Mutation frequency in drug-susceptible clinical isolates of *Mycobacterium tuberculosis* under aerobic and anaerobic conditions

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Submitted in partial fulfilment of the requirements for the degree of Master of Medical Science in the Department of Infection Prevention and Control, Nelson R Mandela School of Medicine, University of KwaZulu-Natal

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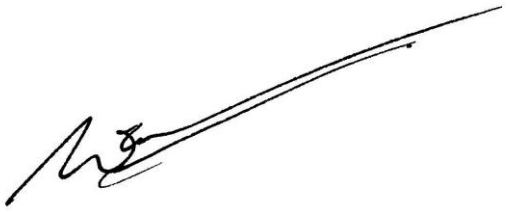
Declaration

I, Lavania Joseph (Student Number: 206508595), declare that this is my own unaided work. This work has not been submitted previously to this or any other University. Where I have used the work or ideas of others, the appropriate referencing conventions have been adhered to.

Lavania Joseph



Prof A. Willem Sturm

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Presentations

POSTER PRESENTATIONS

1. **Mutation frequencies in currently-circulating clinical strains of *Mycobacterium tuberculosis***.
   Lavania Joseph and A. Willem Sturm
   5th FIDSSA Conference 2013: Changing Attitudes, Drakensburg, KwaZulu-Natal

2. **Mutation frequency in a drug-susceptible clinical isolate of *Mycobacterium tuberculosis* belonging to the Beijing genotype family**
   Lavania Joseph and A. Willem Sturm
   UKZN College of Health Sciences Research Symposium 2012

ORAL PRESENTATION

1. **Mutation frequencies in currently circulating clinical strains of *Mycobacterium tuberculosis***
   Lavania Joseph and A. Willem Sturm
   UKZN College of Health Sciences Research Symposium 2013

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List of abbreviations

ACP	Enoyl-acyl carrier protein
CFU	Colony Forming Units
dAMP	Adenine monophosphate
dATP	Adenine triphosphate
DNA	Deoxyribonucelic acid
DOTS	Directly-Observed Treatment, Short Course
EIS	Enhanced intracellular survival
F15/LAM4/KZN	KZN
HIV	Human Immunodeficiency Virus
INH	Isoniazid
IPT	Isoniazid Preventative Therapy
KAN	Kanamycin
KZN	KwaZulu-Natal
LTBI	Latent TB Infection
MIC	Minimum Inhibitory Concentration
MDR	Multidrug-resistant
OD	Optical density
OH·	Hydroxyl radical
OFX	Ofloxacin
PAS	Para-aminosalicylic acid
PBS	Phosphate Buffered Saline
QRDR	Quinolone Resistance Determining Region
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RNA	Ribonucleic acid

RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
STR	Streptomycin
TB	Tuberculosis
TDR	Totally drug-resistant
WHO	World Health Organisation
XDR	Extensively drug-resistant

Ethics

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, under the ethics number BCA274/09.

CHAPTER 1: INTRODUCTION

Tuberculosis (TB) remains the leading cause of death due to a single infectious agent, *Mycobacterium tuberculosis* (*M. tuberculosis*). The disease is a major public health problem and is responsible for approximately 1.7 million deaths annually worldwide (Sandgren et al. 2009). It ranks number one among causes of death in South Africa (Statistics South Africa 2014).

The two main outcomes of infection with *M. tuberculosis* are active disease and latent infection (Parrish et al. 1998). Active disease is characterised by the presence of dividing *M. tuberculosis* bacilli able to accumulate mutations leading to resistance. To avoid treatment failure due to mutations in genes coding for drug targets, active TB is treated with a combination of four drugs in the first two months of a six month treatment course (Bass et al. 1994). In this initiation phase of treatment, the bacterial load is supposed to be high and therefore, the number of mutating bacterial cells is also high. The four drug treatment regimen is based on the reduced likelihood of two or more mutations arising in a single bacterium (Johnson et al. 2006). In contrast with active disease, latent TB infection is asymptomatic (Flynn & Chan 2001) and thought to be characterised by dormant, non-replicating bacilli (Wayne 1994). Since dormant bacilli are thought to lack the capacity to acquire resistance conferring mutations, latent TB is treated by prophylactic therapy using a single drug, usually isoniazid (Sterling 2008).

It is well established that there are differences between strains of *M. tuberculosis* with respect to their ease of spread within the human population. One of the more successful strains

globally is the Beijing strain (Bifani et al. 2002; Glynn et al. 2002; Hanekom et al. 2011), with other strains predominating in different parts of the world. These successfully spreading strains also predominate among the drug resistant cases of TB.

A number of resistant strains are known to circulate within the KwaZulu-Natal province of South Africa. This includes strains of the Beijing genotype and strains of the F15/LAM4/KZN (KZN) genotype (Ashiru et al. 2010). Both are found more commonly in patients with resistant TB, with the Beijing strain being multidrug resistant (MDR) and the KZN strains showing a variety of resistance patterns including extensive drug resistance (XDR) and total drug resistance (TDR) (unpublished).

The frequency of mutation acquisition leading to drug resistance is thought to be strain-dependent (Brown et al. 2010). Based on the epidemiological association of the Beijing strain with multidrug resistance globally, it is presumed to acquire resistance conferring mutations at higher frequencies than others. However, there is significant discordance in the literature regarding strain-based mutation acquisition (Werngren & Hoffner 2003; de Steenwinkel et al. 2012; Ford et al. 2013).

Given the limited clarity regarding the mutational capacity between strain families and during latent infection, the mutation frequencies will be compared between isolates of the Beijing and KZN lineages, each under aerobic and anaerobic conditions. For the work presented here, anaerobic incubation conditions are proposed as a proxy for latency (Wayne & Hayes 1996; Koul et al. 2008; Rao et al. 2008; Tan et al. 2010).

CHAPTER 2: LITERATURE REVIEW

2.1. Burden of tuberculosis in South Africa

Decades after the introduction of the first anti-tuberculosis drugs, tuberculosis (TB) remains the leading cause of death due to an infectious agent in South Africa (Statistics South Africa, 2014). Of particular concern is the emergence of resistant strains, which greatly threaten TB control programmes (Moodley et al. 2011). These include multidrug-resistant (MDR) organisms, which are resistant to the most effective first-line drugs isoniazid and rifampicin, and extensively drug-resistant (XDR) organisms, which are MDR with additional resistance to one of the second-line injectables (amikacin, capreomycin or kanamycin) and the fluoroquinolones (Ioerger et al. 2009). Compounding this, totally drug resistant (TDR) *M. tuberculosis* strains have been reported in Iran (Velayati et al. 2009), Italy (Migliori et al. 2007), India (Udwadia et al. 2012) and most recently, South Africa (Klopper et al. 2013). South Africa ranks amongst the countries with the highest burden of TB (Figure 2.1).

KwaZulu-Natal currently ranks as the province with the highest mortality rate due to TB in South Africa (Gandhi et al. 2014). The two most predominant strains currently circulating within this province are the W-Beijing strain and the F15/LAM4/KZN (KZN) strain (Ashiru et al. 2010).

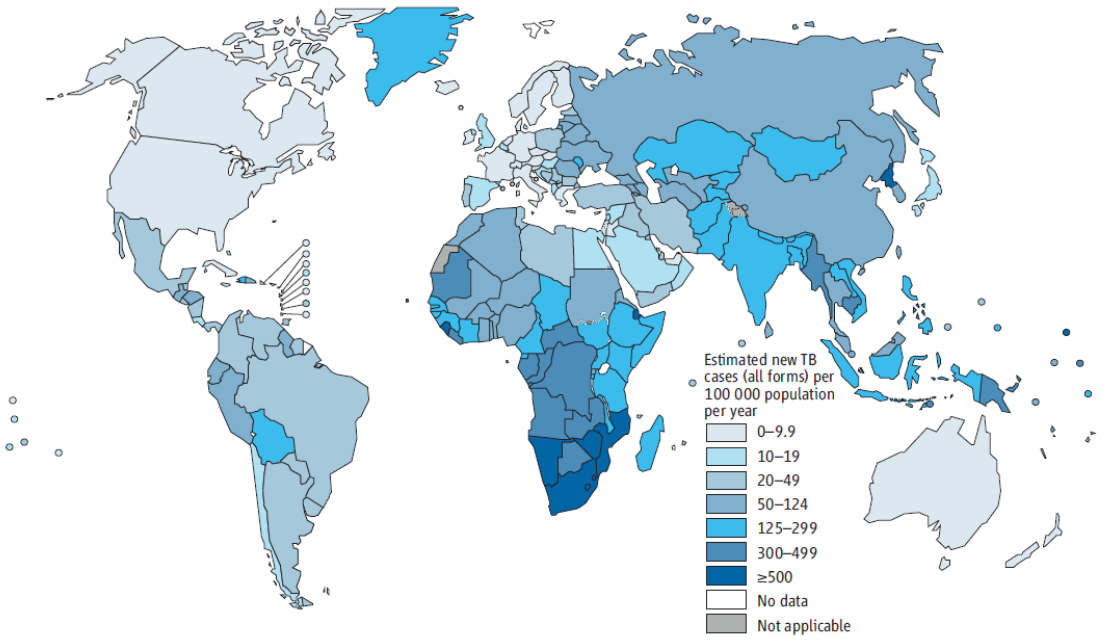

Figure 2.1: Estimated TB incidence including MDR and XDR in 2012

(World Health Organization 2013)

2.2. *Mycobacterium tuberculosis* strain families in South Africa

2.2.1. The Beijing family

The Beijing strain was first identified via IS6110 restriction fragment length polymorphism (RFLP) fingerprint analysis in 1995 in China, and was found to be similarly distributed in adjacent countries including South Korea and Thailand (van Soolingen et al. 1995). Based on the high frequency of isolates observed in the Beijing region, this family of strains was thought to have originated in this city; hence the family name "Beijing" was coined.

The first clonal outbreak of MDR-TB was observed in New York City in the early 1990s (Frieden et al. 1993; Bifani et al. 1996). The causative strain was found to be resistant to first-line therapy, and was associated with rapid spread in populations with a high human immunodeficiency virus (HIV) prevalence, including prisons (Valway et al. 1994). This strain, initially designated strain "W", was later identified as the Beijing strain, and was found to be responsible for numerous outbreaks in South-East Asia (van Soolingen et al. 1995; Agerton et al. 1999).

Many epidemiological studies have demonstrated high levels of resistance associated with the disease outbreaks caused by strains belonging to the Beijing family (Bifani et al. 2002; Brown et al. 2010). The global dissemination of the Beijing family of strains (Figure 2.2), and the concomitant emergence of drug resistance within this family, has led to its classification as the most "successful" family of *M. tuberculosis* strain (Hanekom et al. 2011).

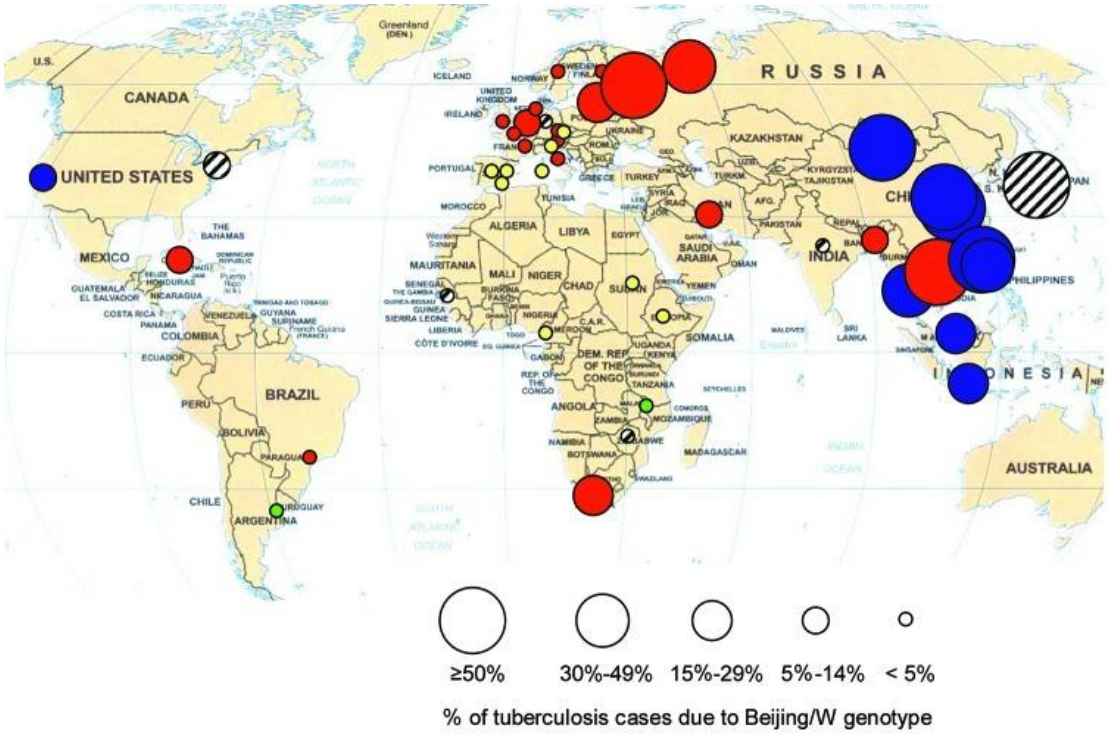

Figure 2.2: Global distribution and percentage of tuberculosis cases caused by Beijing strains. The size of the circle represents the percentage of TB cases due to the Beijing genotype. The colour of the circle represents the distribution and association of these Beijing genotype strains with drug resistance. Blue: stable distribution with no known association with drug resistance. Red: increasing distribution with associated drug resistance. Green: increasing distribution with no known association with drug resistance. Yellow: Beijing genotype is absent. Striped: distribution and association with drug resistance unknown.

In South Africa, such cases are increasing in distribution and are also associated with resistance.

(European Concerted Action on New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis 2006)

2.2.2. The KZN family

Following the advance of the HIV epidemic in South Africa, high rates of TB disease were reported locally, with the highest rate reported in KwaZulu-Natal (Abdool Karim et al. 2009). The first reported cases of XDR-TB in South Africa were identified in KwaZulu-Natal in 2005 (Gandhi et al. 2006). Spoligotyping of isolates from infected patients within KwaZulu-Natal revealed that one particular genotype was responsible for these infections (Gandhi et al. 2006). These isolates were resistant to isoniazid, rifampicin, the fluoroquinolones and kanamycin and were thus termed "extensively drug resistant" (XDR). Based on its origin, the causative strain was termed the "KwaZulu-Natal strain" (World Health Organization 2006; Pillay & Sturm 2007). Based on spoligotype patterns (Streicher et al. 2007), this strain was later classified as the F15/LAM4/KZN strain (Pillay & Sturm 2007), henceforth referred to as the KZN strain.

The first multidrug resistant variant of the KZN strain was identified in 1994, and exhibited resistance to isoniazid, rifampicin and streptomycin (Pillay & Sturm 2007). By means of RFLP analysis, it was shown that this variant acquired resistance to both first and second-line drugs, eventually culminating as extensive drug resistance in 2001 (Figure 2.3).

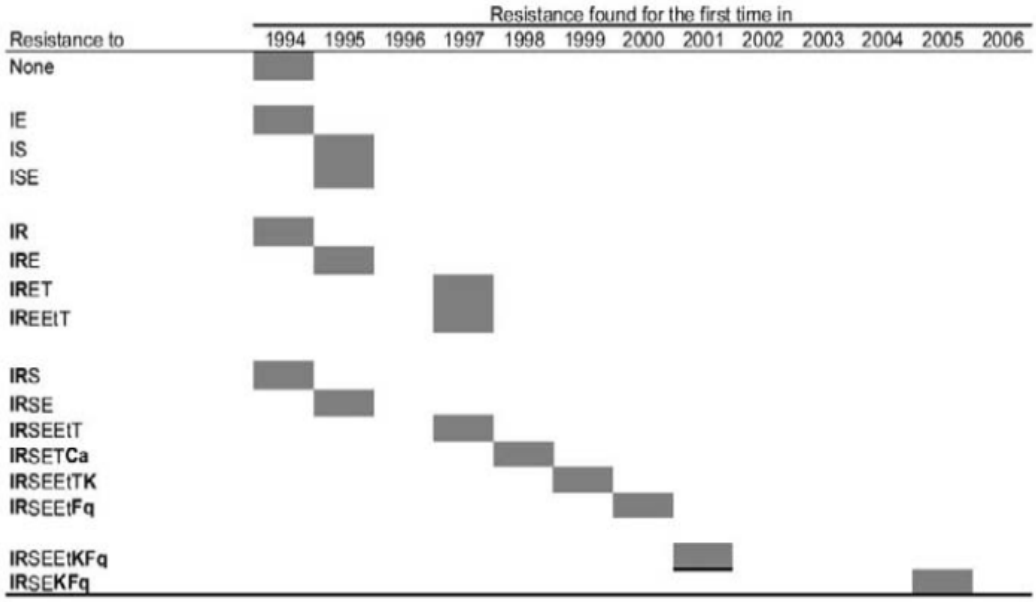

Figure 2.3: Progression of the F15/LAM4/KZN strain from multidrug resistant to extensively drug resistant (Pillay & Sturm 2007)

An important observation of this study was that the first multidrug resistant variant, from which extensive drug resistance developed in 2001, harboured additional resistance to streptomycin. This suggests a possible association between streptomycin resistance and extensive drug resistance within the KZN family. Furthermore, this suggests that the addition of streptomycin to the first-line regimen for patients that needed retreatment was the driving force for the development of the XDR form of the KZN strain (Pillay & Sturm 2007). While epidemiological studies have shown an association between *M. tuberculosis* genotypes and resistance, the direct link between mutational capacity and levels of resistance development has not yet been fully elucidated.

2.3. Development of anti-tuberculosis therapy

Treatment of tuberculosis differs from that of other bacterial infections. *M. tuberculosis* is slow-growing, which may affect drug activity. In addition, the characteristic lipid-rich cell wall of *M. tuberculosis* is thought to render the bacterium impermeable to polar antibiotics, including beta-lactam drugs (Finch 1986).

The discovery of streptomycin in 1946 marked the beginning of antibiotic-based anti-tuberculosis therapy (Youmans & Feldman 1946), with early clinical studies showing a reduction in bacterial load and mortality in patients placed on streptomycin monotherapy. However, this was followed by relapse, accompanied by the appearance of streptomycin resistance (Crofton & Mitchison 1948). This promptly led to the introduction of combination therapy using streptomycin and para-aminosalicylic acid (PAS), in order to reduce the emergence of resistance (Graessle & Pietrowski 1949; Fox & Sutherland 1956).

Isoniazid was discovered shortly afterwards in 1952 (Knox et al. 1952), and has since remained the backbone of anti-tuberculosis therapy due to its effective early bactericidal activity, low minimum inhibitory concentration (MIC) and minimal side effects. Early clinical studies using combinations of streptomycin, PAS and isoniazid led to the design of three-drug regimes, intended to reduce the occurrence of resistance (Stahle 1958; Mitchison & Davies 2012). The introduction of pyrazinamide, rifampicin and ethambutol further increased options for anti-tuberculosis therapy, since each drug targets a unique component essential for mycobacterial growth, minimising the likelihood of resistance development.

After numerous clinical trials using a range of drug combinations to reduce both the occurrence of resistance and the duration of therapy, it was concluded that for anti-tuberculosis therapy to be effective, a minimum of three drugs was needed: isoniazid, rifampicin and pyrazinamide (Iseman 2002; Mitchison & Davies 2012). In areas with high levels of monoresistance to one of these drugs, the addition of ethambutol was recommended. Currently, the WHO recommends a 6-month treatment regime comprising of 2 months of isoniazid, rifampicin, pyrazinamide and ethambutol, followed by a 4-month continuation phase of isoniazid and rifampicin (Bass et al. 1994).

