Lin, 1982). However in our study, incubation for 6 to 9 months would have had its own problems as the tests could then not have been performed in the small volumes of broth used in the MODS test. The media started drying up after 40 days. And also that the duration of efficacy of the drugs in solution after prolonged exposure to a temperature of 37^{o}C duration is questionable.

During hypoxic respiration *M. tuberculosis* changes metabolically and morphologically. Morphological changes include the thickening of the cell wall (Zahrt, 2003; Hett and Rubin, 2008), which has an influence on the accessibility of the drugs to the bacilli. The cell wall components are also important in regulating persistence of *M. tuberculosis* (Wang *et al*., 2011). One of the metabolic changes that occur during hypoxic respiration is the upregulation of certain genes such as the genes that are part of the DosR regulon. In our study we utilized *fdxA* which is part of this regulon. Although this gene was expressed under both aerobic and anaerobic

conditions there was increased expression under the latter conditions in some of the drug resistant isolates. The expression of the genes from the DosR regulon is vital in ensuring survival of the bacilli under the 'lethal' anaerobic conditions (Fang *et al*., 2012).

Killing experiments

The time-kill experiments were conducted to see whether oxygen deprivation made organisms less or more susceptible to antibiotics. We choose to use MDR isolates for this since these isolates could potentially show an increase in susceptibility to INH and RIF because of their known resistance under aerobic conditions. Likewise, the same isolates, being susceptible under aerobic conditions to KAN and OFL, could show an increased tolerance to these drugs under oxygen deprivation.

The effect of anaerobiosis of *M. tuberculosis* on susceptibility to INH and RIF differed. With INH, there was no effect of anaerobiosis as there was no kill of the isolates under anaerobic conditions. As expected, there was also lack of killing under aerobic conditions. The minimal bactericidal activity shown by INH under anaerobic conditions was in agreement with what other studies have found in terms of its decreased potency towards non-replicating bacilli as its targets are mainly the metabolic pathways that are active during replication (Parrish *et al*., 2009). *M. tuberculosis* under anaerobic conditions is known to replicate at a slower rate or to be non-replicating. This would mean that processes such as mycolic acid synthesis which is the target for INH are not less available for anti-TB drug to act on under anaerobic conditions. Since INH would have fewer sites to act on, it will not be able to kill a lot of bacilli, hence less bactericidal activity towards *M. tuberculosis* growing anaerobically.

With RIF which is a protein synthesis inhibitor that binds to the DNA-dependant RNA polymerase during transcription of DNA to RNA (Wayne and Hayes, 1996), the opposite was observed. Bacilli growing under anaerobic conditions showed increased susceptibility when compared to the bacilli growing aerobically. This antimicrobial agent has been said to have better activity against non-replicating bacilli (Deb *et al*., 2009; Joshi, 2011) and has demonstrated some bactericidal activity in environments with minimal oxygen (Wayne and Hayes, 1996). In this study similar activity was observed. The possible reason for the better killing shown by this antibiotic under anaerobic conditions is that there is less gene transcription, resulting in decreased amounts of the drug target e.g. RNA polymerase available under such conditions. This decreased transcription under anaerobic conditions is due to the fact that under oxygen deprived conditions there is only 2% of protein synthesis that is occurring (Filippini *et al*., 2010) and that synthesis of DNA is terminated (Wayne and Hayes, 1996), resulting in decreased transcription. This means there is less target sites for RIF to act on. As a result fewer RIF molecules are needed to stop transcription hence it is more potent against non-replicating bacilli.

With OFL and KAN, the effect of oxygen deprivation on the killing potency differed. By day 2 susceptibility to OFL under anaerobic conditions increased, but by day 7 it decreased. This could be because at day 2, the percentage of cells that went into dormancy was lower than at day 7. OFL as a DNA synthesis inhibitor, will still have an impact (able to kill) on the anaerobic incubated bacilli as they will still be replicating at this stage. The level of dormancy increases, with longer incubation. Hence, the bacilli become more tolerant to OFL (Deb *et al*., 2009) the less active the bacilli are, the less DNA synthesis activity is happening and therefore decreased number of target sites for the drug to act on. With KAN the opposite was seen. By day 2 there

was no change in the susceptibility of the bacilli incubated under anaerobic conditions. But by day 7 there was a decrease in the susceptibility of *M. tuberculosis*. This is to be expected since the transport over the cell wall of aminoglycosides is mainly by proton motive force, a process that requires aerobic metabolism. This class of drugs can also enter the bacterial cell by diffusion but that process is much slower and is affected by the thickness of the cell wall. Changes in the cell wall permeability are highly associated with non-replicating bacilli as the cell wall becomes thicker under anaerobic conditions (Zahrt, 2003). If the cell wall is thicker, this will have an influence in the uptake of the drug into the inside of the bacilli.

It should also be noted that KAN is a protein synthesis inhibitor (Mitchison and Davies, 2007) which binds to the 30S subunit resulting in the inhibition of translation (Kolyva and Karakousis, 2012). Since there is less protein synthesis during anaerobiosis as witnessed in a study done by Filippini *et al.* (2010) where there was only about 2 % of protein synthesis occurring on 50 day old microaerophilic bacilli, then this drug would have less sites to act on.

The anaerobically incubated bacilli were more susceptible to the drugs by day 2 compared to by day 7. This was witnessed with RIF, OFL and KAN. This could be because may be the concentration of the drug does not remain constant throughout the incubation period; it may be inactivated during that period. Therefore, the efficacy of the drugs dissolved in the media and incubated at 37ºC is unknown and questionable. If this is the case, it will mean that the lack of killing that is seen by day 7 with some drugs while there was killing by the second day may be because the drugs concentrations may have reduced to sub-lethal concentration. And this concentration may not be equivalent to the concentration at day 2. If this is true, then that would mean that it is not because increased tolerance but decreased drug concentration during the incubation period.

RIF killed more effectively under anaerobic conditions. Its potency appeared to be time dependant as by day 2 under anaerobic conditions there was killing at lower concentrations while on day 7 kill only occurred at the highest concentration tested. Since the kill at the lower concentrations on day 2 was not 100%, the persisters that were not killed seem to have grown again by day 7.As RIF is a concentration dependant drug (Jayaram *et al*., 2003), this may mean that this drug's potency decreases as incubation continues. OFL displayed the same phenomenon where susceptibility to this antibiotic under anaerobic conditions increased by day 2 but by day 7, it had decreased.

Another possibility is that as the bacilli spend more time under anaerobic conditions, the more their metabolism adapts to being anaerobic hence possessing more anaerobic characteristics. This may mean that the antibiotics will have fewer sites to act on. Bacilli that are surviving under anaerobiosis are said to be tolerant to a number of anti-TB drugs as these target actively growing bacilli (Parrish *et al*., 2009). The longer the bacilli are surviving anaerobically, the more they become dormant, hence the more they become tolerant to the drugs, i.e. the higher the drug concentration that would be able to kill such bacilli. This likely results from low replication rate or absence of replication of the bacilli under anaerobic conditions which results in tolerance to some antibiotics (Gomez *et al*., 2004). This characteristic is phenotypic and is inheritable (Fattorini *et al.*, 2013). In 2004, Gomez *et al.*, postulated that the persistent state assisted drug sensitive *M. tuberculosis* to be resistant or, better tolerant to some drugs (Daniel *et al*., 2011), and in another study done by Deb *et al.*, (2009) they observed that the longer *M. tuberculosis* was grown under stressful conditions the more it became drug tolerant. In this study the decrease in the potency of the drugs was only seen under anaerobic conditions, whereas under aerobic conditions the opposite was witnessed. This reason seems more valid than the former

reason (decrease in the potency over time) as under aerobic conditions the killings were greater by day 7. This was the case with RIF, OFL and KAN.