2.4. Drug resistance in *Mycobacterium tuberculosis*

Following his discovery of the first antibiotic, Sir Alexander Fleming advised that "misuse of antibiotics could result in selection for resistant bacteria" (Bhagwati 2012). To date, despite the range of antituberculosis drugs and treatment options available, multidrug-resistant (MDR), extensively drug-resistant (XDR) and totally drug-resistant (TDR) *M. tuberculosis* strains have emerged. These resistant strains pose a major threat to the global control of tuberculosis.

Drug-resistance in bacteria typically arises via a number of different mechanisms including the horizontal transfer of resistance genes and chromosomal mutations (Martinez & Baquero 2000; Livermore 2003). However, *M. tuberculosis* has a unique cell wall which is thought to render the former mechanism impossible (Pillay & Sturm 2007). Additionally, there exists very little evidence to suggest that *M. tuberculosis* contains plasmids (Zainuddin & Dale 1990), thereby diminishing the possibility of inter-bacterial exchange of resistance genes. In theory, gene transfer between mycobacteria could happen through transduction. However,

the special cell wall of mycobacteria allows only for the specialised bacteriophages known as mycobacteriophages to infect this group of bacteria. Therefore, exchange of genes through transduction with bacteria other than mycobacteria cannot occur. Resistance in *M. tuberculosis* is therefore considered to develop predominantly via spontaneous chromosomal mutations (Gillespie 2002; Ford et al. 2011; Almeida Da Silva & Palomino 2011).

Mutations can arise non-specifically, either directly via oxidative damage, or indirectly via errors in DNA replication (Dwyer et al. 2009). Consequently, drug resistance arises if these mutations occur in regions coding for a specific drug target. Such mutations confer a selective advantage (Martinez & Baquero 2000) and are not directed (Pope et al. 2008). Antimicrobial drugs target functions that are essential for growth and multiplication of the bacterial cell. Mutations in genes coding for drug targets will lead to resistance if the mutation results in a change in the target molecule preventing inactivation by the drug without affecting the viability of the bacterial cell. Since resistance-conferring mutations interfere with functions essential for bacterial growth, many of these are lethal and thus, resistance only becomes apparent if such interferences are non-lethal (Martinez & Baquero 2000). As with all bacterial species, drug resistance in *M. tuberculosis* arises via interplay between bacterial-determined factors and host-determined factors.

2.4.1. Bacterial determinants

2.4.1.1. Prokaryotic DNA Polymerase

Prokaryotic DNA Polymerase is responsible for the incorporation of nucleotides into the growing DNA strand during the process of cell replication. However, this enzyme is known

to misincorporate nucleotides (Fujii et al. 1999), inducing mutations at a frequency of one per 10^5 base pairs (Kunkel 2004). While such low fidelity DNA replication plays a role in maintaining genetic variation, resistance will arise if mutations occur in a gene coding for a specific drug target.

2.4.1.2. Reactive Oxygen Species

The three main sources for oxidative stresses leading to DNA damage are hydrogen peroxide (H_2O_2), superoxide radicals (O_2·-) and hydroxyl radicals (OH·) (Dwyer et al. 2009). These reactive oxygen species (ROS) are typically formed as by-products of catabolism in the presence of oxygen (Sakai et al. 2006). Exposure to endogenous ROS does not necessarily result in mutations, since many bacteria contain detoxifying enzymes to convert these ROS into their non-reactive form (Sakai et al. 2006). These include superoxide dismutase, which hydrolyses superoxide to hydrogen peroxide and oxygen, and catalase, which hydrolyses hydrogen peroxide to oxygen and water (Dwyer et al. 2009). In contrast, there are no enzymes which are able to detoxify OH·, which is consequently able to directly induce DNA damage (Imlay 2003).

2.4.1.3. Reactive Nitrogen Species

An alternative for ROS-induced mutagenesis is mutagenesis caused by reactive nitrogen species (RNS), produced following anaerobic respiration. RNS may alternatively be produced within the host macrophage *in vivo* (Kumar et al. 2011).

2.4.1.4. Defective DNA repair systems

Exposure to endogenous ROS or RNS is known to induce DNA damage resulting in a range of mutations (Bjelland & Seeberg 2003). The most common of these DNA lesions is formation of 8-oxoguanine (Dwyer et al. 2009). This oxidised guanine base is able to erroneously pair with adenine instead of cytosine during DNA replication, and is also subject to further damage by ROS and RNS.

In order to eliminate such bases, the 8-oxoguanine elimination system, comprising of the following components, is employed:

1. **MutM glycosylase:** eliminates 8-oxoguanine adducts
2. **MutY glycosylase:** eliminates misincorporated adenine nucleotides
3. **MutT phosphatase:** converts adenine triphosphate (dATP) to adenine monophosphate (dAMP), thereby preventing the erroneous pairing of dATP with 8-oxoguanine (Maki & Sekiguchi 1992)

Previous *in silico* analysis revealed that common mismatch repair systems, involved in bacterial DNA repair, are absent in *M. tuberculosis*. Defects in DNA repair systems may play a role in mutator phenotypes and multidrug resistance (Oliver et al. 2000). Sequencing of DNA repair genes in select Beijing isolates revealed mutations in *mut*T2 and *mut*T4 (Ebrahimi-Rad et al. 2003), suggesting a role for these mutator genes in multidrug resistance. These mutations were unique to the Beijing family. However, the mutations were not found to be associated with drug resistance (Lari et al. 2006). Representatives of the KZN family, which is also associated with resistance, were not included in this study.

2.4.1.5. Antibiotic-induced ROS formation

Antibiotic exposure may stimulate the production of ROS (Kohanski et al. 2007). Kohanski *et al* (2010) have recently shown that antibiotic therapy at low concentrations may stimulate random mutagenesis and ROS production, leading to drug resistance (Kohanski et al. 2010). Additionally, the authors showed that multidrug resistance may also develop during prolonged exposure to suboptimal antibiotic concentrations (Kohanski et al. 2010). This is particularly problematic in the clinical setting, given patient non-compliance, inadequate antibiotic therapy, and limited drug accessibility to certain tissues (Kohanski et al. 2010).

2.4.1.6. Efflux Pumps

More recently, efflux pumps have been proposed as alternative mechanisms of acquired drug resistance in bacteria (Rouveix 2007), including *M. tuberculosis* (Gumbo et al. 2007; Xin et al. 2008; Louw et al. 2009; Louw et al. 2011). Efflux pumps confer drug resistance by actively extruding antibiotics out of the bacterial cell (Rouveix 2007). The antibiotic resistance arrow of time, proposed by Schmalstieg *et al* (2012), suggests that resistance due to efflux pump activity arises shortly after the onset of antibiotic therapy (Schmalstieg et al. 2012). Efflux pump activity has been demonstrated against the major anti-tuberculosis drugs, namely isoniazid (Gumbo et al. 2007; Machado et al. 2012), rifampicin (Siddiqi et al. 2004; Louw et al. 2011), streptomycin (Spies et al. 2008) and ofloxacin (Louw et al. 2011; Sun et al. 2014).

2.4.2. Host determinants

2.4.2.1. Inadequate therapy

Pharmacokinetic variability can lead to suboptimal antibiotic concentrations within the patient, resulting in inadequate therapy. The consequence of this is the selection of drug-resistant mutants (secondary resistance), which can be followed by the transmission of these resistant mutants to close contacts (primary resistance) (Andrews et al. 2008). In order to reduce the emergence of resistance related to inadequate TB therapy, the Directly-Observed Treatment, Short Course (DOTS) strategy was implemented in the mid-1990s (Kochi 1997).

2.5. Determining mutation frequencies in bacteria

A seminal study by Luria and Delbruck in 1947 addressed the question of whether the basis of bacterial resistance was the presence of pre-existing mutations, or mutations induced by exposure to a selective pressure (Luria & Delbrück 1943). In the study (Figure 2.4), a number of identical, low-density *Escherichia coli* cultures were prepared and incubated until the end of log phase was reached. Each culture was then plated onto media containing phage T1, a bacteriophage that specifically infects *E. coli* cells. The authors hypothesized that if phage resistance-conferring mutations pre-existed prior to exposure of the selective pressure, then there would be a variation in the number of resistant mutants observed between the parallel cultures, dependent on when during the bacterial life-cycle the mutation arose. Conversely, if the mutations were induced on exposure to the selective pressure, then each bacterial cell in the culture would have a similar probability of mutating and thus, there would be a similar number of resistant mutants between the parallel cultures (Luria & Delbrück 1943).

On exposure of the identical *E. coli* cultures to phage T1, variation in the distribution of T1-resistant mutants between the parallel cultures was observed. Based on their hypotheses, this fluctuation was thought to be due to pre-existing mutations. They thus concluded that resistance occurs as a result of random, spontaneous mutations prior to and independently of the selective pressure. This method is currently widely applied in determining resistance-conferring mutation frequencies in bacteria (Werngren & Hoffner 2003).

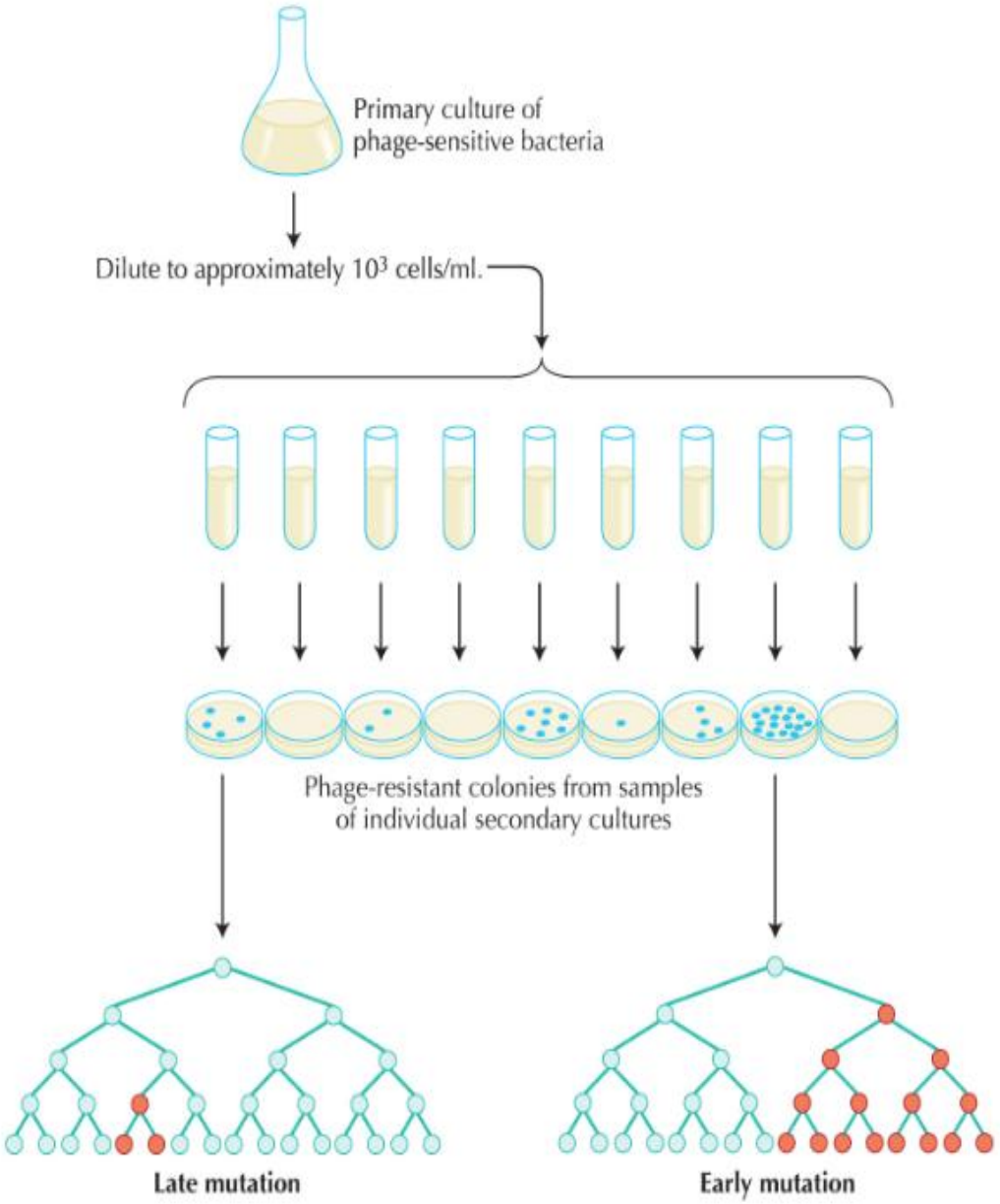

Figure 2.4: Schematic representation of the Fluctuation Analysis. Identical low-density cultures are incubated and plated onto selective media to isolate resistant mutants. Variation observed between individual cultures confirms the occurrence of spontaneous resistance mutations. The number of isolated mutants per culture depends on whether the mutation occurred early (leading to numerous mutants) or late (leading to few or no mutants) during the incubation period.

(Barton et al. 2007)

2.6. Mutation frequency in *Mycobacterium tuberculosis*

Since resistance acquisition in *M. tuberculosis* is chromosomally encoded (Gillespie 2002; Ford et al. 2011; Almeida Da Silva & Palomino 2011), mutation frequencies are commonly used as an indicator of resistance development. The mutation frequency reflects the proportion of resistant mutant bacteria within a total population, regardless of the stage at which the mutations were acquired during growth of the population (Martinez & Baquero 2000).

Resistance in *M. tuberculosis* is acquired to each of the first-line and second-line drugs at frequencies ranging between 10^{-6} for isoniazid, to 10^{-8} to rifampicin (Johnson et al. 2006). Patients with active TB infection are accordingly treated with multiple drugs, in order to eliminate such spontaneously-occurring resistant mutants. This is based on the probability that a single bacterium acquiring resistance against two drugs simultaneously is very low: e.g. the probability of a single bacterium acquiring combined resistance to isoniazid and rifampicin is approximately 10^{-14}, or 1 resistant mutant in every 10^{14} bacteria (Gillespie 2002; Sharma & Mohan 2004).

2.6.1. Beijing strains and the hypermutator phenotype

Using mutation frequency as a marker for drug resistance, the high association of the Beijing strains with drug resistance suggests that this strain acquires mutations more frequently than other strain families. Strains with defective DNA repair systems may readily acquire mutations and consequently, acquire drug resistance. Sequencing of putative mutator genes, known to play a role in DNA repair, revealed missense mutations and polymorphisms in the *mut*T4, *mut*T2 and *ogt* genes (Ebrahimi-Rad et al. 2003). In addition, since these mutations

were unique to the Beijing strains, the idea that strains of this genotype readily acquire mutations leading to drug resistance as a result of a poor or defective DNA repair system was supported. This was further reinforced by recent studies, in which a higher association with multidrug resistance within the Beijing family was demonstrated (de Steenwinkel et al. 2012; Ford et al. 2013).

Challenging this hypothesis, by means of the Fluctuation Analysis, it was found that Beijing strains acquire rifampicin resistance-conferring mutations at similar rates to non-Beijing strains (Werngren & Hoffner 2003). This suggests that the association of the Beijing family with multidrug resistance is not as a result of a higher mutational capacity. The basis of the association of this family with drug resistance is to date unconfirmed.

2.7. Outcomes of *Mycobacterium tuberculosis* infection

Figure 2.5 provides a schematic overview of the different outcomes following infection with *M. tuberculosis*. Tuberculosis infection begins when droplet nuclei containing *M. tuberculosis* expelled from the lungs of a patient with active tuberculosis are inhaled by a healthy human being (Koul et al. 2011). The clinical outcomes of *M. tuberculosis* infection are either active disease or latent infection (Gengenbacher & Kaufmann 2012), each of which is considered mutually exclusive.

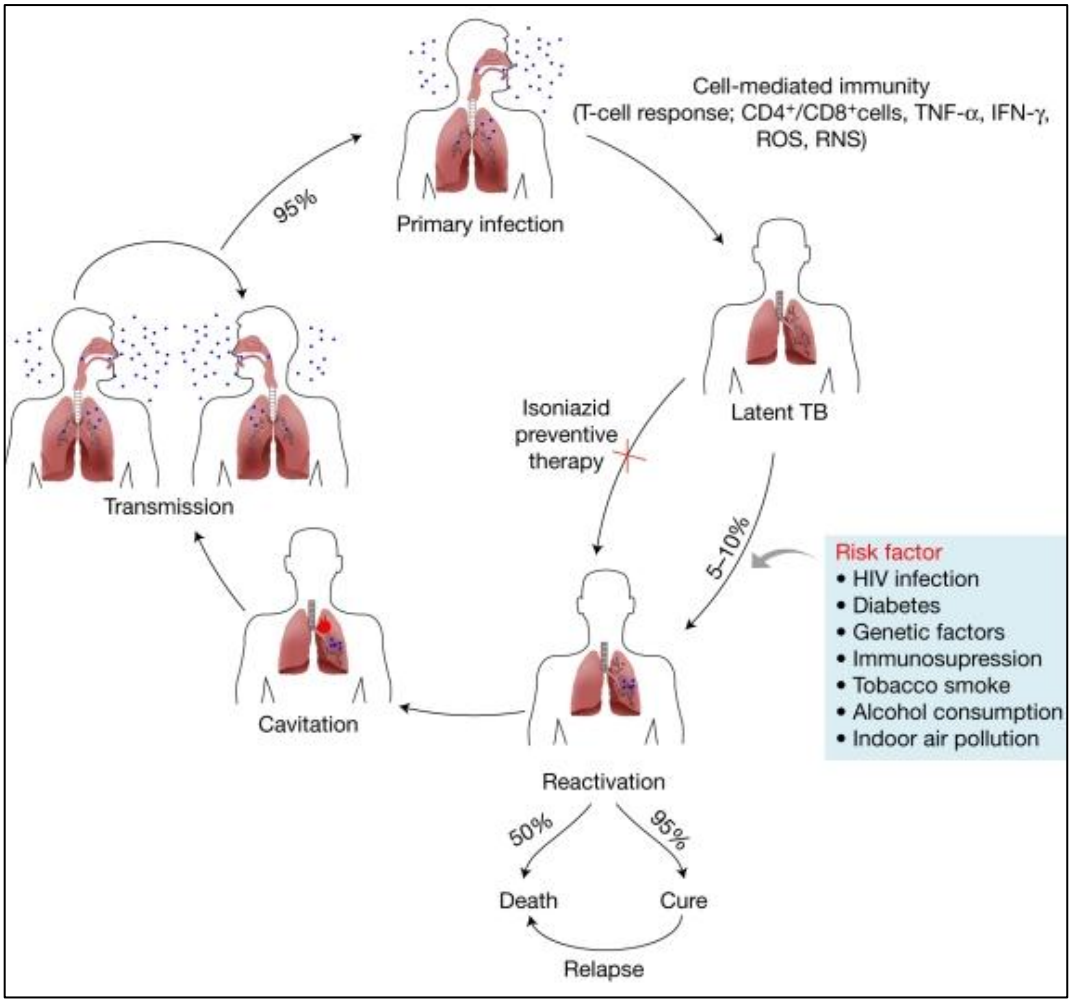

Figure 2.5: Possible outcomes of infection with *M. tuberculosis*.

(Kumar et al. 2011)

2.7.1. Active infection

Active TB infection is characterised as infection with signs of clinical disease and arises due to the inability of the host to develop an immune response able to control the initial infection (Gengenbacher & Kaufmann 2012). In this state, *M. tuberculosis* multiplies and in doing so, is able to accumulate numerous mutations.

To avoid selection of single or dual drug resistance mutants, the patient is treated with a combination of four drugs during the initial two months of a six-month course, in order to eliminate mutants resulting in drug resistance (Bass et al. 1994). This assumption is based on the fact that it is unlikely that two or more mutations will occur in the same bacterial cell (Pillay & Sturm 2007). In the last 4 months, the bacterial load is thought to be too low for mutations to occur at a significant rate. Continuation treatment is therefore with two drugs only.

2.7.2. Latent tuberculosis infection

Early clinical observations of latent TB (LTBI) infection prompted the first definition of latency as "the presence of any tuberculous lesion which fails to produce symptoms of its presence" (Amberson Jr 1938). LTBI is defined as infection with *M. tuberculosis* in the absence of disease indicators, and accounts for approximately 90% of all TB cases. While those harbouring dormant TB are not infectious, they serve as a potential reservoir for disease reactivation, followed by further spread (Parrish et al. 1998). The risk of disease reactivation depends on varied factors including the immune status of the infected individual, the infecting strain and bacterial load, the geographical location, and environmental factors (Sterling 2008).

During LTBI, the bacteria are phagocytosed to form granulomas (Flynn & Chan 2001). Within these lesions, the bacteria are thought to be exposed to hostile conditions including hypoxia, limited nutrient availability, reactive oxidative species and acidity (Tan et al. 2010; Rustad et al. 2009). Granulomas serve to limit the spread of infection and prevent the emergence of active disease (Flynn & Chan 2001). However, in this proportion of patients,

the bacteria are able to avoid the host defence forces and are presumed to lie dormant. As a result, *M. tuberculosis* undergoes far less growth and metabolic activity during latent infection as compared to active infection. Due to the reduced or ceased replication rate, they are presumed to accumulate fewer mutations (Sterling 2008). This provides the basis for prophylaxis to avoid reactivation of TB by a single drug, usually isoniazid (INH), administered over a six to nine month period (Sterling 2008). While numerous studies have afforded a better understanding of the ability of *M. tuberculosis* to adapt during latent infection, the physiological state of the bacterium during this phase in the host remains poorly understood (Gomez & McKinney 2004).

Challenging the classic concept of *M. tuberculosis* infection outcomes, recent evidence suggests that the two *M. tuberculosis* diseases states are not distinct (Barry 3rd et al. 2009; Lawn et al. 2011). This implies that there may be significant overlap between active and latent disease states.

2.8. Anti-mycobacterial drugs

Patients with active TB are treated with a combination of drugs in order to eliminate mutants which may lead to drug resistance, since actively dividing bacilli are more likely to acquire mutations via error-prone DNA polymerases. The drugs used are grouped as first-line and second-line drugs. The latter group consists of drugs that are only used when initial treatment fails, or in case of established drug resistance. They are more toxic and less effective than the first line drugs.

2.8.1. Isoniazid

2.8.1.1. Mode of action

Isoniazid was first demonstrated to show bactericidal activity against *M. tuberculosis* in 1952 (Knox et al. 1952; Rattan et al. 1998). Since then, isoniazid has remained the backbone in the treatment of both active disease and latent infection. Isoniazid is a prodrug which requires activation by bacterial catalase-peroxidase, encoded by *kat*G (Zhang et al. 1992). This results in the generation of reactive oxygen species which are toxic to the infected cell (Zhang et al. 1992). In its active form, isoniazid directly disrupts mycolic acid biosynthesis, which is an essential component for the growing *M. tuberculosis* cell wall. The target of activated isoniazid is inhA, encoded by the *inh*A gene, which is proposed to play a role in elongation of fatty acids during mycolic acid biosynthesis (Banerjee et al. 1994).

The mode of action of isoniazid is summarised in Figure 2.6:

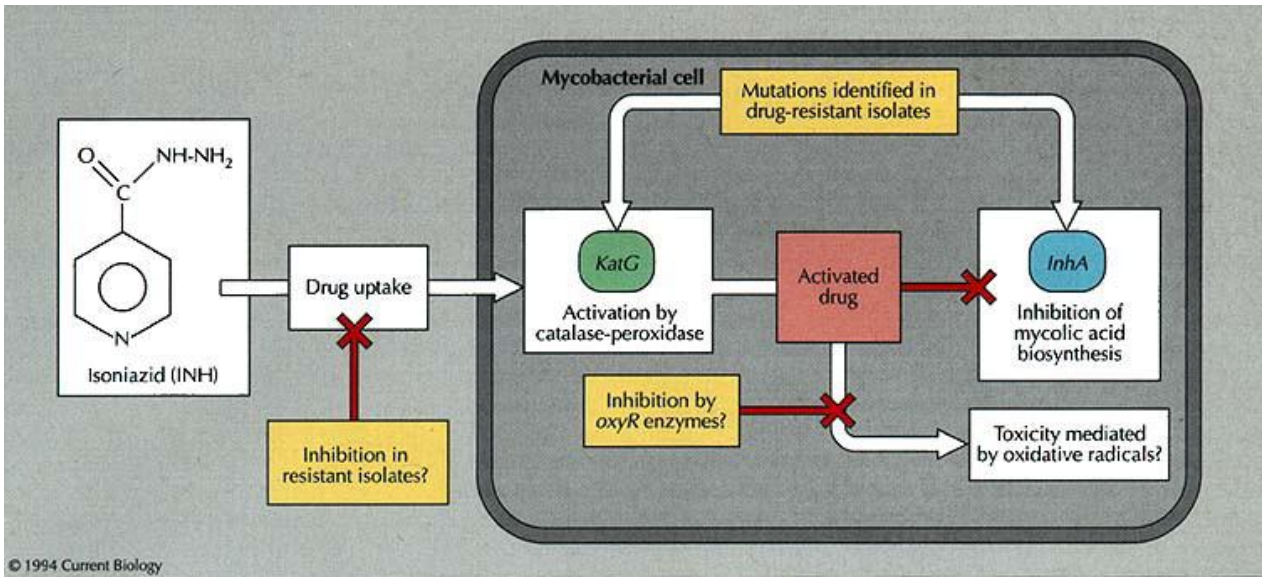

Figure 2.6: Mode of action of isoniazid on *M. tuberculosis*

(Young 1994)

2.8.1.2. Isoniazid Resistance

The development of isoniazid resistance following isoniazid monotherapy has been well documented since its anti-tuberculosis properties were first described (Ogilvie 1954). Since then, numerous studies have shown that *M. tuberculosis* is able to acquire isoniazid-resistance conferring mutations at a frequency of approximately $1x10^{-6}$ (David 1970; Johnson et al. 2006), which is the highest of all the anti-tuberculosis drugs. It is likely that this high mutation frequency is dependent upon the large number of isoniazid resistance-associated genes (Table 2.1), including those genes coding for products involved in isoniazid activation and mycolic acid biosynthesis.

Since catalase-peroxidase is required to activate isoniazid into its toxic form, mutations within the *kat*G gene, which encodes catalase-peroxidase, confer high-level isoniazid resistance (Ramaswamy & Musser 1998). High-level isoniazid resistant mutants defective in *kat*G activity are thought to rapidly acquire further mutations leading to an elevated mutation rate as a result of defective catalase-peroxidase activity, lacking the ability to detoxify ROS intermediates including hydrogen peroxide (Zhang et al. 1992). However, recent data suggest that *M. tuberculosis* isolates with defective catalase-peroxidase activity are able to compensate by the upregulation of *ahp*C (O'Sullivan et al. 2008). The *ahp*C gene encodes an alkyl hydroperoxide reductase protein, which detoxifies oxidative intermediates in the absence of katG activity.

The proposed target of activated isoniazid is inhA, an enoyl-acyl carrier protein (ACP) reductase encoded by the *inh*A gene (Banerjee et al. 1994), which plays a role in mycolic acid biosynthesis. In its active form, isoniazid binds to inhA, disrupting mycolic acid

biosynthesis, eventually leading to cellular death (Timmins & Deretic 2006). Mutations within the *inh*A gene prevent the binding of isoniazid, leading to low-level resistance (Guo et al. 2006).

An additional gene proposed to play a role in low-level isoniazid resistance is *kas*A. This gene encodes a β-ketoacetyl-ACP synthase, which functions in mycolic acid biosynthesis (Mdluli et al. 1998). Previous studies demonstrated the presence of point mutations in the *kas*A gene in low-level isoniazid resistant isolates (Lee et al. 1999; Piatek et al. 2000). However, similar mutations were found in isoniazid susceptible isolates (Lee et al. 1999; Hazbón et al. 2006), casting doubt on the reliability of this gene as a resistance marker.

2.8.2. Rifampicin

2.8.2.1. Mode of action

Rifampicin is a first-line antibiotic (Laurenzo & Mousa 2011) with rapid bactericidal activity against *M. tuberculosis* (Miller et al. 1994). Together with isoniazid, it forms the backbone of first-line anti-TB therapy. The specific target for rifampicin is the β-subunit (rpoB) of prokaryotic RNA polymerase, encoded by *rpo*B (Miller et al. 1994). Upon binding of rifampicin to rpoB, transcription is physically blocked, leading to inhibition of bacterial growth and subsequent death.

2.8.2.2. Rifampicin Resistance

Single step mutations in the *rpo*B gene are associated with high-level rifampicin resistance (Miller et al. 1994). Previous studies indicate that resistance to rifampicin is rare and occurs at a frequency of approximately 10^{-8}, approximately 100 times lower than for isoniazid (David 1970; Johnson et al. 2006; Bergval et al. 2009). This low frequency can be attributed to mutations in *rpo*B as the predominant contributor of rifampicin resistance (Tsukamura 1972). To date, no alternative mutations associated with rifampicin resistance have been identified. Rifampicin resistance serves as an important indicator for multidrug resistant TB, since approximately 90% of rifampicin resistant strains harbour additional resistance to isoniazid (Siddiqi et al. 1981; Somoskovi et al. 2001).

2.8.3. Streptomycin

2.8.3.1. Mode of action

Streptomycin is an alternative first-line drug used during retreatment when first-line therapy is interrupted (Cooksey et al. 1996). The target of streptomycin is 16S ribosomal RNA (rRNA), which forms part of the 30S small subunit prokaryotic ribosome (Nair et al. 1993; Honort & Cole 1994). Following binding of streptomycin to 16S rRNA, structural ribosomal changes are induced (Finken et al. 1993; Sreevatsan et al. 1996), resulting in misreading of mRNA during translation and consequently hindering protein synthesis.

2.8.3.2. Streptomycin Resistance

Resistance to streptomycin is frequently found in *M. tuberculosis* bacilli harbouring mutations in the *rrs* and *rps*L genes, conferring intermediate and high-level resistance

respectively (Cooksey et al. 1996; Meier et al. 1996). More recently, it has been proposed that mutations in the *gid*B gene are associated with low-level streptomycin resistance (Okamoto et al. 2007). The *gid*B gene encodes a SAM-dependent methyltranferase which functions in methylation of the bacterial 16S rRNA and thus provides an additional binding site for streptomycin. Mutations within this gene may likely result in termination in expression of the methyltransferase enzyme. Consequently, in the absence of this additional binding site, strains harbouring this mutation will acquire streptomycin resistance. The reliability of this gene as a marker for streptomycin resistance is questionable however, since mutations in this gene have also been observed in streptomycin susceptible isolates (Wong et al. 2011).

2.8.4. Kanamycin

2.8.4.1. Mode of action

Kanamycin is an aminoglycoside which, similar to streptomycin, binds to the 16S rRNA of the 30S ribosomal subunit, thereby interrupting protein synthesis (Gikalo et al. 2012). Consequently, aminoglycosides are most effective against actively-growing bacilli. In South Africa, kanamycin is the aminoglycoside of choice for the treatment of MDR tuberculosis.

2.8.4.2. Kanamycin Resistance

Mutations in the *rrs* gene, which encodes 16S rRNA, are most frequently associated with high-level kanamycin resistance (Suzuki et al. 1998). However, up to 60% of *M. tuberculosis* isolates exhibit low level kanamycin resistance lack mutation in the *rrs* gene, suggesting alternative resistance mechanisms (Zaunbrecher et al. 2009). Recently, the eis (enhanced

intracellular survival) promoter has been proposed to play a role in kanamycin resistance (Zaunbrecher et al. 2009). This protein was also shown to encode an aminoglycoside acetyltransferase, which acetylates and inactivates kanamycin (Zaunbrecher et al. 2009). Initially observed to play a role in intracellular survival in macrophages (Wei et al. 2000), this gene was found to be expressed in low-level resistant kanamycin isolates. So far, the aminoglycoside modifying enzymes have not been shown to play a role in the resistance of mycobacteria to this group of drugs.

2.8.5. Ofloxacin

2.8.5.1. Mode of action

Ofloxacin is a broad-spectrum, early-generation fluoroquinolone (Ginsburg et al. 2003) and is, in South Africa, the quinolone of choice for the treatment of MDR tuberculosis. DNA gyrase is responsible for mediating supercoiling of DNA within the bacterial cell (Ramaswamy & Musser 1998). The interaction between ofloxacin and DNA gyrase results in double-stranded DNA breakage, preventing supercoiling of DNA (Drlica et al. 2008). Although the precise mechanism of killing remains unclear, the DNA gyrase-ofloxacin complex is known to physically disrupt DNA replication and gene expression, leading to cell death (Kampranis & Maxwell 1998).

2.8.5.2. Ofloxacin Resistance

Resistance to all quinolones, including ofloxacin, results from mutations in the *gyr*A gene. Despite their effective antimicrobial activity (Ginsburg et al. 2003), fluoroquinolones are also

known to induce resistance-conferring mutations via error-prone DNA repair processes (Lopez et al. 2007).

The quinolone resistance determining region (QRDR) in *M. tuberculosis* comprises *gyr*A and *gyr*B, which are each directly involved in interaction between DNA gyrase and fluoroquinolones (Alangaden et al. 1995). Missense mutations within *gyr*A are most frequently associated with clinical resistance (Cambaup et al. 1994; Alangaden et al. 1995; Kocagöz et al. 1996). In contrast, *gyr*B mutations have been predominantly observed in a number of resistant laboratory isolates, but are absent in clinical isolates (Kocagöz et al. 1996; Zhou et al. 2000). These rare mutations are therefore not frequently associated with clinical fluoroquinolone resistance. The level of fluoroquinolone resistance is dependent on the number of mutations acquired within the QRDR (Ouabdesselam et al. 1995). In contrast with single-step mutations conferring high-level rifampicin resistance, high-level fluroquinolone resistance only becomes apparent following successive, accumulative mutations within the *gyr*A gene (Xu et al. 1996; Kocagöz et al. 1996; Zhou et al. 2000).

Table 2.1: Summary of drug targets and resistance-associated genes in *M. tuberculosis*

DRUG	MODE OF ACTION	TARGET	RESISTANCE ASSOCIATED GENE	REFERENCES
Isoniazid (INH)	Bactericidal	Mycolic acid biosynthesis (Cell wall synthesis)	*kat*G, *inh*A, *kas*A, *ahp*C	Zhang et al. 1992; Banerjee et al. 1994; O'Sullivan et al. 2008
Rifampicin (RIF)	Bactericidal	RNA Polymerase (Transcripion)	*rpo*B	Miller et al. 1994
Streptomycin (STR)	Bactericidal	Ribosomal RNA (Protein synthesis)	*rrs*, *rps*L, *gid*B?	Nair et al. 1993; Finken et al. 1993; Okamoto et al. 2007
Kanamycin (KAN)	Bactericidal	Ribosomal RNA (Protein synthesis)	*rrs*, *eis*?	Suzuki et al. 1998; Zaunbrecher et al. 2009
Ofloxacin (OFX)	Bactericidal	DNA gyrase / topoisomerase II	*gyr*A, *gyr*B	Alangaden et al. 1995; Kocagöz et al. 1996

2.9. Treatment of latent tuberculosis infection

2.9.1. Isoniazid Preventative Therapy

Soon after the implementation of curative therapy for active disease using antibiotics, it was found that isoniazid prevented active disease in guinea pigs (Ferebee & Palmer 1956) and in humans (Ferebee et al. 1957). Isoniazid preventative therapy (IPT) remains the preferred treatment for latent tuberculosis, and for the prevention of disease in immunocompromised individuals who are at a greater risk of infection (Lobue & Menzies 2010). More recently, recommendations of a combination of isoniazid and rifapentine administered over 3 months have been suggested (Sterling et al. 2011). Prophylactic therapy is not intended to eliminate *M. tuberculosis*, but instead prevents the progression of latent infection to active infection (Hawkridge 2007). As such, during prophylactic therapy, *M. tuberculosis* growth is inhibited. However, the precise mechanism of activity of isoniazid against *M. tuberculosis* during LTBI remains unclear.

In 1999, Comstock sought to determine the optimal duration of IPT in HIV-infected adults (Comstock 1999) based on the findings of two previous studies (Ferebee 1970; Comstock et al. 1979). He established that treatment for at least 10 months resulted in a 68% reduction in disease, versus only an 18% reduction for treatment of less than 10 months (Figure 2.7). Additionally, there was a steady decrease in the case rate until 9-10 months, after which the number of cases remained constant (Comstock 1999).

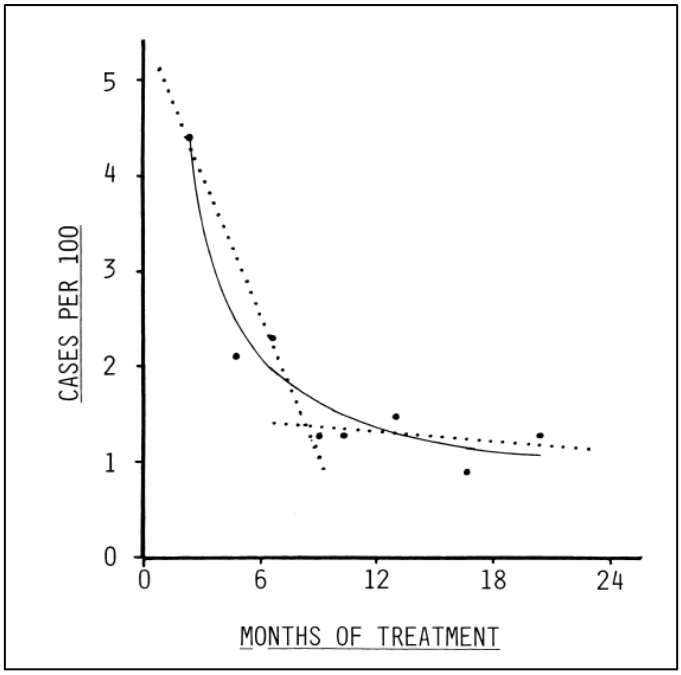

Figure 2.7: Comstock's analysis, showing a reduction in TB cases until 9-10 months, followed by no further increase or decrease in TB cases. Current IPT recommendations are based on this observation. (Comstock 1999)

Comstock's analysis validates the current recommendation of 9-10 months of IPT. This recommendation, however, is challenged by findings of the Thibela TB Study (Churchyard et al. 2014). In this study, it was found that community-wide use of IPT had no effect in controlling TB transmission in gold mines in South Africa by means of mass screening. Similarly, in an IPT trial conducted in Botswana (Samandari et al. 2011), resistance to isoniazid was observed in 17% of new cases on IPT. However, the prevalence of resistance was deemed to be too low to warrant concern.

2.9.2. Drawbacks of IPT for latent infection

The high prevalence of HIV infection, particularly in sub-Saharan Africa, allows for the reactivation of latent infection, or infection with a new strain from a close contact with active disease due to a poor immune response in these individuals. This forms the rationale for the use of a 9-month regime of isoniazid prophylaxis in HIV-infected individuals by the World Health Organisation (Godfrey-faussett 1998). Prophylactic therapy is thought not to be bactericidal, but instead maintains the bacilli in a dormant state. However, poor completion rates have been reported due to the lengthy duration of treatment (Raviglione et al. 2011). In addition, this 9-month regime has not been favourably received, due to concerns of resistance development (Balcells et al. 2006). Despite this, IPT is still the treatment of choice for LTBI, and is particularly recommended in patients with HIV infection that have a positive tuberculin skin test (Raviglione et al. 2011).