There were differences in the drug susceptibilities under anaerobic conditions amongst the isolates that were used for the killing experiments. We hypothesise that since these isolates are from different genetic backgrounds they may adapt to anaerobiosis at different rates; hence this will have an influence in their level of drug tolerance.

There is still a lot of research that needs to be done as the MICs under anaerobic conditions are still unattainable. This makes it hard to evaluate the susceptibility of non-replicating *M. tuberculosis*. Although we were not able to determine the MIC under such conditions we were able to subculture the bacilli that had been exposed to drugs. Although the method used in the time-kill experiment was a more labour intensive and time consuming with a longer turnaround time, we were able to get an insight on the vulnerability of the bacilli towards the drugs. It indicates that bacilli from different genetic lineages take different duration to adapt to oxygen depletion, which may influence the bacilli's drug tolerance.

These results highlight the need for more drugs that are active against both aerobically and anaerobically metabolising *M. tuberculosis* as there was no drug from those tested in the time-kill experiments that showed similar activity against both bacterial sub-populations. There is still a lot that is unknown about this bacilli's anaerobic persistence, which seems to be a major contributor to its success as a pathogen. Hence, this study needs to be done more extensively, in the form of quantitative time-kill experiments as this will give more insight in the changes of the drug susceptibility profiles of the *M. tuberculosis* that is surviving under anaerobiosis.

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Appendix A

A.1 Media

A.1.1 Middlebrook 7H9 Broth for isolation of the single colonies

4.7g Middlebrook 7H9 powder

2ml glycerol

0.5ml Tween 80

900ml distilled water

The above materials were mixed and autoclaved at 121ºC for 15 minutes

100ml OADC was added once the media was cool.

A.1.2 Middlebrook 7H9 Broth

4.7g Middlebrook 7H9 powder

2ml glycerol

900ml distilled water

The above materials were mixed and autoclaved at 121ºC for 15 minutes

100ml OADC was added once the media was cool.

A.1.3 Middlebrook 7H9 Broth MODS Assay

5.9g Middlebrook 7H9 powder

3.1ml glycerol

1.25g casitone

900ml distilled water

The above materials were mixed and autoclaved at 121ºC for 15 minutes

OADC was added on the day of the assay at a quantity of 10%.

A.1.4 Middlebrook 7H11 Agar

21g Middlebrook 7H11 powder

5g L-Asparagine monohydrate

1g casitone

5ml glycerol

900ml distilled water

The above materials were mixed and autoclaved at 121ºC for 15 minutes

100ml OADC was added once the media was cool.

For the selective media the antibiotics mentioned below were added at this point as well.

Antibiotics for selective media:

200.000 units/L of Polymixin B

20 mg/L of Amphotericin B

100 mg/L of Carbenicilin

20 mg/L of Trimethoprim

A.1.5 Storage Media

2g Proteose peptone

16ml glycerol

Make up to 200ml with distilled water

The above reagents were mixed and autoclaved at 121ºC for 15 minutes

Appendix B

B.1: MTT Assay reagents

B.1.1: 20% Sodium Dodecyl Sulphate

10g Sodium Dodecyl Sulphate was dissolved in distilled water to a volume of 50ml.

B.1.2: 50% Dimethylformamide

25ml Dimethylformamide

25ml distilled water

The two reagents were mixed.

B.1.3: MTT Solution

25mg MTT powder was dissolved in Phosphate Buffer Saline to a volume of 5ml.

Appendix C

C.1: Gel Electrophoresis

C.1.1: 2% Agarose gel

4g agarose

200ml 1XTris-acetate-EDTA (TAE) Buffer

C.1.2: 1XTris-acetate-EDTA (TAE) Buffer

20ml 50X TAE Buffer

980ml distilled water