In theory, IPT can drive resistance in two ways: selection of resistant mutants that develop in the patient receiving IPT, and in facilitating preferential spread of resistant strains by preventing new infections with susceptible strains. Of particular concern is the potential for acquisition of resistant strains in latently HIV-infected individuals on IPT. In this regard, IPT can be considered a risk factor in the emergence of isoniazid resistance, including isoniazid monoresistance, MDR or XDR.

The development of resistance through mutation is thought not to occur during IPT (Ferebee 1970). In contrast, by means of mathematical modelling, it was found that the prevention of disease during community-wide IPT was followed by an increase in drug resistance (Cohen et al. 2006). The observation that there is an overlap in the spectrum of disease between full

blown active infection and latent infection highlights the fact that selection of resistant mutants remains a possibility (Ford & Rubin 2010).

2.10. Anaerobic metabolism in *Mycobacterium tuberculosis*

2.10.1. Association between bacterial growth and oxygen availability

M. tuberculosis is classically defined as a strict aerobe (Kumar et al. 2011). As such, it is believed to survive exclusively in the presence of oxygen, lacking the ability to survive anaerobically. Conversely, complete genome sequencing of the reference strain H37Rv revealed the presence of genes known to play a role in anaerobic metabolism (Cole et al. 1998).

A key feature of latency is the ability of *M. tuberculosis* to persist in host granulomas for extended periods of time (Flynn & Chan 2001). Granulomas present hostile conditions to *M. tuberculosis*, including limited oxygen and nutrient availability, acidic pH (Koul et al. 2008) and host immune factors including nitric oxide (Kumar et al. 2011) This suggests that *M. tuberculosis* exhibits a degree of metabolic flexibility, allowing for better adaptation to a range of microenvironments within the host.

Oxygen tension and the growth of *M. tuberculosis* are thought to be closely associated. In humans, the disease rate is higher in coastal regions, where there is a high oxygen tension, than in highland regions (Olender et al. 2003). In an aerated environment, *M. tuberculosis* is

able to undergo active growth and replication, leading to active disease (Lawn et al. 2011). In contrast, the replication rate of *M. tuberculosis* decreases with reduction in oxygen tension. Histological analysis of lung lesions in infected patients revealed high bacterial loads in lesions adjacent to open airways with a greater access to oxygen. The bacterial load and number of visible *M. tuberculosis* bacilli decreased in lesions with restricted access to oxygen (Kaplan et al. 2003). Whether this translates in a reduced rate of active TB disease is unknown.

This association with oxygen tension and *M. tuberculosis* survival may have also formed the basis for artificial lung collapse, prior to the introduction of curative therapy using antibiotics. Artificial lung collapse was found to be effective in reducing the growth of *M. tuberculosis* due to the reduced accessibility of oxygen to the bacilli (Singer 1936). However, this method led to detrimental consequences (Skinner & Sinclair 1992).

2.10.2. Growth of *Mycobacterium tuberculosis* under anaerobiosis

There are several conflicting hypotheses with regards to the replicative state of *M. tuberculosis* under anaerobiosis. Complete genome sequencing of the control strain H37Rv revealed a number of genes known to play a role in anaerobic respiration, including those involved in the nitrate respiratory pathway (Cole et al. 1998). This is affirmed by prior studies which showed that fatty acids are utilised as a primary carbon source during latency in the absence of oxygen (Segal & Bloch 1956; Gomez & McKinney 2004). Despite this, *M. tuberculosis* currently remains classified as a strict or obligate aerobe.

2.11. Latency paradigm

2.11.1. Dormancy of *Mycobacterium tuberculosis*

The most widely accepted hypothesis is that the growth of *M. tuberculosis* is arrested during latency. This is due to the reduced availability of oxygen and nutrients, coupled with exposure to acidic pH and host immune effectors including nitric oxide, following granuloma formation (Rustad et al. 2008).

2.11.2. The Wayne Model

The association between oxygen tension and *M. tuberculosis* growth has prompted the design of a number of *in vitro* hypoxic models representing latent TB infection. The most widely-used of these is Wayne's Hypoxia Model (Wayne & Hayes, 1996). In this model, tightly-sealed cultures are allowed to stand unagitated for a period of time. As the *M. tuberculosis* cells grow and consequently utilise the available oxygen, conditions within the culture flask become anaerobic. Concomitantly, there is significant reduction in metabolic activity within the cells as they adapt to oxygen deprived conditions (Wayne & Hayes 1996). The use of methylene blue indicator dye, which decolourises under anaerobic conditions, confirms that anaerobiosis is maintained under these conditions (Wayne & Hayes 1996).

Gene expression profiles of *M. tuberculosis* cultured under the Wayne Model indicate a downregulation of genes involved in central metabolic pathways, and an upregulation of genes involved in alternate metabolic pathways including fatty acid metabolism (Muttucumaru et al. 2004). These bacilli remain susceptible to rifampicin (Mitchison 2004), and resistant to isoniazid (Paramasivan et al. 2005).

2.11.3. Limitations of dormancy models

Wayne *et al* (1996) refer to cells cultured under such conditions as "non-replicating persisters" which establish latent infection (Wayne & Hayes, 1996). While this may support the theory that *M. tuberculosis* growth is inhibited during latency, it fails to explain how isoniazid, a drug which targets actively dividing cells, is effective in treating latent infection. This raises the question as to whether presumed dormant *M. tuberculosis* serves as a suitable proxy of latent infection *in vitro*.

Additionally, given the fact that *M. tuberculosis* retains susceptibility to rifampicin under oxygen deprivation, this casts doubt as to whether *M. tuberculosis* remains dormant under anaerobiosis. Since rifampicin targets RNA polymerase, this indicates that there is a degree of metabolic activity under anaerobiosis (Wayne & Hayes, 1996; Wayne & Sramek, 1994). This is in contrast with the classical definition of dormancy as a complete metabolic shutdown.

2.11.4. Alternative latency paradigm

While the current paradigm of latent TB infection assumes that the bacteria are in a dormant state, there is ample evidence to suggest that *M. tuberculosis* retains the ability to replicate, albeit at a slow rate, during latency. The two main outcomes of infection with *M. tuberculosis* are either active infection leading to active disease if uncontrolled, or latent infection. These two outcomes are presumed to be mutually exclusive, with bacilli comprising each outcome exhibiting distinct metabolic and replicative profiles. Retrospective analyses, however, suggests that this concept may significantly simplify the replicative capacity of *M. tuberculosis* during LTBI (Barry 3rd et al. 2009; Lawn et al. 2011;

Zumla et al. 2011). A recent study revealed overlap in the host transcriptional response to active and latent infection. The expression profile of such genes is thought to distinct between the two disease states (Fortune & Rubin 2010). This suggests that active infection and latent infection may not be distinct entities.

Furthermore, isoniazid is bactericidal against actively dividing bacilli, yet is effective in preventing disease reactivation in latently infected individuals. It is therefore likely that during latency, the pool of bacilli alternate between spurts of metabolic activity and dormancy (Lawn et al. 2011). Latent tuberculosis can thus be viewed as subclinical infection comprising of bacteria in a variety of metabolic states (Barry 3rd et al. 2009; Lawn et al. 2011).

2.12. Mutational capacity of *Mycobacterium tuberculosis* during latent infection

Clinical observations and classic dormancy models suggest that *M. tuberculosis* lacks the ability to grow during latent infection. This assumption forms the basis for the current treatment of latent tuberculosis infection with prophylactic therapy using a single drug (Sterling 2008). Multidrug therapy is thought to be inessential during latent infection, since the bacilli are presumed to lie dormant and as such, do not mutate.

Challenging this concept, a recent study provided evidence that *M. tuberculosis* does retain the ability to acquire mutations during latent infection (Ford et al. 2011). In this study,

cynomolgus macaques were infected with *M. tuberculosis*, and bacilli isolated from lesions at three distinct disease states: active lesions, latent lesions and reactivated lesions. Whole genome sequencing revealed a higher number of resistance conferring mutations in those bacteria isolated from latent and reactivated lesions than those isolated from active lesions (Ford et al. 2011). These findings are in stark contrast with current latent TB models, in which the bacteria are presumed to lay dormant due to a cessation of replication, and hence acquire few to no mutations.

One of the contributors to the high mutation acquisition during latency could be the accumulation of reactive oxidative species, capable of directly inducing DNA damage. Consistent with this, ten of the fourteen mutations identified were products of oxidative damage, either due to cytosine deamination or 8-oxoguanine formation (Ford et al. 2011). This is thought to occur due to exposure to these oxidative intermediates within the granuloma *in vivo*. Since latency manifests as long-term infection, the duration of exposure to these reactive intermediates increases, resulting in further DNA damage (Ford et al. 2011). Additionally, reduced replication rates during latency may be accompanied by a downregulation in DNA repair (Ford et al. 2011). This could contribute to the high mutation rate since oxidative DNA damage, including 8-oxoguanine, may not readily be repaired.

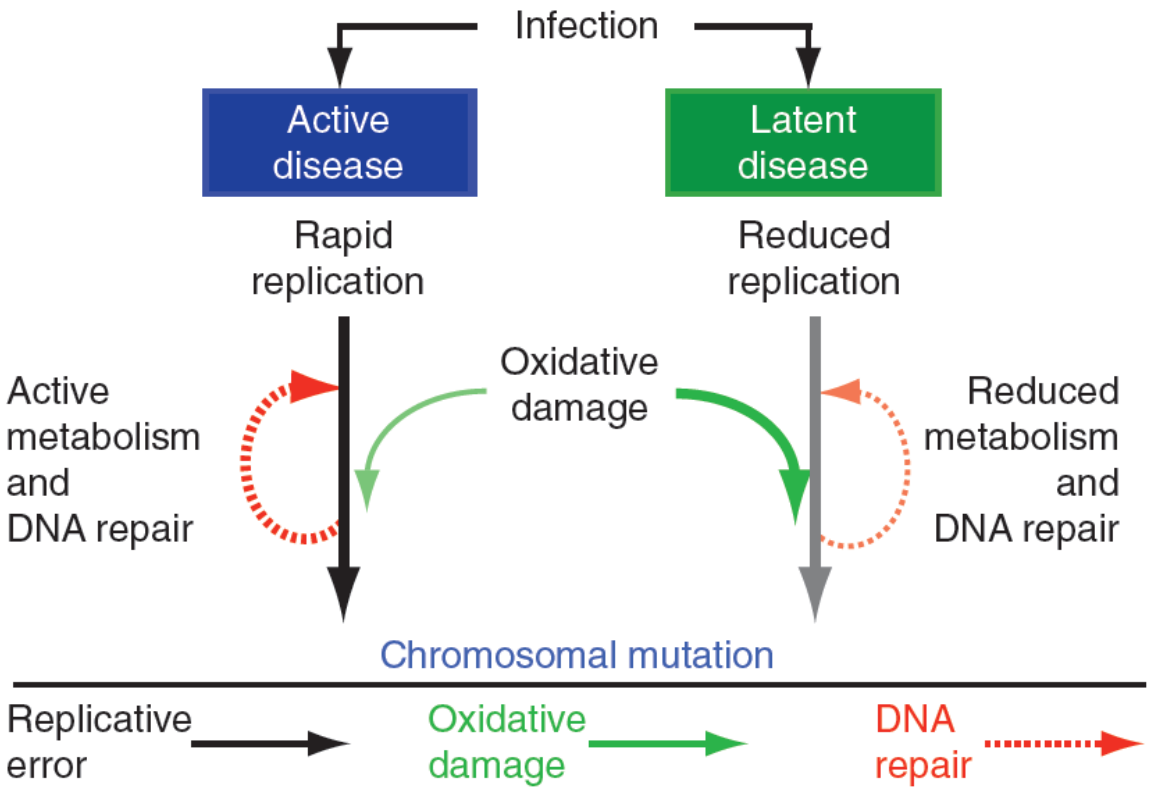

Figure 2.8: Alternative model for mutation acquisition in *M. tuberculosis* during latency (Ford et al. 2011)

Based on the ability of *M. tuberculosis* to survive and metabolise anaerobically (Ramchandra & Sturm 2010), it is possible that reactive nitrogen species produced during anaerobic respiration provide an alternative source for mutations.

CHAPTER 3: PAPER

Mutation frequency in *Mycobacterium tuberculosis* belonging to the Beijing and F15/LAM4/KZN strain families under aerobic and anaerobic conditions

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ABSTRACT

Background: Infection with *Mycobacterium tuberculosis* results in either active disease or latent infection. The two predominant *M. tuberculosis* strain families circulating in KwaZulu-Natal are F15/LAM4/KZN and Beijing. Drug resistance is defined on the basis of mutation acquisition. *M. tuberculosis* is thought to lie dormant and acquire few to no mutations during the course of latent infection. In contrast, recent evidence suggests that *M. tuberculosis* may retain the ability to mutate during latent infection.

Methods: Using the Luria-Delbrück fluctuation analysis, the frequency of resistance-conferring mutations in isolates of *M. tuberculosis* was determined. Two susceptible isolates of each strain family and H37Rv were used. Low-density cultures of each isolate were grown under aerobic and anaerobic conditions. Resistance acquisition frequency to isoniazid (1µg/ml), rifampicin (1µg/ml), streptomycin (2µg/ml), ofloxacin (2µg/ml) and kanamycin (5µg/ml) was expressed as the proportion of resistant CFU/ml to the total CFU/ml. This was used as a proxy for mutation frequency.

Results: Aerobically, F15/LAM4/KZN isolates acquired resistance at higher frequencies than the Beijing isolates and H37Rv against rifampicin, streptomycin and kanamycin ($P<0.0001$). The mutation frequencies for these drugs were similar or higher than for isoniazid. No differences were observed with isoniazid or ofloxacin between the isolates ($P>0.05$). Under anaerobic conditions, the KZN isolates were found to acquire resistance at higher frequencies than the Beijing isolates ($P<0.0001$).

Conclusions: F15/LAM4/KZN isolates acquired resistance at higher frequencies under aerobic and anaerobic conditions as compared to Beijing isolates. This suggests a hypermutator phenotype.

INTRODUCTION

Drug resistance in *Mycobacterium tuberculosis* has reached proportions threatening the success of current TB control programs. Multidrug-resistant (MDR) organisms are resistant to isoniazid and rifampicin, while extensively drug-resistant (XDR) organisms have acquired additional resistance to fluoroquinolones and any of the second-line injectables (capreomycin, amikacin or kanamycin) (Ioerger et al. 2009). More recently, totally drug-resistant isolates have been reported from Italy (Migliori et al. 2007), Iran (Velayati et al. 2009), India (Udwadia et al. 2012) and most recently, South Africa (Klopper et al. 2013).

The development and emergence of drug resistance is determined by bacterial factors, mainly mutation acquisition, and host factors such as non-adherence to treatment. Its cell wall structure makes horizontal gene transfer in *M. tuberculosis* unlikely (Pillay & Sturm 2007) and there exists little evidence that *M. tuberculosis* contains plasmids (Ramaswamy & Musser 1998). Therefore, drug resistance in this speciesis considered to arise mainly via spontaneous mutations (Gillespie 2002). Measurement of the fraction of resistant colony forming units for a specific antimycobacterial agent in a population of bacteria can thus be used as a proxy for mutation frequency.

A recent study by Moodley *et al* (2011) indicates the high prevalence of MDR and XDR tuberculosis within the KwaZulu-Natal province of South Africa (Moodley et al. 2011). Strains associated with resistance within this province are the Beijing and the F15/LAM4/KZN (KZN) strains. The frequency of mutation acquisition is thought to differ

between strains. Due to the widespread distribution of the Beijing strain, it is considered to be the most successful family of strains of *M. tuberculosis* globally (Glynn et al. 2002; Hanekom et al. 2011), and is epidemiologically associated with multidrug-resistance (Brown et al. 2010; de Steenwinkel et al. 2012). It is therefore presumed to acquire mutations at a higher frequency during the course of infection than other strains. In KwaZulu-Natal however, the KZN strain shows a high association with drug resistance. This family of strains has developed from multidrug-resistance to extensive drug-resistance within a decade (Pillay & Sturm 2007). This suggests that both the KZN and Beijing strains acquire resistance-conferring mutations at high frequencies.

Infection with *M. tuberculosis* results in either active disease or latent infection. Each is characterised by the presence of bacteria with vastly different and distinct replicative and metabolic profiles. During latent tuberculosis infection (LTBI), *M. tuberculosis* bacilli enter a state of presumed dormancy, characterised by arrested growth and hypometabolism (Loebel et al. 1933; Wayne & Hayes 1996). LTBI poses a major obstacle in the control of tuberculosis, as it serves as a potential reservoir for future spread of the disease (Parrish et al. 1998).

During LTBI, *M. tuberculosis* is phagocytosed and driven into a quiescent state by host alveolar macrophages to form granulomas (Flynn & Chan 2001). Within the granuloma, *M. tuberculosis* is proposed to be exposed to a range of hostile conditions including limited oxygen and nutrient availability (Wayne 1994), acidity and pH (Tan et al. 2010), each of which are non-conducive to active growth. In lung lesions the number of visible *M. tuberculosis* bacilli decreases in lesions with restricted access to oxygen (Kaplan et al. 2003).

Following this, *in vitro* dormancy models use anaerobiosis as a proxy for latent infection. *In vitro* dormancy models suggest a state of non-replicating persistence characterised by a metabolic shutdown (Wayne 1994). However, this fails to explain how drugs like isoniazid, which targets actively dividing cells, is effective against latent infection.

Challenging common dormancy models, recent evidence suggests that *M. tuberculosis* remains metabolically active, and possibly replicates, during latency (Barry 3rd et al. 2009; Chao & Rubin 2010; Lawn et al. 2011). This suggests that the two outcomes of *M. tuberculosis* infection, active disease and latent infection, are not mutually exclusive. Instead, latent infection may comprise a spectrum of bacilli with a range of metabolic activities. This is supported by a recent study (Ford et al. 2011) in which *M. tuberculosis* isolated from latent lesions in macaques was shown to have acquired resistance-conferring mutations at a higher rate than those isolated from active lesions. This raises the question as to whether *M. tuberculosis* retains the capacity to acquire resistance during anaerobic metabolism, possibly reflecting latency.

We sought to compare the mutation frequencies in the genes coding for the MDR and XDR defining drug targets between isolates from the Beijing and KZN families, under aerobic and anaerobic conditions. Anaerobic incubation is used as a proxy for latency, and frequency of resistance development for mutation frequency.

MATERIALS AND METHODS

Bacterial strains

Two clinical isolates of the Beijing family and two of the KZN family were used as well as reference strain H37Rv. All isolates were susceptible to all drugs tested, and was defined as no growth on Middlebrook 7H10 agar containing the following drugs: isoniazid (1 μg/ml), rifampicin (1 μg/ml), streptomycin (2 μg/ml), kanamycin (5 μg/ml) or ofloxacin (2 μg/ml).

Fluctuation analysis

One hundred μL of frozen *M. tuberculosis* suspension of each isolate was inoculated in enriched 7H9 broth containing 10% OADC, 0.5% glycerol and 0.05% Tween-80 and incubated at 37^{o}C until an OD_{600nm} of 1 was reached. This was used to prepare low-density cultures.

For the aerobic assays, 100 μL of culture was inoculated into 10mL of enriched 7H9 broth. Five hundred μL of this diluted culture was then inoculated into 100mL of 7H9 broth, to yield a final dilution of 1/20,000. Aliquots of 4mL of this diluted culture were then dispensed into 20 inkwell bottles. For the aerobic assays, these cultures were incubated at 37^{o}C in a shaking incubator until an OD_{600nm} of 1, corresponding to the end of log phase, was obtained.

For the anaerobic assays, the same process was followed. Once an OD_{600nm} of 1 was reached in each of the initial cultures, they were transferred to a Forma Anaerobic Chamber. The remaining steps to prepare the 1/20,000 diluted cultures were performed within the Anaerobic Chamber, using prereduced 7H9 broth. The twenty 4 mL aliquots of each isolate were incubated anaerobically within the chamber at 37^{o}C until an OD_{600nm} of 1 was reached. This

took between 6 and 8 weeks. Anaerobic conditions were confirmed using *Bacteroides fragilis* ATCC 25285 and a resazurin indicator strip (Oxoid).

Once the OD_{600nm} reached 1 in both the aerobically and anaerobically incubated cultures, the entire culture from each of the 20 inkwell bottles was sonicated for 10 seconds each at an amplitude of 15 Hz using the Misonix 4000 Ultrasonic Liquid Processor. Six tenfold dilutions of each of the 20 sonicated cultures were prepared in PBS supplemented with 20% Tween. Of each dilution, 200 µL aliquots were plated onto each of two drug-free Middlebrook 7H10 plates.

The remaining 3900 µL of each culture was centrifuged at 4000 r.p.m. for 10 minutes at 25^{o}C. The supernatant was decanted and the pellets were resuspended in 200 µL of 7H9 broth and homogenised by vortexing. The entire volume of each of the 20 resuspended pellets was plated onto five Middlebrook 7H10 plates containing isoniazid (1µg/mL), rifampicin (1µg/mL), streptomycin (2µg/mL), ofloxacin (2µg/mL) and kanamycin (5µg/mL) respectively. A drug-free plate was included as a control, in order to demonstrate that the drug-containing plates permitted growth of mutants only, and that growth on the drug-containing plates was not a result of inactivity of the drugs. Plates were incubated at 37^{o}C in 5% CO_2 for 32 days and the number of resistant colonies as well as the total number was counted for each of the 20 cultures. Resistant and total colony counts obtained from each of the 20 identical cultures were averaged. Select resistant colonies were verified to be acid-fast bacilli by means of the Ziehl-Neelsen stain.

For each isolate, the proportion of resistant colonies of the total colony count against each drug was determined. The proportion of resistant colonies was taken as a proxy for the mutation frequency in the gene coding for the respective drug targets.

Statistical analysis

GraphPad InStat 3.00 was used. The Kruskal-wallis Test (Non-parametric ANOVA) with Dunn's Multiple Comparisons Test was applied for all statistical analyses. *P* values < 0.05 were considered significant.

Ethics

The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BCA27409).

RESULTS

Table 1 shows the number of resistant mutant CFUs in a total population of 10^6 bacilli under aerobic and anaerobic conditions.

There was no significant difference between the frequency of mutation acquisition conferring isoniazid resistance between the KZN and Beijing isolates, and H37Rv (P>0.05) under aerobic conditions. Both Beijing isolates and H37Rv acquired resistance to isoniazid at the highest frequency as compared to the other drugs. The KZN isolates acquired resistance to isoniazid at similar rates to rifampicin (P=0.4772) and kanamycin (P=0.3317). These isolates acquired resistance to streptomycin at significantly higher frequencies than to isoniazid (P<0.0001). The KZN isolates acquired resistance at higher frequencies than those of the Beijing family and H37Rv for rifampicin (P<0.0001), streptomycin (P<0.0001) and kanamycin (P<0.0001 for the Beijing isolates and P = 0.008 for H37Rv). There was no significant difference in mutation frequencies conferring ofloxacin resistance between isolates and H37Rv (P>0.05).

All four clinical isolates as well as H37Rv survived the sudden change from aerobic to anaerobic incubation conditions. All grew well anaerobically, although at a slower rate than aerobically. Both KZN isolates acquired resistance to isoniazid (P<0.0002), rifampicin (P<0.0005), streptomycin (P<0.0001), kanamycin (P<0.0001) and ofloxacin (P<0.0004) at significantly higher frequencies than the Beijing isolates under anaerobic conditions.

DISCUSSION

Fluctuation analysis protocols use low density inocula. This ensures that no pre-existing mutants are present in the inoculum prior to incubation, and that a sufficient number of multiplication rounds can occur before the end of the log phase is reached. This allows for the measurement of spontaneously-occurring mutations during the incubation period.

Isolates of the KZN family acquired resistance to rifampicin, streptomycin and kanamycin more frequently than the Beijing isolates and at frequencies comparable to isoniazid resistance. This implies that strains belonging to the KZN family do have an enhanced capacity to acquire drug resistance compared to those of the Beijing family. Only two susceptible isolates belonging to each strain family were used in this study, since our *M. tuberculosis* collection contains only two confirmed susceptible isolates belonging to the KZN strain family.

The Beijing isolates and H37Rv acquired rifampicin resistance conferring mutations at an approximately 100-fold lower rate than to isoniazid. This is in accordance with most studies (David 1970; Johnson et al. 2006; Bergval et al. 2009). However, both the KZN isolates acquired rifampicin resistance at a much higher frequency than those belonging to the Beijing family and H37Rv. In the KZN isolates, the mutation frequency for rifampicin was similar to that for isoniazid. This suggests that the KZN strain needed shorter exposure to inadequate therapy to develop into MDR.

The mutation frequencies conferring streptomycin resistance in both KZN isolates were approximately 100 fold higher than that of the Beijing strains. The multidrug-resistant KZN variant, from which the first extensively drug-resistant strain developed, revealed early resistance to streptomycin (Pillay & Sturm 2007). Until recently, TB treatment protocols in South Africa included the addition of streptomycin to first line drugs in case of treatment failure before susceptibility tests become available (South African Department of Health 2004), which undoubtedly has added to the resistance selection.

All isolates acquired isoniazid resistance at comparably high frequencies. These high mutation frequencies are in accordance with the number of isoniazid resistance associated genes (Johnson et al. 2006). The true mutation frequency may even be higher since the design of the study did not allow the detection of mutations in the *inh*A gene. Isoniazid is used singly as a prophylactic drug to prevent disease reactivation in latently infected HIV-infected individuals (Raviglione et al. 2011). Given the high mutation frequencies to isoniazid for all strains, the importance of excluding active TB infection prior to administering isoniazid prophylaxis (Reddy et al. 2010) is emphasized.

There was no difference observed in mutation frequencies conferring ofloxacin resistance for the KZN and Beijing isolates. All strains acquired ofloxacin resistance at the lowest frequencies. This may be explained by the fact that high-level fluoroquinolone resistance is observed after stepwise acquisition of mutations in the *gyr*A gene, and not via single mutational events (Ginsburg et al. 2003). Therefore, the methodology applied here may not reflect the true mutation frequency.

Data pertaining to differences in the mutation rates in different strain families is conflicting. One group (Werngren & Hoffner 2003) reported similar mutation rates in the Beijing family as non-Beijing strains. In contrast, others (de Steenwinkel et al. 2012; Ford et al. 2013) showed that Beijing strains acquired rifampicin resistance at significantly higher rates than non-Beijing strains. We report significant differences between the KZN and Beijing strain families.

The reported association of the Beijing family with drug resistance may not necessarily be as a result of its mutational capacity, but instead reflect the high number of people infected with this strain, providing a larger bacterial population that can potentially mutate. This is supported by the observations of Werngren and Hoffner (2003) (Werngren & Hoffner 2003). The high mutational capacity of the KZN strains could be a result of a higher replication rate, leading to a greater possibility of replicative error due to error-prone DNA polymerase (Boshoff et al. 2003), resulting in a higher mutation frequency. However, the Beijing strains were observed to grow faster in 7H9 broth than the KZN strains. Therefore, a more likely possibility is that the KZN family exhibit a mutator phenotype. Mutations have been identified in the DNA repair genes *mut*T2 and *mut*T4 in Beijing strains (Ebrahimi-Rad et al. 2003; Lari et al. 2006).

Mutation frequencies leading to drug resistance are thought to differ between strain families (Brown et al. 2010; Ford et al. 2013). The KZN isolates acquired significantly more mutations conferring resistance to all drugs tested than the Beijing isolates under anaerobic conditions. These results support our observation that KZN strains acquire resistance more

frequently than Beijing strains under aerobic conditions, possibly reflecting defective DNA repair systems within the KZN family.

The mutational capacity of *M. tuberculosis* forms the basis for treatment regimens with multiple drugs, in order to prevent the selection of resistant mutants during treatment. Active TB infection is treated by a combination of drugs, since during this manifestation of disease, *M. tuberculosis* is presumed to undergo active replication and consequently accumulates a number of mutations. In contrast, the rationale to use single drug treatment to prevent reactivation disease is based on previous observations that *M. tuberculosis* lacks the ability to replicate and acquire mutations during the course of latent infection. Using anaerobiosis as a proxy for latent infection *in vitro*, the results of this study indicate that *M. tuberculosis* does retain the ability to multiply, albeit at a slow rate, and consequently acquire mutations anaerobically. As shown in Table 1, clinical *M. tuberculosis* isolates of the Beijing and KZN families, and H37Rv, each acquire resistance at mostly similar or higher frequencies under anaerobic conditions than aerobic conditions. This is in support of the study by Ford *et al* (2011), which shows that *M. tuberculosis* isolated from latent lesions in macaques with *M. tuberculosis* infection, acquire mutations leading to resistance at a higher rate than those isolated from active lesions (Ford et al. 2011).

Factors which may contribute to mutation acquisition include downregulation in DNA repair, accompanying the reduction in bacterial replication under anaerobic conditions (Ford et al. 2011). An alternative source of DNA damage leading to mutation acquisition anaerobically are reactive nitrogen species (Kumar et al. 2011). Prolonged incubation in broth under anaerobic conditions could increase the time of exposure of the bacterial cells to reactive

nitrogen species, a by-product of anaerobic metabolism. This could increase the rate of DNA damage, leading to resistance if damage occurs in genes coding for drug targets. This is supported by the study by Ford *et al* (2011), which showed that ten out of fourteen mutations observed in latent lesions were as a result of oxidative damage (Ford et al. 2011).

The current latency paradigm indicates little to no replication (Wayne, 1994), negating the possibility of mutation acquisition leading to resistance during the course of infection. This forms the basis for isoniazid preventive therapy (IPT). However, the results presented here indicate that *M. tuberculosis* retains the ability to mutate *in vitro* under anaerobic conditions. If anaerobic conditions reflect latent infection, then IPT may result in the generation of *de novo* isoniazid resistance.

In conclusion, the adaptation of *M. tuberculosis* to anaerobic conditions *in vitro* offers a simplified representation of the environment faced by the bacterium as it establishes latent infection. Although the true role of anaerobic multiplication in latent infection remains to be elucidated, an anaerobic environment does exit in the human host and the slower replication rate under these conditions does not prevent mutations to occur.

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Conflict of interest:

The authors declare no conflict of interest.

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REFERENCES

Barry 3rd, C. E., Boshoff, H. I., Dartois, V., Dick, T., Ehrt, S., Flynn, J., … Young, D. (2009). The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol*, *7*(12), 845–855.

Bergval, I. L., Schuitema, A. R., Klatser, P. R., & Anthony, R. M. (2009). Resistant mutants of *Mycobacterium tuberculosis* selected in vitro do not reflect the in vivo mechanism of isoniazid resistance. *J Antimicrob Chemother*, *64*(3), 515–523.

Boshoff, H. I., Reed, M. B., Barry 3rd, C. E., & Mizrahi, V. (2003). DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in *Mycobacterium tuberculosis*. *Cell*, *113*(2), 183–193.

Brown, T., Nikolayevskyy, V., Velji, P., & Drobniewski, F. (2010). Associations between *Mycobacterium tuberculosis* strains and phenotypes. *Emerg Infect Dis*, *16*(2), 272–280.

Chao, M. C., & Rubin, E. J. (2010). Letting Sleeping dos Lie: Does Dormancy Play a Role in Tuberculosis? *Annual Review of Microbiology*, *64*(1), 293–311.

David, H. L. (1970). Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. *Appl Microbiol*, *20*(5), 810–814.

De Steenwinkel, J. E., ten Kate, M. T., de Knegt, G. J., Kremer, K., Aarnoutse, R. E., Boeree, M. J., … Bakker-Woudenberg, I. A. (2012). Drug susceptibility of *Mycobacterium tuberculosis* Beijing genotype and association with MDR TB. *Emerg Infect Dis*, *18*(4), 660–663.

Ebrahimi-Rad, M., Bifani, P., Martin, C., Kremer, K., Samper, S., Rauzier, J., … Gicquel, B. (2003). Mutations in putative mutator genes of *Mycobacterium tuberculosis* strains of the W-Beijing family. *Emerg Infect Dis*, *9*(7), 838–845.

Flynn, J. L., & Chan, J. (2001). Tuberculosis : Latency and Reactivation. *Infect Immun*, *69*(7), 4195–4201.

Ford, C. B., Lin, P. L., Chase, M. R., Shah, R. R., Iartchouk, O., Galagan, J., … Fortune, S. M. (2011). Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. *Nat Genet*, *43*(5), 482–486.

Ford, C. B., Shah, R. R., Maeda, M. K., Gagneux, S., Murray, M. B., Cohen, T., … Fortune, S. M. (2013). *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat Genet*, *45*(7), 784–790.

Gillespie, S. H. (2002). Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. *Antimicrob Agents Chemother*, *46*(2), 267–274.

Ginsburg, A. S., Grosset, J. H., & Bishai, W. R. (2003). Fluoroquinolones, tuberculosis, and resistance. *Lancet Infect Dis*, *3*(7), 432–442.

Glynn, J. R., Whiteley, J., Bifani, P. J., Kremer, K., & van Soolingen, D. (2002). Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. *Emerg Infect Dis*, *8*(8), 843–849.

Hanekom, M., Gey van Pittius, N. C., McEvoy, C., Victor, T. C., Van Helden, P. D., & Warren, R. M. (2011). *Mycobacterium tuberculosis* Beijing genotype: a template for success. *Tuberculosis (Edinb)*, *91*(6), 510–523.

Ioerger, T. R., Koo, S., No, E. G., Chen, X., Larsen, M. H., Jacobs Jr., W. R., … Sacchettini, J. C. (2009). Genome analysis of multi- and extensively-drug-resistant tuberculosis from KwaZulu-Natal, South Africa. *PLoS One*, *4*(11), e7778.

Johnson, R., Streicher, E. M., Louw, G. E., Warren, R. M., van Helden, P. D., & Victor, T. C. (2006). Drug resistance in *Mycobacterium tuberculosis*. *Curr Issues Mol Biol*, *8*(2), 97–111.

Kaplan, G., Post, F. A., Moreira, A. L., Wainwright, H., Kreiswirth, B. N., Tanverdi, M., … Bekker, L. G. (2003). *Mycobacterium tuberculosis* growth at the cavity surface: a microenvironment with failed immunity. *Infect Immun*, *71*(12), 7099–7108.

Kumar, A., Farhana, A., Guidry, L., Saini, V., Hondalus, M., & Steyn, A. J. (2011). Redox homeostasis in mycobacteria: the key to tuberculosis control? *Expert Rev Mol Med*, *13*, e39.

Lari, N., Rindi, L., Bonanni, D., Tortoli, E., & Garzelli, C. (2006). Mutations in mutT genes of *Mycobacterium tuberculosis* isolates of Beijing genotype. *J Med Microbiol*, *55*(Pt 5), 599–603.

Lawn, S. D., Wood, R., & Wilkinson, R. J. (2011). Changing concepts of "latent tuberculosis infection" in patients living with HIV infection. *Clin Dev Immunol*, *2011*.

Loebel, R. O., Shorr, E., & Richardson, H. B. (1933). The Influence of Adverse Conditions upon the Respiratory Metabolism and Growth of Human Tubercle Bacilli. *J Bacteriol*, *26*(2), 167–200.

Migliori, G. B., De Iaco, G., Besozzi, G., Centis, R., & Cirillo, D. M. (2007). First tuberculosis cases in Italy resistant to all tested drugs. *Euro Surveill*, *12*(5), E070517 1.

Moodley, P., Shah, N. S., Tayob, N., Connolly, C., Zetola, N., Gandhi, N., … Sturm, A. W. (2011). Spread of extensively drug-resistant tuberculosis in KwaZulu-Natal province, South Africa. *PLoS One*, *6*(5), e17513.

Parrish, N. M., Dick, J. D., & Bishai, W. R. (1998). Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends Microbiol*, *6*(3), 107–112.

Pillay, M., & Sturm, A. W. (2007). Evolution of the extensively drug-resistant F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa. *Clin Infect Dis*, *45*(11), 1409–1414.

Ramaswamy, S., & Musser, J. M. (1998). Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis*, *79*(1), 3–29.

Raviglione, M., Yew, W. W., & Migliori, G. B. (2011). Treatment of latent infection with *Mycobacterium tuberculosis*: update 2010. *European Respiratory Journal*, *37*(3), 690–711.

Reddy, K. P., Brady, M. F., Gilman, R. H., Coronel, J., Navincopa, M., Ticona, E., … Moore, D. A. (2010). Microscopic observation drug susceptibility assay for tuberculosis screening before isoniazid preventive therapy in HIV-infected persons. *Clin Infect Dis*, *50*(7), 988–996.

South African Department of Health. (2004). *The South African National Tuberculosis Control Programme: Practical Guidelines 2004*.

Tan, M. P., Sequeira, P., Lin, W. W., Phong, W. Y., Cliff, P., Ng, S. H., … Alonso, S. (2010). Nitrate respiration protects hypoxic *Mycobacterium tuberculosis* against acid- and reactive nitrogen species stresses. *PLoS One*, *5*(10), e13356.

Udwadia, Z. F., Amale, R. A., Ajbani, K. K., & Rodrigues, C. (2012). Totally drug-resistant tuberculosis in India. *Clin Infect Dis*, *54*(4), 579–581.

Velayati, A. A., Masjedi, M. R., Farnia, P., Tabarsi, P., Ghanavi, J., Ziazarifi, A. H., & Hoffner, S. E. (2009). Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. *Chest*, *136*(2), 420–425.

Wayne, L. G. (1994). Dormancy of *Mycobacterium tuberculosis* and latency of disease. *Eur J Clin Microbiol Infect Dis*, *13*(11), 908–914.

Wayne, L. G., & Hayes, L. G. (1996). An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun*, *64*(6), 2062–2069.

Werngren, J., & Hoffner, S. E. (2003). Drug-susceptible *Mycobacterium tuberculosis* Beijing genotype does not develop mutation-conferred resistance to rifampin at an elevated rate. *J Clin Microbiol*, *41*(4), 1520–1524.

Table 1: Resistance-conferring mutations acquired in a population 10^6 bacilli under aerobic and anaerobic conditions

	Beijing				F15/LAM4/KZN					
	TF1516		TF2284		V9124		V4207		H37Rv	
	O_2	AnO_2	O_2	AnO_2	O_2	AnO_2	O_2	AnO_2	O_2	AnO_2
INH[1]	15	5.03	9.3	1.08	8.4	14.2	2.24	32.75	7.4	35.8
RIF[2]	0.29	0.573	0.118	0.761	5.8	2.38	3.68	5.76	0.36	1.95
STR[3]	0.12	0.2025	0.2	0.212	11	196	14.4	189.8	1.89	11.5
KAN[4]	0.33	1.95	2.5	0.588	6.9	22.7	6.65	13.2	1.79	9.32
OFX[5]	0.49	0.139	0.079	0.317	2.1	0.339	0.94	1.43	0.475	14.9

O_2 = Aerobic; AnO_2 = Anaerobic

[1]Isoniazid resistance was acquired at similar frequencies for TF1516 (P=0.0851), TF2284 (P=0.5073) and H37Rv (P<0.0001) under aerobic and anaerobic conditions. Isoniazid resistance was acquired at significantly higher frequencies for V4207 (P = 0.0473) and H37Rv (P<0.0001) under anaerobic conditions.

[2] TF1516 acquired rifampicin resistance at a similar frequency under aerobic and anaerobic conditions (P = 0.9892). V9124 acquired rifampicin resistance at a lower frequency anaerobically (P=0.0351). TF2284, V4207 and H37Rv each acquired rifampicin resistance at significantly higher frequencies under anaerobic conditions (P<0.05).

[3] Both of the Beijing isolates acquired streptomycin resistance at similar frequencies under aerobic and anaerobic conditions (P>0.05). Streptomycin resistance was acquired at higher frequencies for each of the KZN isolates (P<0.0001) and H37Rv (P = 0.0084) under anaerobic conditions.

[4] TF1516 was found to acquire kanamycin resistance at a similar frequency under aerobic and anaerobic conditions (P = 0.5941), while TF2284 acquired kanamycin resistance at a lower frequency anaerobically (P<0.05). Each of the KZN isolates and H37Rv acquired kanamycin resistance at significantly higher frequencies anaerobically (P<0.005).

[5] TF1516, V4207 and H37Rv acquired ofloxacin resistance at significantly higher frequencies anaerobically (P<0.05). Ofloxacin resistance was acquired at similar frequencies for TF2284 (P=0.9238) and V9214 (P=0.0764) under aerobic and anaerobic conditions.

CHAPTER 4: DISCUSSION

4.1. Mutation acquisition under aerobic conditions

The findings presented in this dissertation support epidemiological association of the Beijing strain with multidrug resistance (MDR), and the KZN strain with extensive drug resistance (XDR). Given the high frequencies of resistance acquisition of the KZN strain, we provide an explanation for the overrepresention of this strain in patients with XDR-TB in KwaZulu-Natal.

4.2. Mutation acquisition under anaerobic conditions

The classical definition of *M. tuberculosis* dormancy is a cessation of bacterial replication accompanied by complete metabolic shutdown (Wayne 1994; Wayne & Sohaskey 2001). Dormancy is the state the bacteria are in while the term latency refers to the state of the disease. However, "dormancy" is a much disputed term in "latency" (Chao & Rubin 2010). We used the Hypoxia Model to imitate conditions encountered by *M. tuberculosis* within the granuloma during latency. In contrast with the Wayne Model (Wayne & Hayes 1996), we immediately exposed the cultures to anaerobic conditions.

Table 1 shows that *M. tuberculosis* retains the ability to acquire resistance-conferring mutations at either similar or higher frequencies under anaerobic conditions as compared to aerobic conditions. This is supported by the findings of Ford *et al* (2011) (Ford et al. 2011).

If anaerobic conditions are a proxy for latent infection, then the mutation frequencies of *M. tuberculosis* is at least higher as in acute infection.

4.3. Antibiotic-mediated mutation acquisition

Exposure to bactericidal antibiotics is known to stimulate ROS production via the generation of hydroxyl radicals (OH·), subsequently inducing mutations (Kohanski et al. 2007). In contrast, exposure to bacteriostatic drugs does not lead to the production of OH·. Since all five antimicrobial agents used are bactericidal (Table 2.1), increase in the mutation frequency on exposure after plating is possible. Differences in resistance development between drugs may be related to differences in mutation induction by these compounds.

4.4. Technical considerations

4.4.1. Clumping

Estimation of mutation frequencies relies extensively on accurate colony counts following incubation on agar. *M. tuberculosis* is known to clump during replication as a result of its cording factor (Darzins & Fahr 1956), and this may greatly hamper accurate colony counts. In order to control for clumping, all cultures were incubated in 7H9 broth containing 0.05% Tween-80, a detergent known to reduce clumping (Jacobs et al. 1991). Following incubation in broth under both aerobic and anaerobic growth conditions, all cultures were sonicated to further disperse clumps. Prior to plating of both the diluted cultures onto drug-free 7H10 agar, and resuspended pellets onto drug-containing 7H10 agar, cultures were briefly vortexed in order to obtain homogenous suspensions containing minimal clumps.

Suspensions from select cultures were stained via the Ziehl-Neelsen method and viewed microscopically to determine whether cultures were free of contaminating bacteria. Microscopic observation revealed that the development of clumps during incubation with Tween-80 were minimal.

4.4.2. Confirmation of resistance

Following incubation on antibiotic-containing 7H10 agar, select resistant mutant colonies were picked and used to prepare a 1 MacFarland suspension for susceptibility testing using the absolute proportion method. All pick-offs were tested for all five drugs. Monoresistance solely to the antibiotic used for selection was observed, confirming phenotypic resistance for the relevant antibiotic. In addition, this supports prior observations that resistance is not acquired to more than one drug simultaneously.

4.5. Limitations

Only two susceptible isolates belonging to each strain family were used in this study, since our *M. tuberculosis* collection contains only two confirmed susceptible KZN isolates. Based on this, we cannot draw a specific conclusion on strain-dependent resistance development. These observations could be strengthened by including representative strains belonging to different families. However, our results do support the existing association of the KZN family with drug resistance, possibly due to an enhanced mutational capacity.

Mutation acquisition leading to resistance was defined by the appearance of growth on antibiotic-containing agar. While these resistance-conferring mutations were confirmed phenotypically via susceptibility testing, sequencing to determine the presence of resistance-associated mutations in the genes coding for the drug targets would have provided additional, genetic confirmation of resistance. However, this would have increased the body of work beyond that of a Masters degree project.

4.6. Conclusions

Mutation acquisition between strain families may vary under both aerobic and anaerobic growth conditions. *M. tuberculosis* retains the ability to acquire mutations leading to resistance under both aerobic and anaerobic growth conditions.

4.6.1. Suggestions for further research

This study focussed on phenotypic resistance only. Sequencing of the whole genome, or previously defined resistance-associated genes, in spontaneously-generated mutants would allow the identification of potential alternative resistance mechanisms or associated genes. Given the high mutational capacity of the KZN family, genes known to encode DNA repair machinery should be sequenced in order to identify mutations contributing to defective DNA repair activity. Sequencing of genes encoding products known to repair oxidative damage under anaerobic conditions could provide insight in the observed differences between strains.

Finally, gene expression could be performed on aerobically and anaerobically incubated cultures in order to establish whether DNA repair genes are upregulated or downregulated under oxygen deprivation. This could serve as an indication of the mutational capacity of *M. tuberculosis* during latency.

References

Abdool Karim, S. S., Churchyard, G. J., Karim, Q. A., & Lawn, S. D. (2009). HIV infection and tuberculosis in South Africa: an urgent need to escalate the public health response. *Lancet*, *374*(9693), 921–933.

Agerton, T. B., Valway, S. E., Blinkhorn, R. J., Shilkret, K. L., Reves, R., Schluter, W. W., … Onorato, I. M. (1999). Spread of strain W, a highly drug-resistant strain of *Mycobacterium tuberculosis*, across the United States. *Clin Infect Dis*, *29*(1), 85.

Alangaden, G. J., Manavathu, E. K., Vakulenko, S. B., Zvonok, N. M., & Lerner, S. A. (1995). Characterization of fluoroquinolone-resistant mutant strains of *Mycobacterium tuberculosis* selected in the laboratory and isolated from patients. *Antimicrob Agents Chemother*, *39*(8), 1700–3.

Almeida Da Silva, P. E., & Palomino, J. C. (2011). Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs. *Journal of Antimicrobial Chemotherapy* , *66* (7), 1417–1430.

Amberson Jr, J. B. (1938). The Significance of Latent Forms of Tuberculosis. *N Engl J Med*, *219*, 572–576.

Andrews, J. R., Gandhi, N. R., Moodley, P., Shah, N. S., Bohlken, L., Moll, A. P., … Sturm, A. W. (2008). Exogenous reinfection as a cause of multidrug-resistant and extensively drug-resistant tuberculosis in rural South Africa. *J Infect Dis*, *198*(11), 1582–1589.

Ashiru, O. T., Pillay, M., & Sturm, A. W. (2010). Adhesion to and invasion of pulmonary epithelial cells by the F15/LAM4/KZN and Beijing strains of *Mycobacterium tuberculosis*. *J Med Microbiol*, *59*(Pt 5), 528–533.

Balcells, M. E., Thomas, S. L., Godfrey-faussett, P., & Grant, A. D. (2006). Isoniazid Preventive Therapy and Risk for Resistant Tuberculosis. *Emerg Infect Dis*, *12*(5), 744–751.

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Sun, K., Wilson, T., … Um, K. S. (1994). *inh*A, a Gene Encoding a Target for Isoniazid and Ethionamide in *Mycobacterium tuberculosis*. *Science*, *263*(5144), 227–230.

Barry 3rd, C. E., Boshoff, H. I., Dartois, V., Dick, T., Ehrt, S., Flynn, J., … Young, D. (2009). The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol*, *7*(12), 845–855.

Barton, N. H., Briggs, D. E. G., Eisen, J. A., Goldstein, D. B., & Patel, N. H. (2007). *Evolution* (Figure 12.22). *Cold Spring Harbour Press*.

Bass, J. B., Farer, L. S., Hopewell, P. C., O'Brien, R., Jacobs, R. F., Ruben, F., … Thornton, G. (1994). Treatment of tuberculosis and tuberculosis infection in adults and children. *American Journal of Respiratory and Critical Care Medicine*, *149*(5), 1359–1374.

Bergval, I. L., Schuitema, A. R., Klatser, P. R., & Anthony, R. M. (2009). Resistant mutants of *Mycobacterium tuberculosis* selected *in vitro* do not reflect the *in vivo* mechanism of isoniazid resistance. *J Antimicrob Chemother*, *64*(3), 515–523.

Bhagwati, A. M. (2012). Antibiotic resistance: need for rationale use. *J Assoc Physicians India*, *60*, 9.

Bifani, P. J., Mathema, B., Kurepina, N. E., & Kreiswirth, B. N. (2002). Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. *Trends Microbiol*, *10*(1), 45–52.

Bifani, P. J., Plikaytis, B. B., Kapur, V., Stockbauer, K., Pan, X., Lutfey, M. L., … Kreiswirth, B. N. (1996). Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. *JAMA*, *275*(6), 452–457.

Bjelland, S., & Seeberg, E. (2003). Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. *Mutat Res*, *531*(1-2), 37–80.

Boshoff, H. I., Reed, M. B., Barry 3rd, C. E., & Mizrahi, V. (2003). DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in *Mycobacterium tuberculosis*. *Cell*, *113*(2), 183–193.

Brown, T., Nikolayevskyy, V., Velji, P., & Drobniewski, F. (2010). Associations between *Mycobacterium tuberculosis* strains and phenotypes. *Emerg Infect Dis*, *16*(2), 272–280.

Cambaup, E., Sougakoff, W., Besson, M., Truffot-Pernot, C., Grosset, J., & Jarlier, V. (1994). Selection of a *gyr*A Mutant of *Mycobacterium tuberculosis* Resistant to Fluoroquinolones during Treatment with Ofloxacin. *Journal of Infectious Diseases* , *170* (2), 479–483.

Chao, M. C., & Rubin, E. J. (2010). Letting Sleeping dos Lie: Does Dormancy Play a Role in Tuberculosis? *Annual Review of Microbiology*, *64*(1), 293–311.

Churchyard, G. J., Fielding, K. L., Lewis, J. J., Coetzee, L., Corbett, E. L., Godfrey-Faussett, P., … Grant, A. D. (2014). A Trial of Mass Isoniazid Preventive Therapy for Tuberculosis Control. *The New England Journal of Medicine*, *370*(4), 301–10.

Cohen, T., Lipsitch, M., Walensky, R. P., & Murray, M. (2006). Beneficial and perverse effects of isoniazid preventive therapy for latent tuberculosis infection in HIV-tuberculosis coinfected populations. *Proc Natl Acad Sci U S A*, *103*(18), 7042–7047.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., … Barrell, B. G. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature*, *393*(6685), 537–544.

Comstock, G. W. (1999). How much isoniazid is needed for prevention of tuberculosis among immunocompetent adults? *Int J Tuberc Lung Dis*, *3*(10), 847–850.

Comstock, G. W., Baum, C., & Snider, D. E. (1979). Isoniazid prophylaxis among Alaskan Eskimos: a final report of the bethel isoniazid studies. *The American Review of Respiratory Disease*, *119*(5), 827–830.

Cooksey, R. C., Morlock, G. P., McQueen, A., Glickman, S. E., & Crawford, J. T. (1996). Characterization of streptomycin resistance mechanisms among *Mycobacterium tuberculosis* isolates from patients in New York City. *Antimicrob Agents Chemother*, *40*(5), 1186–1188.

Crofton, J., & Mitchison, D. A. (1948). Streptomycin resistance in pulmonary tuberculosis. *Br Med J*, *2*(4588), 1009–1015.

Darzins, E., & Fahr, G. (1956). Cord-Forming Property, Lethality and Pathogenicity of Mycobacteria. *Chest*, *30*(6), 642–8.

David, H. L. (1970). Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. *Appl Microbiol*, *20*(5), 810–814.

De Steenwinkel, J. E., ten Kate, M. T., de Knegt, G. J., Kremer, K., Aarnoutse, R. E., Boeree, M. J., … Bakker-Woudenberg, I. A. (2012). Drug susceptibility of *Mycobacterium tuberculosis* Beijing genotype and association with MDR TB. *Emerg Infect Dis*, *18*(4), 660–663.

Drlica, K., Malik, M., Kerns, R. J., & Zhao, X. (2008). Quinolone-mediated bacterial death. *Antimicrobial Agents and Chemotherapy*, *52*(2), 385–92.

Dwyer, D. J., Kohanski, M. A., & Collins, J. J. (2009). Role of reactive oxygen species in antibiotic action and resistance. *Curr Opin Microbiol*, *12*(5), 482–489.

Ebrahimi-Rad, M., Bifani, P., Martin, C., Kremer, K., Samper, S., Rauzier, J., … Gicquel, B. (2003). Mutations in putative mutator genes of *Mycobacterium tuberculosis* strains of the W-Beijing family. *Emerg Infect Dis*, *9*(7), 838–845.

European Concerted Action on New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis. (2006). *Beijing/W Genotype Mycobacterium tuberculosis and Drug Resistance*. *Emerg Infect Dis* (2006/05/18 ed., Vol. 12, pp. 736–743).

Ferebee, S. H. (1970). Controlled chemoprophylaxis trials in tuberculosis. A general review. *Bibl Tuberc*, *26*, 28–106.

Ferebee, S. H., & Palmer, C. E. (1956). Prevention of experimental tuberculosis with isoniazid. *Am Rev Tuberc*, *73*(1), 1–18.

Ferebee, S., Mount, F. W., & Anastasiades, A. (1957). Prophylactic effects of isoniazid on primary tuberculosis in children; a preliminary report. *Am Rev Tuberc*, *76*(6), 942–963.

Finch, R. (1986). Beta-lactam antibiotics and mycobacteria. *J Antimicrob Chemother*, *18*(1), 6–8.

Finken, M., Kirschner, P., Meier, A., Wrede, A., & Böttger, E. C. (1993). Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Molecular Microbiology*, *9*(6), 1239–1246.

Flynn, J. L., & Chan, J. (2001). Tuberculosis: Latency and Reactivation. *Infect Immun*, *69*(7), 4195–4201.

Ford, C. B., Lin, P. L., Chase, M. R., Shah, R. R., Iartchouk, O., Galagan, J., … Fortune, S. M. (2011). Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. *Nat Genet*, *43*(5), 482–486.

Ford, C. B., Shah, R. R., Maeda, M. K., Gagneux, S., Murray, M. B., Cohen, T., … Fortune, S. M. (2013). *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat Genet*, *45*(7), 784–790.

Fortune, S. M., & Rubin, E. J. (2010). Host transcription in active and latent tuberculosis. *Genome Biology*, *11*(9), 135.

Fox, W., & Sutherland, I. (1956). A five-year assessment of patients in a controlled trial of streptomycin, para-aminosalicylic acid, and streptomycin plus para-aminosalicylic acid, in pulmonary tuberculosis. *Q J Med*, *25*(98), 221–243.

Frieden, T. R., Sterling, T., Pablos-Mendez, A., Kilburn, J. O., Cauthen, G. M., & Dooley, S. W. (1993). The emergence of drug-resistant tuberculosis in New York City. *N Engl J Med*, *328*(8), 521–526.

Fujii, S., Akiyama, M., Aoki, K., Sugaya, Y., Higuchi, K., Hiraoka, M., … Maki, H. (1999). DNA replication errors produced by the replicative apparatus of *Escherichia coli*. *J Mol Biol*, *289*(4), 835–850.

Gandhi, N. R., Brust, J. C. M., Moodley, P., Weissman, D., Heo, M., Ning, Y., … Shah, N. S. (2014). Minimal diversity of drug-resistant *Mycobacterium tuberculosis* strains, South Africa. *Emerging Infectious Diseases*, *20*(3), 426–33.

Gandhi, N. R., Moll, A., Sturm, A. W., Pawinski, R., Govender, T., Lalloo, U., … Friedland, G. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*, *368*(9547), 1575–1580.

Gengenbacher, M., & Kaufmann, S. H. E. (2012). *Mycobacterium tuberculosis*: success through dormancy. *FEMS Microbiology Reviews*, *36*(3), 514–32.

Gikalo, M. B., Nosova, E. Y., Krylova, L. Y., & Moroz, A. M. (2012). The role of eis mutations in the development of kanamycin resistance in *Mycobacterium tuberculosis* isolates from the Moscow region. *The Journal of Antimicrobial Chemotherapy*, *67*(9), 2107–9.

Gillespie, S. H. (2002). Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. *Antimicrob Agents Chemother*, *46*(2), 267–274.

Ginsburg, A. S., Grosset, J. H., & Bishai, W. R. (2003). Fluoroquinolones, tuberculosis, and resistance. *Lancet Infect Dis*, *3*(7), 432–442.

Glynn, J. R., Whiteley, J., Bifani, P. J., Kremer, K., & van Soolingen, D. (2002). Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. *Emerg Infect Dis*, *8*(8), 843–849.

Godfrey-faussett, P. (1998). Policy statement on preventive therapy against tuberculosis in people living with HIV. World Health Organization (Geneva), February 1998.

Gomez, J. E., & McKinney, J. D. (2004). *M. tuberculosis* persistence, latency, and drug tolerance. *Tuberculosis*, *84*(1-2), 29–44.

Graessle, O. E., & Pietrowski, J. J. (1949). The in vitro effect of para-aminosalicylic acid in preventing acquired resistance to streptomycin by *Mycobacterium tuberculosis*. *J Bacteriol*, *57*(4), 459–464.

Gumbo, T., Louie, A., Liu, W., Ambrose, P. G., Bhavnani, S. M., Brown, D., & Drusano, G. L. (2007). Isoniazid's Bactericidal Activity Ceases because of the Emergence of Resistance, Not Depletion of *Mycobacterium tuberculosis* in the Log Phase of Growth. *Journal of Infectious Diseases*, *195*, 194–201.

Guo, H., Seet, Q., Denkin, S., Parsons, L., & Zhang, Y. (2006). Molecular characterization of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from the USA. *Journal of Medical Microbiology*, *55*(Pt 11), 1527–31.

Hanekom, M., Gey van Pittius, N. C., McEvoy, C., Victor, T. C., Van Helden, P. D., & Warren, R. M. (2011). *Mycobacterium tuberculosis* Beijing genotype: a template for success. *Tuberculosis (Edinb)*, *91*(6), 510–523.

Hawkridge, T. (2007). Tuberculosis contacts and prophylaxis. *South African Medical Journal*, *97*(10), 998–1000.

Hazbón, M. H., Brimacombe, M., Bobadilla del Valle, M., Cavatore, M., Guerrero, M. I., Varma-Basil, M., … Alland, D. (2006). Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, *50*(8), 2640–9.

Honort, N., & Cole, S. T. (1994). Streptomycin Resistance in Mycobacteria. *Antimicrob Agents Chemother*, *38*(2), 238–242.

Imlay, J. A. (2003). Pathways of Oxidative Damage. *Annual Review of Microbiology*, *57*(1), 395–418.

Ioerger, T. R., Koo, S., No, E. G., Chen, X., Larsen, M. H., Jacobs Jr., W. R., … Sacchettini, J. C. (2009). Genome analysis of multi- and extensively-drug-resistant tuberculosis from KwaZulu-Natal, South Africa. *PLoS One*, *4*(11), e7778.

Iseman, M. D. (2002). Tuberculosis therapy: past, present and future. *Eur Respir J Suppl*, *36*, 87s–94s.

Jacobs, W. R., Kalpana, G. V., Cirillo, J. D., Pascopella, L., Snapper, S. B., Udani, R. A., … Bloom, B. R. (1991). *Bacterial Genetic Systems*. *Methods in Enzymology* (Vol. 204, pp. 537–555).

Johnson, R., Streicher, E. M., Louw, G. E., Warren, R. M., van Helden, P. D., & Victor, T. C. (2006). Drug resistance in *Mycobacterium tuberculosis*. *Curr Issues Mol Biol*, *8*(2), 97–111.

Kampranis, S. C., & Maxwell, A. (1998). The DNA Gyrase-Quinolone Complex : ATP Hydrolysis and the Mechanism of DNA Cleavage. *Journal of Biological Chemistry*, *273*(35), 22615–22626.

Kaplan, G., Post, F. A., Moreira, A. L., Wainwright, H., Kreiswirth, B. N., Tanverdi, M., … Bekker, L. G. (2003). *Mycobacterium tuberculosis* growth at the cavity surface: a microenvironment with failed immunity. *Infect Immun*, *71*(12), 7099–7108.

Klopper, M., Warren, R. M., Hayes, C., Gey van Pittius, N. C., Streicher, E. M., Muller, B., … Trollip, A. P. (2013). Emergence and spread of extensively and totally drug-resistant tuberculosis, South Africa. *Emerg Infect Dis*, *19*(3), 449–455.

Knox, R., King, M. B., & Woodroffe, R. C. (1952). The in-vitro action of isoniazid on *Mycobacterium tuberculosis*. *The Lancet*, *260*(6740), 854–858.

Kocagöz, T., Hackbarth, C. J., Unsal, I., Rosenberg, E. Y., Nikaido, H., Hackbarth, C. J., & Ibrahim, U. (1996). Gyrase mutations in laboratory-selected, fluoroquinolone-resistant mutants of *Mycobacterium tuberculosis* H37Ra. *Antimicrob Agents Chemother*, *40*(8), 1768–74.

Kochi, A. (1997). Tuberculosis control--is DOTS the health breakthrough of the 1990s? *World Health Forum*, *18*(3-4), 225–247.

Kohanski, M. A., DePristo, M. A., & Collins, J. J. (2010). Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol Cell*, *37*(3), 311–320.

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell*, *130*(5), 797–810.

Koul, A., Arnoult, E., Lounis, N., Guillemont, J., & Andries, K. (2011). The challenge of new drug discovery for tuberculosis. *Nature*, *469*(7331), 483–490.

Koul, A., Vranckx, L., Dendouga, N., Balemans, W., Van den Wyngaert, I., Vergauwen, K., … Andries, K. (2008). Diarylquinolines are bactericidal for dormant mycobacteria as a result of disturbed ATP homeostasis. *The Journal of Biological Chemistry*, *283*(37), 25273–80.

Kumar, A., Farhana, A., Guidry, L., Saini, V., Hondalus, M., & Steyn, A. J. (2011). Redox homeostasis in mycobacteria: the key to tuberculosis control? *Expert Rev Mol Med*, *13*, e39.

Kunkel, T. A. (2004). DNA replication fidelity. *J Biol Chem*, *279*(17), 16895–16898.

Lari, N., Rindi, L., Bonanni, D., Tortoli, E., & Garzelli, C. (2006). Mutations in *mut*T genes of *Mycobacterium tuberculosis* isolates of Beijing genotype. *J Med Microbiol*, *55*(Pt 5), 599–603.

Laurenzo, D., & Mousa, S. A. (2011). Mechanisms of drug resistance in *Mycobacterium tuberculosis* and current status of rapid molecular diagnostic testing. *Acta Trop*, *119*(1), 5–10.

Lawn, S. D., Wood, R., & Wilkinson, R. J. (2011). Changing concepts of "latent tuberculosis infection" in patients living with HIV infection. *Clin Dev Immunol*, *2011*.

Lee, A. S. G., Lim, I. H. K., Tang, L. L. H., Lee, A. N. N. S. G., & Telenti, A. (1999). Contribution of kasA Analysis to Detection of Isoniazid-Resistant *Mycobacterium tuberculosis* in Singapore. *Antimicrob Agents Chemother*, 12–15.

Livermore, D. M. (2003). Bacterial resistance: origins, epidemiology, and impact. *Clin Infect Dis*, *36*(Suppl 1), S11–23.

Lobue, P., & Menzies, D. (2010). Treatment of latent tuberculosis infection: An update. *Respirology (Carlton, Vic.)*, *15*(4), 603–22.

Loebel, R. O., Shorr, E., & Richardson, H. B. (1933). The Influence of Adverse Conditions upon the Respiratory Metabolism and Growth of Human Tubercle Bacilli. *J Bacteriol*, *26*(2), 167–200.

Lopez, E., Elez, M., Matic, I., & Blazquez, J. (2007). Antibiotic-mediated recombination: ciprofloxacin stimulates SOS-independent recombination of divergent sequences in *Escherichia coli*. *Mol Microbiol*, *64*(1), 83–93.

Louw, G. E., Warren, R. M., Gey van Pittius, N. C., McEvoy, C. R. E., Van Helden, P. D., & Victor, T. C. (2009). A Balancing Act: Efflux/Influx in Mycobacterial Drug Resistance. *Antimicrobial Agents and Chemotherapy*, *53*(8), 3181–3189.

Louw, G. E., Warren, R. M., Pittius, N. C. G. Van, Leon, R., Hernandez-pando, R., Mcevoy, C. R. E., & Grobbelaar, M. (2011). Rifampicin Reduces Susceptibility to Ofloxacin in Rifampicin-resistant *Mycobacterium tuberculosis* through Efflux. *American Journal of Respiratory and Critical Care Medicine*, *184*(2), 269–276.

Luria, S. E., & Delbrück, M. (1943). Mutations of Bacteria from Virus Sensitivity to Virus Resistance. *Genetics*, *28*(6), 491–511.

Machado, D., Couto, I., Perdigão, J., Rodrigues, L., Portugal, I., Baptista, P., … Viveiros, M. (2012). Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. *PloS One*, *7*(4), e34538.

Maki, H., & Sekiguchi, M. (1992). MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature*, *355*(6357), 273–275.

Martinez, J. L., & Baquero, F. (2000). Mutation frequencies and antibiotic resistance. *Antimicrob Agents Chemother*, *44*(7), 1771–1777.

Mdluli, K., Slayden, R. A., Zhu, Y., Ramaswamy, S., Pan, X., Mead, D., … Barry, C. E. (1998). Inhibition of a *Mycobacterium tuberculosis* β-Ketoacyl ACP Synthase by Isoniazid. *Science* , *280* (5369), 1607–1610.

Meier, A., Sander, P., Schaper, K. J., Scholz, M., & Böttger, E. C. (1996). Correlation of molecular resistance mechanisms and phenotypic resistance levels in streptomycin-resistant *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, *40*(11), 2452–4.

Migliori, G. B., De Iaco, G., Besozzi, G., Centis, R., & Cirillo, D. M. (2007). First tuberculosis cases in Italy resistant to all tested drugs. *Euro Surveill*, *12*(5), E070517 1.

Miller, L. P., Crawford, J. T., Shinnick, T. M., Miller, L. P., Crawford, J. T., & Shinnick, T. M. (1994). The rpoB Gene of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*, *38*(4).

Mitchison, D. A. (2004). The search for new sterilizing antituberculosis drugs. *Frontiers in Bioscience*, *9*, 1059–72.

Mitchison, D., & Davies, G. (2012). The chemotherapy of tuberculosis: past, present and future. *Int J Tuberc Lung Dis*, *16*(6), 724–732.

Moodley, P., Shah, N. S., Tayob, N., Connolly, C., Zetola, N., Gandhi, N., … Sturm, A. W. (2011). Spread of extensively drug-resistant tuberculosis in KwaZulu-Natal province, South Africa. *PLoS One*, *6*(5), e17513.

Muttucumaru, D. G. N., Roberts, G., Hinds, J., Stabler, R. A., & Parish, T. (2004). Gene expression profile of *Mycobacterium tuberculosis* in a non-replicating state. *Tuberculosis*, *84*(3), 239–246.

Nair, J., Rouse, D. A., Bai, G.-H., & Morris, S. L. (1993). The *rps*L gene and streptomycin resistance in single and multiple drug-resistant strains of *Mycobacterium tuberculosis*. *Molecular Microbiology*, *10*(3), 521–527.

O'Sullivan, D. M., McHugh, T. D., & Gillespie, S. H. (2008). The effect of oxidative stress on the mutation rate of *Mycobacterium tuberculosis* with impaired catalase/peroxidase function. *J Antimicrob Chemother*, *62*(4), 709–712.

Ogilvie, C. M. (1954). Bacterial resistance to isoniazid and marsilid in treatment of pulmonary tuberculosis; results of trials. *Br Med J*, *2*(4892), 829–833.

Okamoto, S., Tamaru, A., Nakajima, C., Nishimura, K., Tanaka, Y., Tokuyama, S., … Ochi, K. (2007). Loss of a conserved 7-methylguanosine modification in 16S rRNA confers low-level streptomycin resistance in bacteria. *Mol Microbiol*, *63*(4), 1096–1106.

Olender, S., Saito, M., Apgar, J., Gillenwater, K., Bautista, C. T., Lescano, A. G., … Gilman, R. H. (2003). Low prevalence and increased household clustering of *Mycobacterium tuberculosis* infection in high altitude villages in Peru. *The American Journal of Tropical Medicine and Hygiene*, *68*(6), 721–7.

Oliver, A., Canton, R., Campo, P., Baquero, F., & Blazquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science*, *288*(5469), 1251–1254.

Ouabdesselam, S., Hooper, D. C., Tankovic, J., Soussy, C. J., Ouabdesselam, S., Hooper, D. C., … Henri, U. (1995). Detection of *gyr*A and *gyr*B mutations in quinolone-resistant clinical isolates of *Escherichia coli* by single-strand conformational polymorphism analysis and determination of levels of resistance conferred by two different single *gyr*A mutations. *Antimicrob Agents Chemother*, *39*(8), 1667–70.

Paramasivan, C. N., Sulochana, S., Kubendiran, G., Venkatesan, P., & Mitchison, D. A. (2005). Bactericidal Action of Gatifloxacin, Rifampin, and Isoniazid on Logarithmic- and Stationary-Phase Cultures of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, *49*(2), 627–631.

Parrish, N. M., Dick, J. D., & Bishai, W. R. (1998). Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends Microbiol*, *6*(3), 107–112.

Piatek, A. M. Y. S., Telenti, A., Murray, M. R., El-hajj, H., Jacobs, W. R., Kramer, F. R., & Alland, D. (2000). Genotypic Analysis of *Mycobacterium tuberculosis* in Two Distinct Populations Using Molecular Beacons: Implications for Rapid Susceptibility Testing, *44*(1), 103–110.

Pillay, M., & Sturm, A. W. (2007). Evolution of the extensively drug-resistant F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa. *Clin Infect Dis*, *45*(11), 1409–1414.

Pope, C. F., O'Sullivan, D. M., McHugh, T. D., & Gillespie, S. H. (2008). A practical guide to measuring mutation rates in antibiotic resistance. *Antimicrob Agents Chemother*, *52*(4), 1209–1214.

Ramaswamy, S., & Musser, J. M. (1998). Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis*, *79*(1), 3–29.

Ramchandra, P., & Sturm, A. W. (2010). Expression of the naphthoate synthase gene in *Mycobacterium tuberculosis* in a self-generated oxygen depleted liquid culture system. *Anaerobe*, *16*(6), 610–613.

Rao, S. P. S., Alonso, S., Rand, L., Dick, T., & Pethe, K. (2008). The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating *Mycobacterium tuberculosis*. *PNAS*, *105*(33), 11945–11950.

Rattan, A., Kalia, A., & Ahmad, N. (1998). Multidrug-Resistant *Mycobacterium tuberculosis*: Molecular Perspectives. *Emerg Infect Dis*, *4*(2), 195–209.

Raviglione, M., Yew, W. W., & Migliori, G. B. (2011). Treatment of latent infection with *Mycobacterium tuberculosis*: Update 2010. *European Respiratory Journal*, *37*(3), 690–711.

Reddy, K. P., Brady, M. F., Gilman, R. H., Coronel, J., Navincopa, M., Ticona, E., … Moore, D. A. (2010). Microscopic observation drug susceptibility assay for tuberculosis screening before isoniazid preventive therapy in HIV-infected persons. *Clin Infect Dis*, *50*(7), 988–996.

Rouveix, B. (2007). Clinical implications of multiple drug resistance efflux pumps of pathogenic bacteria. *Journal of Antimicrobial Chemotherapy*, *59*(6), 1208– 1209.

Rustad, T. R., Harrell, M. I., Liao, R., & Sherman, D. R. (2008). The enduring hypoxic response of *Mycobacterium tuberculosis*. *PloS One*, *3*(1), e1502.

Rustad, T. R., Sherrid, A. M., Minch, K. J., & Sherman, D. R. (2009). Hypoxia: a window into *Mycobacterium tuberculosis* latency. *Cellular Microbiology*, *11*(8), 1151–9.

Sakai, A., Nakanishi, M., Yoshiyama, K., & Maki, H. (2006). Impact of reactive oxygen species on spontaneous mutagenesis in *Escherichia coli*. *Genes to Cells*, *11*(7), 767–78.

Samandari, T., Agizew, T. B., Nyirenda, S., Tedla, Z., Sibanda, T., Shang, N., … Wells, C. D. (2011). 6-month versus 36-month isoniazid preventive treatment for tuberculosis in adults with HIV infection in Botswana: a randomised, double-blind, placebo-controlled trial. *The Lancet*, *377*(9777), 1588–1598.

Sandgren, A., Strong, M., Muthukrishnan, P., Weiner, B. K., Church, G. M., & Murray, M. B. (2009). Tuberculosis drug resistance mutation database. *PLoS Med*, *6*(2), e2.

Schmalstieg, A. M., Srivastava, S., Belkaya, S., Deshpande, D., Meek, C., Leff, R., … Gumbo, T. (2012). The Antibiotic Resistance Arrow of Time: Efflux Pump Induction Is a General First Step in the Evolution of Mycobacterial Drug Resistance. *Antimicrobial Agents and Chemotherapy*, *56*(9), 4806–15.

Segal, W., & Bloch, H. (1956). Biochemical Differentiation of *Mycobacterium tuberculosis* grown in vivo and in vitro. *J Bacteriol*, *72(2)*, 132–141.

Sharma, S. K., & Mohan, A. (2004). Multidrug-resistant tuberculosis. *Indian J Med Res*, *120*(4), 354–376.

Siddiqi, N., Das, R., Pathak, N., Banerjee, S., Ahmed, N., Katoch, V., & Hasnain, S. (2004). *Mycobacterium tuberculosis* Isolate with a Distinct Genomic Identity Overexpresses a Tap-Like Efflux Pump. *Infection*, *32*(2), 109–111.

Siddiqi, S. H., Aziz, A., Reggiardo, Z., & Middlebrook, G. (1981). Resistance to rifampicin and isoniazid in strains of *Mycobacterium tuberculosis*. *J Clin Pathol*, *34*(8), 927–929.

Singer, J. J. (1936). Collapse therapy in pulmonary tuberculosis. *Western Journal of Medicine*, *45*(2), 120–125.

Skinner, J. S., & Sinclair, D. J. M. (1992). Fatal mediastinal compression as a late complication of surgical plombage. *Thorax*, *47*, 321–322.

Somoskovi, A., Parsons, L. M., & Salfinger, M. (2001). The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in *Mycobacterium tuberculosis*. *Respir Res*, *2*(3), 164–168.

South African Department of Health. (2004). *The South African National Tuberculosis Control Programme: Practical Guidelines 2004*.

Spies, F. S., da Silva, P. E. A., Ribeiro, M. O., Rossetti, M. L., & Zaha, A. (2008). Identification of Mutations Related to Streptomycin Resistance in Clinical Isolates of *Mycobacterium tuberculosis* and Possible Involvement of Efflux Mechanism. *Antimicrobial Agents and Chemotherapy*, *52*(8), 2947–2949. doi:10.1128/AAC.01570-07

Sreevatsan, S., Pan, X., Stockbauer, K. E., Williams, D. L., Kreiswirth, B. N., & Musser, J. M. (1996). Characterization of *rps*L and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. *Antimicrob Agents Chemother*, *40*(4), 1024.

Stahle, I. (1958). Combined treatment of pulmonary tuberculosis with cycloserine & other tuberculo-statics: preliminary report. *Sven Lakartidn*, *55*(16), 1117–1125.

Statistics South Africa. (2014). *Statistical release Mortality and causes of death in South Africa , 2011 : Findings from death notification*.

Sterling, T. R. (2008). New Approaches to the Treatment of Latent Tuberculosis. *Seminars in Respiratory and Critical Care Medicine*, *29*(5), 532–541.

Sterling, T. R., Villarino, M. E., Borisov, A. S., Shang, N., Gordin, F., Bliven-sizemore, E., … Chaisson, R. E. (2011). Three Months of Rifapentine and Isoniazid for Latent Tuberculosis Infection. *The New England Journal of Medicine*, *365*(23), 2155–2166.

Streicher, E. M., Victor, T. C., van der Spuy, G., Sola, C., Rastogi, N., van Helden, P. D., & Warren, R. M. (2007). Spoligotype signatures in the *Mycobacterium tuberculosis* complex. *J Clin Microbiol*, *45*(1), 237–240.

Sun, Z., Xu, Y., Sun, Y., Liu, Y., Zhang, X., Huang, H., & Li, C. (2014). Ofloxacin Resistance in *Mycobacterium tuberculosis* Is Associated with Efflux Pump Activity Independent of Resistance Pattern and Genotype. *Microbial Drug Resistance*, *20*(6), 525–532.

Suzuki, Y., Katsukawa, C., Tamaru, A., Abe, C., Makino, M., Mizuguchi, Y., & Taniguchi, H. (1998). Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. *Journal of Clinical Microbiology*, *36*(5), 1220–5.

Tan, M. P., Sequeira, P., Lin, W. W., Phong, W. Y., Cliff, P., Ng, S. H., … Alonso, S. (2010). Nitrate respiration protects hypoxic *Mycobacterium tuberculosis* against acid- and reactive nitrogen species stresses. *PLoS One*, *5*(10), e13356.

Timmins, G. S., & Deretic, V. (2006). Mechanisms of action of isoniazid. *Molecular Microbiology*, *62*(5), 1220–7.

Tsukamura, M. (1972). The pattern of resistance development to rifampicin in *Mycobacterium tuberculosis*. *Tubercle*, *53*(2), 111–117.

Udwadia, Z. F., Amale, R. A., Ajbani, K. K., & Rodrigues, C. (2012). Totally drug-resistant tuberculosis in India. *Clin Infect Dis*, *54*(4), 579–581.

Valway, S. E., Greifinger, R. B., Papania, M., Kilburn, J. O., Woodley, C., DiFerdinando, G. T., & Dooley, S. W. (1994). Multidrug-resistant tuberculosis in the New York State prison system, 1990-1991. *J Infect Dis*, *170*(1), 151–156.

Van Soolingen, D., Qian, L., de Haas, P. E., Douglas, J. T., Traore, H., Portaels, F., … van Embden, J. D. (1995). Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. *J Clin Microbiol*, *33*(12), 3234–3238.

Velayati, A. A., Masjedi, M. R., Farnia, P., Tabarsi, P., Ghanavi, J., Ziazarifi, A. H., & Hoffner, S. E. (2009). Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. *Chest*, *136*(2), 420–425.

Wayne, L. G. (1994). Dormancy of *Mycobacterium tuberculosis* and latency of disease. *Eur J Clin Microbiol Infect Dis*, *13*(11), 908–914.

Wayne, L. G., & Hayes, L. G. (1996). An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun*, *64*(6), 2062–2069.

Wayne, L. G., & Sohaskey, C. D. (2001). Nonreplicating Persistence of *Mycobacterium tuberculosis*. *Annual Review of Microbiology*, *55*(1), 139–163.

Wayne, L. G., & Sramek, H. A. (1994). Metronidazole Is Bactericidal to Dormant Cells of *Mycobacterium tuberculosis*, *38*(9), 2054–2058.

Wei, J. U. N., Dahl, J. L., Moulder, J. W., Roberts, E. A., Gaora, P. O., Young, D. B., … Acteriol, J. B. (2000). Identification of a *Mycobacterium tuberculosis* Gene That Enhances Mycobacterial Survival in Macrophages, *182*(2), 377–384.

Werngren, J., & Hoffner, S. E. (2003). Drug-susceptible *Mycobacterium tuberculosis* Beijing genotype does not develop mutation-conferred resistance to rifampin at an elevated rate. *J Clin Microbiol*, *41*(4), 1520–1524.

Wong, S. Y., Lee, J. S., Kwak, H. K., Via, L. E., Boshoff, H. I., & Barry 3rd, C. E. (2011). Mutations in *gid*B confer low-level streptomycin resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*, *55*(6), 2515–2522. doi:10.1128/AAC.01814-10

World Health Organization. (2006). WHO Global Task Force outlines measures to combat XDR-TB worldwide. *http://www.who.int/mediacentre/news/notes/2006/np29/en/index.html*.

World Health Organization. (2013). *World Health Organization: Global Tuberculosis Report 2013* (p. 14).

Xin, J., Wenhong, Z., Ying, Z., Feng, G., Chanyi, L., Xuelian, Z., & Honghai, W. (2008). Assessment of efflux pump gene expression in a clinical isolate *Mycobacterium tuberculosis* by real-time reverse transcription PCR. *Microbial Drug Resistance*, *1*, 7–11.

Xu, C., Kreiswirth, B. N., Sreevatsan, S., Musser, J. M., & Drlica, K. (1996). Fluoroquinolone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*. *The Journal of Infectious Diseases*, *174*(5), 1127–30.

Youmans, G. P., & Feldman, W. H. (1946). The sensitivity of tubercle bacilli in vitro to streptomycin. *J Bacteriol*, *51*, 608.

Young, D. B. (1994). Tuberculosis: Beating the bacillus. *Current Biology*, *4*(4), 351–353.

Zainuddin, Z. F., & Dale, J. W. (1990). Does *Mycobacterium tuberculosis* have plasmids? *Tubercle*, *71*(1), 43–49.

Zaunbrecher, M. A., Sikes, R. D., Metchock, B., Shinnick, T. M., & Posey, J. E. (2009). Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(47), 20004–9.

Zhang, Y., Heym, B., Allen, B., Young, D., & Cole, S. (1992). The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature*, *358*(6387), 591–593.

Zhou, J., Dong, Y., Zhao, X., Lee, S., Amin, A., Ramaswamy, S., … Drlica, K. (2000). Selection of antibiotic-resistant bacterial mutants: allelic diversity among fluoroquinolone-resistant mutations. *The Journal of Infectious Diseases*, *182*(2), 517–25.

Zumla, A., Atun, R., Maeurer, M., Mwaba, P., Ma, Z., O'Grady, J., … Grange, J. (2011). Viewpoint: Scientific dogmas, paradoxes and mysteries of latent *Mycobacterium tuberculosis* infection. *Trop Med Int Health*, *16*(1), 79–83.

APPENDICES

Appendix A: Drug stocks

Antibiotic	Diluent	Final concentration
Isoniazid	Distilled water	1 µg/ml
Rifampicin	30% methanol	1 µg/ml
Streptomycin	Distilled water	2 µg/ml
Ofloxacin	Distilled water	2 µg/ml
Kanamycin	Distilled water	5 µg/ml

Appendix B: Culture media

7H9 broth

4.7g 7H9 powder was dissolved in 900ml distilled water containing 2ml glycerol and autoclaved for 15 minutes at 121^{o}C. After autoclaving, the media was cooled down to 55^{o}C and supplemented with 100ml OADC and 1.25ml 20% tween (to prevent clumping). In order to confirm sterility prior to use, two drops of the media was inoculated onto a blood agar plate and incubated overnight.

7H10 agar (Non-selective media)

38g of 7H10 powder was dissolved in 1800ml distilled water in a 3-litre flask containing10ml glycerol and 2g casitone. The media was autoclaved for 15 minutes at 121^{o}C. After autoclaving, the media was placed in a water bath and cooled to 55^{o}C. After cooling, the media was supplemented with 200ml OADC and rapidly poured into 90mm plates. Each plate received 20-22ml agar. Once the media had cooled and solidified, sterility was confirmed by placing one plate in a CO_2 incubator overnight. These plates were used to obtain the total CFU count.

7H10 agar (Selective media)

7H10 agar was prepared as described above. After autoclaving, the agar was placed in a water bath and cooled to 45^{o}C. At this temperature, the agar was then supplemented with 200ml OADC and 2ml of antibiotic (either isoniazid, rifampicin, streptomycin, ofloxacin or kanamycin), and rapidly poured into 150mm plates. Each plate received 90ml agar. Sterility was confirmed as described above. These plates were used to obtain the resistant (mutant) CFU count.

Appendix C: Confirmatory Tests

Ziehl-Neelsen staining

1. 1-2 drops of suspensions of culture presumed to be *M. tuberculosis* were placed onto fresh slides.
2. The slides were heat-fixed on a hot plate at 65˚C under UV light for 1 hour. This also permitted the heat-killing of*M. tuberculosis*.
3. The fixed slides were then flooded with carbol fuchsin dye and heated gently, in order to allow the dye to enter all the bacterial cells. The slides were then rinsed.
4. The slides were decolourised with 3% acid alcohol for 1 minute and rinsed.
5. The slides were counterstained with methylene blue for 1-2 minutes and rinsed.
6. All slides were allowed to air-dry.
7. Stained slides were viewed under a light microscope at 100x magnification to confirm the presence of *M. tuberculosis*, the absence of contamination, and to directly confirm that no large clumps had developed during incubation in liquid 7H9 broth.

Drug susceptibility testing

1. Single colonies were inoculated into 4ml tween buffer with beads and vortexed for 1-2 minutes. Dense particles were allowed to settle.
2. These suspensions were adjusted to a No. 1 McFarland standard using distilled water and vortexed.
3. Each of the suspensions was then serially diluted by inoculating 500 µl of the no.1 McFarland to 4.5ml distilled water, vortexing until a dilution of 10^{-4} was reached.
4. The control (drug-free) and drug-containing quadrants were each inoculated with 3 drops of the 10^{-4} dilution, and the plates gently tilted to spread inoculum
5. The plates were allowed to dry in the biosafety cabinet and then packed into CO_2 permeable plastic packets, and incubated at $37^{o}C$ for 3 weeks
6. Strains were only defined as susceptible strictly in the absence of colonies on the drug-containing medium compared to the drug-free control.

Appendix D: Colony Counts

TF1516: AEROBIC

Resistant CFU counts

INH	84	111	142	173	163	0	217	0	4	190	35	0	45	0	512	1	252	304	126	398
RIF	2	0	1	3	8	12	1	32	12	3	15	27	11	12	5	4	18	7	4	8
OFX	4	12	5	9	22	9	2	21	13	22	24	5	47	23	11	16	12	11	28	3
STR	4	22	7	9	43	5	17	10	9	15	8	10	4	19	20	10	18	8	21	7
KAN	49	34	30	42	36	2	0	48	14	17	22	16	47	142	39	44	13	33	26	29

Mutation Frequencies

Drug	Resistant CFU/ml	Total CFU/ml	Mutation Frequency
INH	689.25	$4.496X10^{7}$	$\mathbf{1.53X10^{-5}}$
RIF	46.25	$1.6x10^{8}$	$\mathbf{2.9X10^{-7}}$
STR	66.5	$5.39x10^{8}$	$\mathbf{1.23X10^{-7}}$
OFX	74.75	$1.644X10^{8}$	$\mathbf{4.55X10^{-7}}$
KAN	170.75	$5.13X10^{8}$	$\mathbf{3.33X10^{-7}}$

TF1516: ANAEROBIC

Resistant CFU Counts

INH	253	4	301	0	387	52	100	46	143	10	9	108	0	2	78	1	291	1	53	0
RIF	4	32	14	2	1	0	0	2	15	2	6	0	0	1	0	4	0	8	1	9
STR	1	3	10	1	3	2	0	0	1	0	1	0	2	0	0	1	2	1	1	4
OFX	0	3	3	1	0	0	0	3	0	1	1	1	0	0	0	0	4	3	0	4
KAN	3	4	0	5	45	0	44	2	0	7	0	5	0	0	0	1	0	76	0	0

Mutation Frequencies

Drug	**Resistant CFU/ml**	**Total CFU/ml**	**Mutation Frequency**
INH	459.75	9.15×10^{7}	**5.025×10^{-6}**
RIF	25.25	4.411×10^{7}	**5.73×10^{-7}**
STR	8.25	4.075×10^{7}	**2.025×10^{-7}**
OFX	6	4.31×10^{7}	**1.392×10^{-7}**
KAN	48	2.46×10^{7}	**1.95×10^{-6}**

TF2284: AEROBIC

Resistant CFU Counts

INH	0	156	135	0	140	171	68	122	120	95	114	115	31	720	224	278	401	126	98	86
RIF	3	9	5	0	6	2	3	2	4	1	1	3	2	3	2	4	7	4	1	2
OFX	2	4	3	3	13	20	16	57	7	5	10	2	0	6	5	0	1	22	3	0
STR	0	28	8	4	9	11	6	11	3	16	8	21	198	17	11	5	4	8	9	3
KAN	0	209	126	138	114	165	188	208	140	104	124	198	150	312	106	166	142	0	132	152

Mutation Frequencies

Drug	Resistant CFU/ml	Total CFU/ml	Mutation Frequency
INH	850	$9.1X10^{7}$	**$9.5X10^{-6}$**
RIF	16	$8.165X10^{8}$	**$1.96X10^{-8}$**
STR	100	$5.03x10^{8}$	**$2.0X10^{-7}$**
OFX	44.7	$5.66x10^{8}$	**$7.9x10^{-8}$**
KAN	718.5	$2.9x10^{8}$	**$2.5x10^{-6}$**

TF2284: ANAEROBIC

Resistant CFU Counts

INH	119	63	29	31	27	66	87	129	60	100	62	13	71	102	104	7	0	87	153	0
RIF	5	0	0	10	0	2	6	8	0	2	1	3	8	2	2	16	2	2	1	0
STR	4	14	12	5	12	1	1	8	114	1	15	0	31	2	1	1	6	4	25	60
OFX	4	0	1	3	0	1	11	0	2	55	1	0	0	0	2	0	1	0	1	2
KAN	0	0	0	0	0	86	12	0	0	62	169	2	154	139	0	0	221	0	18	21

Mutation Frequencies

Drug	**Resistant CFU/ml**	**Total CFU/ml**	**Mutation Frequency**
INH	327.5	$3.57X10^{8}$	$\mathbf{1.077X10^{-6}}$
RIF	17.5	$2.3x10^{7}$	$\mathbf{7.609x10^{-7}}$
STR	79.25	$3.74X10^{8}$	$\mathbf{2.117X10^{-7}}$
OFX	21	$6.68x10^{7}$	$\mathbf{3.17x10^{-7}}$
KAN	221	$3.76X10^{8}$	$\mathbf{5.88X10^{-7}}$

V9124: AEROBIC

Resistant CFU Counts

INH	154	140	284	1180	1280	908	265	283	293	272	281	406	198	340	256	72	0	64	498	267
RIF	33	16	11	0	104	8	44	39	118	232	0	360	116	238	46	39	3	23	9	37
OFX	3	7	5	3	1	5	9	15	1	32	7	23	2	18	2	1	3	4	17	393
STR	24	7	131	160	51	86	36	137	49	24	46	187	160	93	130	244	346	230	402	535
KAN	13	27	6	6	4	212	52	14	564	1	25	11	17	10	185	36	114	27	5	374

Mutation Frequencies

Drug	**Resistant CFU/ml**	**Total CFU/ml**	**Mutation Frequency**
INH	1860.25	$2.574x10^{8}$	**$8.13x10^{-6}$**
RIF	369	$6.35x10^{7}$	**$5.8x10^{-6}$**
OFX	769.5	$6.891x10^{7}$	**$1.1X10^{-5}$**
STR	137.35	$6.3x10^{7}$	**$2.18X10^{-6}$**
KAN	425.75	$6.098X10^{7}$	**$6.98X10^{-6}$**

V9124: ANAEROBIC

Resistant CFU Counts

INH	465	10	137	62	210	190	216	149	0	45	110	0	487	169	79	14	8	59	30	18
RIF	2	35	63	60	0	1	5	37	0	55	5	0	10	0	1	35	0	0	0	1
STR	79	290	124	84	126	201	115	34	206	82	93	345	87	198	129	256	139	267	24	38
OFX	2	0	1	2	1	14	2	1	1	1	2	3	6	3	0	0	2	3	4	1
KAN	0	158	133	152	107	60	75	56	108	82	104	176	87	84	127	67	85	125	117	5

Mutation Frequencies

Drug	**Resistant CFU/ml**	**Total CFU/ml**	**Mutation Frequency**
INH	614.5	$4.325X10^{7}$	$\mathbf{1.42X10^{-5}}$
RIF	77.5	$3.26X10^{7}$	$\mathbf{2.38X10^{-6}}$
STR	729.25	$3.725x10^{6}$	$\mathbf{1.96X10^{-4}}$
OFX	12.25	$3.6175X10^{7}$	$\mathbf{3.39X10^{-7}}$
KAN	477	$2.1X10^{7}$	$\mathbf{2.27X10^{-5}}$

V4207: AEROBIC

Resistant CFU Counts

INH	219	213	220	318	339	270	492	394	195	261	153	205	489	178	209	152	220	227	96	650
RIF	44	31	174	46	3	15	1	2	11	8	99	57	98	69	1	39	52	36	86	55
OFX	1	1	1	2	0	2	1	0	1	4	1	1	0	3	2	3	2	0	13	4
STR	119	256	228	73	272	153	418	174	260	142	171	442	127	94	128	0	151	128	168	145
KAN	136	104	168	142	80	101	185	120	97	65	0	61	146	35	70	88	124	71	124	99

Mutation Frequencies

Drug	**Resistant CFU/ml**	**Total CFU/ml**	**Mutation Frequency**
INH	1375	6.47×10^{7}	$\mathbf{2.13X10^{-5}}$
RIF	231.75	6.29×10^{7}	$\mathbf{3.68X10^{-6}}$
STR	912.25	6.68×10^{7}	$\mathbf{1.37X10^{-5}}$
OFX	10.5	5.71×10^{7}	$\mathbf{1.83x10^{-7}}$
KAN	504	7.58×10^{7}	$\mathbf{6.65x10^{-6}}$

V4207: ANAEROBIC

Resistant CFU Counts

INH	388	297	301	119	349	397	456	340	372	15	163	125	0	0	0	273	0	466	0	0
RIF	14	12	13	0	64	8	10	7	5	13	19	2	20	14	17	16	0	31	11	28
STR	183	184	56	51	39	164	209	98	252	121	194	72	246	116	179	108	172	183	1	3
OFX	2	9	0	0	2	2	8	11	54	27	6	12	0	1	4	17	1	12	0	0
KAN	153	69	67	84	135	144	214	96	267	161	134	60	236	86	93	100	124	201	63	106

Mutation Frequencies

Drug	**Resistant CFU/ml**	**Total CFU/ml**	**Mutation Frequency**
INH	1015.25	$3.1x10^{7}$	**$3.275x10^{-5}$**
RIF	76	$1.32x10^{7}$	**$5.7576x10^{-6}$**
STR	657.75	$3.465x10^{6}$	**$1.898x10^{-4}$**
OFX	42	$2.9375x10^{7}$	**$1.43x10^{-6}$**
KAN	129.65	$4.9x10^{7}$	**$1.32x10^{-5}$**

H37Rv: AEROBIC

Resistant CFU Counts

INH	432	411	321	222	304	374	397	296	315	318	259	0	369	520	402	0	345	350	351	286
RIF	5	9	4	18	15	12	16	5	10	6	4	11	3	3	10	22	1	7	11	1
OFX	16	31	40	4	10	2	38	19	9	12	5	64	15	45	18	9	10	6	10	13
STR	80	86	72	259	202	146	110	86	138	201	48	66	101	145	127	129	224	112	163	146
KAN	228	250	164	172	241	140	94	96	122	128	99	64	72	83	100	89	84	113	134	55

Mutation Frequencies

Drug	**Resistant CFU/ml**	**Total CFU/ml**	**Mutation Frequency**
INH	1568	$2.13x10^{8}$	$\mathbf{7.36x10^{-6}}$
RIF	42.75	$1.16x10^{8}$	$\mathbf{3.6x10^{-7}}$
STR	660.25	$3.47X10^{8}$	$\mathbf{1.91x10^{-6}}$
OFX	94	$1.97X10^{8}$	$\mathbf{4.78x10^{-7}}$
KAN	632	$3.525x10^{8}$	$\mathbf{1.79x10^{-6}}$

H37Rv: ANAEROBIC

Resistant CFU Counts

INH	0	209	43	174	155	211	44	65	87	73	81	95	138	283	95	94	53	155	150	132
RIF	12	9	16	4	8	8	10	26	4	24	11	15	16	10	6	18	5	11	7	7
STR	5	74	0	95	140	20	46	29	8	25	0	135	0	4	12	9	26	107	139	24
OFX	139	166	38	14	7	135	67	106	91	96	165	160	134	17	164	163	151	77	1	5
KAN	50	20	26	39	36	63	62	95	39	74	31	8	80	35	107	27	1	1	0	0

Mutation Frequencies

Drug	**Resistant CFU/ml**	**Total CFU/ml**	**Mutation Frequency**
INH	584.25	$1.63X10^{7}$	**$3.58X10^{-5}$**
RIF	56	$2.875x10^{7}$	**$1.95x10^{-6}$**
STR	224.45	$1.95x10^{7}$	**$1.15x10^{-5}$**
OFX	474	$31.75x10^{6}$	**$1.49x10^{-5}$**
KAN	198.5	$21.3x10^{6}$	**$9.32x10^{-6}$**